TECHNISCHE UNIVERSITÄT MÜNCHEN
Lehrstuhl für Genetik

Origin of $CD8^+$ effector and memory T cell subsets

Christian Stemberger

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr. S. Scherer
Prüfer der Dissertation:	1. Univ.-Prof. Dr. A. Gierl
	2. Univ.-Prof. Dr. D. Busch
	3. Univ.-Prof. Dr. D. Haller

Die Dissertation wurde am 03.07.2008 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 21.09.2008 angenommen.

.

Parts of this thesis have already been published:

Stemberger, C., Huster, KM., Busch, DH. (2007).
Defining correlates of T cell protection against infection.
Discov Med 6(34), 148-152.

Stemberger, C., Neuenhahn, M., Buchholz, V. R. and Busch, D. H. (2007).
Origin of $CD8^+$ effector and memory T cell subsets.
Cell Mol Immunol 4(6), 399-405.

Stemberger, C., Huster, K. M., Koffler, M., Anderl, F., Schiemann, M., Wagner, H. and Busch, D. H. (2007)
A single naïve T cell precursor can develop into diverse effector and memory subsets. Immunity 27(4), 985-997.

1 TABLE OF CONTENTS

2 INDEX OF FIGURES

3 ABBREVIATIONS

Ab	Antibody
APC	Allophycocyanin
APC(s)	Antigen-presenting cell(s)
ATP	Adenosine 5'-triphosphate
BCR	B cell receptor
β_2m	β_2-microglobuline
BHI	Brain heart infusion
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFSE	5-(and-6) Carboxyfluorescein succinimidyl ester
CFU	Colony forming units
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
d	day
DC	Dendritic cell
ddH_2O	Distilled, deionized water
DTT	Dithiothreitol
DNA	Deoxyribonucleic acid
EBV	Eppstein-Barr virus
E. coli	*Escherichia coli*
EDTA	Ethylendiaminetetraacetate
EMA	Ethidiummonazid-bromide
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein-isothiocyanat
FoxP3	Forkhead box P3
FPLC	Fast performance liquid chromatography

GPI	Glycosylphosphatidylinositol
GvH	Graft versus Host
h	Hour / human
hCMV	*Human cytomegalovirus*
HIV	Human immunodeficeny virus
HLA	Human leukocyte antigen
IFN	Interferon
IL	Interleukin
i.p.	Intraperitoneal
IPTG	Isopropylthiogalactoside
ITAM	Immunoreceptor tyrosine activatory motif
ITIM	Immunoreceptor tyrosine inhibitory motif
i.v.	Intravenous
kDa	Kilo Dalton
ko	Gene knockout
KLRG1	Killercell lectin-like receptor G1
LAMP1	Lysosome-associated membrane protein 1
LB	Luria-Bertoni medium
LCMV	Lymphocytic choriomeningitis virus
LD	Lethal dose
L. m.	*Listeria monocytogenes*
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MACS	Magnetically activated cell sorting
MHC-I/II	Major histocompatibility complex class I/II
MPEC	Memory precursor effector cell
mRNA	Messenger RNA
NKT cell	Natural killer-like T cell
dNTP	Deoxynucleoside-triphosphate

OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Programmed death
PE	Phycoerythrin
PECy7	Phycoerythrin-Cy7
PFA	Paraformaldehyde
PI	Propidium iodide
PLC	Phospholipase C
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Room temperature
SA	Streptavidin
SDS	Sodium dodecyl sulfate
TBE	Tris/Borate/EDTA buffer
T_{CM}	central memory T cell
TCR	T cell receptor
T_{EC}	effector cell
T_{EM}	effector memory cell
TEMED	N, N, N‘, N‘-Tetramethylethylendiamine
TGF-β	Transforming growth factor-β
T_h	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tr1	antigen-induced regulatory T cell 1
T_{reg}	regulatory T cell
Tris	Tris-(Hydroxylmethyl-)Aminomethane
wk(s)	Week(s)
wt	Wildtype

4 INTRODUCTION

4.1 The immune system

During the course of evolution, nature developed a complex system of cells and molecules to defend organisms in a concerted action against multiple pathogens like bacteria, viruses, parasites as well as certain tumors. To achieve this challenging task, the mammalian immune system has developed two main branches: the innate and the adoptive immune system (Abbas and Lichtman, 2007; Janeway et al., 2005).

The innate immune system is evolutionary more ancient, and represents the first immediate line of defense against invading pathogens. To achieve this task, the innate immune system employs next to physical epithelial barriers and antimicrobial or inflammatory substances also a wide range of leukocytes. These include macrophages, granulocytes, dendritic cells (DCs) natural killer cells (NK), and NK-T cells that recognize pathogens via a limited set of germline encoded receptors. Many of these receptors guarantee self/non-self discrimination by specific recognition of evolutionary conserved microbe-derived structures, so-called pathogen associated molecular patterns (PAMPs) which are not expressed by the host (Janeway and Medzhitov, 2002). There are several types of pattern recognition receptors including intracellular Nod proteins, Toll-like receptors and receptors for complement, glucans and mannose.

As PAMPs are also common to commensals, this definition appears misleading and specific mechanisms must exist that further discriminate between beneficial colonization and potentially harmful invasive infection. In order to achieve this task, intramucosal and intercellular compartmentalization seems to be very important (Lotz et al., 2007), but the underlying mechanisms are not yet well understood.

Once cells of the innate immune system detect a harmful microbe, a complex set of intracellular signaling cascades is triggered that results in phagocytosis and secretion of antimicrobial and proinflammatory factors (e.g. cytokines and chemokines). In turn, these signals act in an auto- or paracrine fashion to the primary producing cell itself or activate other immune cells of both the innate and adoptive branch.

As innate defense mechanisms are usually regulated by conserved signaling pathways, repeated stimuli with the same pathogen result in similar responses that are often circumvented by an evading pathogen. In addition, innate immunity shows no signatures of immunological memory able to confer long-lasting protection, which is the most characteristic feature of adoptive immunity (Janeway et al., 2005).

4.2 Adoptive immunity

Today it is well accepted that cells of the innate immune system, most important DCs, also play a pivotal role in the initiation and orchestration of adoptive immune responses (Banchereau et al., 2000; Liu et al., 2007; Neuenhahn and Busch, 2007). Due to the tremendous diversity of their receptors, cells of the adoptive (or acquired) immune system are able to generate immune reactions specifically directed against non-self molecules (so called antigens) of virtually any foreign protein. The necessary receptor diversity is thereby generated during lymphocyte development by modular rearrangement of gene segments specifically selected to recognize "non-self" and tolerate "self". Whereas antibodies and B cell receptors (BCR) recognize the antigen in its native form, T cells require processing and presentation of the antigen before they get activated. The complex task of antigen processing is in most cases fulfilled by specialized phagocytic antigen presenting cells (APCs) of the innate immune system, indicating that tight and complex interactions between innate and adoptive immunity are required.

The development of specific immunity to a pathogen is based on vigorous clonal expansion of a small number of naïve precursor lymphocytes whose antigen receptor binds pathogen-derived oligopeptides (so called epitopes) in the context of autologous major histocompatibility complex (MHC) molecules (Pamer and Cresswell, 1998). One outstanding feature of adoptive immunity is the ability to form an immunological memory that can provide enhanced and in many cases (life-) long protection against reinfection with the same pathogen. The potential to rapidly proliferate, mount accelerated effector functions, and thereby blunt the severity of secondary infection also makes the generation of memory cells, especially $CD8^+$ T cells, an important goal of vaccination (Pulendran and Ahmed, 2006).

The two main effector cells of the adoptive immune system are B- and T cells, which provide humoral and cellular immunity (Janeway et al., 2005).

After B cell receptor (BCR) mediated recognition of antigen, activated B cells start to proliferate and differentiate in the B cell regions of secondary lymphatic organs into antibody (Ab) producing plasma cells (PCs). Secreted antibodies can directly bind to antigen, and Ab/antigen complexes may subsequently activate the complement system, neutralize bacterial toxins or opsonize pathogens to facilitate their recognition and elimination (by various effector mechanisms e.g. promotion of phagocytosis).

Yet despite this tight blood borne control, several pathogens developed efficient strategies to escape the humoral defense mechanisms by hiding inside host cells. Not only all viruses that are strictly dependent on the hosts´ synthesis machinery for replication belong to this group of "intracellular pathogens", also several bacteria and parasites escape antibody recognition by

surviving in intracellular compartments. Elimination or control of infections with intracellular pathogens is often strictly dependent on T cells which recognize short pathogen-derived peptides that are presented on the surface of infected host cells (Abbas and Lichtman, 2007).

4.3 T cells

As mentioned above T cells recognize their cognate antigenic peptides presented by professional APCs via a heterodimeric T cell receptor (TCR) usually consisting of an α and a β chain. Peptide-MHC (pMHC) complexes on the surface of APCs present peptide-epitopes from pathogen-derived antigens, which are bound in tandem by the TCR. T cells also express two different types of coreceptors, CD4 and/or CD8, which are used to phenotypically discriminate the two major $\alpha\beta$ T cell subsets. These coreceptors play an important role in antigen recognition, as they directly bind to MHC molecules and thereby contribute to the binding strength of TCR-ligand interactions. $CD8^+$ T cells recognize peptides bound to MHC class I molecules (MHC-I), and $CD4^+$ T cells recognize peptides bound to MHC-II.

4.3.1 $CD4^+$ T cells

$CD4^+$ T cells, also known as T helper cells (T_h), can be subdivided into different subtypes based on their cytokine profile and their exerted effector functions. These subsets include T_h0, T_h1, T_h2 and T_h17 cells (Murphy and Reiner, 2002; Stockinger and Veldhoen, 2007). When T_h0 cells, which comprise naïve and T cells not yet assigned to one of the other subsets, respond to antigenic stimulation, the encountered cytokine milieu can direct subsequent T cell differentiation. For example, when local APCs produce interleukin (IL) IL-12 and IL-18, (especially in the presence of IFN-γ), $CD4^+$ T cells will preferentially differentiate into IFN-γ secreting T_h1 cells, promoting effective cell-mediated immune responses to intracellular pathogens.

In contrast to the T_h1 situation, under the exposure of IL-4 $CD4^+$ T cells preferentially develop into IL-4-, IL-5- and IL-13-producing T_h2 cells. These can for example promote B cell-dependent humoral immune responses to extracellular pathogens (Vinuesa et al., 2005). Recent studies have provided evidence for a third effector $CD4^+$ T_h pathway that differs in function and phenotype from the classical T_h1 and T_h2 populations. Differentiation of this novel T cell subset is directed by a combination of the cytokines TGF-β and IL-6 and sometimes IL-23 secreted

from APCs. These T cells have been designated "T_h17" based on their production of IL-17A/F cytokines, which are not produced by any of the other T_h subsets. The precise function of T_h17 cells in host defense is still elusive but seems to be associated with antibacterial responses. Furthermore T_h17 cells seem to play a major role in the development of autoimmune diseases (Castellino and Germain, 2006; Stockinger and Veldhoen, 2007; Weaver et al., 2007).

Very different to the above-mentioned effector T_h-subsets is the compartment of $CD4^+$ regulatory T cells (T_{regs}), which seems to be mainly involved in the (down-) modulation and termination of immune responses. At least two types of T_{regs} exist, that can be divided into "naturally occurring" and "antigen-induced" T_{regs}.

About 10% of peripheral $CD4^+$ T cells belong to "naturally occurring" T_{regs} which are strongly dependent on IL-2 for their survival and express self-reactive TCRs. The generation of these $CD4^+CD25^+$ T_{reg} cells is thymus-dependent and this subset is characterized by constitutive expression of the transcription factor Foxp3 (Sakaguchi and Powrie, 2007). Foxp3, whose expression can be turned on by TGF-β engagement, acts as a master switch in the regulation of their development and specific functions (Fontenot and Rudensky, 2005). $CD4^+$ T cells committed to other helper subtypes, like T_h1/T_h2 T cells, in contrast fail to express Foxp3. Naturally occurring regulatory T cells reside mainly in secondary lymphoid organs and are believed to recognize self-peptide/MHC molecules, which are constitutively expressed on all nucleated cells or only on specialized APCs. In contrast, antigen-induced T_{regs} recognize foreign antigens in the periphery and develop during prolonged antigen exposure such as chronic infections. There are at least two types of antigen-induced T_{regs} referred to as T helper 3 cells (T_h3) and Tr1 cells. Tr1 cells produce high levels of IL-10 and TGF-β and require IL-10 for their differentiation and function. On the other hand T_h3 cells produce IL-10, TGF-β and IL-4, and depend on TGF-β for their suppressive function (Oldenhove et al., 2003; Vieira et al., 2004). The mechanisms used by T_{reg} cells used to suppress over-shooting immune responses and autoreactive cells are not well understood. It was demonstrated that these mechanisms can either be indirectly, e.g. by production of cytokines such as IL-10 and TGF-β, or directly through inhibition via contact with CTLA-4 (Tang and Bluestone, 2008). Also combined multifactorial inhibition events are very important. Together the potent suppressive activity of T_{regs} seems to play a crucial role in preventing the development of autoimmunity and allergy (Sakaguchi, 2005; Ziegler, 2006).

4.3.2 $CD8^+$ T cells

Immunity towards a variety of intracellular viral, bacterial and protozoal pathogens as well as some tumors is strongly dependent on the generation of robust $CD8^+$ T cell responses. The major tasks of specific $CD8^+$ immunity, comprise the generation of effector cells that are responsible for acute elimination of the pathogen as well as memory cells that survive long-term and continuously patrol various tissues in search for evidence of re-attack (Reiner et al., 2007; Williams and Bevan, 2006).

The course of a $CD8^+$ T cell response can generally be divided into four phases: (I) the "priming phase" in which naïve $CD8^+$ T cells are primed, get activated and vigorously proliferate, followed by an "effector phase" (II) which is terminated by a dramatic "contraction phase", leaving behind only 5-10% of the original burst size (III). The remaining cells are often maintained at relatively stable numbers during the "memory phase" (IV).

Prior to infection the precursor frequency of naïve $CD8^+$ (as well as $CD4^+$) T cells for different epitope specificities is very low and has been estimated to lie very constantly in the range of 50-200 cells per individual (Arstila et al., 1999; Blattman et al., 2002; Hataye et al., 2006; Moon et al., 2007). In order to run across their antigen, naïve $CD8^+$ T cells need to constitutively migrate through lymphoid tissues, where they eventually encounter their cognate antigen (*e.g.* a processed pathogen-derived peptide) presented by APCs. If this recognition ("signal 1") is accompanied by appropriate co-stimulation referred to as "signal two" (*e.g.* by CD28-CD80/CD86 interaction), this event can result in the activation ("priming") of naïve T cells. Other important co-stimulators for T cells comprise other members of the TNFR family such as interactions between CD40L-CD40, ICOS with B7RP-1, OX40-OX40L, 4-1BB-4-1BBL or CD70-CD27, just to name a few (Croft, 2003; Lipscomb and Masten, 2002).

In order to result in successful T cell priming, it is generally believed that both signals, "signal 1" and "signal 2", have to be provided by specialized and pre-activated APCs such as mature DCs. The modular administration of signals 1 and 2 are necessary to provide specificity (through signal 1) and control mechanisms to prevent unwanted T cell activation (signal 2). Delivery of only a single signal (mainly signal 1) in the absence of additional "licensing" signals is thought to lead to functional tolerance (Schwartz, 2003). Some "costimulatory" molecules, such as CTLA-4 and PD-1, can also play a role in the active suppression of $CD8^+$ T cell responses (Carreno and Collins, 2002).

Although both TCR signals and costimulation play important roles in priming and clonal expansion of T cells, most $CD8^+$ T cell responses require further signals ("signal 3") to improve their efficacy (Mescher et al., 2006). These signals can for example be provided by

(inflammatory) cytokines like IL-2, IL-12, IL-15, type-I INFs, INF-γ. Recognition of PAMPS by TLRs seems to be an important mechanism to induce such cytokines by APCs and accessory innate immune cells, helping to explain how TLR-ligands can act as strong adjuvants for the induction of strong T cell responses. In addition, also $CD4^+$ T cell help can contribute in providing "signal 3". Whether $CD4^+$ T cell help acts directly on the responding $CD8^+$ T cells or indirectly through APCs is still controversially discussed (Castellino and Germain, 2006; Huster et al., 2004; Kolumam et al., 2005; Mescher et al., 2006; Whitmire et al., 2005; Zeng et al., 2005).

Efficient $CD8^+$ T cell priming initiates vigorous expansion of few selected precursor cells through which the overall population size of antigen-reactive T cells gets enormously enhanced. In addition, proliferation and expansion is accompanied by the generation of T cells with distinct phenotypical characteristics that correlate with functionally discrete $CD8^+$ T cell subsets. During this process, a large proportion of T cells acquire effector functions like lysis of target cells via cytotoxic molecules (e.g. by perforin and granzymes) and/or the production of effector cytokines (like INF-γ and TNF-α); only such "licensed" effector T cells can subsequently participate actively in protective immunity towards given pathogens (Stemberger et al., 2007; Williams and Bevan, 2006).

Shortly after clonally expanded antigen-reactive T cells reach a maximal frequency during the effector phase, occurring quite constantly about ~7-10 days after initial antigen challenge (Busch and Pamer, 1999), the overall population collapses, with frequencies decreasing dramatically. This contraction phase is mediated by events inducing programmed cell death and is not directly linked to the clearance of antigen (Badovinac et al., 2002). Evan if an infection is entirely cleared during the primary effector phase, a small fraction of long-lived memory cells can persist in the absence of further antigen/MHC-I encounter for the lifetime of a mouse (Ahmed and Gray, 1996; Lau et al., 1994; Murali-Krishna et al., 1999). In humans it was shown that in certain vaccination-induced T cell responses, $CD8^+$ memory T cells can be maintained for up to 75 years post vaccination, presumably providing a lifelong protection (Hammarlund et al., 2003).

Memory T cells have generally been described as a population of specialized T cells that - in contrast to naïve T cells - can (re-) activate specific effector functions extremely fast, eventually within minutes. Additionally, some memory T cells are able to rapidly expand upon antigen re-encounter, giving rise to enlarged recall effector cell populations. For long-term maintenance of memory T cells weak self-renewing cell divisions seem to keep the overall frequency of memory T cells remarkably constant. The slow but constant homeostatic proliferation is believed to be mainly controlled by the two cytokines IL-7 and IL-15. The two members of the common γ-chain

(γc) family were shown to be essential for promoting survival and proliferation of long-lived memory cells (Altman et al., 1996; Carrio et al., 2007; Goldrath et al., 2002; Kearney et al., 1994; Knabel et al., 2002; Schluns et al., 2000; Tan et al., 2002; Tuma and Pamer, 2002). Most importantly, maintenance of memory cells was found to be completely independent of antigen. Even cells transferred into MHC-I deficient mice were maintained and retained their ability to respond to secondary challenge (Lau et al., 1994). In addition, homeostatic proliferation was unaffected by the absence of TCR/MHC-I interactions what might implicate the sufficient role of IL-7 and IL-15 in sustaining survival of memory T cells (Murali-Krishna et al., 1999).

4.3.3 $CD4^+$ T cell help for the generation of $CD8^+$ T cell responses

Although earlier experiments suggested that $CD4^+$ T cell help is generally important for robust $CD8^+$ T cell responses, more recent work challenged this view. It was demonstrated that in many infection and vaccination models strong inflammatory signals on APCs, e.g. mediated via TLRs, can fully compensate for a potential need of $CD4^+$ T cell help in the generation of robust $CD8^+$ effector responses (Castellino and Germain, 2006; Shedlock and Shen, 2003; Sun et al., 2004).

However, in the absence of direct activation of APCs by the pathogen itself or the inflammatory milieu, $CD4^+$ T cells can fill this gap and condition antigen-presenting DCs for instance via a CD40/CD40L dependent pathway. DCs interacting with a $CD4^+$ T cell thereby receive a “licensing signal” which subsequently renders DCs able to prime $CD8^+$ T cell responses (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998).

In contrast to the largely dispensable role of $CD4^+$ T cell in the generation of primary $CD8^+$ T cell responses, a different role for $CD4^+$ T cell help was recently described for $CD8^+$ memory responses. In the absence of $CD4^+$ helper T cells during the priming phase, subsequent memory $CD8^+$ T cell responses were found to be functionally impaired, which correlated with inefficient control of secondary infections (Huster et al., 2004; Janssen et al., 2003; Shedlock and Shen, 2003; Sun and Bevan, 2003). In defining factors that are involved in helper signals participating in memory T cell generation, an important part is often provided by cytokines. IL-2 as a major product of $CD4^+$ T cells might be a potential link to the impaired functionality of unhelped memory $CD8^+$ T cells. A recent study demonstrated the importance of IL-2 signals during $CD8^+$ priming for the generation of robust and functional secondary responses. In contrast, the primary response of $CD8^+$ T cells in absence of IL-2 derived signals was identical to T cells, which had an intact IL-2 receptor (Bachmann et al., 2007; Williams et al., 2006b).

$CD4^+$ T cells might also be necessary for efficient reactivation and secondary expansion of memory $CD8^+$ T cells. Schoenberger and colleagues provided evidence, that $CD8^+$ memory cells in the absence of $CD4^+$ T cell help could be more prone to commit activation induced cell death after antigen reencounter. This was linked to the observation, that TRAIL (tumour-necrosis factor-related apoptosis-inducing ligand) and its ligand were upregulated on unhelped $CD8^+$ T cells, which induced apoptosis (Janssen et al., 2005).

In addition to their important role in memory generation and re-expansion, $CD4^+$ T cells may also be involved in the long-term maintenance of memory $CD8^+$ T cells. This was deduced from the finding that $CD8^+$ memory T cells are gradually lost in $CD4^+$ T cell deficient mice (Northrop et al., 2006; Williams et al., 2006a). Nevertheless, the special functions of bystander $CD4^+$ T cells to maintain memory $CD8^+$ T cell remains poorly understood (Williams et al., 2006a).

4.4 $CD8^+$ T cell subsets and the concept of "job sharing"

Over the last decade, knowledge about $CD8^+$ T cell biology was boosted through technological advances like MHC-multimer/streptamer reagents, intracellular staining of cytokines or the use of TCR transgenic mice for adoptive transfer studies (Altman et al., 1996; Jung et al., 1993; Kearney et al., 1994; Knabel et al., 2002). Aided by these techniques it was not only possible to track polyclonal antigen-specific $CD8^+$ T cells during the course of an immune response but also to assess their phenotypical and functional differentiation upon activation (Busch et al., 1998; Stemberger et al., 2007).

Intensive research demonstrated that heterogeneity is a hallmark of antigen-specific T cells. It was shown that naïve $CD8^+$ T cells not only develop into either short-lived effector or long-lived memory cells, but that cells within these subsets are themselves diverse in terms of effector functions, homing potential and proliferative capacity (Masopust et al., 2001; Pantaleo and Koup, 2004; Sallusto et al., 1999).

Today, three distinct major subsets of antigen specific $CD8^+$ T cells have been identified. Short-living effector T cells (T_{EC}) dominate the expansion phase, migrate to peripheral organs, and display immediate effector functions (Reinhardt et al., 2001). While some T_{EC} respond with a very restricted functional repertoire, others yield multiple effector functions in parallel, such as production of IFN-γ, TNF-α, and/or IL-2. The latter T_{EC} have been termed multifunctional or pluripotent T cell subpopulations and might be of special importance for T cell-mediated protection against a variety of infectious diseases, including HIV, tuberculosis and malaria (Pantaleo and Koup, 2004; Seder et al., 2008).

Long-lived human and murine memory cells can largely be assigned to two classes: effector memory- (T_{EM}) and central memory (T_{CM}) T cells. T_{EM} have been described to preferentially home to peripheral tissues and respond to antigen encounter with immediate effector functions but poor numeric expansion (Huster et al., 2004; Masopust et al., 2001). On the other hand, central memory T cells (T_{CM}) home to and re-circulate through secondary lymphoid organs and expand vigorously upon antigen re-encounter. Most studies have characterized T_{CM} as cell populations producing large quantities of IL-2 (Huster et al., 2004; Sallusto et al., 1999). Central memory cells have also been identified to display a slow but constant self-renewal (Geginat et al., 2003). Acquisition of full effector functions by T_{EM} and T_{CM} requires further – partly still enigmatic – differentiation signals that could be derived from the inflammatory milieu, repeated antigenic contacts and/or help by other cellular components such as $CD4^+$ T cells (Huster et al., 2006a).

The existing functional diversity of $CD8^+$ T cells complicates their analysis, and much effort has been made to correlate functional properties with phenotypical appearance. Next to “classical” markers expressed by all memory cells (such as CD44 and Ly6C), several surface expressed proteins have been identified that characterize the three major subsets outlined above. These most importantly include the lymph node homing receptors CCR7 and CD62L as well as the α chain of the IL-7 receptor (CD127) (Huster et al., 2004; Kaech et al., 2003; Sallusto et al., 1999). Initially, Sallusto et al. described that loss of CCR7 expression is accompanied by gain of effector function on human $CD8^+$ T cells. Unfortunately, CCR7 expression on murine cells does not correlate similarly with functionally distinct $CD8^+$ T cell subsets. Instead CD62L (that is functionally comparable to CCR7) can be used to distinguish between T_{CM} ($CD62L^{high}$) and T_{EM}/T_{EC} ($CD62L^{low}$, (Huster et al., 2006a; Wherry et al., 2003). However, the sole expression of CD62L does not allow discrimination of short- or long-lived T_{EC} or T_{EM} cells making the usage of additional markers indispensable.

Two recent findings showed that analysis of CD127 expression along with CD62L allows further discrimination between $CD8^+$ subsets. Several subsets expressing distinct patterns of CD62L and CD127 expression were found that correlated functionally with the properties reported for T_{CM} ($CD62L^{high}CD127^{high}$), T_{EM} ($CD62L^{low}CD127^{high}$), and T_{EC} ($CD62L^{low}CD127^{low}$) in mice as well as humans (Huster et al., 2004; Kaech et al., 2003; Sallusto et al., 1999). CD127 as a marker discriminating effector and memory cells was further confirmed by the reported function of IL-7. Long-lived ($CD127^{high}$) naïve as well as $CD8^+$ memory cells require IL-7 for their survival (Goldrath et al., 2002; Prlic et al., 2002). High expression of the IL-7 receptor α chain (IL-7Rα) is therefore necessary for efficient uptake and response to the cytokine (Goldrath et al., 2002). A similar dependency is also very likely in naïve and memory $CD4^+$ T cells (Dooms et al., 2007).

Although IL-7 signals may play a positive role in survival during the contraction phase, enhanced IL-7 signaling does not necessarily lead to enhanced generation of $CD8^+$ memory cells (Hand et al., 2007; Sun et al., 2006). Thus, IL-7 seems not to be a key factor in driving differentiation towards distinct memory subpopulations. Analogous to CD127, also the expression of CD27, a member of the tumor necrosis factor (TNF) receptor superfamily, can be used to discriminate between effector and memory cells. In both humans and mice, loss of CD27 expression might represent a terminal effector phenotype (T_{EC}) whereas high CD27 expression is believed to be important in memory generation and maintenance (Croft, 2003; Hamann et al., 1997; Hendriks et al., 2000; Hikono et al., 2007; Marzo et al., 2007).

Heterogeneity might also affect the functional properties of $CD8^+$ T cells subsets: it has been documented by several groups that at least some memory T cells may exert immediate effector functions (like production of IFN-γ), although phenotypically appearing as T_{CM} cells (Busch et al., 1998; Joshi et al., 2007; Kamimura and Bevan, 2007; Sarkar et al., 2008). These differences might already imply that a certain heterogeneity within the subsets themselves may exist (Joshi et al., 2007; Kaech and Wherry, 2007; Unsoeld and Pircher, 2005). One study demonstrated that more complex T cell memory phenotype patterns could be revealed by co-expression of CD62L and CCR7 that were different from the classical T_{CM} and T_{EM} subset. In line with this observation a recent publication showed that three distinct $CD8^+$ memory subpopulations could be defined based on the expression of the activation marker CD43 in combination with the chemokine receptor CXCR3. These subsets were shown to be distinct from $CD62L^{low}$ effector- and $CD62L^{high}$ central-memory subsets in their efficiency to mediate recall responses to viruses (Hikono et al., 2007). Contrasting to the findings above, Lacombe et al. found that CD127 expression does not necessarily identify memory cells. However, this interpretation might be strongly influenced by the experimental system, since CD127 expression underlies dynamic regulation during T cell activation. When naïve T cells encounter Ag during infection, IL-7Rα is downregulated to avoid competition between clonally expanding cells (Park et al., 2004). Several groups have shown that during acute LCMV and *Listeria monocytogenes* infections the majority of Ag-specific T cells temporarily express very low levels of IL-7Rα at the peak of expansion, whereas expression becomes more prominent during the contraction phase. Lacombe only analyzed IL-7Rα expression during the early effector phase and therefore analyzed T cells T cells at a stage where IL-7Rα expression is physiologically downregulated. Therefore, it has to be stressed out that phenotypical analyses of effector and memory T cell subset segregation at early stages of immune responses have to be interpreted with caution, as the currently used marker combinations clearly have some experimental limitations.

Despite some uncertainties, until today most studies showed clear correlations between expression of CD62L/CD127 and the functional properties of $CD8^+$ subsets in mice and humans (Huster et al., 2006b; Shin et al., 2007; Stemberger et al., 2007). Furthermore, analysis of IL-7Rα expression allows distinction between short-lived effector and memory cell (precursors) already early after *in vivo* priming (Huster et al., 2004; Kaech et al., 2003).

4.5 The protective capacity of memory subsets

In line with their distinct phenotypical and functional characteristics, the three major subsets (T_{EC}, T_{EM}, and T_{CM}) also display major differences in their ability to respond to infection. Most importantly, their different functional "behavior" is also tightly connected to the ability to confer protection.

T_{EC} are the principal mediators of protection early during primary infection, whereas T_{CM} exhibit significant proliferative potential upon re-infection and are therefore seen by several investigators as the crucial population conferring long-lasting protective immunity against infection (Wherry et al., 2003). However, proliferative capacity does not necessarily correlate with protection: for example, the specific induction of $CD62L^{high}$ T_{CM}-like cells (after immunization with heat-killed *Listeria monocytogenes*) results in vigorous proliferation and expansion of the cells after challenge with live *Listeria*, but they are unable to protect against infection as determined by clearance of the bacteria (Huster et al., 2006a; Lauvau et al., 2001). Obviously, for rapidly replicating intracellular bacteria such as *Listeria*, T_{CM} are not capable of conferring effective protection. Interestingly, a recent study demonstrated that antigen-specific T_{CM} could confer protection against infection with lymphocytic choriomeningitis virus (LCMV) but not against vaccinia virus. On the other hand, T_{EM} protected against both types of viruses. It is noteworthy that LCMV is a pathogen that replicates rather slowly in lymphoid organs, a behavior that most likely gives T_{CM} the time and a suitable environment to develop into effector cells. In contrast, vaccinia virus replicates rapidly in peripheral organs like the ovaries. In this situation, protection relies mostly on immediate effector functions, and the functional transition from T_{CM} into protective T_{EM} or T_{EC} cannot occur quickly enough or is not possible at all outside lymphatic tissues. Indeed, at least one study showed that $CD62L^{high}$ memory cells had to go through several rounds of proliferation in the lymph nodes before they appeared as effector cells in the lung. Thus, immediate protection against rapidly replicating peripheral pathogens seems to crucially depend on the presence of sufficient numbers of long-lived effector memory cells, standing guard at the specific entry sites of the pathogen (Bachmann et al., 2005b; Cerwenka et

al., 1999; Hogan et al., 2001; Huster et al., 2006a). Protection against slowly replicating pathogens, however, may also be fulfilled by T_{CM} (Wherry et al., 2003).

4.6 Differentiation pathways, lineage relationship and "stemness"

In addition to resolving the heterogeneity of $CD8^+$ T cell subsets with respect to their phenotypical appearance and function, much effort has been applied to understand the differentiation pathway from naïve to memory $CD8^+$ T cells. Identification of the time point of memory induction is especially important for the analysis of memory-determining conditions or factors (Huster et al., 2006b). It was shown that activation and expansion of $CD8^+$ T cells can be initiated by brief exposure to antigen: *in vitro*, stimulation of bulk naïve $CD8^+$ T cells with APCs engineered to present cognate antigen for as little as two hours is already sufficient to induce proliferation (van Stipdonk et al., 2001). In addition, clonal expansion and acquisition of cytolytic function occurred without the need for further antigenic stimulation. *In vivo*, 24 hours of antigen exposure are sufficient to induce development of effector functions and memory characteristics of $CD8^+$ populations (Kaech and Ahmed, 2001; Mercado et al., 2000). When infection with the intracellular bacterium *Listeria monocytogenes* was abrogated by antibiotic treatment, expansion of Ag-specific T lymphocyte populations and the generation of T cell memory was already sufficiently programmed during the first day. Comparable results were obtained when short-time *in vitro* primed T cells were adoptively transferred into naïve recipient mice. These findings might implicate that after a short initial trigger, $CD8^+$ T cells might run on "autopilot" independent of further antigenic or inflammatory signals (Bevan and Fink, 2001).

A slight modification of this observation was introduced by two recent studies using different in vivo models. They reported that extending the duration of the initial TCR stimulus increases the magnitude of the responding antigen specific $CD8^+$ T cell population but does not alter the generation of memory. Cells that have been stimulated with antigen for a defined short period of time (seven hours), displayed a limited potential to accumulate in the primary response but became fully functional memory cells. These cells were also capable to mount robust secondary responses (Prlic et al., 2006). Interestingly, prolonged antigen contact during the priming phase did not influence their functionality during secondary antigenic reencounter (Prlic et al., 2006; Tewari et al., 2006).

Taken together, the initial priming contact between mature APCs and naïve $CD8^+$ T cells thus seems to represent a crucial event for subsequent differentiation, whereas further signals e.g. by continued antigenic contact, inflammation or even location might have further influence. In

addition to early signaling events, the question concerning the ontogeny and the exact factors that promote the generation of memory T cells are controversially discussed. Some studies suggested a progressive development of memory cells out of effector T cells while others favor an early bifurcation between commitment to memory and effector lineages (Reiner et al., 2007). To determine "when", during or after priming, lineage fate decisions that truly distinguish short-living effector and long-living memory T cells (and their respective subsets) are really made, is currently one of the most outstanding questions in the field of T cell immunology.

To explain the development of memory T cells on the basis of measurable cellular properties (i.e. potential of subsets to proliferative, self-renewal and protect) two contradictory models have been proposed. According to the "linear differentiation model", memory T cells develop out of naïve cells after acquisition of effector functions in a continuum from T_{EC} over T_{EM} and finally T_{CM} (Kaech et al., 2002; Opferman et al., 1999; Wherry et al., 2003). The time point of memory T cell commitment would in this case most likely take place during the contraction phase. This is opposed by recent data in both murine and human infection models, suggesting that memory cells do not have to pass through a fully differentiated effector phase (Joshi et al., 2007; Lanzavecchia and Sallusto, 2000; Lauvau et al., 2001; Manjunath et al., 2001; Wong et al., 2004; Zhang et al., 2007; Zhang et al., 2005).

According to the "progressive differentiation model", differentiation depends on applied signal strength early in the immune response. During the priming period, naïve antigen-reactive $CD8^+$ T cells might receive stochastic stimuli that are not of identical "strength". Signal strength was defined as a term subsuming a composition of TCR-MHC/peptide affinity, antigen concentration, access to co-stimulatory molecules, and cytokines and even different anatomic locations. As a consequence, T cells are driven through hierarchical thresholds of differentiation that define their phenotype and fate: weak signals would generate primarily T_{CM}, whereas strong signals drive differentiation of effector cells. T_{EM} could be generated by intermediate stimulation (Gett et al., 2003; Lanzavecchia and Sallusto, 2002). In the most extreme, priming by a signal of suboptimal strength (such as for example provided by immature DCs) induces CD8 T cell proliferation but the expanded cells fail to upregulate antiapoptotic molecules and receptors for homeostatic cytokines. Upon *in vivo* transfer such T cells are "unfit" and die by neglect (Gett et al., 2003).

This model would place lineage commitment events most crucially into the priming phase. As a consequence, it has to be postulated that memory cells should already be detectable very early during an immune response. First evidence for the early presence of memory cells were derived from an activation dependent transgenic reporter model. In this study, a small number of cells positive for the reporter gene were readily present at the peak of the expansion phase. By

further analysis of the immune response, reporter-positive cells survived the contraction phase and persisted into the memory phase (Jacob and Baltimore, 1999). Other hints for the early generation of memory cells was obtained by the finding that stable numbers of activated $CD127^+$ cells are readily detectable starting on day four after infection with *Listeria* or LCMV. This is of special interest as it was shown that later during the response $CD127^+$ populations mostly contain memory cells. Functional evidence for the early presence of memory cells were also first obtained from experiments in the *Listeria* infection model: re-infection as early as 5 days after primary infection resulted in typical memory expansion of antigen-specific T cells. These cells were as efficient in providing protection against subsequent challenge with *Listeria* as memory cells generated late after primary infection (Busch et al., 2000; Huster et al., 2004; Kaech et al., 2003; Wong et al., 2004). In summary, many recent data indicate that generation of memory cells might be an event that occurs very early during the development of $CD8^+$ T cells.

Although it is clear that staining for CD62L/CD127 really allows discrimination of different $CD8^+$ T cell subsets already early during an immune response, it is a matter of definition whether a recently activated T cell should already be called a memory (precursor) T cell.

Other groups have provided evidence that all memory $CD8^+$ T cells first transit through a stage during which they acquire at least some effector functions (such as effector cytokine production or cytotoxicity) before they further develop into T_{CM} and T_{EM}. This view was also expressed by the suggestion of some groups to change the terminology from MPCs to MPECs (memory precursor effector cells) (Joshi et al., 2007; Kaech and Wherry, 2007; Sarkar et al., 2008). At first glance, the concept of MPECs challenges the “progressive differentiation model”, which postulates that T_{CM} originate from weakly differentiated T cells. However, it has been documented by several groups that a substantial proportion of memory T cells expressing markers associated with the T_{CM} subset can exert some immediate effector functions (such as production of IFN-γ) (Busch et al., 1998), which may indicate that there is still a lack of appropriate markers to distinguish “true” T_{CM} from T cells that have acquired some effector functions or that the pool of T_{CM} itself contains more heterogeneity than commonly believed. Despite the difficulties in identifying proper lineage markers, it could recently be demonstrated using extensive adoptive transfer studies that MPCs with a T_{EM}-associated phenotype ($CD127^{high}/CD62L^{low}$) cannot (re-) convert into T cells with phenotypical or functional characteristics of T_{CM} in vivo (Huster et al., 2006a). In contrast, MPCs with a T_{CM}-associated phenotype ($CD127^{high}/CD62L^{high}$) were capable of efficiently differentiating into T_{EM} upon antigen restimulation (Bachmann et al., 2005a; Huster et al., 2006a). These data indicate that it is very unlikely that after acquisition of effector functions, T cells can revert into cell populations with

high proliferative capacity upon antigen re-challenge (a hallmark of the T_{CM} subset) and the potential for self-renewal by homeostatic proliferation.

Despite the controversies regarding the timing of memory T cell generation that can be deducted from the above discussed models, further insights into the development of subset diversification and linage fate decision heavily depend on understanding at which clonal level different subsets really emerge (Reiner et al., 2007; Stemberger et al., 2007). Activating signals to a naïve T cell could elicit rapid changes in gene expression, even before commitment to the cell division cycle, converting the fate of a cell to something other than naïve (Bird et al., 1998; Yeh et al., 2008). If a distinct cell subset fate is consolidated and determined prior to the first cell division, then clonal expansion of a single precursor T cell would produce uniformly fated, homogenous progeny. According to the progressive differentiation model and the autopilot theory, the signal strength during the initial priming of naïve T cells (before the first cell division) might induce a program determining the extent of differentiation of subsequent daughter cells (Gett et al., 2003). Strong and weak signals might therefore be critical for the modulation of differentiation patterns shifted either towards a phenotype of terminally differentiated T_{EC} or intermediately and less differentiated cells, such as T_{EM} and T_{CM} among clonally related T cells (Fig. 1-①). According to this model, any given naïve precursor cell could be signaled in a unique manner that would result in genetic imprinting of a developmental program. As a result similar characteristics would be transferred to subsequent daughter cells. Whether all daughters derived from a single naïve precursor T cell differentiate exactly to the same deterministic extent or whether the offspring is characterized by a limited range of T cell subsets is unknown (“one cell - one subset model”, (Reiner et al., 2007; Stemberger et al., 2007). The finding that the overall T_{EM} and T_{CM} compartments have substantially different T cell receptor (TCR) repertoires has been interpreted as support for recruitment from distinct naïve precursor cells (Baron et al., 2003). On the other hand, experiments showing that individual clonotypes of epitope-specific TCRs can be found in different subsets, supports a model in which variable subsets can arise from naïve T cells bearing the same TCR (Bouneaud et al., 2005). However, none of these observations can discriminate between origin from a single precursor cell or a small pool of clonotypic naïve T cells (Reiner et al., 2007)

The model scenario described above might be particularly useful in explaining the properties of “latecomers” in the T cell response (Fig. 1-②). It has been reported that T_{CM} preferentially develop when naïve T cells are recruited at later stages into the immune response by gaining only access to already diminished signals provided by the priming environment (Catron et al., 2006; D'Souza and Hedrick, 2006). This would shift the development of responding naïve T cells, in contrast to early recruited cells, away from effector- towards weakly differentiated T_{CM} cells.

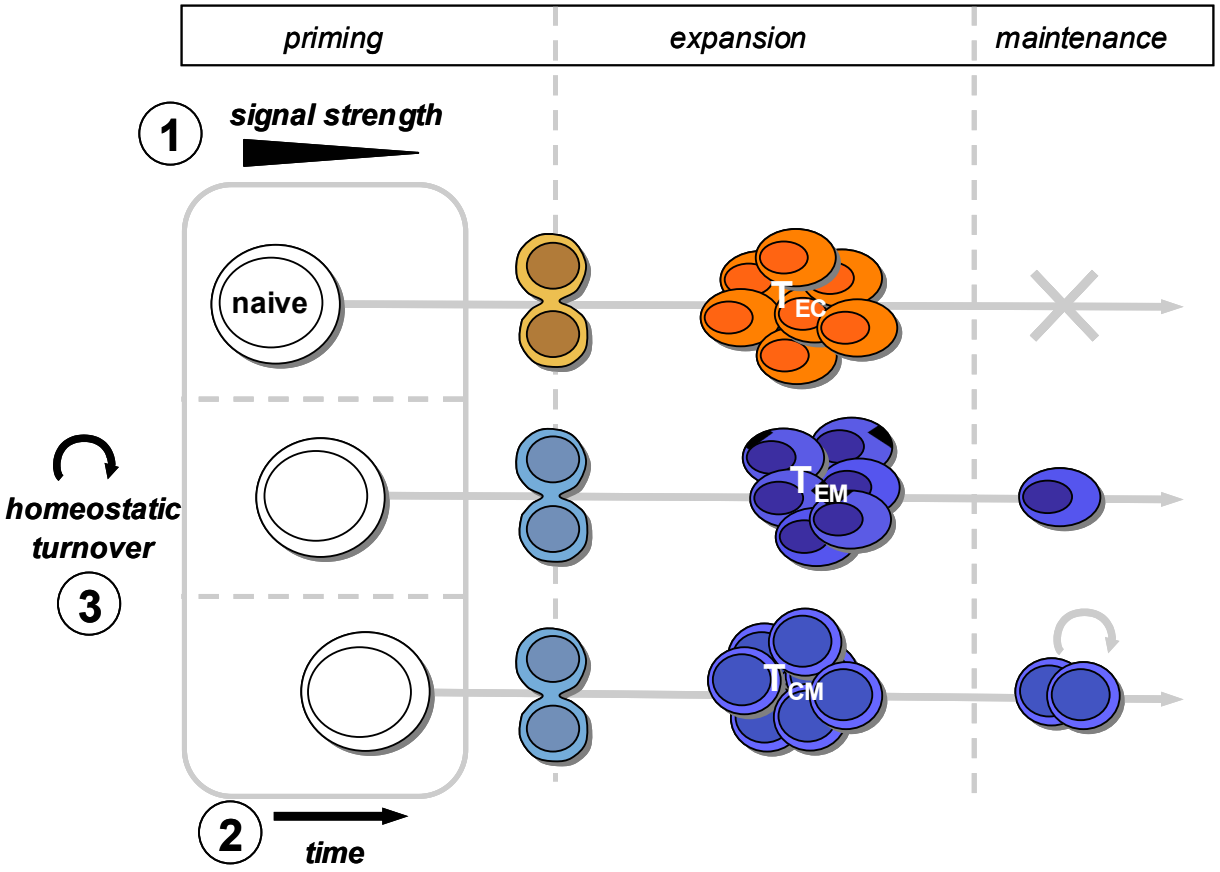

Figure 1: Models of T cell subset diversification.

Naïve antigen specific T cells take a mono-directional lineage fate decision *before* the first antigen driven cell division (grey box indicates time frame for lineage fate decision). Daughter cells are terminally tied to this decision yielding a homogenous phenotype of the ensuing progeny. ① **"Signal strength model":** Depending on the combined intensity of antigen dependent, co-stimulatory and cytokine driven signals, acting upon a naïve T cell, a specific effector phenotype is imprinted onto this cells progeny – with strong signals turning differentiation towards short lived T effector cells (T_{EC}) and weak signals imprinting T effector memory (T_{EM}) or T central memory (T_{CM}) fate (cyclic arrow indicating high homeostatic proliferative potential of T_{CM} cells). ② **"Latecomer model":** During the course of an immune response the availability of stimulatory signals rises, peaks and declines. Naïve T cells joining late into an ongoing response are exposed to mainly weak signals and thus tend towards acquisition of a memory fate. ③ **"Homeostatic proliferation model":** Lineage fate decisions taken stochastically during homeostatic proliferation gradually create phenotypic diversity within a clonotypic T cell population. Antigen and inflammatory signals then select individual predetermined T cells for expansion.

Some investigators may even favor a scenario in which the whole T_{CM} compartment is generated by T cells participating only during late stages (Catron et al., 2006). A possible drawback of these studies was the use of very high numbers of adoptively transferred TCR-tg cells that ranged between 1 x 10^4-1 x 10^6 cells. As this number is up to 10.000-fold larger than the naïve ag-specific repertoire, increasing the precursor frequency to such "unphysiologically" high numbers alters the kinetics, survival and differentiation patterns of $CD8^+$ T cells with a substantial shift towards $CD62L^{high}$ T_{CM}-phenotypes (Badovinac et al., 2007; Hataye et al., 2006;

Marzo et al., 2005). In transfer experiments with high numbers of antigen-specific cells, single analysis of CD62L expression might be misleading. Some transferred naïve $CD8^+$ T cells (that like T_{CM} also express high levels of CD62L) might not even have been recruited in the response. As CD62L is dynamically regulated after activation, mostly resulting in downregulation, unrecruited cells would appear as an unproportional high frequency of "false" $CD62L^{high}$ T_{CM}.

In contrast to the models that relate subset diversification to time points during the priming phase, heterogeneity could already be embedded to some extent within the pool of naïve ag-specific precursor cells (Fig. 1-③). Together with numeric differences of naïve precursor cells, some degree of phenotypical and functional diversification could pre-form within precursor populations specific for the same antigen making lineage decisions (at least partially) independent of the priming condition. Several years ago, it was discovered that naïve $CD8^+$ T cells undergo slow, MHC-I dependent proliferation in the absence of antigen (Ernst et al., 1999; Nesic and Vukmanovic, 1998). $CD8^+$ T cells that undergo homeostatic proliferation were found to upregulate molecules like CD44 and Ly6C, making them indistinguishable from "true" antigen-experienced memory cells (Goldrath et al., 2000; Hamilton et al., 2006; Kieper and Jameson, 1999; Murali-Krishna et al., 1999). Interestingly, these "homeostatic" memory cells also showed increased cytolytic capacity, INF-γ production and even were able to confer protection after antigen stimulation (Murali-Krishna and Ahmed, 2000). These observations indicate that a certain degree of diversification can arise already before the first contact with antigen. In this respect, heterogeneity in cell fate could be the result of recruitment of different daughter cells from single thymic naïve emigrants, which were differently shaped by homeostatic proliferation, leading to diversity on the population level.

Alternatively to the models discussed above, antigen-experienced cell fates may not be adopted or at least finalized until a naïve cell undergoes division or has divided (Yeh et al., 2008). If this would be the case, then one naïve precursor T cell should have cellular progeny that can adopt different lineage fates (Fig. 2). This subsequent development of diversity could still be of deterministic or stochastic origin (Dustin and Chan, 2000; Reiner et al., 2007). A recent study indicated that fate decision accompanied by segregation into short-living effector and long-living memory cells might occur by a deterministic mechanism during the first cell division through asymmetric cell division ("one cell – multiple fates"; (Chang et al., 2007). This observation was based on the finding that by maintaining prolonged contact with its antigen-presenting cell, a newly activated naïve T cell might unequally distribute cellular components to its two daughter cells, imparting distinct consequences towards development into (fixed) effector and memory fates.

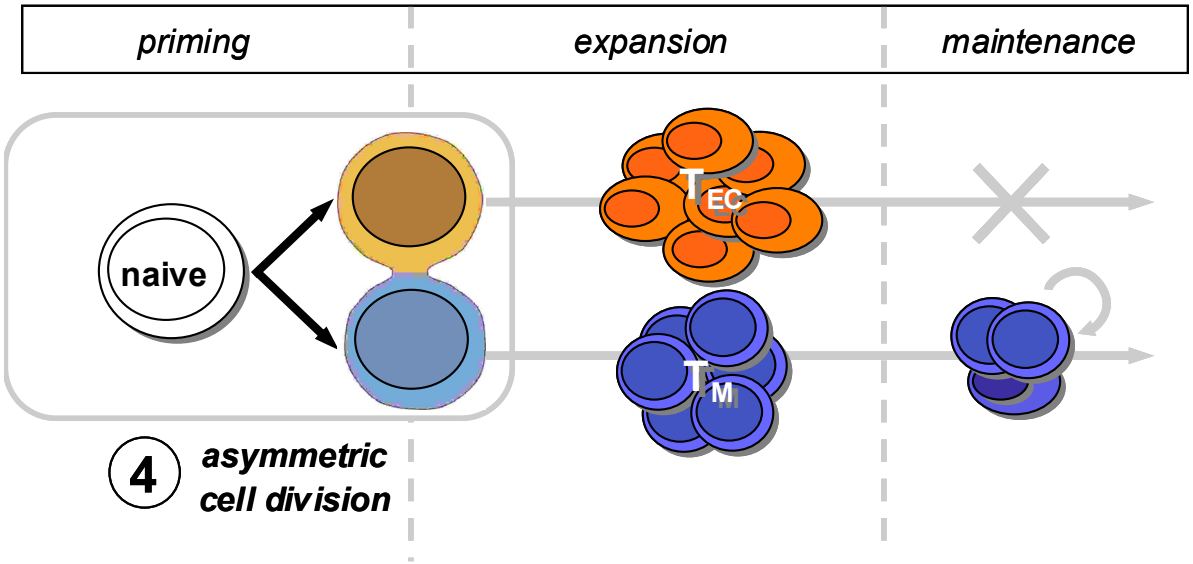

Figure 2: Naïve antigen specific T cells take a bi-directional lineage fate decision during the first cell division.

"Asymmetric cell division model": the first antigen-induced cell division of naïve T cells leads to an uneven distribution of cellular components onto the first two daughter cells. The daughter proximal to the APC-T cell interphase becomes precursor for effector cells whereas the distal daughter gives rise to memory T cells.

Unfortunately the experimental systems used in this study did not allow to analyze the T cells more closely beyond the first cell division, yet making it difficult to argue that the full spectrum of diversity is already determined by a single asymmetric cell division. In addition, it is important to consider that the duration of stimulation for most acute infections is prolonged throughout the effector response until pathogen clearance. Although it is now widely accepted that T cells acquire a differentiation program shortly after an initial "activation hit" (Bevan and Fink, 2001), the program might not be absolutely independent of extrinsic factors. As proliferation and differentiation occur in tandem, segregation into more diverse T cell subsets could follow the initial priming period, promoted for example by additional antigen contacts or recruitment into different anatomical sites that provide their own unique factors. The degree of stimulation provided by the innate immune system or the inflammatory milieu could then further contribute in shaping T cell responses continuously during the expansion phase.

Taken together, it is currently unknown at which level subset diversification really occurs and at what stage lineage fate decisions are really made. In order to obtain deeper insights into these aspects, it would be necessary to determine and track the differentiation patterns derived from single naïve precursor T cells.

5 AIM OF THIS PhD THESIS

It is well accepted that $CD8^+$ T cells play an important role in conferring effective protection during primary infections with intracellular pathogens, as well as in providing long-lasting protection against re-encounter with the same invader (immunological memory). Over the past years, also based on work from our group, it has become more and more evident that in order to fulfill these multiple tasks, distinct subsets of effector and memory T cells have to be generated. However, until today only little is known about the mechanisms underlying subset differentiation and lineage fate decisions. As the different subsets generated during an immune response also contribute differently to protective immunity and therefore have different implications for the development of vaccination strategies or T cell-based immunotherapies, one major task lies in resolving how subset diversity is generated.

In this context, it is of great interest at which clonal level - starting from a naïve T cell - functional and phenotypical heterogeneity is achieved. However, so far it has only been possible to tackle questions regarding the origin of subset diversification through analysis of indirect parameters such as TCR repertoires or transgenic reporter gene models. In addition, these experiments were often limited to global analyses of unphysiologically high amounts of transferred clonotypic T cells.

To improve our understanding of T cell subset generation on a cellular level *in vivo*, we attempted to develop a novel adoptive transfer system that allows for the first time to track the fate of a single antigen-specific naïve T cell in a normal (wild-type) recipient mouse.

Besides the technical challenges to develop necessary technical tools for the isolation and transfer of single T cells, the expected extremely low frequencies of recoverable cell populations derived from single progenitor cells required the development of novel flow-cytometric acquisition procedures. By monitoring the progeny derived from single naïve T cells at different stages during infection or other immunization regimens, further insights into the developmental properties, subset diversification and linage fate decisions should be gained.

We specifically attempted to develop an adoptive transfer system where single cell-derived T cell populations can be studied in an otherwise normal wild type environment. Further advantage of such a natural experimental setting is that the “behavior” of genetically labeled T cells can directly be compared to the endogenously developing polyclonal T cell population with the same epitope-specificity within the same individual mouse.

Ultimate goal of this study was to analyze the progeny derived from a single naïve progenitor T cell for its capability to generate functional and phenotypical diversity, with a special emphasis on the development of different long-lasting memory T cell subsets.

6 MATERIALS AND METHODS

6.1 Materials

6.1.1 *Equipment*

Equipment	Supplier
Flow cytometer	Cyan Lx, Beckman Coulter, Fullerton
	Cyan ADP, Beckman Coulter, Fullerton
	MoFlo Cell Sorter, Beckman Coulter, Fullerton
	FACSAria, Becton Dickinson, Heidelberg
	FACSCalibur, Becton Dickinson, Heidelberg
Microscopes	Axiovert S100, Carl Zeiss, Jena
	Zeiss LSM 510, confocal microscope, Carl Zeiss, Jena
	Leica SP 5, confocal microscope, Leica, Bensheim
Camera	AxioCam HSm, Carl Zeiss, Jena
Centrifuges	Biofuge fresco, Heraeus, Hanau
	Multifuge 3 SR, Heraeus, Hanau
	Sorvall® RC 26 Plus, Heraeus , Hanau
FPLC	Amersham Biosciences, Europe GmbH, Freiburg
Heating block	Thermomixer compact, Eppendorf, Hamburg
HE33 agarose gel casting system	Hoefer, San Francisco
Incubator	Cytoperm 2, Heraeus, Hanau
Laminar flow hood	HERA safe, Heraeus, Hanau
MightySmall SE245 gel casting system	Hoefer, San Francisco
Neubauer counting device	Schubert, München
Photometer	BioPhotometer, Eppendorf, Hamburg
NanoDrop spectrophotometer	NanoDrop, Baltimore
Shaker	Multitron® Version 2, INFORS AG, Bottmingen
Superdex 200HR	Amersham, München
Thermocycler	T3 Thermocycler, Biometra, Göttingen
Waterbath	LAUDA ecoline 019, Lauda-Königshofen

6.1.2 Chemicals and reagents

Reagent	Supplier
α-Methylmannopyranoside (α-MM)	Calbiochem, Darmstadt, Germany
Ammoniumchloride (NH_4Cl)	Sigma, Taufkirchen, Germany
Ampicillin	Sigma, Taufkirchen, Germany
BCA assay reagents	Interchim, Montlucon, France
β-Mercaptoethanol	Sigma, Taufkirchen, Germany
Bovine serum albumin (BSA)	Sigma, Taufkirchen, Germany
CELLection Biotin Binder kit	Dynal, Oslo, Norway
CFSE	Sigma, Taufkirchen, Germany
Chromium-51 (^{51}Cr)	Amersham, Freiburg, Germany
Collagenase VIII	Sigma, Taufkirchen, Germany
CpG 1826 (phosphorothioate modified)	Coley Pharmaceuticals, Wellesley, USA
Cytofix / Cytoperm	BD Biosciences, Heidelberg, Germany
d-Biotin	Sigma, Taufkirchen, Germany
Dimethylformamid (DMF)	Sigma, Taufkirchen, Germany
DMSO	Sigma, Taufkirchen, Germany
DNase I	Sigma, Taufkirchen, Germany
Ethanol	Klinikum rechts der Isar, Munich, Germany
Ethidium-monazide-bromide (EMA)	Molecular Probes, Leiden, The Netherland
Fetal calf serum (FCS)	Biochrom, Berlin, Germany
Gentamycin	GibcoBRL, Karlsruhe, Germany
Gluthathione (oxidized)	Sigma, Taufkirchen, Germany
Gluthathione (reduced)	Sigma, Taufkirchen, Germany
GM-CSF	Pepro Tec, Hamburg, Germany
Golgi-Plug	BD Biosciences, Heidelberg, Germany
Golgi-Stop	BD Biosciences, Heidelberg, Germany
Guanidine-HCl	Sigma, Taufkirchen, Germany
HBSS	Gibco BRL, Karlsruhe, Germany
HCl	Roth, Karlsruhe, Germany
Heparin (Liquemin® N 25000)	Roche, Basel, Swizerland
HEPES	Gibco BRL, Karlsruhe, Germany
recombinant mouse IL-15	R & D Systems, Minneapolis, USA

IPTG	Sigma, Taufkirchen, Germany
L-Arginine	Roth, Karlsruhe, Germany
L-Glutamine	Gibco BRL, Karlsruhe, Germany
Leupeptin	Sigma, Taufkirchen, Germany
Lysozyme	Sigma, Taufkirchen, Germany
Lympholite	Cedarlane, Burlington, USA
NaOH	Roth, Karlsruhe, Germany
Chicken ovalbumin	Sigma, Taufkirchen, Germany
Paraformaldehyde	Sigma, Taufkirchen, Germany
Penicillin	Roth, Karlsruhe, Germany
Pepstatin	Sigma, Taufkirchen, Germany
PermWash	BD Biosciences, Heidelberg, Germany
pET3a, pET27b expression vectors	Novagen, Darmstadt, Germany
Paraformaldehyde (PFA)	Sigma, Taufkirchen, Germany
Phosphate buffered saline (PBS)	Biochrom, Berlin, Germany
RPMI 1640	GibcoBRL, Karlsruhe, Germany
Propidiumiodide (PI)	Molecular Probes, Invitrogen, UK
Sodiumacetate	Sigma, Taufkirchen, Germany
Sodiumazide (NaN_3)	Sigma, Taufkirchen, Germany
Sodiumchloride (NaCl)	Roth, Karlsruhe, Germany
Sodium-EDTA (Na-EDTA)	Sigma, Taufkirchen, Germany
Streptomycin	Sigma, Taufkirchen, Germany
Streptactin-PE	IBA, Göttingen, Germany
Streptavidin-PE	IBA, Göttingen, Germany
Tris-hydrochloride (Tris-HCl)	Roth, Karlsruhe, Germany
Triton X-100	Biorad, Munich, Germany
Trypan Blue solution	Sigma, Taufkirchen, Germany
T-Stim culture supplement	BD Biosciences, Heidelberg, Germany

6.1.3 Buffers and Media

All buffers prepared for FACS or sterile preparations were filtered using a Stericup 0.22 µM vacuum filtering system (Millipore, Bedfore, USA). Proper pH adjustment was performed with NaOH or HCl.

All used buffers and contents are listed below:

Buffer	Composition	
Ammoniumchloride-Tris (ACT)	0.17 M	NH_4Cl
	0.3 M	Tris-HCl, pH 7.5
Collagenase VIII Buffer	95 ml	RPMI
	20,33 mg	$MgCl_2$
	14,7 mg	$CaCl_2$
	5 ml	FCS
DNase I Buffer	75 mM	NaCl
	in 50% (v/v)	Glycerol
d-biotin 10 M stock solution	244.31 g	d-biotin
	ad 100 ml H_2O, pH was brought to ≈ pH 11.0 to facilitate solution of d-biotin, then back down to pH 7.0	
FACS staining buffer, pH 7.45	1x	PBS
	0.5% (w/v)	BSA
FPLC buffer	20 mM	Tris-HCl
	50 mM	NaCl
	ad 1 L	H_2O, pH 8.0 or pH 7.3
Guanidine solution	3 M	Guanidine-HCl
	10 mM	Na-Acetate
	10 mM	Na-EDTA
	ad 100 ml	H_2O, pH4.2
HBSS	1x	HBSS
	1,3 mM	EDTA
Lysis buffer, pH 7.4	150 mM	NaCl
	50 mM	HEPES
	1 mM	EDTA
	10% (v/v)	Glycerol
	1% (v/v)	Triton-X-100
	10 mM	$Na_4P_2O_7$
	1 Tabl.	Protease inhibitors/100ml
	5 ml	Tween20
$RP10^+$ cell culture medium	1x	RPMI 1640
	10% (v/v)	FCS
	5% (v/v)	SC^+

Refolding buffer	100 mM	Tris-HCl
	400 mM	L-Arginin
	2 mM	NaEDTA
	0.5 mM	ox. Gluthathione
	5 mM	red. Gluthathione
	ad 1 L	H_2O, pH 8.0
2 x Sample Buffer (SB)	10 ml	ddH_2O
	10 µl	0.5M Tris, pH 6.8
	20 µl	10% SDS
	10 µl	Glycerol
	1.543 g	DTT
	0.1 g	Bromophenol blue
SC^+ (supplement complete, in 1 L RPMI)	1 ml	β-Mercapto-ethanol
	20 ml	Gentamycin
	23.83 g	HEPES
	4 g	L-Glutamin
	200 ml	Penicillin/Streptomycin
4x Running buffer (2 L)	24 g	Tris
	115.2 g	Glycine
	8 g	SDS
5x TBE buffer (2 L), pH 8.0	108 g	Tris Ultra
	55 g	Boric acid
	40 ml	EDTA (0.5M)
10x Tris-Glycine (1 L)	29 g	Glycine
	58 g	Tris

6.1.4 Gels

Buffer	**Composition**	
SDS-PAGE running gel 10%	7 ml	ddH_2O
	4.38 ml	1.5 M Tris, pH 8.8
	5.8 ml	30% Acryl 1% Bisacrylamide
	170 µl	10% SDS
	8.8 µl	Temed

	170 µl	10% APS
SDS-PAGE stocking gel	6.2 ml	ddH_2O
	2.5 ml	0.5M Tris, pH 6.8
	1.2 ml	30% Acryl 1% bis
	100 µl	SDS
	5 µl	Temed
	100 µl	10% APS
Agarose gel 1.1%	0.45 g	Agarose
	40 ml	1x TBE
	1 µl	Ethidium bromide

6.1.5 Antibodies

Unless otherwise declared, all antibodies are directed against mouse antigens and have been titrated for optimal dilutions.

Antibody / reagent	**Clone**	**Supplier**
CD3ε APC	145-2C11	BD Pharmingen, San Diego, USA
CD3ε PE	145-2C11	BD Pharmingen, San Diego, USA
CD4 APC	L3T4	BD Pharmingen, San Diego, USA
CD4 Pacific Blue	L3T4	BD Pharmingen, San Diego, USA
CD8α APC	5H10	Caltag, Hamburg, Germany
CD8α Alexa405	5H10	Caltag, Hamburg, Germany
CD8α FITC	5H10	Caltag, Hamburg, Germany
CD8α Pacific Blue	53-6.7	BD Pharmingen, San Diego, USA
CD8α PE	5H10	Caltag, Hamburg, Germany
CD16/32 (Fcγ-RII/III, Fc-block)	2.4G2	BD Pharmingen, San Diego, USA
CD19 FITC	1D3	BD Pharmingen, San Diego, USA
CD27 PE	LG.3A10	BD Pharmingen, San Diego, USA
CD28 biotin	37.51	BD Pharmingen, San Diego, USA
CD43 APC	1B11	Bio Legend, San Diego, USA
CD44 APC	IM7	BD Pharmingen, San Diego, USA
CD44 FITC	IM7	BD Pharmingen, San Diego, USA

CD44 PECy7	IM7	BD Pharmingen, San Diego, USA
CD45.1 APC	A20	BD Pharmingen, San Diego, USA
CD45.1 FITC	A20	BD Pharmingen, San Diego, USA
CD62L FITC	MEL-14	BD Pharmingen, San Diego, USA
CD62L PECy7	MEL-14	eBioscience, San Diego, USA
CD69 FITC	H1.2F3	BD Pharmingen, San Diego, USA
CD90.1 (Thy1.1) APC	HIS51	eBioscience, San Diego, USA
CD90.1 (Thy1.1) FITC	OX7	BD Pharmingen, San Diego, USA
CD127 APC	A7R34	eBioscience, San Diego, USA
CXCR3 APC	220803	R & D Systems, Minneapolis, USA
IFN-γ APC	XMG1.2	eBioscience, San Diego, USA
IFN-γ FITC	XMG1.2	eBioscience, San Diego, USA
TNF-α FITC	MP6-XT22	BD Pharmingen, San Diego, USA
TNF-α PECy7	MP6-XT22	BD Pharmingen, San Diego, USA
IL-2 PE	JES6-5H4	eBioscience, San Diego, USA
CD107a (LAMP1) FITC	1D4B	BD Pharmingen, San Diego, USA

6.1.6 Peptides

All peptides were purchased from Biosynthan GmbH, Berlin, Germany. Lyophilized peptides were dissolved in DMSO at a concentration of 1 µg/µl.

Following peptides were used for restimulation of ag-specific cells:

$Ova_{257-264}$: SIINFEKL

gp_{33-41}: KAVYNFATM

6.1.7 MHC multimers

Conventional MHC-I multimers for the detection of Ag-specific $CD8^+$ T cells were routinely produced in our laboratory according to well established protocols (Busch et al., 1998). Depending on the respective MHC allele, the following peptide-loaded MHC-I multimers were used:

H2-K^b #45 / mβ_2m/ $Ova_{257-264}$ Streptavidin-PE

H2-K^d #45 / mβ_2m/ gp_{33-41} Streptavidin-PE

6.1.8 MHC streptamers

Reversible MHC-I streptamers were generated as described (see 6.2.2, (Knabel et al., 2002). The following MHC-I streptamers were used:

H2-K^b StreptagIII / mβ_2m / $Ova_{257-264}$ Streptactin-PE

H2-K^d StreptagIII / mβ_2m / gp_{33-41} Streptactin-PE

6.1.9 Artificial antigen presenting cells (aAPCs)

To stimulate TCR transgenic OT-I $CD8^+$ T cells antigen-specifically, artificial APCs conjugated with peptide-loaded MHC-I molecules (signal 1) in combination with a stimulating aCD28 antibody (signal 2) were used. Artificial antigen presenting cells were generated as described under 6.2.8.

6.1.10 Mice

Mice from the inbred mouse strain C57BL/6 were purchased from Harlan-Winkelmann (Borchen, Germany). $Rag1^{-/-}$ mice (on the C57BL/6 background) were obtained from The Jackson Laboratory (Bar Harbor, USA) and bred under SOPF conditions. $Ova_{257-264}$ peptide-specific TCR transgenic C57BL/6 OT-I, C57BL/6 CD45.1 and C57BL/6 CD45.1 OT-I mice were derived from in house breeding. Thy1.1 P-14 TCR-transgenic mice, specific for gp_{33-41} from LCMV, were obtained from H.-W. Pircher (Freiburg, Germany). All Mice were bred under specific pathogen-free conditions at the mouse facility at the Technical University of Munich, and age / sex-matched mice were used at an age of 6-8 weeks for transfer- and infection experiments.

All animal experiments were approved by the local authorities.

6.1.11 Software

FlowJo	Treestar, Ashland, USA
Summit V.4.3	Dako, Fort Collins USA
FACS Diva	BD Biosciences, Heidelberg, Germany
MetaMorph Offline	Molecular Devices, Downingtown, USA
Prism V.5.0a	GraphPad Software, San Diego, USA
Sigma Plot 10.0	Systat, San Jose, USA
Microsoft Office for OS X	Microsoft, Redmond, USA

6.2 Methods

6.2.1 Preparation of cells from different organs

For FACS analysis, FACS sorting or *in vitro* cultures of T cells, organs from either naïve or infected animals were removed and cells were isolated as previously described (Pope et al., 2001).

Briefly, splenocytes and lymph node cells (pool of mesenteric, axillar, inguinal and cranial LNs) were harvested by dissociation of entire organs through nylon cell strainers (BD Bioscience, San Diego, USA). Subsequently homogenized organs were resuspended in $RP10^+$ medium.

Bone marrow was obtained from the femurs and tibiae of the two lower extremities by perfusion with $RP10^+$. Liberated cells were subsequently filtered through nylon mesh with $RP10^+$ medium.

Peripheral blood lymphocytes were obtained from blood samples taken by bleeding mice from the tail vein.

Erythrocytes of the spleen, bone marrow, lung and LNs were lysed with ACT buffer for 6 min at room temperature. Blood samples were lysed for 10 min. After lysis, cells were pelleted, washed (1500 rpm, 7 min) and resuspended in $RP10^+$ or FACS buffer for further usage.

Prior to lymphocyte isolation from livers and lungs, blood was removed by perfusion of the organs with either $RP10^+$ or heparinized PBS (75 U/ml Roche, Basel, Switzerland), respectively. After dissociation of livers (over nylon cell strainers), cell suspensions were pelleted at 300 rpm for 3 min to remove coarse tissue particles. A digestion mix containing 0.2 mg/ml collagenase type VIII (Sigma, Taufkirchen, Germany) and 0.02 mg/ml DNase I (Sigma, Taufkirchen, Germany) was used to liberate lymphocytes from hepatic tissue (30 min, 37°C under constant

motion). After washing with PBS, liver lymphocytes were separated over a Lympholyte density gradient (2500 rpm, 20 min, 4°C Cedarlane, Burlington, USA). After washing, cells were resuspended in $RP10^+$ or FACS buffer.

After perfusion with heparinized PBS, lungs were cut into small pieces and incubated for 30 min in HBSS/1.3 mM EDTA (Gibco, Karlsruhe, Germany) at 4°C. After washing with $RP10^+$ lungs were digested for 30 min at 37°C using 0.3 mg/ml collagenase type VIII (Sigma) in $RP10^+$. Lungs were then washed with $RP10^+$ and cells were dissociated through a nylon cell strainer followed by filtration, erytrothyte lysis and washing as mentioned above.

Prior to use, cells were filtered again through nylon mesh to remove remaining tissue aggregates.

Numbers of cells were determined by counting appropriate dilutions in a Neubauer counting chamber. Live/dead discrimination was performed using Trypan Blue staining.

6.2.2 Production of MHC-I multimers / streptamers

All MHC class I molecules employed for the generation of multimers/streptamers used in this study were expressed in *E. coli*, purified, refolded *in vitro* and conjugated with fluorescent dyes.

6.2.2.1 Protein production

Expression vectors (pET3a, pET27b; Novagen, Darmstadt, Germany) encoding the murine MHC class I heavy chains H2-K^b, H2-K^d, all lacking the transmembrane region but having a C-terminal biotinylation or streptagIII sequence, as well as a vector encoding the mβ_2m, were available in the lab.

Expression of proteins after transformation of the vectors in *E. coli* BL21 (DE3) CP (Novagen, Darmstadt, Germany) was done in 6 L cultures (LB, 0.4% Glucose, Carbenicillin 100 µg/ml). Expression was induced by addition of 0.4 mM IPTG at an OD_{600} of 0.7 for 3 h. Purification of the recombinantly expressed insoluble protein inclusion bodies was done by enzymatic and mechanic lysis (Lysozym, ultrasound, Triton X-100, freeze-thawing). The proteins were then solved in 8 M urea.

6.2.2.2 Refolding and fluorescence conjugation of MHC class I molecules

Heavy chain and mβ_2m proteins in 8 M urea were diluted into refolding buffer containing high concentrations of the respective synthetic peptide epitope (60 µg/ml).

Aliquots of the proteins were first diluted in 3 M guanidine buffer, injected directly into 200 ml refolding buffer every 8 h while vortexing heavily and incubated under constant agitation for 48 h at 10°C. The refolding buffer contained a glutathione redox system to facilitate optimal formation of disulfide-bridges. After 48 h, the protein solution was concentrated to a volume of 10-20 ml over a 10 kDa membrane (Millipore, Eschborn, Germany), and then further reduced to 1 ml using 10 kDa concentrator columns (Millipore, Eschborn, Germany). The flow through of the first concentration step still contained large amounts of peptide and could be used for a second refolding.

Correctly folded MHC-I molecules were purified by gel filtration (Superdex 200HR, Amersham, Munich, Germany) over a FPLC system (FPLC basic, Amersham, Munich, Germany); pooled and incubated over night in a buffer containing NaN_3, protease inhibitors (1 mM NaEDTA, Leupeptin, Pepstatin) and 0,1 mM DTT. The next day, the buffer was exchanged against FPLC buffer and the protein concentration determined by a standard BCA-assay.

6.2.2.3 Multimerization

For each staining with streptamers (up to 5 x 10^6 cells), 2 µg MHC-I/peptide and 5 µl (= 5 µg) streptactin-PE was multimerized in a final volume of 50 µl FACS buffer and incubated over night or for at least one h in the dark and cold. For conventional MHC-I multimers 0.4 µg MHC-I/peptide was incubated with 1 µl streptavidin-PE, filled up to 50 µl and incubated over night in the dark at 4°C.

6.2.3 Antibody and multimer staining

For antibody and MHC multimer staining, about 5 x 10^6 cells were used per sample. For rare event analysis, at least 5 x 10^7 cells were used and the amount of staining volumes and reagents was scaled up respectively. Staining was usually performed in 96 well plates. To block FcγII/III receptors and for live/dead discrimination, cells were first incubated for 20 min under

light in 100 µl of Fc block (1:400) and EMA (1:2000) solution. Alternatively, live dead discrimination was performed by mixing the cells with propidium iodide (end concentration 1: 500 in FACS buffer) at the end of the staining procedure.

Cells were then washed once with FACS buffer in a total volume of 200 µl, pelleted for 2 min at 460 x g, and resuspended in 50 µl MHC multimer or streptamer solution (see 6.2.2). Cells were then incubated on ice in the dark for 45 min. Antibodies at the appropriate dilution were added for the last 20 min of the staining procedure. If only antibodies were used, cells were stained for 20 min as mentioned above. Finally, samples were washed three times in FACS buffer and optionally fixed in 1% PFA and stored at 4°C in the dark until further analysis.

6.2.4 CFSE staining

For CFSE proliferation assays, murine splenocytes were washed twice with warm PBS and 5 x 10^7 cells were labeled with CFDA-SE (Sigma, Taufkirchen, Germany) at a concentration of 5 µM for 10 min at 37°C in a large volume (usually 5 ml). Staining was stopped by adding warm $RP10^+$ and further incubation for 5 min at 37°C. Cells were then washed twice with $RP10^+$ and counted again.

To stimulate splenocytes *in vitro*, 1 x 10^6 CFSE-labeled or unlabeled cells were cultured in 1 ml $RP10^+$ in 48 well plates. Naive transgenic OT-I $CD8^+$ T cells were stimulated with artificial APCs loaded with 0,05 µg MHC-I (H2-K^b / $Ova_{257-264}$) and 0,5 µg αCD28 / 10^7 beads. After three to four days of culture (usually 72-96 h), cells were stained and analyzed as described under 6.2.3 and 6.2.6. For *in vivo* proliferation after adoptive transfer, naïve T cells were stimulated by infection with *Listeria monocytogenes*. For assessment of homeostatic proliferation, mice were left untreated after adoptive transfer

6.2.5 Intracellular cytokine staining and degranulation assay

To measure the production of T cell-derived cytokines like IFN-γ, TNF-α or IL-2, T cells were (re-) stimulated *in vitro* with their cognate antigen. For each stimulation, at least 2 x 10^7 cells (splenocytes, LN, liver- or lung cells) were stimulated in the presence of 10^{-6} M of the respective peptide (OT-I cells: $Ova_{257-264}$; P14 cells: gp_{33-41}) or pure DMSO in 48 well plates at 37°C. After 2 h of incubation, Brefeldin-A (Golgi-Plug, BD Biosciences) was added at a concentration of 2 µg/ml, to inhibit the export of intracellular cytokines from the Golgi apparatus leading to intracellular accumulation. After three additional hours of incubation cells were spun down and

stained in 50 µl Fc-block/EMA mix (Fc-block 1:500, stock at 2.5 µg/µl, EMA 1:2000, stock at 2 µg/µl, 20 min under light on ice). The cells were washed once in FACS buffer in the dark, and subsequently stained in 50 µl FACS buffer for 20 min on ice against surface expressed markers. After washing, cells were fixed and permeabilized in 100 µl Cytofix/Cytoperm (BD Biosciences, San Diego, USA) for 20 min (dark, on ice), then washed twice in Perm/Wash buffer (BD Biosciences, San Diego, USA), and finally stained with the respective antibodies against intracellular cytokines (in 50 µl Perm/Wash buffer for 30 min, in the dark, on ice). After three final washing steps in Perm/Wash (2x) and FACS buffer, cells were fixed in 1% PFA and stored in the dark at 4°C until FACS analysis.

Degranulation of cells was measured using a modified intracellular staining protocol against CD107a (LAMP1, 1:500). In brief, cells were (re-) stimulated as mentioned above. In addition anti-CD107a antibody was added at the beginning of restimulation. Endosomal acidification was further blocked from the beginning of restimulation by adding 1 µl/ml Monensin (Golgi Stop, BD Biosciences, San Diego, USA). Staining for live/dead discrimination, surface antigens and intracellular cytokines as well as data acquisition and analysis were performed as mentioned above.

6.2.6 FACS acquisition and analysis

For FACS analysis, at least 1 x 10^5 cells of the populations of interest were acquired on a CyAn LX or ADP flow cytometer (Dako, Fort Collins USA). For rare event analysis (like tracking the fate of single cells), Summit V4.3 was used to generate files that contained at least 1 x 10^7 events. Data analysis was performed using FlowJo software (Treestar, Ashland, USA).

6.2.7 FACS sorting

FACS sorting was used to highly purify distinct cell populations. Freshly prepared cells from various organs were stained in FACS buffer at a concentration of 1 x 10^8 / 1000 µl. For large cell numbers, cells were incubated in 15 ml tubes in the dark with the respective antibodies at 4°C under permanent rotation in the cold room. For smaller cell numbers, cells were stained as described above (see 6.2.6). Staining was followed by three washing steps (7 or 2 min, 1500 rpm), and finally cells were resuspended at 2 x 10^8 cells per ml. Just before sorting on a MoFlo (Dako, Fort Collins, USA) or FACSAria cytometer (BD Biosciences, San Diego, USA), cells

were incubated with propidium iodide to allow live/dead discrimination. Fluorescence-positive and -negative cells were sorted directly into cooled FCS. When using reversible MHC-I streptamers, cells were sorted directly into FCS containing 1 mM d-Biotin to allow immediate detachment of bound streptamers. Purity of sorted subsets was confirmed by flow cytometry.

6.2.8 Stimulation of naïve T cells with aAPCs

For the Ag-specific stimulation of TCR transgenic T cells *in vitro*, artificial antigen presenting cells were used. With the help covalently surface immobilized streptavidin these uniform paramagnetic polysterene beads could be easily loaded with biotinylated MHC-I/peptide and stimulatory antibodies.

For generation of aAPCs, 12.5 µl of the CELLection stock (Dynal, Oslo, Norway) was used, that translated to about 5 x 10^6 particles. Prior to loading with MHC-I/peptide or antibodies, beads were washed twice with PBS, pH 7.5 with the help of strong magnets. aAPCs were then loaded stepwise with 0.05 µg of biotinylated H2-K^b / $Ova_{257-264}$ and 0.5 µg of biotinylated αCD28-ab for 30 min at room temperature and under permanent motion. To remove unbound molecules, aAPCs were washed twice and finally resuspended in 100 µl PBS.

All amounts of MHC-I/peptide or antibodies were carefully titrated to sub–saturating concentration. For *in vitro* stimulation, 4 – 6 x 10^5 naïve T cells were seeded in a volume of 1 ml in 48 well plates and stimulated with aAPCs at a ratio of about five beads per one cell. In addition 5% T-Stim / α-MM (BD Biosciences, San Diego, USA) or 150 ng/ml IL-15 (R&D Systems, Minneapolis, USA) was added to the medium.

6.2.9 Listeria monocytogenes infection and immunization

6.2.9.1 Infection

Infection experiments were performed by intravenous (i.v.) injection of recombinant ovalbumin-expressing *Listeria monocytogenes* (*L.m.*-Ova, kindly provided by H. Shen, Philadelphia, PA) (Pope et al., 2001). For primary infection with *L.m.*-Ova, mice were injected i.v. with 0.1 x LD_{50} (5000 bacteria). Alternatively, *L.m.* expressing gp33 from LCMV (*L.m.*-gp33, kindly provided by A. Diefenbach, Freiburg, Germany) was used for experiments using Thy1.1 P14 TCR-tg T cells.

Doses were as mentioned above. Rechallenge experiments were performed after 5 weeks with a second injection of *L.m.*-Ova / -gp33 with 5 x LD_{50}.

For growing bacteria, 20 µl glycerol stock of bacteria was added to 5 ml BHI medium. Bacteria were cultured at 37°C at gentle shaking (90 rpm) until bacteria entered the exponential growth phase as determined by OD measurement (OD_{600} ≈ 0.05). The amount of bacteria was calculated using standard curves. The exact infection dose was further controlled by plating out dilutions of the *L.m.* suspension used (on BHI plates, incubation over night, counting CFU and calculating the original bacteria concentration).

6.2.9.2 Immunization and vaccination

Subcutaneous immunizations were performed by application of 100 µl physiological sodium chloride solution containing 200 µg chicken ovalbumin (Ova, Sigma, Taufkirchen, Germany) and 10 nM phosphoro-thioate-modified CpG-DNA oligonucleotide (1826, 5´-TCCATGACGTTCCTGACGTT-3´, Coley, Wellesley, USA) at the base of the tail immediately after cell transfer.

Alternatively, i.v. vaccinations were performed by injecting 200 µl physiological sodium chloride solution containing 1 x 10^8 IU of an ovalbumin expressing strain of the viral vector MVA (modified vaccinia virus Ankara, MVA-Ova was kindly provided by I. Drexler, Munich, Germany) into the tail vein. Vaccination was performed immediately after cell transfer. For protection experiments, transferred cells were further expanded by an i.v. boost immunization with 1 x 10^8 MVA-Ova 14 days after initial vaccination.

6.2.10 Adoptive cell transfers

6.2.10.1 Transfer of large cell numbers

T cells for adoptive transfer after cell culture were counted using a Neubauer counting chamber, excluding dead cells by trypan blue staining. The indicated amount of T cells was adjusted to 200 µl injection volume and injected into the tail vein.

For titration experiments using adoptive transfer of enriched T cells, C57BL/6 mice ($CD45.2^+$) received varying numbers (10-10.000) of naïve $CD45.1^+$ OT-I $CD8^+$ T cells either i.v. or by i.p. application. The given cell numbers were adjusted as mentioned above, and the true cell count

was confirmed by recounting aliquots of injection solutions (accuracy +/- 10%). Immediately after transfer, recipient mice were infected with *L.m.-Ova*.

6.2.10.2 Single cell transfer

For transferring single cells, FACS-enriched naïve ($CD44^{lo}$) $CD45.1^{+}$ OT-I $CD8^{+}$ T cells were diluted to an approximate concentration of 20 cells per 20 µl in pure FCS, and a 20 µl drop was applied onto a glass slide. Under microscopic control (Axiovert S100, Zeiss), a single cell was aspirated into the tip of a hand-drawn glass microinjection needle, and the presence of only a single cell was further confirmed by extensive microscopy of the needle content. With the same needle, the cell was immediately transferred into the peritoneal cavity of a recipient mouse by flushing the needle with 200 µl of a physiological sodium chloride solution. Transferred cells were analyzed after either 7 or 12 days or 5 weeks after primary infection or 5 days after recall infection.

6.2.11 Measurement of bacterial load

As readout for protective capacity of transferred T cells, numbers of live bacteria were determined in the spleens and livers of infected animals.

The organs were harvested three days after challenge with 2 x 10^5 bacteria, homogenized through steel-grids and resuspended in 5 ml sterile PBS. 100 µl of the cell suspensions were diluted 1:10, 1:100 and 1:1000 in 0.1% Triton X-100 to release intracellular *Listeria monocytogenes* from the cells. Aliquots of 10 µl per respective dilution were plated out in triplicates on BHI plates and incubated over night at 37°C. CFU were counted on the following day, and the amounts of *L.m.* per organ were calculated according to the respective dilutions. Using this approach, the living bacteria could be enumerated with a detection limit of approximately 5.000 *Listeria* per organ.

6.2.12 Microscopy

All adoptive transfers of single T cells were done using a Zeiss AxioVert S100 microscope.

All microscopic images and movies were taken on a Zeiss LSM 510 or a Leica SP5 confocal laser scanning microscope. The microscope was equipped with a Zeiss HSm high-speed camera and a climate box for keeping constant temperatures. For confocal images, $CD8^+$ T cells were stained with anti-CD8α-ab as described under 6.2.6.

Images were analyzed using either MetaMorph image analysis software (Molecular Devices, Downingtown, USA) or AxioVison Software (Zeiss, Jena, Germany).

6.2.13 Statistics

All statistics were done with Sigma Plot 10.0 software (Systat, San Jose, USA) or Prism (GraphPad Software, La Jolla, USA) using students t tests or ANOVA tests.

7 RESULTS

7.1 Development of an adoptive transfer system for single cells

Primary T cell responses to infectious agents are often characterized by the induction of robust expansion of antigen-reactive T cell populations, although the number of precursor cells is thought to be very low, ranging between 50-200 cells. Therefore, we hypothesized that supplementation of the precursor repertoire with only a few naïve T cells of a defined specificity should be sufficient to subsequently detect expanded daughter cells during the expansion (effector) phase. In order to verify this assumption, we first chose to perform titration experiments with genetically labeled CD45.1$^+$ OT-I cells. These TCR-transgenic cells specifically recognize the immunodominant $Ova_{257-264}$ peptide derived from chicken ovalbumin presented by H2-K^b. Decreasing numbers of approximately 10.000, 1.000, 100 and 10 FACS-sorted, naïve (CD44^{low}) cells were adoptively transferred by intravenous (i.v.) injection into naïve congenic CD45.2$^+$ C57/BL6 mice, followed by subsequent infection of recipient mice with 5.000 (0.1 x LD_{50}) ovalbumin-expressing *Listeria monocytogenes (L.m.*-Ova*)*. Seven days after transfer and infection, splenocytes were analyzed by flow cytometry. We found that expanded CD45.1$^+$ OT-I T cells were readily detectable in all recipient mice, even including the “10 cell group” (Fig. 3, upper row).

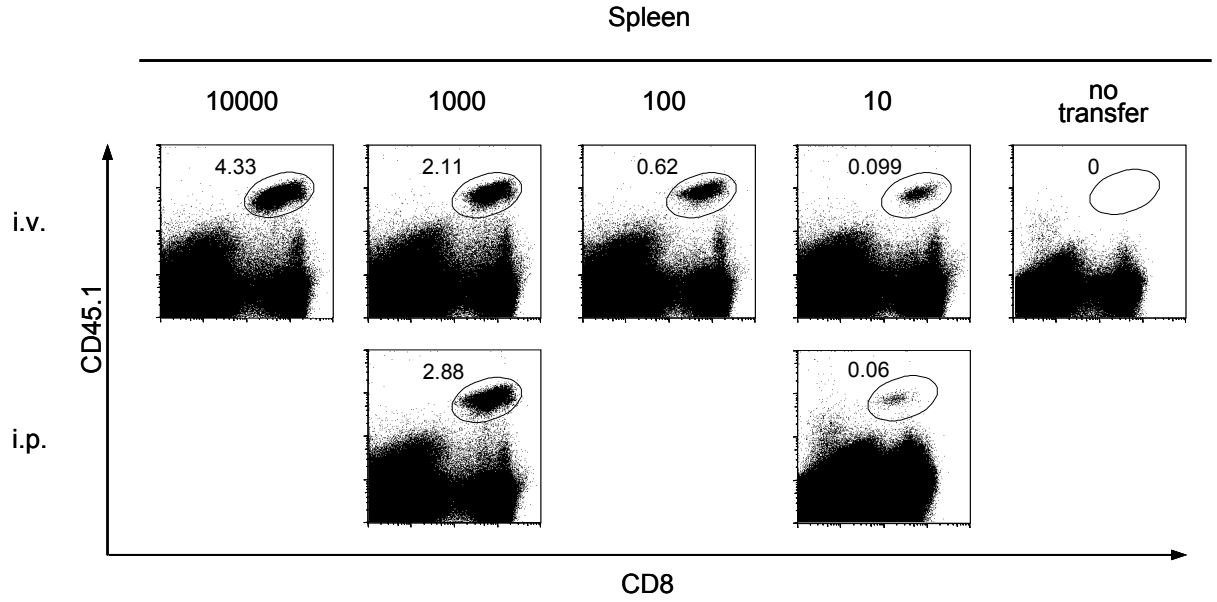

Figure 3: Adoptive transfer of single T cells.

C57BL/6 mice (CD45.2$^+$) received varying numbers (10-10.000) of highly FACS-enriched (selected for propidium iodide negative (live)/CD3$^+$/CD8$^+$/CD44^{low}) CD45.1$^+$ OT-I CD8$^+$ T cells by intravenous (i.v.) injection. Alternatively, some mice received cells by intraperitoneal (i.p.) application (lower row). The given cell numbers were adjusted to 200 µl injection volume, and

the true cell content was confirmed by recounting aliquots of injection solutions (accuracy +/– 10%). Immediately after transfer, recipient mice were infected with *L.m.*-Ova, and 7 days later splenocytes were analyzed by flow cytometry (acquisition of 1 x 10^7 events). Representative dot pots of splenocytes (gated on live cells by exclusion of propidium iodide-positive events) stained for CD8 and CD45.1 for each group are shown. Percentages among all splenocytes are indicated for the shown gates. Mice without adoptive T cell transfer served as a negative control.

To simplify the application route for T cell transfer, we additionally tested the engraftment efficiency of donor T cells after adoptive transfer into the peritoneal cavity. Interestingly, intraperitoneal (i.p.) application of naïve T cells showed very similar transfer efficiency compared to i.v. application (Fig. 3, lower row). Further kinetic studies demonstrated that naïve T cells applied via the intraperitoneal route migrate within a few hours into lymphoid organs including the spleen. Migration into the spleen is suggested to be important for the subsequent *Listeria* infection experiments, as it is generally believed that the majority of T cell priming occurs in this particular organ (Fig. 4) (Neuenhahn et al., 2006; Wong and Pamer, 2003). To exclude that i.p injected OT-I cells undergo homeostatic proliferation during the small time window needed for migration into the spleen, which could potentially result in pre-activation and some phenotypical changes, we investigated cell division patterns by analysis of CFSE dilution. These experiments showed that 48h after adoptive transfer in the absence of infection none of the transferred OT-I cells had undergone homeostatic proliferation while migrating into the spleen (Fig. 4 B). As (homeostatic) proliferation may also result in changes of the "naïve phenotype", we further assessed surface expression of CD62L and CD127. We could not detect a change in the expression of these markers. Indeed all cells appeared to be $CD62L^+CD127^+$, indicating that the cells stably maintained their naïve phenotype (Fig. 4 A).

Taken together, the above summarized data indicate that the T cell repertoire of normal wildtype recipient mice can be supplemented with very low numbers of naïve TCR-tg T cells (as low as 10 cells) and that this procedure allows to recover the progeny of very few TCR-tg T cells during the immune response to infection with *Listeria*.

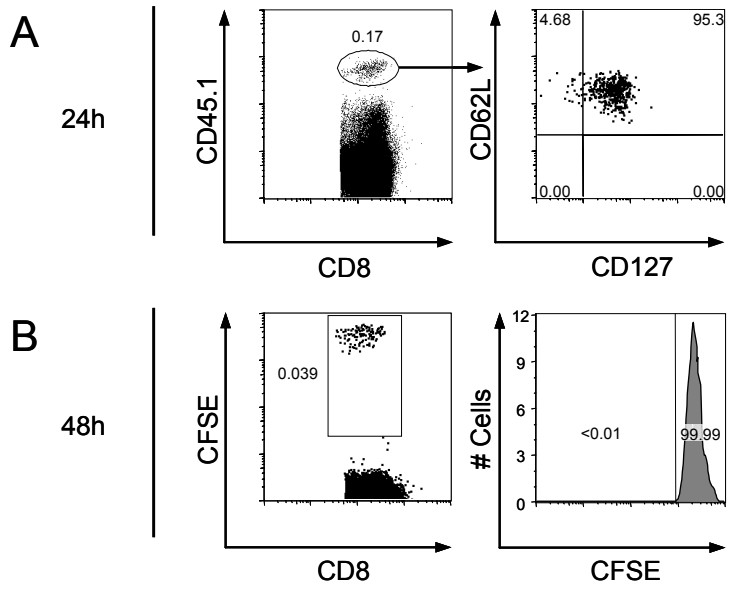

Figure 4: Rapid migration to the spleen and lack of homeostatic proliferation early after intraperitoneal injection of OT-I cells.

1 x 10^6 naïve CFSE labeled or unlabeled CD45.1$^+$ OT-I cells were transferred into C57/BL6 mice by intraperitoneal injection. **(A)** 24 hours later, transferred cells were readily detectable among splenocytes by staining for CD45.1 and CD8 (left; CD8$^+$ gated cells are shown). CD45.1$^+$CD8$^+$ cells were additionally analyzed for their surface expression of CD62L and CD127 (right). **(B)** 48 hours after adoptive transfer, CFSE dilution of transferred CD45.1$^+$CD8$^+$ cells was analyzed in the spleen (parental gates are shown). Left histogram depicts CFSE dilution of transferred cells in the spleen. Right dot plot represents parental gate on CD8$^+$ cells.

These data motivated us to perform similar experiments by transferring only a single CD8$^+$ OT-I T cell. We developed an injection system in which a single naive OT-I T cell is aspirated under microscopic control into a glass injection needle, from which it is subsequently flushed into the peritoneal cavity. First, naïve (CD3$^+$CD8$^+$CD44^{low}PI^{neg}) CD45.1$^+$ OT-I T cells were highly enriched from blood samples or splenocytes by FACS sorting. Usually, obtained purities were above 99% as confirmed by FACS (re-) analysis (Fig. 5, right dot plot). Subsequently, enriched cells were diluted to an approximate concentration of 20 cells per 20 µl, and a 20 µl drop was applied onto a glass slide. Under microscopic control a single cell was aspirated into the tip of a glass microinjection needle, and the presence of only a single cell was further confirmed by extensive microcopy of the needle content. With the same needle, the cell was immediately transferred into the peritoneal cavity of a recipient mouse by flushing the needle with 200 µl physiological sodium chloride solution finally followed by infection with *L.m.*-Ova.

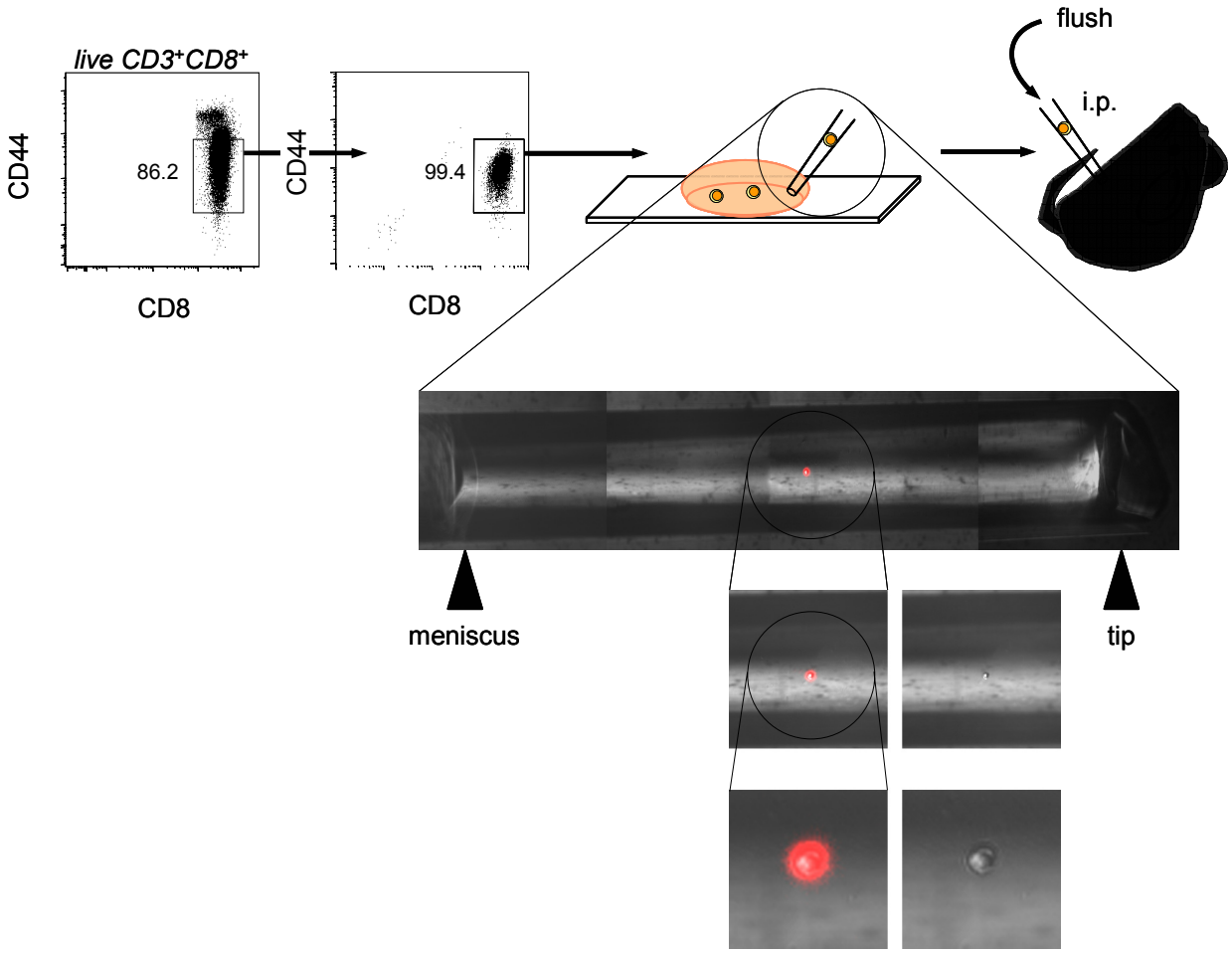

Figure 5: Principles of the single cell microinjection method.

Splenocytes from naïve OT-I TCR-transgenic mice were enriched for naïve $CD8^+$ T cells by flow cytometry-based cell sorting. Only PI negative (live)/$CD3^+$/$CD8^+$/$CD44^{low}$ cells were selected (purity >99%, right dot plot). The exact sort gate is indicated (left dot plot). Subsequently, enriched cells were diluted to an approximate concentration of 20 cells per 20 µl, and a 20 µl drop was applied onto a glass slide. Under microscopic control (Axiovert S100, Zeiss), a single cell was aspirated into the tip of a glass microinjection needle, and the presence of only a single cell was further confirmed by extensive microcopy of the needle content. An example is shown as a digital overlay picture; the cell was stained with anti-CD8-PE, and PE emission is shown in red. The area of location of the single cell is further amplified. Both fluorescence and white light images are shown. With the same needle, the cell was immediately transferred into the peritoneal cavity of a recipient mouse by flushing the needle with 200 µl physiological sodium chloride solution.

The precision of this novel method was further confirmed by microscopically monitoring the time course of single cell aspiration. To do this, we controlled selection and aspiration of a single cell with the help of high-speed microscopic video imaging and compared it to the behavior of cells lying in close proximity to the picked one.

As depicted in Figure 6, usage of fine injection capillaries and appropriate dilutions allowed precise manipulation and aspiration of only a single cell without contamination of adjacent cells.

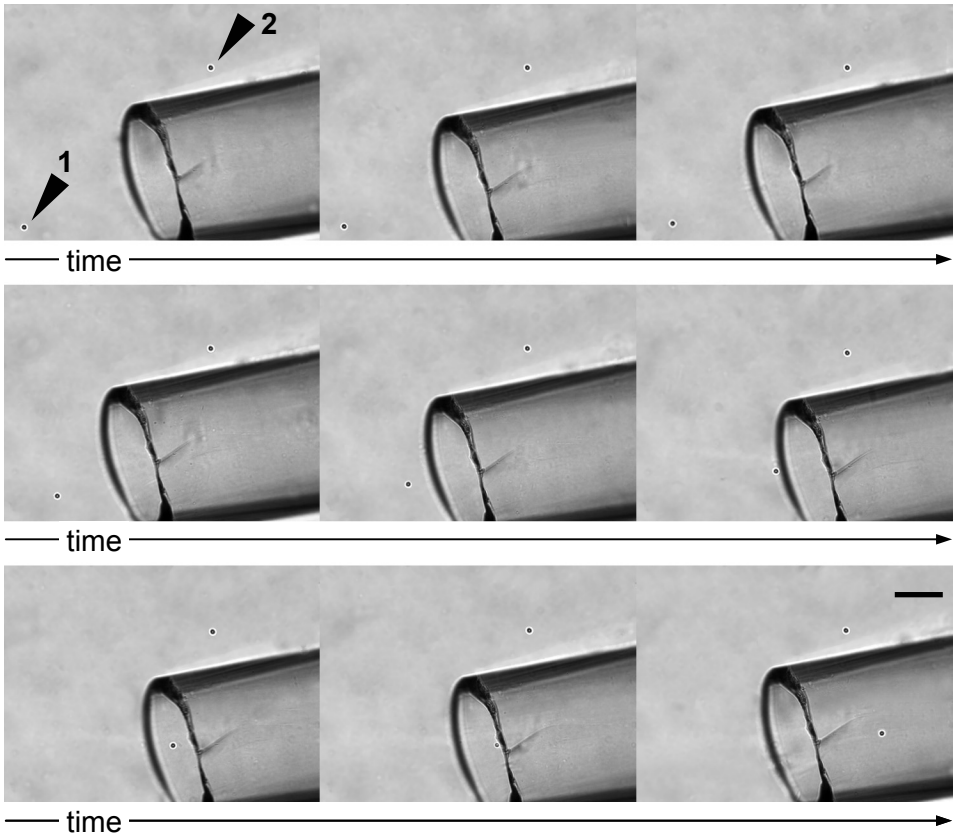

Figure 6: Precision of single cell aspiration.

In order to determine the precision of the single cell aspiration technique, selection of a single cell was monitored via microscopic video imaging. Approximately 20 cells per 20 µl of FACS enriched PI negative (live)/CD3$^+$/CD8$^+$/CD44^{low} CD45.1$^+$ OT-I cells were applied onto a glass slide. A single cell was subsequently selected, monitored by high speed imaging via an Axiovison HSm camera with 50 images per second. Selected pictures display the chronological sequence over time (one picture for every 0.75 seconds). The aspirated cell (1) is marked with a circle; a neighboring resting cell is marked with (2). The scale in lower right picture represents 100 µm.

7.2 Diverse effector T cell populations arise from single naïve precursor cells

By using this technique, we were able to recover in average 15-20% of recipient mice (149 mice transferred, 22 mice found to be positive for transferred cells) an expanded CD45.1$^+$ OT-I population on day 7 (Fig. 7, see below) or day 12 upon immunization (Fig. 8, see below), which could be subjected to further analysis. These data demonstrate for the first time that it is possible to transfer a single naïve T cell and experimentally track the progeny of this cell upon clonal expansion *in vivo*.

As shown in Figure 7, single cell-expanded populations after primary *Listeria* monocytogenes infection demonstrated a broad spectrum of heterogeneity indicated by scattered distribution of surface markers as well as migration to different organs (spleen, lymph nodes, lung and liver).

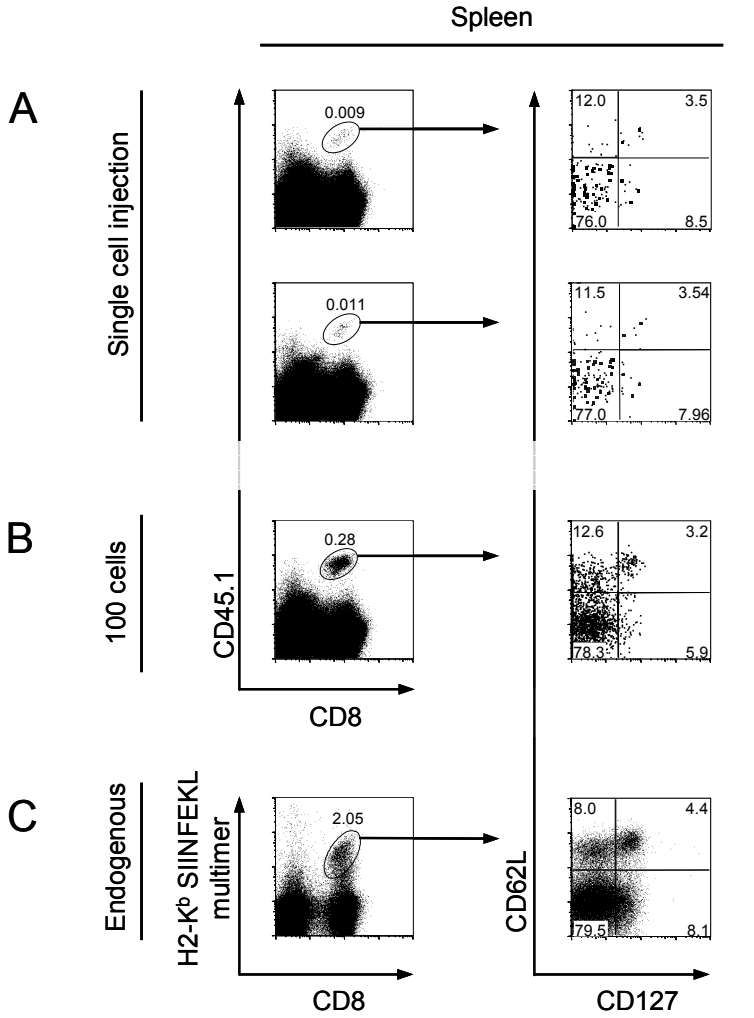

Figure 7: Phenotypical diversity of T cells expanded from a single naïve precursor cell during the effector phase (day 7).

C57BL/6 mice (CD45.2$^+$) received **(A)** a single naïve CD45.1$^+$/CD8$^+$ OT-I T cell (two representative mice, upper two rows) by intraperitoneal microinjection or **(B)** 100 naive OT-I cells intravenously. Immediately after transfer, recipient mice were infected with *L.m.*-Ova (0.1 x LD_{50}), and 7 days later T cells were analyzed in the spleen by flow cytometry. Each row summarizes data from an individual mouse. Dot plots to the left show the identification of transferred OT-I cells in the spleen by staining for CD45.1 and CD8. Percentages among total splenocytes are indicated for the shown gates. Dot plots in the middle and right show CD62L and CD127 expression patterns of *in vivo*-expanded CD45.1$^+$ OT-I cells in the spleen (parental gate is shown). **(C)** Analyses of endogenous SIINFEKL-specific T cells in the spleen identified by MHC multimer (Streptamer) staining 7 days after infection with *L.m.*-Ova (0.1 x LD_{50}). Identification of the cell populations is indicated in the left dot plot (gated on CD45.1^{negative} cells).

A substantial part of single cell-derived subpopulations seven days after primary *Listeria* infection belonged to $CD62L^{low}CD127^{low}$ T_{EC}, which are typically the dominant cell population at the peak of the effector phase.

As staining for CD62L and CD127 surface expression starts to become most reliable around 10-12 days post *L.m.* infection (Huster et al., 2004), analysis of single cell progeny was analyzed in more detail at this slightly later time point. These experiments confirmed the above described segregation of single cell-derived progeny into phenotypically distinct subsets. This could for example be demonstrated by a prominent increase in cells expressing CD127 and/or CD62L (Fig. 8 A).

As experiments were placed in an otherwise normal wildtype mouse, we were also able to compare subset diversification of transferred and endogenous Ag-specific T cells in the same individual mice. The endogenous SIINFEKL-specific cell population was visualized by MHC multimer staining and analyzed for surface expression of various molecules parallel to the single cell-derived ($CD45.1^{positive}$) population. To our surprise, the endogenous SIINFEKL-specific T cell populations showed a very similar – if not identical – phenotypical differentiation pattern as compared to single cell-derived population in the same mouse (Fig. 8 C). Also when the number of adoptively transferred T cells was slightly increased to higher numbers (100 cells) of naïve precursor cells (Fig. 8 B), the phenotypical differentiation patterns compared to natural polyclonal SIINFEKL-reactive $CD8^+$ T cell populations still remained very similar.

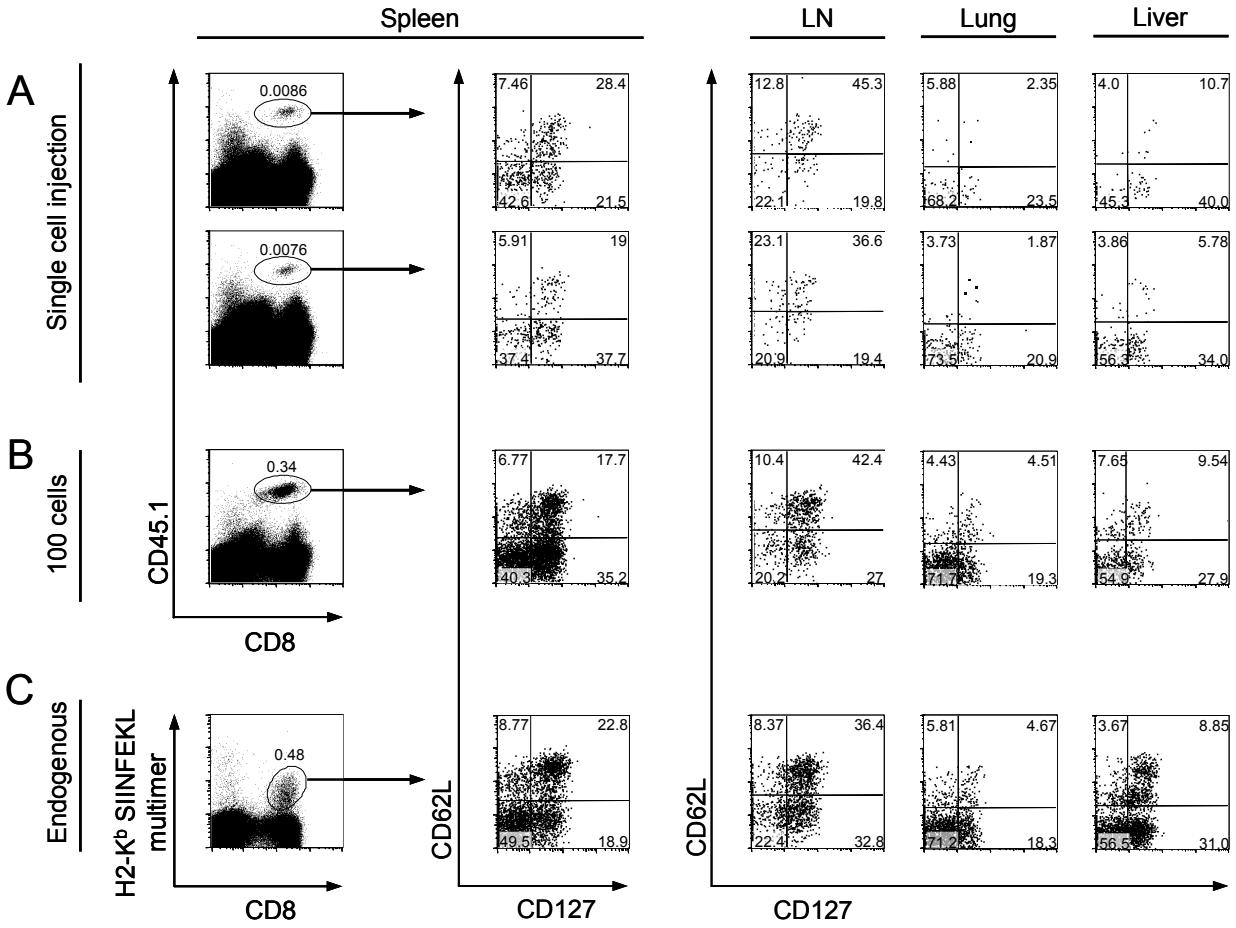

Figure 8: Phenotypical diversity of T cells expanded from a single naïve precursor cell during the effector phase (day 12).

C57BL/6 mice (CD45.2$^+$) received **(A)** a single naïve CD45.1$^+$ CD8$^+$ OT-I T cell (two representative mice, upper two rows) by intraperitoneal microinjection or **(B)** 100 naive OT-I cells intravenously. Immediately after transfer, recipient mice were infected with *L.m.*-Ova (0.1 x LD_{50}), and 12 days later T cells were analyzed in the spleen and lymph nodes (LN) by flow cytometry. Each row summarizes data from an individual mouse. Dot plots to the left show the identification of transferred OT-I cells in the spleen by staining for CD45.1 and CD8. Percentages among total splenocytes are indicated for the shown gates. Dot plots in the middle and right show CD62L and CD127 expression patterns of *in vivo*-expanded CD45.1$^+$ OT-I cells in the spleen and LN, respectively (parental gate is shown). **(C)** Analyses of endogenous SIINFEKL-specific T cells in spleen and LN identified by MHC multimer (Streptamer) staining 12 days after infection with *L.m.*-Ova (0.1 x LD_{50}). Identification of the cell populations is indicated in the left dot plot (gated on CD45.1^{negative} cells). Within each quadrant percentages for defined subpopulations referring to the indicated parental gates are shown.

The observed phenotypical similarities between endogenous and single cell-derived populations indicate that naïve OT-I T cells reliably reflect differentiation capabilities of normal (non-TCR-tg) naïve T cells.

Next we decided to analyze whether single cell-derived daughter cells also functionally diversify. Indeed, in addition to the phenotypical heterogeneity discussed above, intracellular staining for INF-γ, TNF-α and IL-2 revealed the presence of subsets with variable functional properties (Fig. 9). Further insight into the repertoire of effector functions alongside with heterogeneity was obtained by degranulation assays (by measuring surface exposed CD107a, (Betts et al., 2003) as well as analysis of cytokine coexpression from different subsets. Single cell-derived daughter cells are characterized by variable amounts or combinations of INF-γ, IL-2 or TNF-α and a distinct subpopulation showed the potential to expose CD107a on the cell surface (Fig. 9 E), confirming the presence of polyfunctional subsets with variable effector functions (Fig. 9 B).

Like for phenotypical surface markers, also the patterns in effector function of single-cell derived populations were almost identical when compared to endogenous epitope-reactive T cells (Fig. 9 B and D) or when increasing the precursor frequency to about 100 cells (Fig. 9 C). In summary, our data demonstrate for the first time that phenotypically and functionally heterogeneous subpopulations can arise from a single naïve precursor cell; furthermore, the patterns of diversification into distinct subsets appeared to mirror the diversification of endogenous epitope-specific T cell populations.

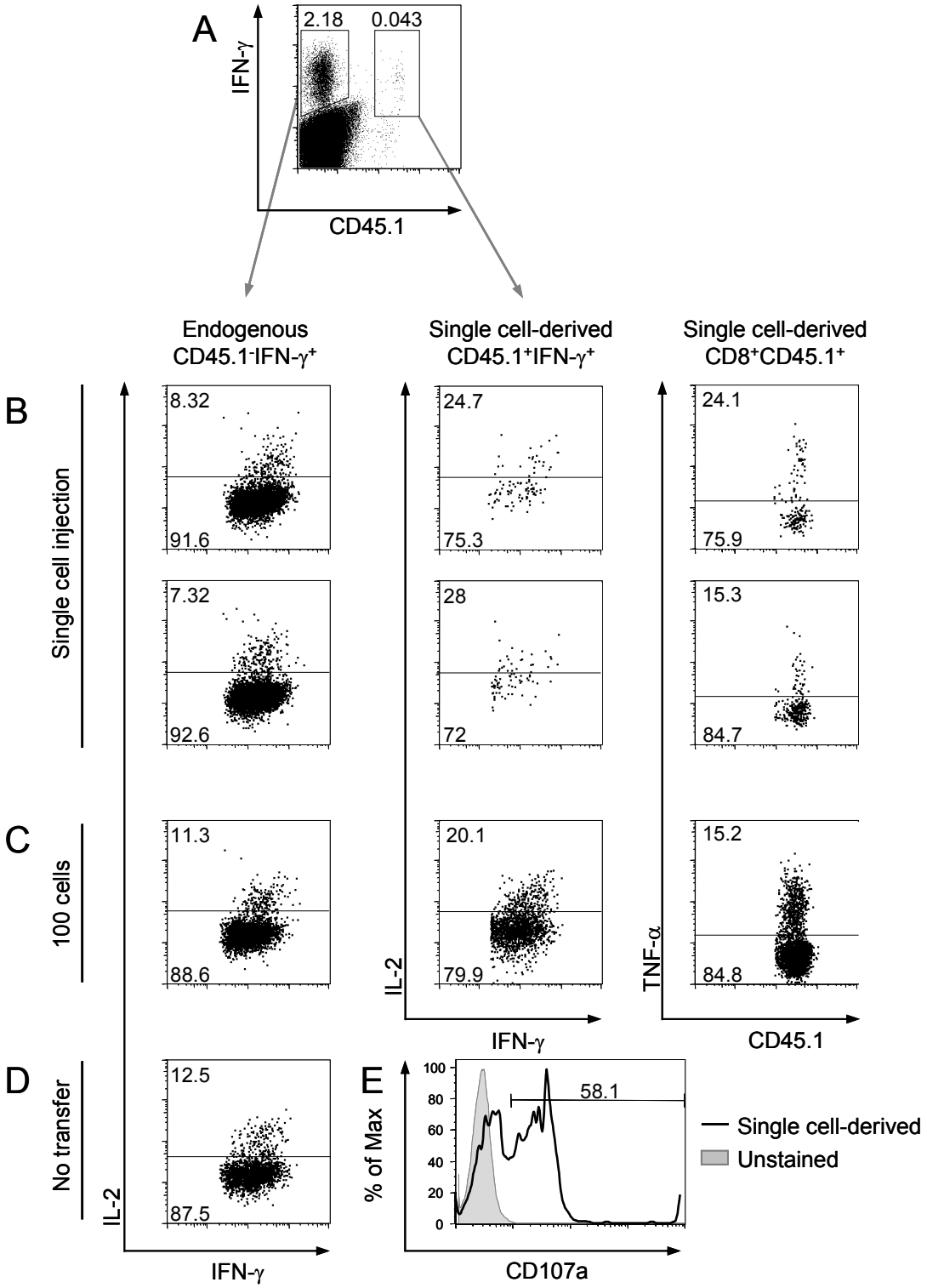

Figure 9: Functional diversity of T cells expanded from a single naïve precursor cell during the effector phase.

C57BL/6 mice (CD45.2$^+$) received **(B)** a single naïve CD45.1$^+$/CD8$^+$ OT-I T cell (two representative mice) by intraperitoneal microinjection or **(C)** 100 naive OT-I cells intravenously. **(D)** Control mice did not receive any adoptively transferred cells but were otherwise treated identically. Immediately after transfer, recipient mice were infected with *L.m.*-Ova (0.1 x LD_{50}), and 12 days later T cells from the spleen were analyzed by intracellular cytokine staining upon

in vitro restimulation in the presence of $Ova_{257-264}$ peptide (SIINFEKL, 10^{-6} M). **(A)** Representative dot plot (pre-gated on $CD8^+$ T cells) indicating the gating strategy for identification of endogenous ($CD45.1^{negative}$) and single cell-derived ($CD45.1^+$) IFN-γ producing effector populations. Cells were pre-gated on live $CD8^+$ cells, and staining for IFN-γ versus CD45.1 is displayed. **(B–D)** Analysis of subpopulations defined as described in (A) for IFN-γ versus IL-2 are shown in the left two dot plots. The dot plots to the far right show TNF-α production by $CD45.1^+$ single cell-derived cells. Gates segregating subpopulations are indicated, and percentages for each area are shown. **(E)** Degranulation upon peptide restimulation in the presence of Brefeldin A was determined by anti-CD107a staining. Histogram shows $CD45.1^+$ cells from a single cell injection experiment (black line) whereas the unstained control is show in the overlap mode (filled grey histogram).

7.3 $CD8^+$ memory cells develop from single naïve precursor T cells

As shown above, seven days and even more pronounced twelve days post *L.m.* inoculation, CD62L and CD127 staining patterns indicated the possibility of memory T cell precursor formation (cells that are $CD127^{high}$, Fig. 7 and 8). Furthermore, the presence of diverse CD62L/CD127 expression patterns suggested that both major types of long-lived T_{CM} and T_{EM} subsets might arise from a single naïve T cell. As staining for CD62L/CD127 during early *Listeria* infection might not be without its limitations, we next sought to demonstrate the presence of memory cells more directly.

As a first experimental setup, mice that had received a single naïve OT-I cell together with primary infection were rested and five weeks later recipient mice were rechallenged with a high dose of *L.m.*-Ova. Similar to analysis during the primary effector phase, we could detect a $CD45.1^+$ single cell-derived OT-I population in about 25% of mice upon reinfection (Fig. 10). This result clearly showed that long-lived cells with the typical characteristic to mount rapid recall responses (analysis five days after rechallenge) have emerged from a single naïve progenitor cell.

Re-expanded single cell-derived populations again closely paralleled the diversity observed for the endogenous SIINFEKL-specific recipient populations according to their phenotypical (compare Fig. 10 A to B and C) and functional properties (Fig. 10 D).

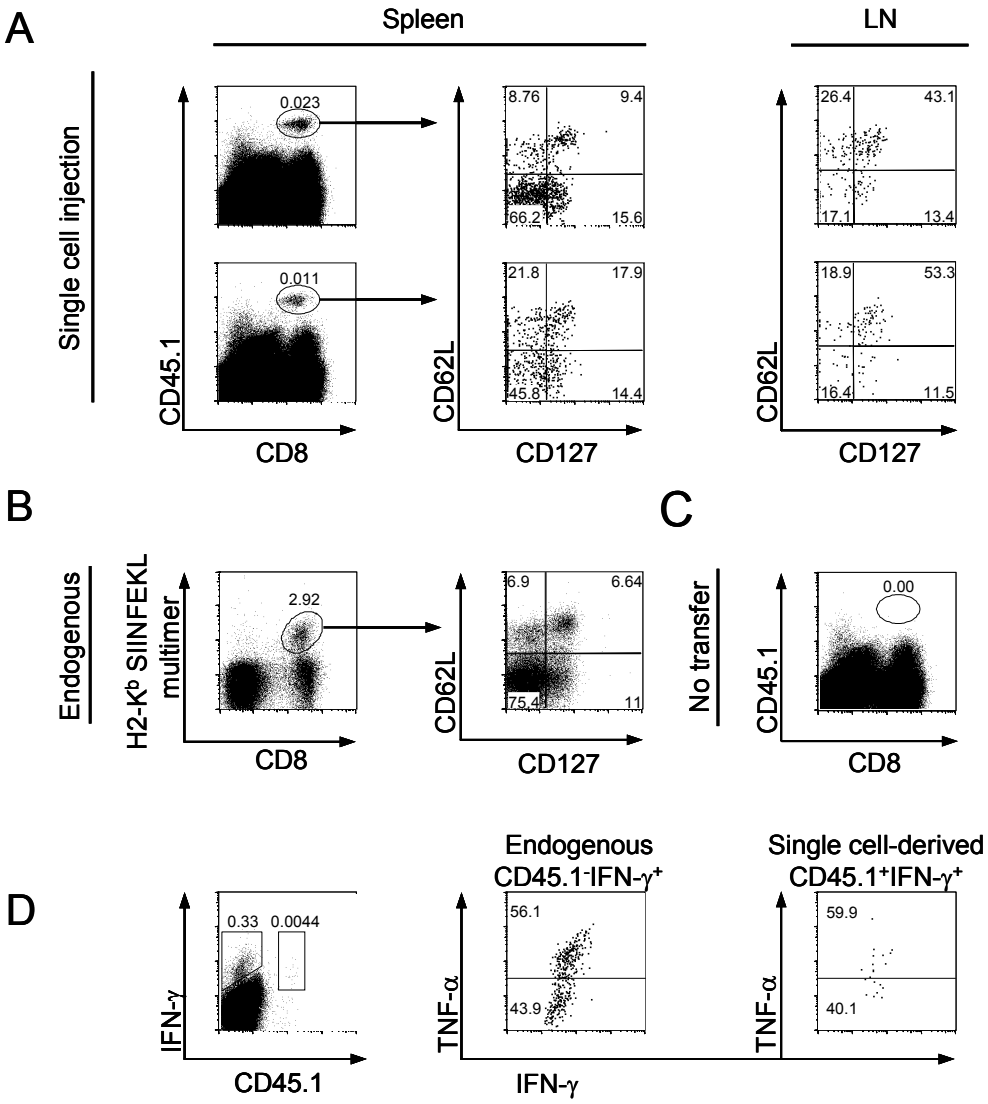

Figure 10: Phenotypical diversity of T cells expanded from a single naïve precursor cell upon recall expansion.

C57BL/6 mice (CD45.2$^+$) received a single naïve CD45.1$^+$ CD8$^+$ OT-I T cell by intraperitoneal microinjection. Immediately after transfer, recipient mice were subsequently infected with *L.m.*-Ova (0.1 x LD_{50}) Mice received a second injection with *L.m.*-Ova (5 x LD_{50}) 5 weeks later, and T cells were analyzed in the spleen and lymph nodes by flow cytometry on day 5 post-infection. **(A)** Each row summarizes data from an individual mouse. Dot plots to the left show the identification of transferred OT-I cells by staining for CD45.1 and CD8. Percentages among all splenocytes are indicated for the shown gates. Dot plots in the middle show CD62L and CD127 expression patterns of *in vivo*-expanded CD45.1$^+$ OT-I cells in the spleen (parental gate is shown). Dot plots to the right display corresponding stainings in LNs. **(B)** Similar analyses as described in (A) were performed for endogenous SIINFEKL-specific T cells identified by MHC multimer (Streptamer) staining. Identification of cell populations is indicated in the left dot plot (gated on CD45.1^{negative} cells). The displayed data belong to the same mouse shown in the upper row in (A). **(C)** Example of a control mouse that did not receive any CD45.1$^+$ CD8$^+$ OT-I T cells. **(D)** Intracellular cytokine staining: the two dot plots to the right show expression of IFN-γ versus TNF-α on endogenous SIINFEKL-reactive CD45.1^{negative} (middle) and transferred CD45.1$^+$ OT-I cells (right) as described in Fig. 7. Shown populations are gated on IFN-γ^+ cells (left).

When compared to the primary response, recall population sizes from single transferred CD45.1$^+$ OT-I cells were about eight times larger than their respective primary effector populations (Fig. 11, 3.1±1.02 x 10^3 compared to 2.6±0.7 x 10^4 cells for transferred). This corresponded to an almost identical increase in the number of endogenous SIINFEKL-specific T cells in the same cohort of mice (3.05±0.07 x10^5 compared 2.52±0.4 x 10^6 cells). Corresponding to the measured absolute CD45.1$^+$ cell numbers (seven days after *L.m.* infection, also compare Fig. 7), the transferred single CD8$^+$ OT-I T cell divided about 13-14 times that related to an approximately 8.000-10.000-fold increase in cell numbers. These data indicated that the two populations (OT-I and endogenous SIINFEKL-specific T cells) did not compete with each other in their expansion and survival kinetics (during primary [d12] and recall [d5] response the single cell-derived CD45.1$^+$ OT-I population represented about 1% within the total population of SIINFEKL-specific cells, Fig. 11).

Following the assumption that single cell-derived and endogenous T cell populations have similar *in vivo* kinetics of clonal expansion, it should be possible to calculate the initial precursor frequency for the endogenous population. As deduced from Fig. 11, comparing the absolute numbers of single cell-derived and endogenous antigen specific populations, the size of the naïve endogenous repertoire of SIINFEKL-specific naïve T cells must comprise approximately 100 cells (107±13 cells).

In summary, the data analyzing recall responses indicate that a single naïve precursor cell indeed harbors the potential to give rise to memory T cells. These cells can survive and rapidly re-expand upon antigen re-exposure, which is believed to be mainly a characteristic of central memory T cells. Population sizes of re-call expanded single naïve T cell-derived populations were enhanced as compared to primary responses and developed into a diverse phenotypical and functional pattern of cell subsets during the secondary effector phase.

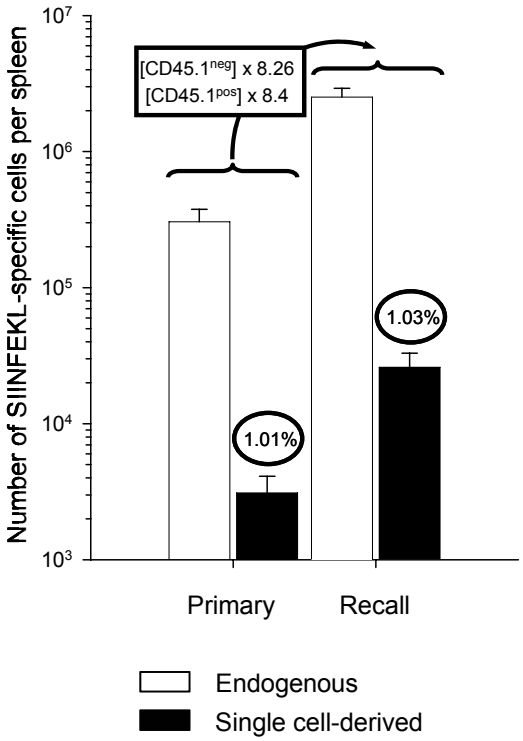

Figure 11: Absolute numbers of single-cell derived and endogenous SIINFEKL-specific T cells during primary and recall immune responses.

A cohort of C57BL/6 mice (CD45.2$^+$) received a single naïve CD45.1$^+$/CD8$^+$ OT-I T cell per individual mouse by intraperitoneal microinjection. Immediately after transfer, recipient mice were infected with *L.m.*-Ova (0.1 x LD_{50}); five weeks later, mice received a second injection with *L.m.-Ova* (5 x LD_{50}). Splenocytes were analyzed 12 days after primary infection or 5 days after recall infection) and absolute numbers of single cell-derived (CD8$^+$/CD45.1$^+$; black bars) or endogenous (H2-K^b/SIINFEKL multimer$^+$/CD8$^+$; white bars) SIINFEKL-reactive cells were determined per spleen. Data for each time point are expressed as mean values, and standard deviations are shown (6 mice per group). The numbers in the box indicate the fold differences when comparing the mean values of primary and recall population sizes. Numbers labeled with an oval express the percentages of single cell-derived (CD45.1$^+$) cells within the total population of SIINFEKL-specific cells at the indicated time points.

7.4 Different CD8$^+$ memory subsets develop from single naïve precursor T cells

Although rechallenge experiments could clearly identify the generation of memory T cells derived from single naïve precursors, the phenotypical and functional diversity of secondary expanded effector cells does not necessarily reflect the presence of both major subsets (T_{CM} and T_{EM}) of memory T cells. In fact, despite hints deducted from CD62L expression analyses during the primary contraction phase, reported differences in the proliferative capacities

between T_{CM}, as the main extensively proliferating subset (Bachmann et al., 2005b; Huster et al., 2006a) and T_{EM}, complicates conclusions about existing memory subsets after antigen reencounter.

In order to examine whether both T_{CM} and T_{EM} subsets are present in recipients of a single adoptively transferred T cell, we attempted to directly visualize single cell-derived memory T cells five weeks after primary immunization without reinfection. Because of the very low frequencies of expected memory T cells we used an improved version of flow cytometry acquisition software allowing us to examine up to 7 x 10^7 cells per organ. By this method, we were able to visualize memory cells derived from a single naïve precursor cell in several different organs like the spleen, the lymph nodes or the lung five weeks after primary infection (Fig. 12). As shown in Figure 12 A, all detected memory cells in the spleen were exclusively positive for CD127, a typical characteristic for memory T cells. When additionally analyzing the expression of CD62L we could observe two clearly distinguishable populations indicating that both subsets of memory $CD8^+$ T cells were present at this time point (T_{EM} as $CD62L^{low}$, T_{CM} as $CD62L^{high}$). The presence as well as the observed subset distribution was also typical for the spleen at this time point as reported previously (Busch et al., 1998; Huster et al., 2004). In addition, the phenotype of endogenous SIINFEKL-specific T cells detected by MHC multimer staining was again similar (Fig. 12 D). Intracellular cytokine staining for IFN-γ, TNF-α and IL-2 in the spleen (in all other organs frequencies were too low for analysis) further confirmed the presence of different memory T cell subsets within single cell-derived memory populations (Fig. 12 A). Like during acute primary or recall infections, the complexity found within the endogenous SIINFEKL-specific T cell population was comparable (Fig. 12 D).

T_{CM} have been described as cells that preferentially migrate to lymphatic organs like the LNs. In line with this definition, most of the single cell-derived $CD45.1^+$ memory OT-I cells found in the LNs were expressing CD62L at high levels (Fig. 12 B). In contrast, exactly the opposite has been reported for T_{EM}: they appear as $CD62L^{low}$ and migrate into peripheral organs. Also in our experiments we could observe that more than 70% of the single cell-derived progeny found in the lung were negative for CD62L (Fig. 12 C). We observed the same CD62L expression ratios for single cell-derived T_{CM} and T_{EM} subpopulations, similar to endogenous SIINFEKL-specific T cells in the same individual mouse (data not shown).

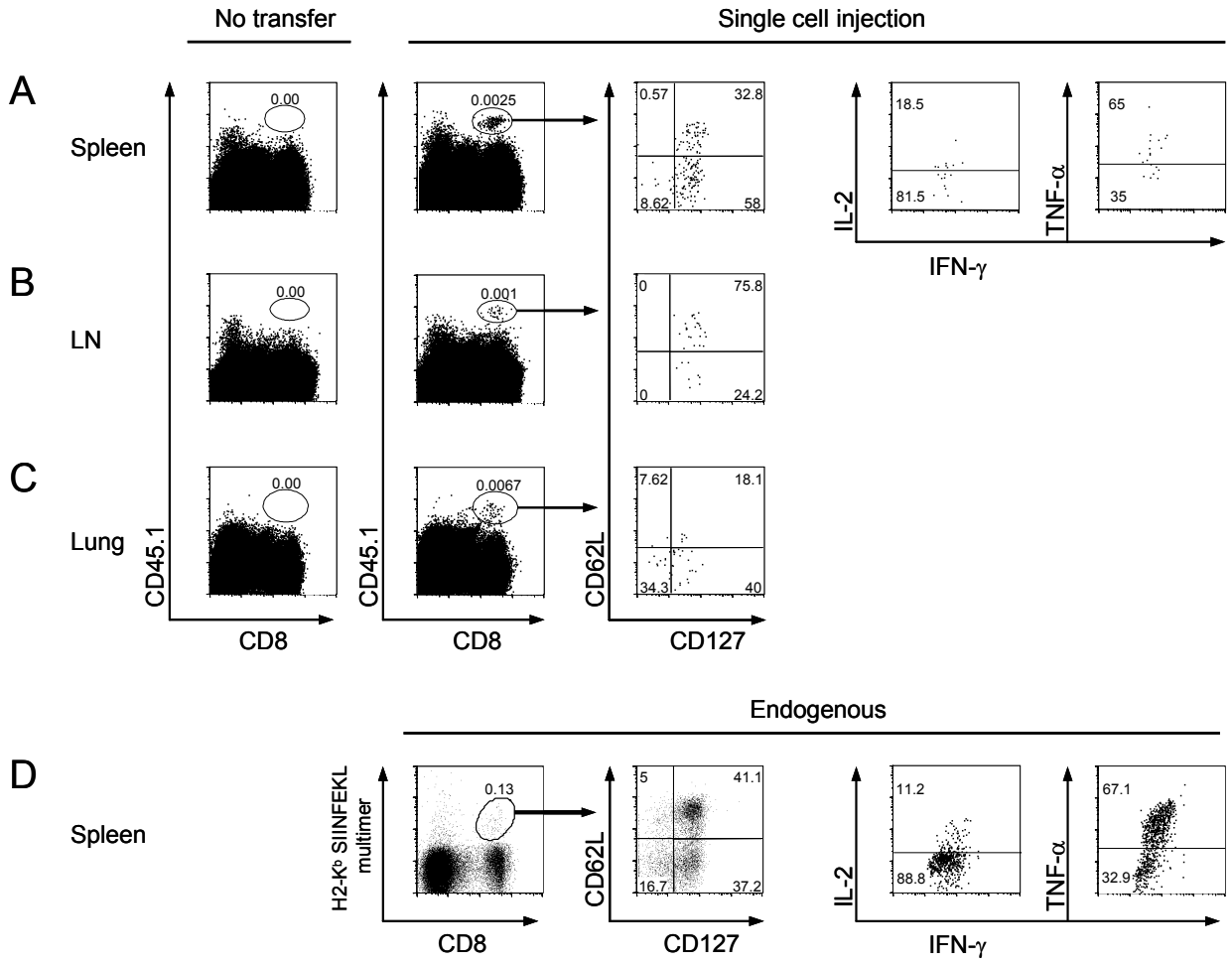

Figure 12: Phenotypical and functional diversity of memory T cells generated from a single naïve precursor cell.

C57BL/6 mice (CD45.2$^+$) received a single naïve CD45.1$^+$/CD8$^+$ OT-I T cell by intraperitoneal microinjection. Immediately after transfer, recipient mice were infected with *L.m.*-Ova (0.1 x LD_{50}), and 5 weeks later T cells were analyzed by flow cytometry in different organs as indicated (all displayed data belong to the same individual mouse). **(A–C)** Analysis of single cell-derived populations in different organs. Left dot plots show stainings for a control mouse without any T cell transfer. All other dot plots show memory T cells from a mouse after single cell transfer. OT-I cells were identified by staining for CD45.1 and CD8. Percentages among viable cells are indicated for the shown gates. CD45.1$^+$/CD8$^+$ T cells were further analyzed for CD62L and CD127 expression. The two dot plots to the right show IFN-γ and IL-2 or TNF-α expression patterns of IFN-γ$^+$ CD45.1$^+$ cells. **(D)** Identification of endogenous SIINFEKL-specific T cells in the spleen by MHC multimer (streptamer) staining. The relevant cell population is indicated in the left dot plot (gated on CD45.1^{negative} cells). Gated antigen-specific cells were further analyzed for CD62L and CD127 expression. The two dot plots to the right show expression of IFN-γ versus IL-2 or TNF-α on endogenous SIINFEKL-reactive IFN-γ$^+$ cells as described in Fig. 7.

A recent publication by Woodland and colleagues tried to identify three subsets of memory CD8$^+$ T cells that might be distinct from the classical T_{CM} and T_{EM} phenotypes in terms of

mounting secondary immune responses (Hikono et al., 2007). They detected three memory populations one month post infection by analyzing the surface expression of the T cell activation marker CD43 together with the chemokine receptor CXCR3. To assess whether these distinct subsets also develop out of a single naïve T cell, we analyzed CD43 and CXCR3 expression of single transferred CD45.1$^+$ OT-I cells six weeks after *L.m.*-Ova infection. Very similar to the observation by Hikono *et al.*, we could identify heterogeneity among expanded cells in different organs, discriminating three distinct subsets: CD43^{high}/CXCR3^{high}, CD43^{low}/CXCR3^{high}, CD43^{low}/CXCR3^{low}. Again the observed subset distributions between transferred (single cell-derived as well as 100 transferred cells) and endogenous polyclonal SIINFEKL-specific T cells were quite comparable (Fig. 13).

In summary, these data demonstrate, that five weeks after clearance of infection phenotypically and functionally different T_{CM} and T_{EM} subsets are present, which can arise from the same naïve precursor T cell.

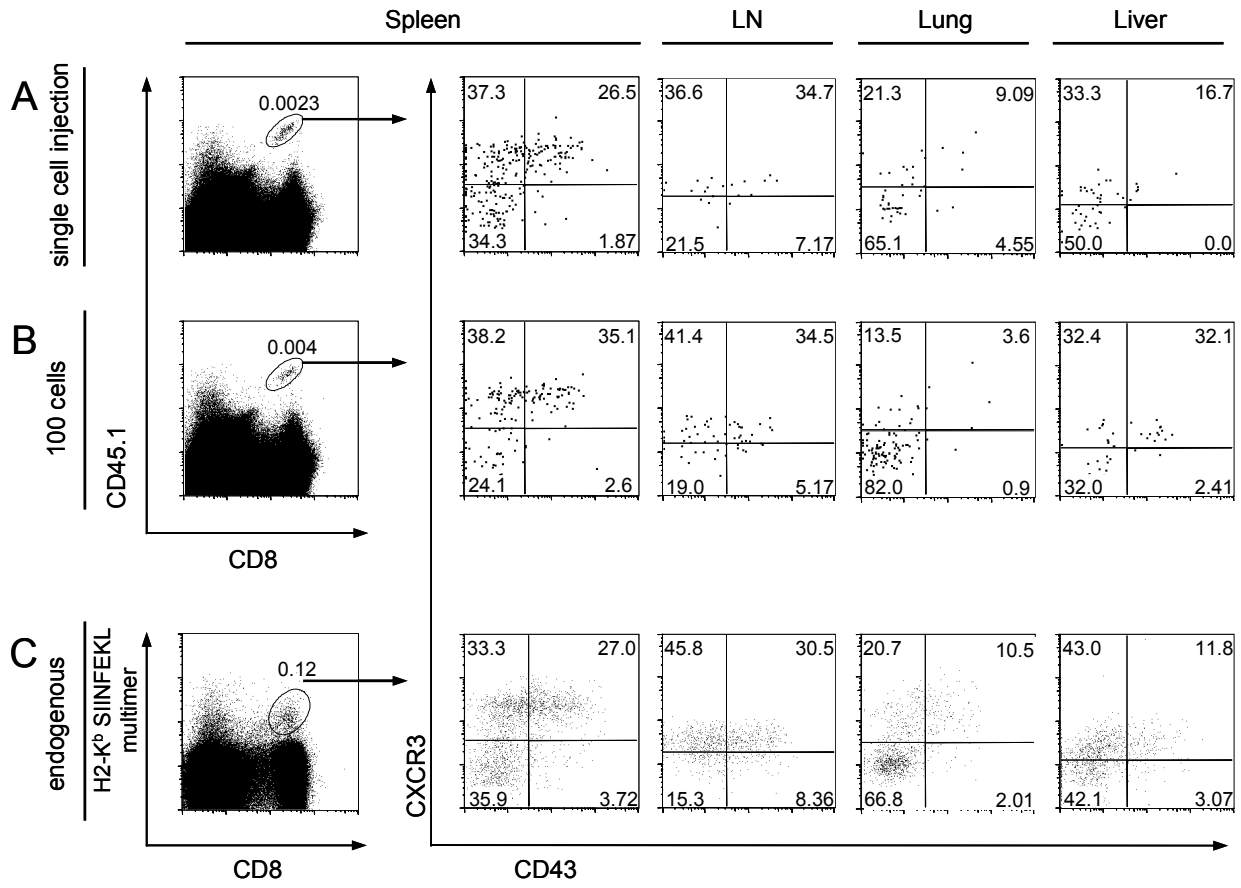

Figure 13: Phenotypical diversity of memory T cells generated from a single naïve precursor cell.

C57BL/6 mice (CD45.2$^+$) received a single naïve CD45.1$^+$/CD8$^+$ OT-I T cell by intraperitoneal microinjection. Immediately after transfer, recipient mice were infected with *L.m.*-Ova (0.1 x

LD_{50}), and 6 weeks later T cells were analyzed by flow cytometry in different organs as indicated). **(A–B)** Analysis of transferred $CD45.1^+$ OT-I populations in different organs as indicated. OT-I cells were identified by staining for CD45.1 and CD8. Percentages among viable cells for single cell-derived or 100 transferred cells are indicated for the shown gates. $CD45.1^+/CD8^+$ T cells were further analyzed for CD43 and CXCR3 expression. Identification of endogenous SIINFEKL-specific T cells in the spleen by MHC streptamer staining. The relevant cell population is indicated in the left dot plot (gated on $CD45.1^{negative}$ cells). Gated antigen-specific cells were further analyzed for CD43 and CXCR3 expression.

7.5 Effector and memory subsets originate from the same single naïve precursor cells

Single cell-derived memory T cells were detectable in *Listeria* infected recipient mice with a similar recovery rate as compared to analyses during the effector phase (Fig. 11), which indicates that both observations are directly linked to each other. However, as experiments assessing the generation of either effector or memory subsets were performed in independent experiments as well as individual mice, these data cannot directly prove that a single precursor cell gave rise to both effector and memory T subsets concurrently.

In order to demonstrate this directly, we performed repetitive adoptive transfer experiments that allowed following the fate of single cell-derived progeny at all stages of the immune response (Fig. 14 A).

Twelve days after single cell transfer and primary *Listeria* infection, splenocytes and lymph node cells from individual mice were pooled. One third of these cells was subsequently used to demonstrate the presence of diversified single cell-derived progeny during the effector phase (Fig. 14 B). The remaining cells were then adoptively transferred a second time into a naïve recipient mouse, which was rested and after five weeks challenged with *L.m*-Ova subsequently followed by analysis for re-expanded T cell responses (6 days after recall infection). Mice that received cells from an initially untransferred mouse served as control.

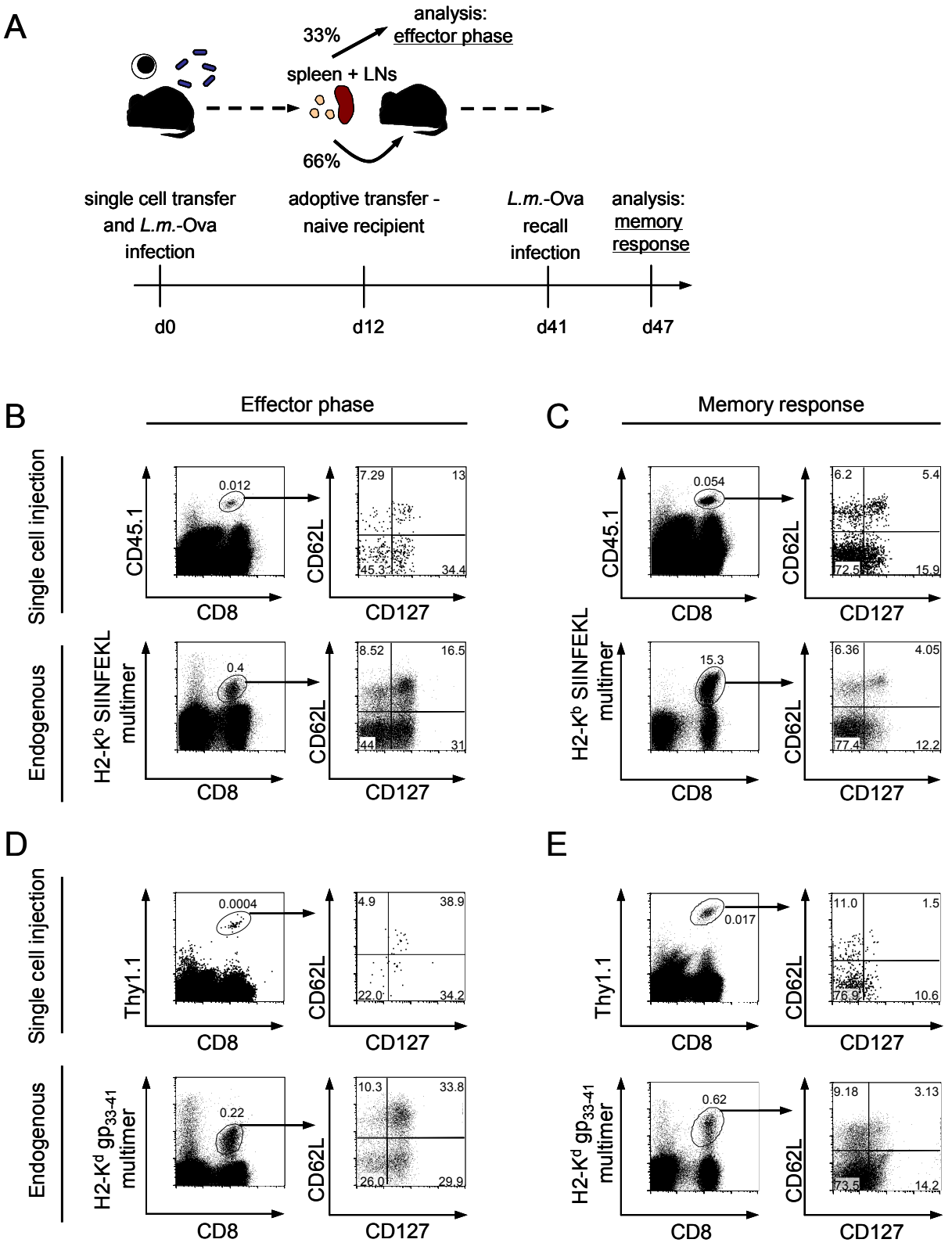

Figure 14: Analysis of single cell-derived effector and memory T cells after primary or secondary adoptive transfer.

(A) Schematic outline of the experimental setup used for directly tracking the concurrent generation of effector and memory cells out of a single naïve T cell. C57BL/6 mice (CD45.2$^+$) received a single naïve CD45.1$^+$ CD8$^+$ OT-I T cell by intraperitoneal microinjection. Immediately

after transfer, mice were infected with *L.m.*-Ova (0.1 x LD_{50}). Twelve days after infection, cells from the spleen and LNs were pooled. One third of the cells was rejected to flow cytometric analysis, while the remaining cells were used for a second adoptive transfer into a naïve CD45.2$^+$ C57/BL6 mouse. After five weeks, recipient mice were (re-) challenged with *L.m.*-Ova and the presence of (secondary) transferred CD45.1$^+$ OT-I T cells was confirmed by flow cytometry.
Twelve days after adoptive transfer of a single cell and *L.m.* infection, spleen and LNs were pooled and either analyzed by flow cytometry **(B, effector phase)** or transferred into a second naïve CD45.2$^+$C57BL/6 mouse. After more than 5 weeks post primary transfer recipient and control mice (primary infected but no transfer of a single cell) were infected with 5 x LD_{50} *L.m.*-Ova and after 6 days splenocytes were analyzed by flow cytometry **(C, memory response)**. Each row in A + B represents data from an individual mouse. Dot plots to the left show the identification of transferred OT-I cells by staining for CD45.1 and CD8 or SIINFEKL-specific CD45.1^{negative} T cells identified by MHC multimer (streptamer) staining. Percentages among all splenocytes are indicated for the shown gates. Dot plots to the right show CD62L and CD127 expression patterns of *in vivo*-expanded SIINFEKL specific (transferred or endogenous) cells in the spleen (parental gate is shown) after primary and secondary infection.
(D and E) Identical experiments were performed as described in **(A-C)** using Thy1.1 P14 TCR-tg T cells. After single cell transfer, recipient mice were infected with 0.1 x LD_{50} with gp33 expressing *Listeria monocytogenes* (*L.m.*-Gp_{33}). After retransfer of T cells, secondary recipient mice were challenged with 5 x LD_{50} *L.m.*-Gp_{33} and transferred P14 cells were identified by staining for Thy1.1. gp_{33-41} specific Thy1.1^{negative} T cells were identified by MHC multimer staining.

As depicted in Figure 14 C *Listeria* rechallenge resulted in a strong expansion of SIINFEKL-specific memory cells. These contained endogenous as well as transferred CD45.1$^+$ T cells, which were initially derived from a single cell precursor. The secondary emerging ovalbumin-specific immune reaction protected the (naïve) recipient mouse from the otherwise lethal challenge with 5 x LD_{50} of *L.m.*-Ova. Although the control mouse received antigen reactive T cells via the second transfer (endogenous SIINFEKL reactive cells from the primary infected mouse) these mice died from the overwhelming infection (not shown), indicating that the additional T cells derived from the single cell were needed for protection.

The transfer of protection against an otherwise lethal infection was in line with the observed distribution of subsets, which was characterized by a strong increase in CD62L^{low}CD127^{low} effector cells (Fig. 14 C) compared to the primary response (Fig. 14 B). In addition, descendants of single cell-derived and endogenous CD8$^+$ T cells again demonstrated almost identical phenotypical differentiation patterns during the primary as well as the secondary response (Fig. 14 B and C). One important question is whether subset diversification is comparable also for other TCR-tg systems or if this observation is limited to OT-I cells. To address this question, we performed retransfer experiments with Thy1.1$^+$ P14 cells that are specific for the LCMV epitope gp_{33-41}. Comparable to data obtained for the OT-I system, we observed similar differentiation patterns in effector and memory subsets for primary and secondary infections, further proposing a general mechanism of subset diversification (Fig. 14 D and E). Although frequencies between

progenies of the two different TCR-tg cells were highly different, the original founder cells differentiated comparably into diverse effector and memory subsets. Most importantly, we again could observe highly resembling phenotypical differentiation patterns between transferred and endogenous gp_{33-41} specific populations

These data demonstrate that short-living as well as long-living memory subsets can develop out of the same single naïve precursor T cell.

7.6 Transferred- and endogenous $CD8^+$ T cell subset diversification is synchronized independently of immunization or infection

Besides the finding that a vast plethora of different effector and memory subsets can develop out of a single naïve founder T cell, the observation that differentiation patterns for transferred and endogenous antigen-specific populations synchronize quite strongly was perhaps even more surprising. Because different vaccination strategies have been described that mediate distinct differentiation patterns (Bachmann et al., 2005b; Harty and Badovinac, 2008; Heit et al., 2008; Huster et al., 2006a), we decided to analyze whether a similar correlation could be found in a different immunization model.

For this purpose, mice received subcutaneous vaccinations with 100 μg ovalbumin together with 10 nmol CpG as adjuvant, immediately after adoptive transfer of a single naïve OT-I cell. Twelve days later lymphocytes were screened in different organs for the presence of single-cell derived daughter cells, followed by subsequent analysis of subset diversification. As shown in Figure 15 A for splenocytes from two representative mice, it was again possible to detect single cell-derived T cell populations with a similar recovery rate as described for *Listeria* infection experiments (approx. 20-25%). However, in contrast to the previous experiments (Fig. 8), the phenotypical differentiation patterns on day 12 after immunization differed substantially. The observed patterns were especially characterized by an enlarged subpopulation of $CD62L^{low}$ $CD127^{low}$ cells accompanied by diminished $CD127^{high}$ subsets (Fig. 15 A).

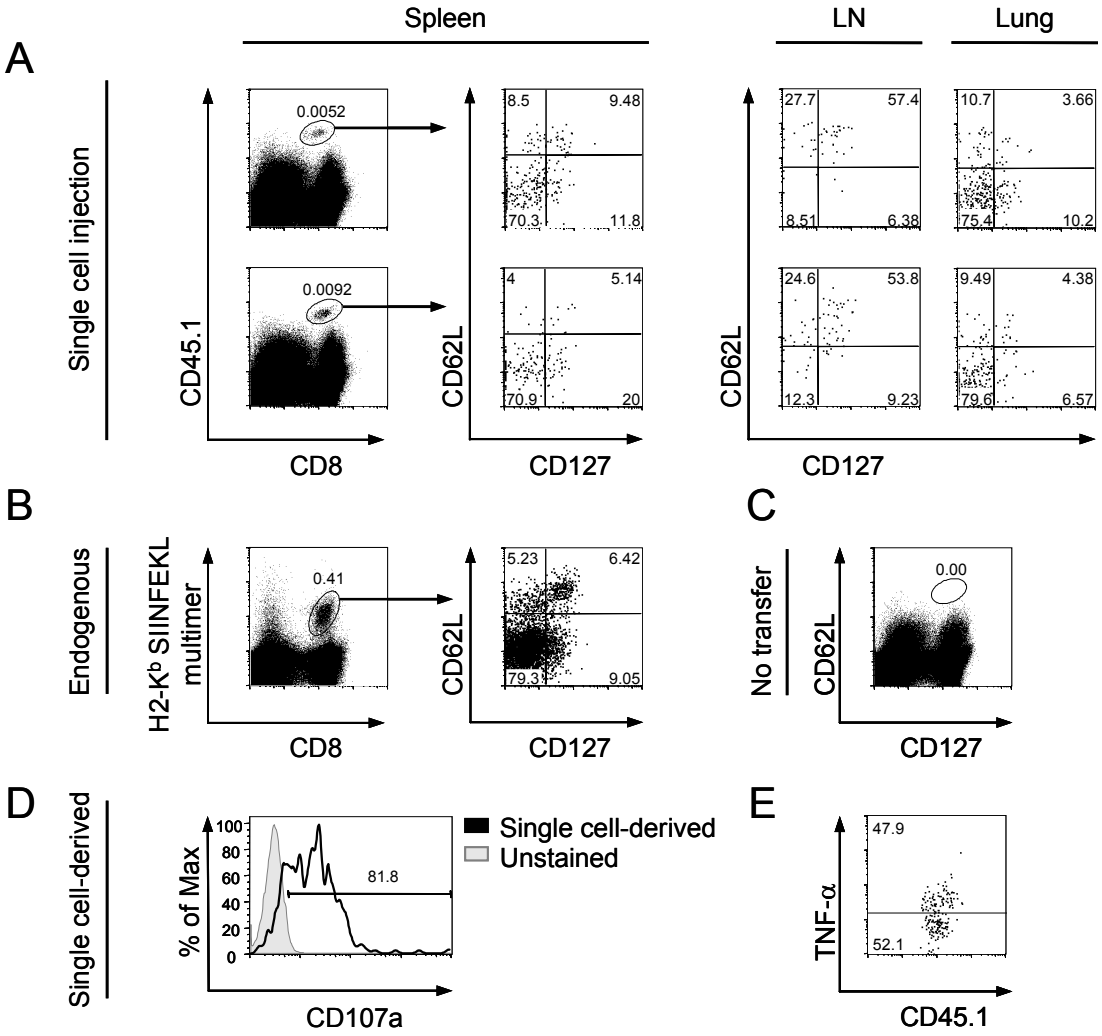

Figure 15: Phenotypical diversity and subset differentiation of single cell-derived and endogenous antigen-reactive T cells upon immunization with Ovalbumin and CpG.

C57BL/6 mice (CD45.2$^+$) received a single naïve CD45.1$^+$ CD8$^+$ OT-I T cell by intraperitoneal microinjection (two representative mice are shown). Immediately after transfer, recipient mice were immunized subcutaneously with Ovalbumin and CpG, and 12 days later T cells were analyzed in the spleen and LN **(A)**. Dot plots to the left show the identification of transferred OT-I cells by staining for CD45.1 and CD8 or **(B)** of endogenous (CD45.1^{negative}) cells by MHC multimer (streptamer) staining (displayed data correspond to the same mouse shown in the upper row of (A)). Percentages among total splenocytes are indicated for the shown gates. Dot plots to the right show CD62L and CD127 expression patterns of *in vivo*-expanded transferred or endogenous SIINFEKL reactive cells in the different organs (parental gates are shown). **(C)** Example of a control mouse that did not receive any CD45.1$^+$CD8$^+$ OT-I T cells. **(D)** Surface expression of CD107a from single-cell derived CD45.1$^+$OT-I cells. Splenocytes from one representative mouse are shown. **(E)** TNF-α production of CD107a positive cells from (D).

The phenotypically different subset distribution as compared to the *Listeria* infection model also correlated functionally with increased numbers of degranulating cells (>80% are positive for

CD107a; Fig. 15 D) and T cells producing TNF-α (Fig. 15 E). But once again, when comparing single cell-derived T cells with endogenous SIINFEKL-specific T cell populations (Fig. 15 B), an obvious difference in the differentiation patterns could not be detected.

This became even more clear and could be further backed up by statistical analyses when comparing the percentages of subset prevalence in larger cohorts of mice (between 6 and 10 mice per group level) that underwent single cell transfer and different immunization procedures (Fig. 16). Whereas both immunizations resulted in substantially distinct subset distributions, the diversification patterns of single cell-derived and endogenous T cells within each group were remarkably similar.

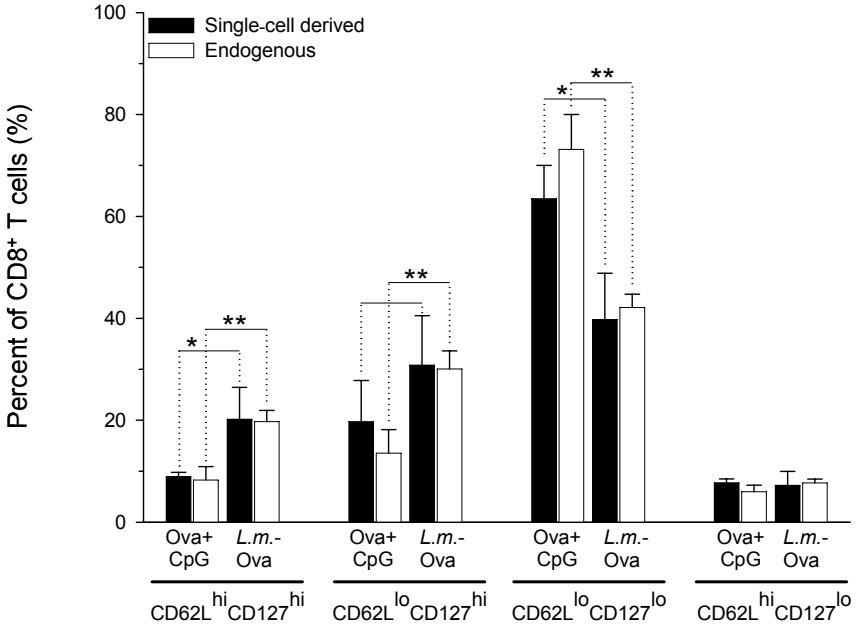

Figure 16: Statistical comparison of CD62L/CD127 expression patterns of single-cell derived progeny vs. endogenous antigen specific populations upon immunization or *L.m.* infection.

C57BL/6 mice received a single naïve OT-I T cell subsequently followed by subcutaneous immunization with Ovalbumin + CpG or *L.m.-Ova* challenge (0.5 x LD_{50}). After 12 days, different CD62L and CD127 expressing subsets ($CD62L^{hi}CD127^{hi}$, $CD62L^{lo}CD127^{hi}$, $CD62L^{lo}CD127^{lo}$ and $CD62L^{hi}CD127^{lo}$) were analyzed by flow cytometry and plotted as percentage of $CD8^+$ cells. Black bars represent single cell derived-, white bars cells from endogenous antigen reactive cells (* $p < 0.02$; ** $p < 0.003$). Group levels ranged between n = 6-10 mice per group.

7.7 "Latecomer" cells still have the potential to differentiate into diverse subsets

It has recently been suggested that $CD8^+$ and $CD4^+$ T cells recruited late into an immune response (e.g. 2-3 days after infection) preferentially give rise to memory cells, especially T_{CM} (Catron et al., 2006; D'Souza and Hedrick, 2006). These latecomer cells might encounter priming conditions that differ from early inflammatory conditions in such a way that they are not sufficient any more to drive full activation of naïve cells. The changed priming milieu would then only be sufficient to drive development of "latecomer" T cells to weakly activated, but long-lived memory cells rather than end-stage differentiated effector cells.

Such a correlation, especially measured by the expression of CD62L, has for example been demonstrated by adoptive transfer experiments using relatively high numbers (10^4-10^6 cells) of naïve $CD8^+$ T cells and subsequent infection with VSV or *Listeria monocytogenes*. Some recent reports clarified already the importance to use physiological cell numbers in adoptive transfer experiments (already 500 cells are seen as unphysiological (Badovinac et al., 2007; Marzo et al., 2005)), since this can alter differentiation patterns substantially. Therefore, we decided to re-evaluate the question of fate of lately recruited T cells by tracking the progeny of a single naïve latecomer T cell.

Figure 17 A depicts the experimental setup: a single naïve $CD45.1^+$ OT-I T cell was adoptively transferred into day two infected C57BL/6 recipient mice (48h after infection with *L.m.*-Ova). As controls, some mice received 100 naïve T cells and in all cases the differentiation patterns were compared to endogenous SIINFEKL-specific $CD8^+$ T cell populations. Twelve days after infection, which translates to ten days of activation and proliferation for the latecomers, we could observe that although the overall size of single cell-derived daughter cell populations from "latecomers" were substantially lower compared to adoptive transfer directly together with infection (Fig. 7, 8 and Fig. 17 B and C), in all cases the phenotypical and functional CD62L/CD127 differentiation patterns were highly diverse. This observation was strengthened by the fact that single cell-derived populations were composed of multifunctional subsets, producing different amounts of IFN-γ and TNF-α (Fig.17).

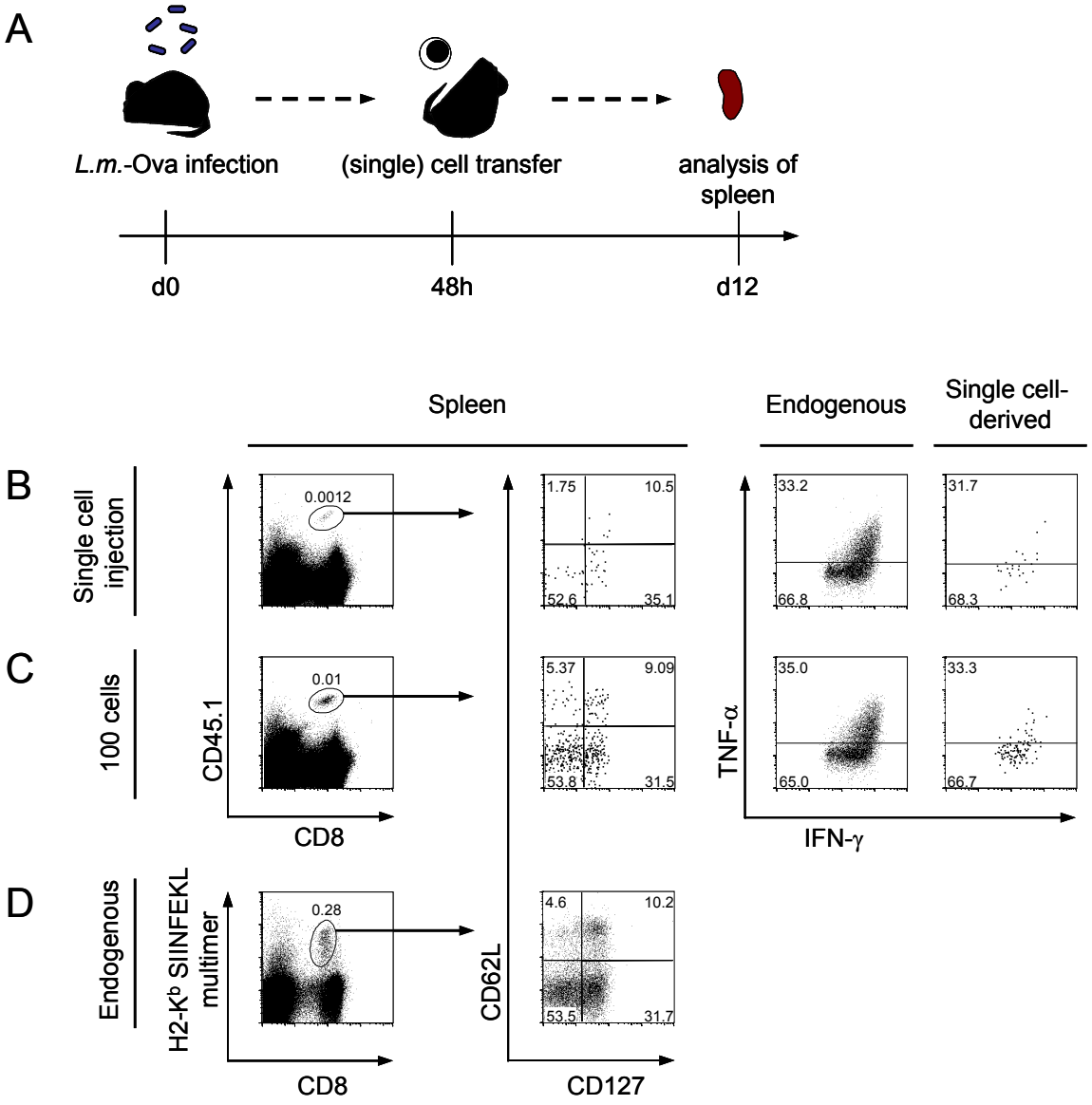

Figure 17: Functional diversity of daughter cells from "latecomer" precursor cells.

(A) Schematic outline of the experimental setup used for the phenotypical analysis of latecomer cells. C57BL/6 mice (CD45.2$^+$) were infected with *L.m.-Ova* (0.1 x LD_{50}) and received naïve CD45.1$^+$ CD8$^+$ OT-I T cells 48h post infection. At twelve days post infection, single cell-derived and endogenous Ova-reactive CD8$^+$ T cells were analyzed by flow cytometry.
C57BL/6 mice (CD45.2$^+$) were infected with *L.m.-Ova* (0.1 x LD_{50}) and received naïve CD45.1$^+$ CD8$^+$ OT-I T cells 48h post infection. At day twelve post infection, single cell-derived and endogenous Ova-reactive CD8$^+$ T cells were analyzed by flow cytometry. **(B)** Transfer of a single naïve CD45.1$^+$ CD8$^+$ OT-I T cell or **(C)** 100 naive OT-I cells by intraperitoneal microinjection. OT-I cells were identified by staining for CD45.1 and CD8. Percentages among viable cells are indicated for the shown gates. CD45.1$^+$ CD8$^+$ T cells were further analyzed for CD62L and CD127 expression. The two dot plots to the right show IFN-γ versus TNF-α expression patterns of IFN-γ$^+$ CD45.1$^+$ OT-I cells (right) compared to IFN-γ$^+$ CD45.1$^-$ endogenous SIINFEKL responsive cells (left) **(D)** Analyses of endogenous SIINFEKL-specific T cells in the spleen (shown data correspond to the same individual mouse as in (B)) identified by

MHC multimer (Streptamer) staining 12 days after infection with *L.m.-Ova* (0.1 x LD_{50}). Identification of the cell populations is indicated in the left dot plot (gated on $CD45.1^{negative}$ cells).

Furthermore, also single cell-derived "latecomer" T cell populations demonstrated a phenotypical and functional differentiation pattern of daughter cells, which closely resembled the endogenous polyclonal SIINFEKL-reactive T cells (Fig. 17 B and D). In addition, transfer experiments with 100 naïve T cells 2 days (48h) post infection demonstrated exactly the same picture (Fig. 17 C), and no statistically significant trend towards larger frequencies of $CD62L^{high}$ subsets could be observed, which has been associated with T_{CM}-like characteristics (see above; Fig. 18). These data strongly support the interpretation that subset diversification is both shaped and synchronized continuously during the expansion phase, and making it unlikely that the T_{CM} subset is mainly derived from latecomer T cells.

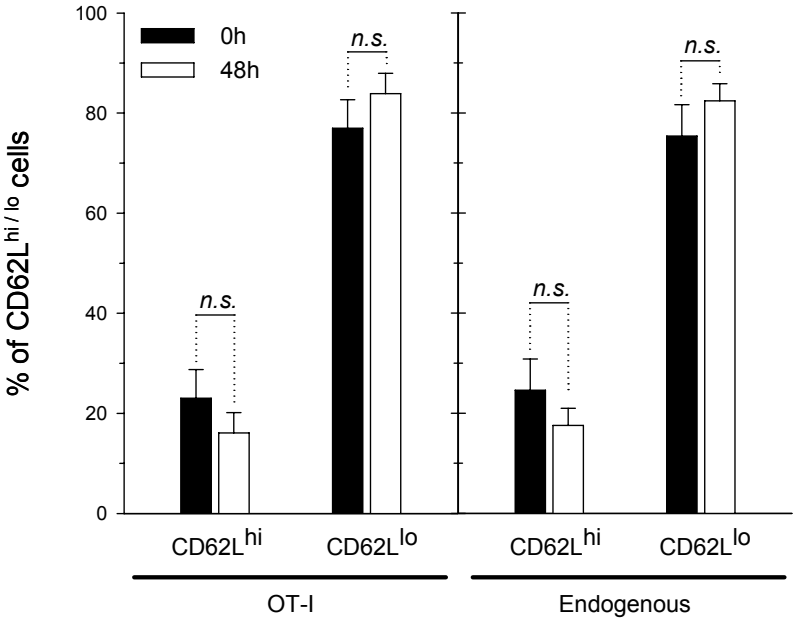

Figure 18: CD62L surface expression of adoptively transferred "latecomer" cells.

$CD45.2^+$ C57BL/6 mice received single naïve $CD45.1^+/CD8^+$ OT-I cells by intraperitoneal microinjection either immediately before or 48h after infection with *L.m.*-Ova (0.5 x LD_{50}). Twelve days after infection, $CD62L^{hi}$ or $CD62L^{lo}$ expressing subsets of transferred or endogenous antigen specific cells were analyzed by flow cytometry. Subsets were plotted as percentage of $CD8^+$ cells. Black bars represent the percentage of $CD62L^{high/lo}$ OT-I cells transferred immediately before infection or after 48h (white bars). The corresponding endogenous cells are depicted on the right. Plots represent 4 mice per group. *n.s.* = no statistically significant difference.

7.8 The descendants of a single naive T cell confer protection against *Listeria monocytogenes*

Our data demonstrate the enormous plasticity of single naïve T cells, allowing them to develop into highly heterogenic populations of $CD8^+$ effector and memory subsets. This observation reminds of the characteristics of "stem cells", which – besides providing highly differentiated progeny – still maintain daughter cells that keep a high capacity of proliferation and self-renewal. If the degree of plasticity and self-renewal would be really that high, one might even envision that adoptive transfer of a single "stem cell-like" T cell is already sufficient to generate enough daughter cells to provide protective immunity towards infection. This question cannot be addressed with the previously described adoptive transfer model using wildtype recipient mice, as their endogenous T cells will also substantially participate in protective immunity. To circumvent the participation of endogenously arising antigen-specific $CD8^+$ T cells in clearing the infection, we designed a model in which complete protection relies on the transferred single $CD8^+$ T cell and its descendants. We transferred a single naïve OT-I T cell into the peritoneum of Rag1 deficient ($Rag1^{-/-}$) mice that are completely devoid of B and T cells. Control mice either received 100 cells or were left without cell transfer. Immediately after transfer, mice were vaccinated i.v. with 1 x 10^8 IU of an ovalbumin expressing strain of the viral vector MVA (modified vaccinia virus Ankara, MVA-Ova) to provide a non-infectious expansion of the transferred cells. Two weeks after vaccination, the expanded single cell-derived populations were further expanded by boost vaccination with MVA-Ova (1 x 10^8 IU i.v., Fig. 19 A). At this time point single cell-derived populations were readily detectable in the blood of about 25% of recipient mice (not shown). Seven days after the second MVA vaccination, all mice were challenged with a normally lethal dose of 5 x LD_{50} of *L.m.*-Ova (Pamer, 2004). Three days after *Listeria* challenge we determined numbers of viable bacteria in the spleens and livers to measure the quality of protection. As shown in Figure 19 B, the obtained result was very clear cut: mice that initially received just a single naïve OT-I T cell were completely protected compared to untransferred mice. In fact, mice repopulated with single cell-derived OT-I T cells showed no viable bacteria at any dilution. This was also confirmed by the macroscopic appearance of spleens. Spleens of untransferred mice were covered with granulomas but appeared normal in single cell-transferred mice (not shown). In addition, transferred $CD45.1^+$ T cell populations were detectable at considerable frequencies in both organs (Fig. 19 B).

Taken together, these data demonstrate that a single naïve T cell gives rise to different subsets of daughter cells that are necessary and sufficient to confer protection to an otherwise poorly protected individual mouse. In addition, transferring as little as one single pathogen-reactive T

cell can be sufficient to fully rescue the compromised immune status of Rag1$^{-/-}$ mice for a defined infection, which otherwise lack their own endogenous CD8^{+} T cells.

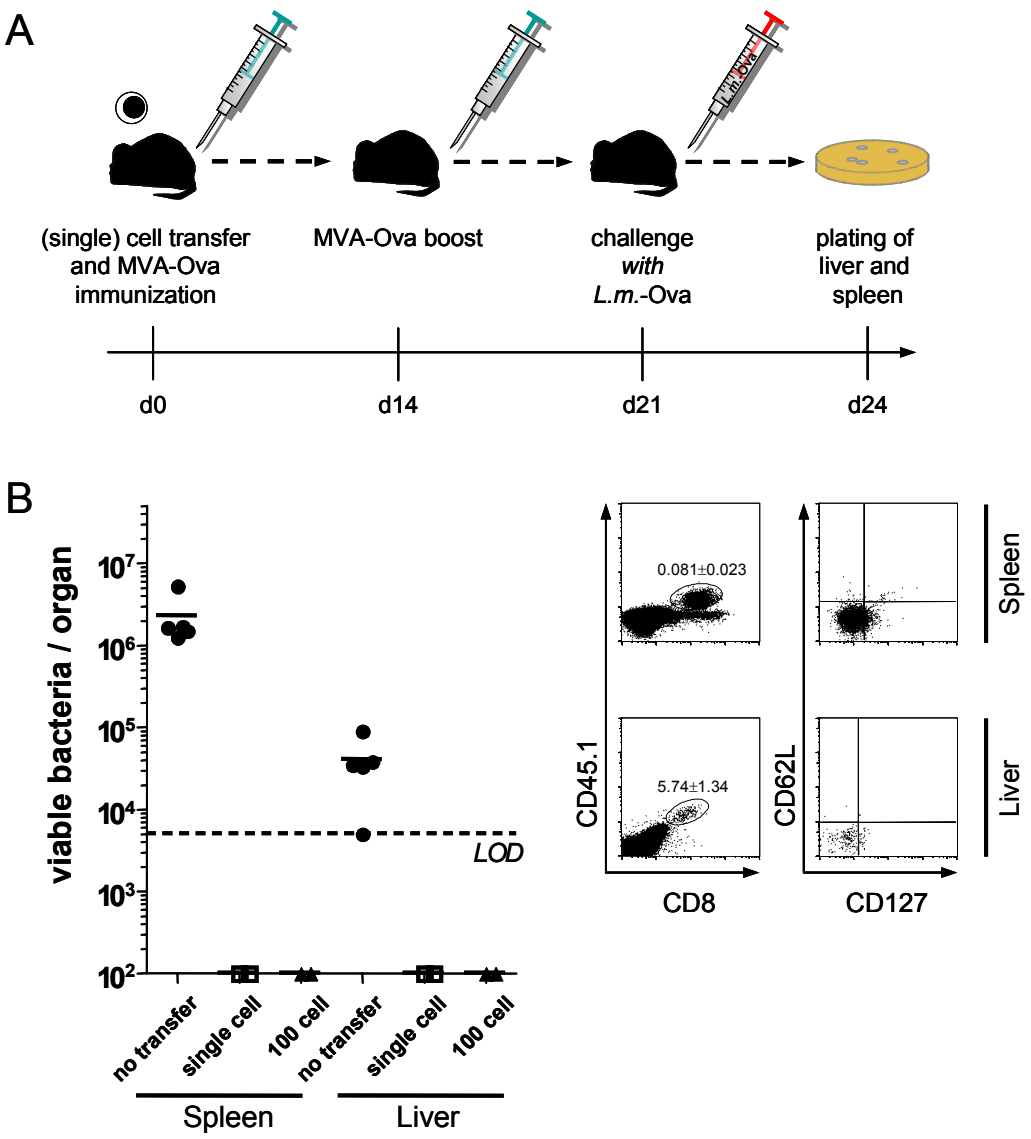

Figure 19: Protective capacity of descendants of single naïve precursors.

(A) Schematic outline of the experimental setup used to analyze the protective capacity of single cell-derived daughter cells. **(B)** Rag1$^{-/-}$ mice (CD45.2^{+}, C57BL/6 background) received varying amounts (either single- or 100 cells) of naïve CD45.1^{+} CD8^{+} OT-I T cells by intraperitoneal microinjection or were left without transfer. Immediately after transfer, recipient mice were vaccinated with 1 x 10^{8} IU MVA-Ova. Two weeks post primary MVA-Ova administration, recipient mice received a second MVA-Ova immunization. On day seven after MVA boost, all mice were challenged by i.v. injection of 5 x LD_{50} *L.m.*-Ova. Protection was assayed by quantification of living bacteria in the spleens and livers three days after *L.m.* challenge. Individual bacterial counts are plotted for each group (n = 2-5 mice per group). Horizontal bars represent mean values. LOD = level of detection.

7.9 Plasticity of antigen-experienced memory OT-I T cell subsets

Considering the enormous plasticity residing within single naïve T cells, we were interested in whether such characteristics are also found within the long-lived memory compartment of antigen-experienced cells. This would be of special interest for the improvement of adoptive T cell therapies in which available donor cells would most probably consist of a mixture of antigen-experienced (memory) cells instead of naïve T cells. As such $CD8^+$ memory T cells are themselves composed of heterogeneous subsets (e.g. T_{CM} vs. T_{EM} subsets), we first analyzed whether we could observe differences in the characteristics of major memory subpopulations. According to the described CD62L expression profiles for T_{CM} and T_{EM} cells (Huster et al., 2006b; Stemberger et al., 2007), we adoptively transferred varying amounts of highly FACS-enriched $CD62L^{high}$ or $CD62L^{low}$ expressing $CD45.1^+$ OT-I memory cells. Decreasing amounts of approximately 1.000, 100 and 10 cells were adoptively transferred by i.p. injection into naïve congenic $CD45.2^+$ C57BL/6 mice, followed by subsequent infection with *Listeria*. As seen in Figure 20 A, twelve days after transfer we could readily detect $CD45.1^+$ OT-I T cells derived from $CD62L^{high}$ expressing memory cells that expanded even better than naïve OT-I T cells. Most important – as for naïve Ag-specific cells – we also could recover a substantial population of these cells even in the “10 cell group”. Interestingly these cells still displayed a pluripotent differentiation potential as seen by the presence of diverse CD62L / CD127 expressing subsets. In addition, twelve days after transfer the recovered $CD45.1^+$ OT-I T cells were different from the arising endogenous SIINFEKL-specific T cell response (Fig. 20 B), resembling the typical subset distribution patterns found during recall responses. Although most cells displayed the $CD62L^{low}$ phenotype of effector- and effector memory cells, we could still find a clear population of cells that again expressed high amounts of CD62L. In strong contrast, transferred $CD62L^{low}$ cells could only be recovered from the “1.000 cell group”. In addition to their poor proliferative capacity, these cells failed to generate the pluripotent differentiation patterns observed before. The $CD62L^{low}$ memory-derived $CD45.1^+$ cells almost exclusively expressed low amounts of CD62L and CD127 associated with terminal effector cells.

These data imply that exclusively $CD62L^{high}$ memory OT-I T cells or a subset within this compartment proliferates vigorously upon antigen reencounter while they are still reflecting the pluripotent potential required for subset diversification. On the other hand, this seems not to be an conceptual property of memory cells themselves, as certain subsets, like for example $CD62L^{low}$ memory cells, do not display this characteristic. Taken together, the observed properties including an enormous plasticity of certain memory subsets reminds – in analogy to naïve $CD8^+$ T cells – of the characteristics of “(memory) stem cells”.

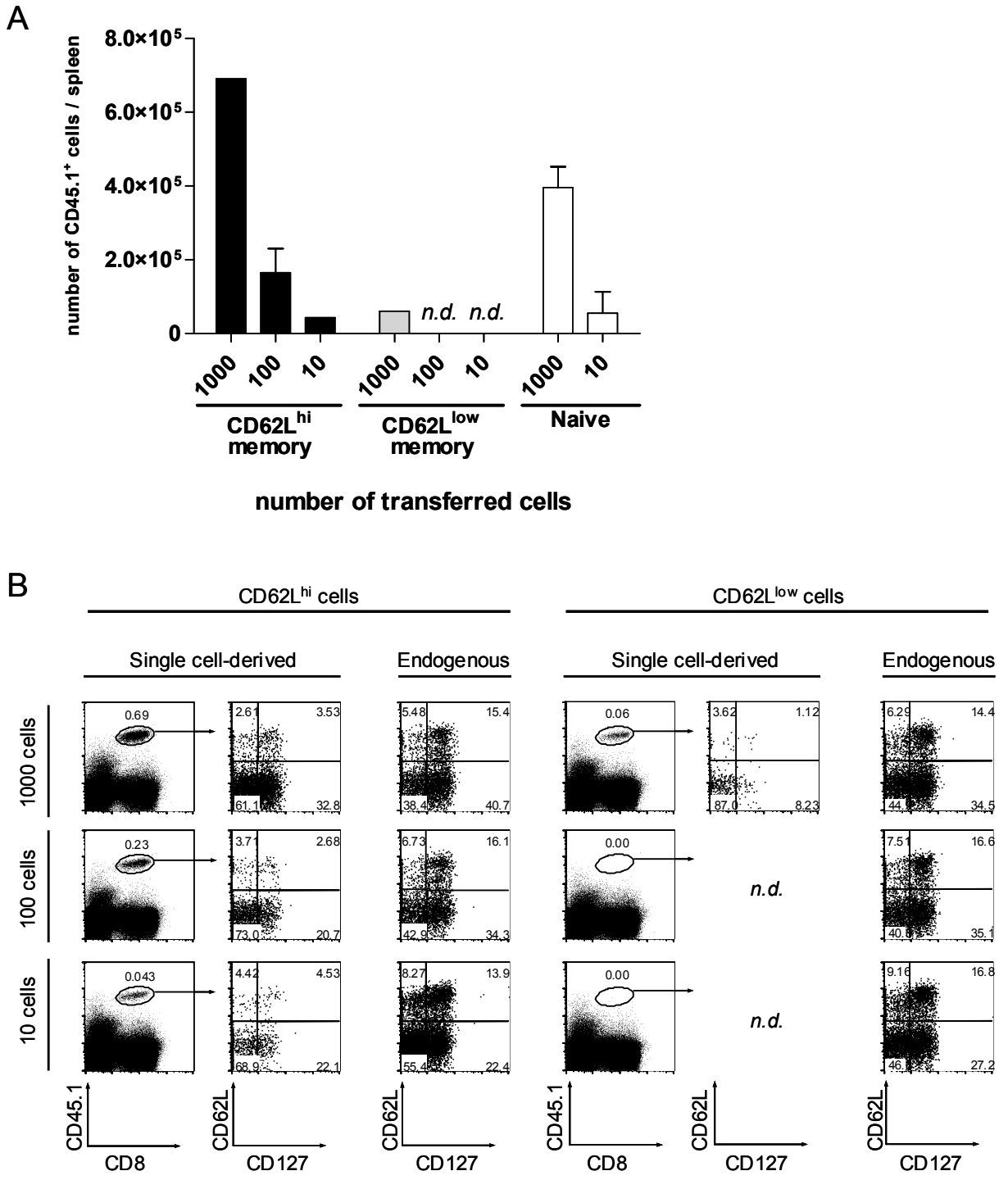

Figure 20: Transfer of CD62$^{low\ /\ high}$ antigen-experienced memory OT-I T cells.

Naïve C57BL/6 mice (CD45.2$^+$) received varying numbers (10-1.000) of highly FACS-enriched (selected for CD45.1$^+$/CD8$^+$/CD44^{high}) CD62L high or low expressing memory CD45.1$^+$ OT-I CD8$^+$ T cells by intraperitoneal microinjection. Memory cells were initially generated by adoptive transfer of 1.000 naïve CD45.1$^+$ OT-I T cells into congenic C57BL/6 mice cells that resolved *Listeria* infection and were rested for three additional months. Naïve (CD44^{low}) CD45.1$^+$ OT-I CD8$^+$ T cells served as reference. Immediately after transfer of memory cells, recipient mice were infected with *L.m.*-Ova (0.1 x LD_{50}), and 12 days later splenocytes were analyzed by flow

cytometry. **(A)** Absolute numbers of CD45.1$^+$ OT-I T cells were calculated in the spleen and plotted against the respective groups. **(B)** Representative dot pots for recovered CD45.1$^+$ OT-I T cells (derived from CD62L high (right half) and low (left half) memory cells) for each group are shown. Percentages among all splenocytes (right) or from the indicated gates are shown (middle). In addition, CD62L/CD127 expression patterns of endogenous SIINFEKL-specific T cells are shown as indicated (right). *n.d.* = not detected.

8 DISCUSSION

Immunity to intracellular pathogens is often dependent on the generation of effective $CD8^+$ T cell responses, most importantly memory T cells, which provide enhanced protection against reinfection (Huster et al., 2006b; Williams and Bevan, 2006). Due to this outstanding feature, these cells have become an important goal for vaccination (Pantaleo and Koup, 2004; Seder and Ahmed, 2003). However, effective vaccine design is hampered by our limited understanding of the generation of $CD8^+$ memory T cells.

One of these crucial factors concerns the origin of subset diversity in terms of generating different effector and memory subsets. Many groups have tackled this task experimentally, but all attempts were limited by several technical drawbacks that anticipated direct analysis of cellular parameters or function, or were settled in non-physiological environments (Baron et al., 2003; Bouneaud et al., 2005; Chang et al., 2007).

During this work we attempted to design an improved experimental model, allowing direct analysis of the generation of $CD8^+$ memory cells. We developed a system that enables us to transfer one single naïve $CD8^+$ T cell into the natural polyclonal environment of naïve recipient mice. Using this model we could demonstrate that it is possible to track the immune response evolving from a single cell after subsequent infection with the intracellular bacterium *Listeria monocytogenes* or protein immunization. Furthermore we could determine the differentiation capabilities of single T cells into effector and memory subsets. After clonal expansion from a single naïve founder cell we were able to recover distinct phenotypes among progeny cells. During the peak and late effector phase (seven or twelve days post infection / immunization) cells were diverse according to the expressed surface markers CD62L and CD127 allowing phenotypic characterization of subsets. Daughter cells were also found to be functionally heterogenic in terms of the varying repertoire of produced effector cytokines INF-γ, TNF-α and IL-2. In addition, daughter cells of a single naïve T cell developed into both major subsets of central- (T_{CM}) and effector memory cells (T_{EM}) that were able to generate robust secondary immune responses against antigenic rechallenge. The single cell-derived immune responses were also sufficient to confer full protection to an otherwise lethal challenge with *Listeria*.

These data directly demonstrate for the first time that subset heterogeneity can arise from a single naïve precursor cell. This means that the “one cell - multiple fate” model is sufficient to explain the origin of subset diversification. Furthermore our novel experimental approach directly visualized the enormous plasticity of naïve $CD8^+$ T cells.

In addition to the findings outlined above, we uncovered that in all observed cases the phenotypical and functional diversity of single cell-derived progeny closely resembled the heterogeneity among the endogenous (polyclonal) antigen-specific pool. This finding might include factors operative during the expansion phase as important shapers of ongoing $CD8^+$ T cell responses.

8.1 When during an immune response is heterogeneity achieved?

Given the extremely small number of T cells specific for a given epitope (estimates range between 100-200 cells/mouse (Arstila et al., 1999; Blattman et al., 2002) and the robust differentiation patterns observed during infections, at least some degree of intraclonal diversification might have already been expected from published work. However, it was only recently that – in line with our observations outlined above – a first report argued that identical TCR sequences are indeed present within different memory subset compartments, such as T_{CM} and T_{EM}, supporting a model in which variable subsets can arise from T cells bearing the same TCR (Bouneaud et al., 2005). Despite the found diversity between T cells bearing the same TCR, the performed experiments were carried out via measurement of indirect parameters that were also not linked to functional cellular properties. In addition, these studies could not discriminate whether heterogeneity originates from a single naïve precursor cell or a small pool of clonotypic (bearing the same TCR) T cells. Finally, these types of analysis did not allow addressing the question of when during an immune response (before, during or after priming) diversification is actually achieved.

During or shortly after thymic selection and subsequent emigration, cell proliferation might have already increased the number of cells derived from individual clones bearing the same TCR. This number could then be further amplified by homeostatic proliferation in the periphery, especially if sufficient “space” is present for example as seen under lymphopenic conditions or in neonates (Ernst et al., 1999; Schuler et al., 2004; Viret et al., 1999). This latent antigen-independent turnover has been studied in detail and was shown to be associated with the development of diversity (Goldrath et al., 2000; Goldrath et al., 2002; Hamilton et al., 2006; Kieper and Jameson, 1999; Murali-Krishna and Ahmed, 2000). The observed diversifications included upregulation of activation markers (e.g. CD44, Ly6C and CD122), acquisition of effector functions and the development of memory cell characteristics even in the absence of antigen encounter. Instead homeostatic differentiation was shown to require interactions with self peptides in the context of self MHC molecules (Ernst et al., 1999; Kieper and Jameson,

1999). These findings indicate that the naïve pool of antigen specific $CD8^+$ T cells could already show a predetermined diversity between clones, which also might have dictating consequences in subset development after recruitment into an immune response.

To exclude diversity already established before the onset of an immune reaction, we designed our experimental setup in that way that (1.) a single and (2.) highly pure FACS enriched naïve $CD44^{low}$ TCR-tg T cell is adoptively transferred per individual mouse, which is (3.) immediately challenged with cognate antigen. As we observed in all cases the development of diverse subsets (T_{EC}, T_{CM} and T_{EM}) from a single naïve T cell, the impact of predetermined heterogeneity – marked by prevalence of one / a few exclusive subset(s) – is highly unlikely.

In particular, uttered concerns about the influence of homeostatic proliferation on single transferred T cells prior to the priming event are highly unlikely for the experiments presented in this thesis work for the following reasons:

- 48h after transfer of CD45.1 OT-I T cells into the peritoneal cavity, these cells were recruited very fast and efficiently to the spleen.
- Transfer experiments with CFSE-labeled T cells also showed no signs of homeostatic proliferation within this time frame (48h).
- Transfer experiments were performed in a completely natural and fully immune competent setting (wild-type C57BL/6 recipient mice), whereas homeostatic proliferation is strongly enhanced only in lymphopenic settings (Goldrath et al., 2000; Hamilton et al., 2006).
- Recipient mice were infected / immunized immediately after cell transfer to minimize the time frame between transfer and recruitment into the response. During *Listeria* infection, recent data show compelling evidence that the vast majority of priming happens early (within 24h) after onset of infection (Mercado et al., 2000; Prlic et al., 2006).
- Differentiation of single cell-transferred $CD8^+$ T cells was robust and comparable, which should have been much more variable if homeostatic proliferation had increased diversity of transferred cells before priming.
- In all observed cases we could observe nearly identical differentiation patterns between transferred and endogenous Ag-specific responses (see below).

A possible role for programmed diversification has been suggested by the recent observation that a naïve T cell getting primed by a *Listeria* infected dendritic cell may undergo in a stem cell-

like behavior a first asymmetric cell division (Chang and Reiner, 2007). By maintaining prolonged contact to its APC prior to division, a primarily activated $CD8^+$ T cell may unequally partition fate determinants to its two daughter cells initiating the process of diversification and fixed generation of diverse effector and memory subsets (Chang et al., 2007; Chang and Reiner, 2007; Yeh et al., 2008). This intriguing finding is still not without its rational limitations. Is a lineage fate decision between two opposing fates like effector and memory cells really achieved as early as during this initial first division? In other words, by which mechanisms for example does an asymmetric cell division as early as “division one” transform a 50:50 cell-fate ratio into only 5-10% of T cells from the burst size that usually survive as memory cells. Furthermore, how can the generation of a broader heterogeneity among the generated subsets themselves be explained (e.g. multifunctional effector cells or central vs. effector memory cells)? Is the initial stimulus driving the generation of a memory cell (a daughter cell lying distal to the presenting DC) sufficient to maintain its fate also in an antigen-devoid environment when only the inflammatory milieu is present? Although antigenic presentation by DCs is restricted through several mechanisms (Ferlazzo et al., 2002; Guarda et al., 2007; Yang et al., 2006) the time window of presentation lasts for several days rendering repeated T cell:DC contacts very likely (Bousso and Robey, 2003; Mempel et al., 2004; Stoll et al., 2002; Wong and Pamer, 2003). In combination with the general inflammatory environment of cytokines and chemokines induced during infection (Kolumam et al., 2005; Mescher et al., 2006), daughter cells might encounter even further (stochastic rather than deterministic) signals that could drive the generation of additional branching steps. Additional asymmetric divisions in turn could form these bifurication steps, while symmetric divisions would increase effector cells forming the immense number of this T cell subset during the peak response.

Indeed, next to the above-summarized findings of subset diversification and plasticity of naïve single cells, we could also detect a close relation in subset distribution between adoptively transferred and endogenous T cells. In all observed cases, subset heterogeneity of single cell derived progeny was almost identical to the polyclonal endogenous pool of antigen specific cells. The synchrony in differentiation was also observed when switching from *Listeria* infection to a noninfectious immunization model with ovalbumin and adjuvant. This synchrony between transferred and endogenous subsets recognizing the same epitope was surprising, since it is very unlikely that all selected clones encountered identical priming conditions. In fact, this observation is not completely in line with the importance of (fate determining) factors acting either prior to the first cell division like differential signal strengths during priming (Gett et al., 2003; Lanzavecchia and Sallusto, 2005) or during the first cell division (Chang et al., 2007), which would lead to imprinting of a developmental program transferred throughout subsequent divisions to daughter cells (compare also Fig. 1 and 2).

The latter finding of synchronous appearance between single cell-derived and endogenous cells is particularly interesting, as it suggests an important role of signals provided to responding cells during the proliferation phase (after the first cell division). We therefore currently favor a model in which exposure to external environmental factors during the expansion phase – such as repeated contact to antigen, cytokines and chemokines – continuously shapes $CD8^+$ subset heterogeneity that develops during an immune response (Fig. 21). These later environmental influences during the expansion phase reduce potential intrinsic stochastical variabilities, explaining the distinct and conserved patterns of $CD8^+$ (memory) T cells.

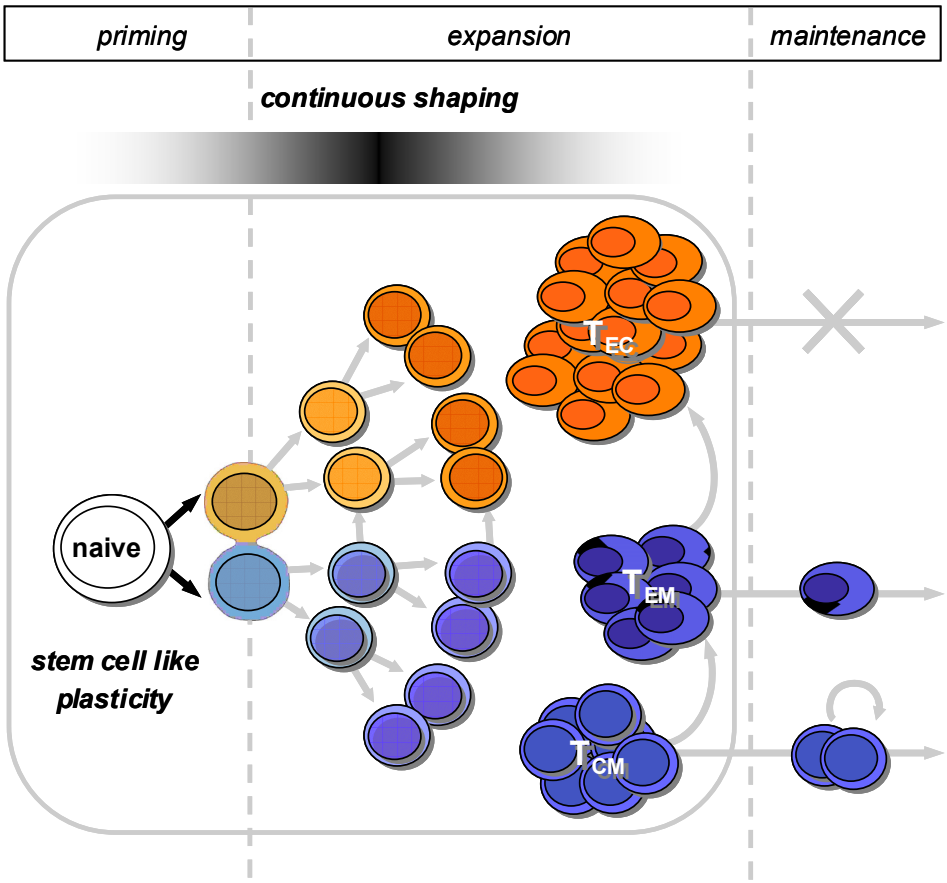

Figure 21: Memory differentiation of a single naive CD8 T cell.

After antigen-specific stimulation *in vivo*, a single naive CD8 T cell develops into all major T cell subsets (T_E, T_{EM} and T_{CM}) and finally survives as long-living memory T cells (**stem cell-like *plasticity***). Furthermore, the single cell-derived progeny is found to be synchronized with the endogenous antigen-specific polyclonal T cell response. This may be explained by an ongoing differentiation capacity of proliferating T cells beyond the first cell division (grey box) sequentially influenced by the immunization-dependent environment (e.g. infection or protein/adjuvant immunization). Later influences during the expansion phase reduce potential intrinsic stochastical variabilities, explaining the distinct and conserved patterns of $CD8^+$ memory T cells (***continuous shaping***).

The influence of the external environment could be derived from “active” differentiation factors acting directly on T cells to develop into special subsets with its respective functional and phenotypical properties, or by “passive” factors promoting the survival of certain subsets while others are committed to disappear. Even when “latecomer cells” were generated by transfer of $CD8^+$ T cells two days after *L. m.* infection, we could observe close synchrony to endogenous T cells recruited much earlier. A recent report has suggested a different outcome for “latecomer cells” (D'Souza and Hedrick, 2006). Here, cells that were recruited later, displayed a strong tendency towards the generation of cells expressing high amounts of CD62L starting 6-9 days after onset of infection. This finding was correlated to an increased development of central memory T cells. In our experiments we could not observe such an increase. Even more importantly, the observed CD62L / CD127 based subset distribution of single cell-derived cells closely mirrored the heterogeneity observed by day 0 transferred or endogenous cells although observed population sizes were strongly reduced. This even further strengthens our interpretation, that factors during the expansion phase beyond priming must strongly influence shaping and thereby synchronize subset diversification. Even in experiments where antigen presentation can be tightly regulated by rapid depletion of DTR transgenic DCs, increasing the time of antigen presentation only influenced the burst size of the CD8 T cell response but not the development of effector function or commitment to memory (Jung et al., 2002; Prlic et al., 2006), further indicating the influence of factors during the expansion phase.

What could be the reason for the observed differences for T cell differentiation of latecomer cells? Our data might not be entirely comparable to the study of D´Souza who used a different infection model as well as adoptive transfer of high numbers of TCR transgenic cells per mouse. It has been shown by several recent reports that numeric differences in precursor frequency have profound influence on critical aspects of the CD8 T cell response. In these experiments changes in the initial precursor frequency to “unphysiologically” high numbers altered kinetics, proliferative expansion, phenotype (e.g. high expression of CD62L), competition for antigen and lineage commitment (Badovinac et al., 2007; Garcia et al., 2007; Hataye et al., 2006; Marzo et al., 2005; van Faassen et al., 2005). Interestingly in one study, transfer of as few as 500 cells was still seen as unphysiological, resulting in an abnormal immune reaction which was only restored to normal values by seeding approximately 10-50 cells (Badovinac and Harty, 2007). Unfortunately in the studies supporting the “latecomer origin” of T_{CM} subsets up to $1x10^6$ cells (in contrast to one cell per mouse in our experiments) were transferred (Catron et al., 2006; D'Souza and Hedrick, 2006). The observed shift towards CD62L high expressing cells might therefore be due to strongly reduced activation (on a per cell basis) and recruitment into an immune response. Given that the T cell response evolved to initiate from rare precursor cells, these results may not be surprising. However, the data suggest that the use of TCR-tg T cells to

model the endogenous $CD8^+$ T cell response may only be reliable under conditions where these cells represent only a fraction of the endogenous repertoire.

8.2 Clinical relevance for the improvement of immunotherapies

The findings discussed above are likely to have impact on the development of T cell-based vaccination strategies and immunotherapies. The importance of shaping factors acting after the initial priming, especially during the expansion phase of responding T cells, might be particularly important. In line with this finding, it was recently shown that developing $CD8^+$ T cell responses are not fixed in their subset patterns independent of the encountered antigenic context (e.g. live vs. inactivated pathogens, type of adjuvant) but can be modulated to distinct subset distributions (Bachmann et al., 2006; Badovinac et al., 2005; Huster et al., 2006a). We could now confirm this on a single cell basis, which further strengthens the importance of environmental factors for the specific profile of diverse subsets. Taking influence on these factors that are still operative at later stages during an immune response might not only harbor the advantage of better accessibility by different therapeutical methods (e.g. longer time window for application, application of certain cytokine cocktails, precise steering of inflammation signals) but might also allow "custom-made" development of $CD8^+$ T cell responses (e.g. favored development of certain protective memory subsets, redirection of non-protective $CD8^+$ T cell responses).

The here described "stem cell-like" plasticity of naïve $CD8^+$ T cells may also play an important role for the design of adoptive immunotherapies against viruses, tumors and intracellular pathogens. For example, patients that received hematopoietic stem cell transplantation often suffer from reactivation of latent, antiviral-therapy resistant CMV or EBV infections (Gattinoni et al., 2006; Moss and Rickinson, 2005). Reactivation of such viruses arises because of absent virus-specific $CD8^+$ T cells normally present in immunocompetent healthy individuals and that are constitutively necessary for efficient viral control (Heslop and Rooney, 1997; Moss and Rickinson, 2005). Recent therapeutic protocols for CMV treatment with T cells often included the application of extremely high doses (up to 10^9 cells/kg bodyweight) of *in vitro* cultured CMV-specific clones derived from transplant donors (Riddell and Greenberg, 1995). Although this procedure has proven to be effective (Dudley et al., 2002; Riddell et al., 1992; Walter et al., 1995), it suffers from high financial and technical demands and in addition is very time consuming. Furthermore, long and intensive *in vitro* cloning and activation may result in T cell subsets that do not anymore display the natural properties of *in vivo* generated clones concerning their persistence, migratory- and differentiation potential as well as their protective

capacity (Dudley et al., 2001; Yee et al., 2002). Given the here described enormous "stem cell-like" plasticity of T cell subset diversification, transfer of undifferentiated antigen-specific T cell subpopulations might circumvent these severe drawbacks. For this purpose, Ag-specific $CD8^+$ T cells may be isolated directly *ex vivo*. e.g. by MHC multimer/streptamer guided sorting strategies (Cobbold et al., 2005; Knabel et al., 2002; Neudorfer et al., 2007). Following adoptive transfer of these cells it should then be possible to expand robust T cell responses even from very low numbers of initially transferred cells (as already shown in this thesis). The emerging response would consist of potent and persistent effector and memory populations, which in turn efficiently control the virus. As MHC based sorting strategies also allow for a very gentle isolation of transplantable donor T cells (even under GMP conditions), the obtained cell quality in terms of cell stress, viability, low pre-activation state and preserved T cell function could outcompete sheer quantity (of *in vitro* manipulated clones) by far (Neudorfer et al., 2007). In addition, *ex vivo* purified T cells would be ready for use directly at the appearance of CMV / EBV reactivation and should moreover reduce graft versus host complications by potentially alloreactive T cells to a minimum.

In most cases in which adoptive T cell transfer may provide a suitable therapeutic tool, the available donor material will most likely not contain enough cells in a naïve state of activation. This aspect is of great interest as thymus atrophy and consecutive antigenic re-encounters with several ubiquitous antigens progressively decreases the existence of naïve T cells over time (Blais et al., 2006; Taub and Longo, 2005). Considering these limitations, which subset of antigen-experienced should then be best used for therapeutic adoptive transfers? Certainly a subset of antigen experienced (memory) $CD8^+$ T cells is needed that has a high potential to engraft, but even more important it should still reflect the full potential to differentiate into functionally diverse subsets. In recent experiments we found that highly-purified $CD62L^{high}$ ($CD127^+$) memory $CD8^+$ T cells showed nearly comparable properties in their engraftment and differentiation capacity (Huster et al., 2006a) as do naïve T cells. These cells engrafted as well as naïve T cells after adoptive transfer and we could recover substantial populations even when transferred in very small numbers (10 cells). In addition, these cells generated a robust pool of phenotypically and functionally diverse progeny. The observed subset distribution patterns from $CD62L^{high}$ memory-derived effector cells were markedly different from the endogenous Ag-specific response. This is particularly interesting as besides the development of robust effector subsets ($CD62L^{negative}$ populations), a substantial population of expanded cells still displayed a T_{CM}-like phenotype ($CD62L^+$ $CD127^+$) twelve days after *Listeria* rechallenge, indicating a certain potential for self-renewal. Importantly this was not found to be true for all memory subpopulations, as $CD62L^{low}$ memory $CD8^+$ T cells purified from the same donor mice did not show these attributes. Taken together, these characteristics closely resemble characteristics

defined for stem cells: longevity associated with self-renewal and asymmetric differentiation into identical and further differentiated subsets (Knoblich, 2008).

In addition to our observations, a recent report identified in an experimental murine graft-versus-host model a subset of $CD8^+$ T cells that persisted throughout the course of disease and was also responsible for host injury (Zhang et al., 2005). Astonishingly, this subset was found to be negative for the memory marker CD44 but expressed high levels of CD62L and Sca-1; the latter is known as a marker found on self-renewing (stem) cells. Furthermore this particular subset harbored the capacity to generate diverse effector and memory (T_{CM} and T_{EM}) cells. Whether this observation not only holds true for the $CD62L^{hi}$ T cell subset within an alloreactive model but also for cells responding to conventional infection needs further analysis. Furthermore, if the alloreactive GvH-reactive subset truly represents $CD44^{negative}$ memory cells or a naïve T cell mimicking as memory cell still remains to be proven. The concept of an oligopotent memory stem cell would be further fueled by additional markers that allow precise detection and manipulation of theses cells. In this respect, we found that expression of another stem cell-associated marker, CD34, may serve as a potential candidate. CD34 was expressed on a small subset of $CD62L^{high}$ T_{CM} memory cells residing in LNs. As T_{CM} are known to self-renew, vigorously proliferate after antigen re-encounter and differentiate into diverse effector populations the expression of CD34 on a small proportion of these cells could further hint to the existence of “memory stem cells”. Confirming this idea, first adoptive transfer experiments showed a direct correlation between CD34 expression and good engraftment and proliferation, but further analysis of memory cells are necessary. In this respect, experiments that comprise sequential retransfers of sorted single cell-derived subsets (according to CD62L expression) are currently ongoing to further analyze the existence of cells with a stem cell-like character (communicated with F. Gebhardt).

8.3 Development of $CD4^+$ T cell subsets

Heterogeneity is not only a hallmark of $CD8^+$ T cells but is also a cardinal feature of $CD4^+$ T cells. By extending the fact of protective capacities of T cells in adoptive immunotherapy to $CD4^+$ T cells would strongly increase the application range of this technique for example in treatment of leishmaniasis or tuberculosis. Next to the generation of short- or long-lived memory responses, $CD4^+$ T cells further have to make effector choices to become committed to either T_h1, T_h2, T_h17 fates or even choose to become antigen-specific regulatory T cells (Murphy and Reiner, 2002; Sakaguchi and Powrie, 2007; Stockinger and Veldhoen, 2007).

In parallel to our findings for $CD8^+$ T cells, the most crucial question is whether $CD4^+$ T cells show a comparable plasticity on a single cell level after clonal selection. According to recent findings that $CD4^+$ T cells may also exhibit asymmetric cell divisions (Chang et al., 2007; Yeh et al., 2008), that $CD4^+$ T cells may reengage with DCs after they have divided (Celli et al., 2005), and that T_h1 / T_h2 fates are not adopted until the first divisions, may already point in that direction (Bird et al., 1998). Furthermore, these findings may also imply that at least certain $CD4^+$ effector subsets – comparable to their $CD8^+$ counterparts – might be shaped by additional signals during the expansion phase.

In contrast, whether for example peripherally induced $Foxp3^+$ regulatory T cells or $CD4^+$ T cells belonging to the just recently discovered T_h17 lineage also develop from single naïve precursors or whether they represent separate precursor-derived lineages still remains unknown. Also the influence of lineage determining factors such as polarizing cytokines or the interactions between opposing fate determining transcription factors, the precise time point of their action and the potential to reconvert already programmed differentiation patterns, could be precisely measured by single cell transfer experiments.

In contrast, recent evidence indicates that the requirements for effector and memory differentiation may vary between $CD4^+$ and $CD8^+$ T cells. Although both $CD4^+$ and $CD8^+$ T cells tremendously proliferate after antigen encounter, the kinetics and efficiency of $CD8^+$ T cell proliferation differs substantially from those of $CD4^+$ T cells. The time of antigen exposure required to launch the proliferative program for naïve $CD8^+$ T cells seems to be much shorter than that required for naïve $CD4^+$ T cells and the latter might also need sequential DC contacts. $CD8^+$ T cells also divide sooner and have a faster rate of cell division than do $CD4^+$ T cells (Breart and Bousso, 2006; Garcia et al., 2007; Obst et al., 2005; Seder and Ahmed, 2003). Furthermore, in some instances murine $CD4^+$ memory T cells have shown a gradual decline over time after acute infection – in strong contrast to the stability of $CD8^+$ memory T cell populations (Homann et al., 2001; Schiemann et al., 2003). An additional aspect that might be different from $CD8^+$ T cells concerns the origin of $CD4^+$ memory T cells. Whether $CD4^+$ memory cells arise independently from effector cells early during immune responses or whether they emerge from effector cells is still debated. One very recent publication argued for the emergence of memory cells directly from $CD4^+$ effector cells although the employed reporter gene model fails to formally proof this (Harrington et al., 2008).

We have recently succeeded in transferring the single cell transfer technique to the $CD4^+$ TCR-tg OT-II model specific for the $I\text{-}A^b$ restricted $Ova_{323\text{-}339}$ peptide from chicken ovalbumin (communicated with V. R. Buchholz), allowing us to analyze the development of $CD4^+$ T cell responses. Nevertheless, due to the extremely small population sizes of single cell-derived

$CD4^+$ compared to $CD8^+$ T cell populations, further modifications in acquisition techniques are required for precise characterization of single-cell derived $CD4^+$ T cell responses.

9 SUMMARY

Immunity to intracellular pathogens and some tumors is often dependent on the generation of effective $CD8^+$ T cell responses. Upon first encounter with their cognate antigen, naïve T cells get activated ("primed"), clonally expand, and develop into very distinct subsets. These comprise short-living effector T cells able to confer immediate protection by different types of effector functions, and memory T cell subsets such as effector- and central memory T cells that provide long-lasting enhanced protection against reinfection. Due to these outstanding features, especially memory $CD8^+$ T cells have become an important target population for vaccination. However, effective vaccine design is hampered by our limited understanding of the generation of $CD8^+$ memory T cells.

To obtain further insights into the generation of complex $CD8^+$ T cell responses especially in the development of $CD8^+$ memory, it is of outstanding interest to understand how subset diversity is generated. In particular, the precise knowledge concerning the origin of subset diversity is of special interest. However, so far it has only been possible to tackle questions regarding the origin of diversification and subset development via analysis of indirect parameters. Previous experiments were also limited to global analyses of clonotypic T cell populations without detailed analysis of single cell fates.

To design an experimental system that allows direct analysis of T cell subset generation on a cellular level *in vivo*, we developed a novel adoptive transfer system that allowed for the first time to trace the fate of a single antigen-specific naïve T cell in an otherwise normal (wild-type) recipient mouse. Upon different infection and immunization models, we were able to monitor the differentiation of single cell-derived daughter cells after clonal expansion in an otherwise completely natural *in vivo* setting. The novel experimental approach further allowed us to directly co-analyze and compare the development of adoptively transferred single cells with the endogenously arising Ag-specific $CD8^+$ T cell responses present in the same recipient mouse.

Direct phenotypical and functional analyses of single cell-expanded populations demonstrated that a wide range of diversity could develop out of a single naïve precursor cell. These included the generation of different types of effector cells as well as long-living memory T cell subsets. Most interestingly, we uncovered that subset diversification derived from a single cell strictly reflects the differentiation pattern also found for the polyclonal endogenous repertoire of $CD8^+$ T cells (in the same individual mouse) with the same antigen-specificity. This finding indicated that the origin of subset heterogeneity is already embedded in the intrinsic plasticity within naïve clonal precursor T cells. In addition, the high phenotypical and functional homology between

transferred and endogenous T cells implicates an important role for factors operative during the expansion phase that lead to an immunization dependent shaping of Ag-specific $CD8^+$ T cell populations.

Perhaps even more important, single cell-derived $CD8^+$ T cell populations were found to confer protection to immunocompromised mice lacking endogenous T and B cells. The functional capacity of these cells was sufficient to completely clear infection with an intracellular pathogen and prevent recipient mice to succumb to infection with an otherwise lethal infection dose.

In conclusion, we could establish a novel adoptive transfer system that for the first time allowed direct visualization and analysis of single-cell derived $CD8^+$ T cell populations participating in an immune response. This novel experimental method is not only a valuable tool for basic T cell research, but also demonstrated important implications in T cell biology as well as for clinical applications in the field of adoptive T cell therapy and vaccination.

10 REFERENCES

Abbas, A. K., and Lichtman, A. H. (2007). Cellular and Molecular Immunology, 5th edn, Saunders - Elsevier Science).

Ahmed, R., and Gray, D. (1996). Immunological memory and protective immunity: understanding their relation. Science *272*, 54-60.

Altman, J. D., Moss, P. A., Goulder, P. J., Barouch, D. H., McHeyzer-Williams, M. G., Bell, J. I., McMichael, A. J., and Davis, M. M. (1996). Phenotypic analysis of antigen-specific T lymphocytes. Science *274*, 94-96.

Arstila, T. P., Casrouge, A., Baron, V., Even, J., Kanellopoulos, J., and Kourilsky, P. (1999). A direct estimate of the human alphabeta T cell receptor diversity. Science *286*, 958-961.

Bachmann, M. F., Beerli, R. R., Agnellini, P., Wolint, P., Schwarz, K., and Oxenius, A. (2006). Long-lived memory CD8+ T cells are programmed by prolonged antigen exposure and low levels of cellular activation. Eur J Immunol *36*, 842-854.

Bachmann, M. F., Wolint, P., Schwarz, K., Jager, P., and Oxenius, A. (2005a). Functional properties and lineage relationship of CD8+ T cell subsets identified by expression of IL-7 receptor alpha and CD62L. J Immunol *175*, 4686-4696.

Bachmann, M. F., Wolint, P., Schwarz, K., and Oxenius, A. (2005b). Recall proliferation potential of memory CD8+ T cells and antiviral protection. J Immunol *175*, 4677-4685.

Bachmann, M. F., Wolint, P., Walton, S., Schwarz, K., and Oxenius, A. (2007). Differential role of IL-2R signaling for CD8+ T cell responses in acute and chronic viral infections. Eur J Immunol *37*, 1502-1512.

Badovinac, V. P., Haring, J. S., and Harty, J. T. (2007). Initial T Cell Receptor Transgenic Cell Precursor Frequency Dictates Critical Aspects of the CD8(+) T Cell Response to Infection. Immunity.

Badovinac, V. P., and Harty, J. T. (2007). Manipulating the Rate of Memory CD8+ T Cell Generation after Acute Infection. J Immunol *179*, 53-63.

Badovinac, V. P., Messingham, K. A., Jabbari, A., Haring, J. S., and Harty, J. T. (2005). Accelerated CD8+ T-cell memory and prime-boost response after dendritic-cell vaccination. Nat Med *11*, 748-756.

Badovinac, V. P., Porter, B. B., and Harty, J. T. (2002). Programmed contraction of CD8(+) T cells after infection. Nat Immunol *3*, 619-626.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B., and Palucka, K. (2000). Immunobiology of dendritic cells. Annu Rev Immunol *18*, 767-811.

Baron, V., Bouneaud, C., Cumano, A., Lim, A., Arstila, T. P., Kourilsky, P., Ferradini, L., and Pannetier, C. (2003). The repertoires of circulating human CD8(+) central and effector memory T cell subsets are largely distinct. Immunity *18*, 193-204.

Bennett, S. R., Carbone, F. R., Karamalis, F., Flavell, R. A., Miller, J. F., and Heath, W. R. (1998). Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature *393*, 478-480.

Betts, M. R., Brenchley, J. M., Price, D. A., De Rosa, S. C., Douek, D. C., Roederer, M., and Koup, R. A. (2003). Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J Immunol Methods *281*, 65-78.

Bevan, M. J., and Fink, P. J. (2001). The CD8 response on autopilot. Nat Immunol *2*, 381-382.

Bird, J. J., Brown, D. R., Mullen, A. C., Moskowitz, N. H., Mahowald, M. A., Sider, J. R., Gajewski, T. F., Wang, C. R., and Reiner, S. L. (1998). Helper T cell differentiation is controlled by the cell cycle. Immunity *9*, 229-237.

Blais, M. E., Louis, I., and Perreault, C. (2006). T-cell development: an extrathymic perspective. Immunol Rev *209*, 103-114.

Blattman, J. N., Antia, R., Sourdive, D. J., Wang, X., Kaech, S. M., Murali-Krishna, K., Altman, J. D., and Ahmed, R. (2002). Estimating the precursor frequency of naive antigen-specific CD8 T cells. J Exp Med *195*, 657-664.

Bouneaud, C., Garcia, Z., Kourilsky, P., and Pannetier, C. (2005). Lineage relationships, homeostasis, and recall capacities of central- and effector-memory CD8 T cells in vivo. J Exp Med *201*, 579-590.

Bousso, P., and Robey, E. (2003). Dynamics of CD8+ T cell priming by dendritic cells in intact lymph nodes. Nat Immunol *4*, 579-585.

Breart, B., and Bousso, P. (2006). Cellular orchestration of T cell priming in lymph nodes. Curr Opin Immunol *18*, 483-490.

Busch, D. H., Kerksiek, K. M., and Pamer, E. G. (2000). Differing roles of inflammation and antigen in T cell proliferation and memory generation. J Immunol *164*, 4063-4070.

Busch, D. H., and Pamer, E. G. (1999). T lymphocyte dynamics during Listeria monocytogenes infection. Immunol Lett *65*, 93-98.

Busch, D. H., Pilip, I. M., Vijh, S., and Pamer, E. G. (1998). Coordinate regulation of complex T cell populations responding to bacterial infection. Immunity *8*, 353-362.

Carreno, B. M., and Collins, M. (2002). The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. Annu Rev Immunol *20*, 29-53.

Carrio, R., Rolle, C. E., and Malek, T. R. (2007). Non-redundant role for IL-7R signaling for the survival of CD8+ memory T cells. Eur J Immunol *37*, 3078-3088.

Castellino, F., and Germain, R. N. (2006). Cooperation between CD4+ and CD8+ T cells: when, where, and how. Annu Rev Immunol *24*, 519-540.

Catron, D. M., Rusch, L. K., Hataye, J., Itano, A. A., and Jenkins, M. K. (2006). CD4+ T cells that enter the draining lymph nodes after antigen injection participate in the primary response and become central-memory cells. J Exp Med *203*, 1045-1054.

Celli, S., Garcia, Z., and Bousso, P. (2005). CD4 T cells integrate signals delivered during successive DC encounters in vivo. J Exp Med *202*, 1271-1278.

Cerwenka, A., Morgan, T. M., and Dutton, R. W. (1999). Naive, effector, and memory CD8 T cells in protection against pulmonary influenza virus infection: homing properties rather than initial frequencies are crucial. J Immunol *163*, 5535-5543.

Chang, J. T., Palanivel, V. R., Kinjyo, I., Schambach, F., Intlekofer, A. M., Banerjee, A., Longworth, S. A., Vinup, K. E., Mrass, P., Oliaro, J.*, et al.* (2007). Asymmetric T lymphocyte division in the initiation of adaptive immune responses. Science *315*, 1687-1691.

Chang, J. T., and Reiner, S. L. (2007). Protection one cell thick. Immunity *27*, 832-834.

Cobbold, M., Khan, N., Pourgheysari, B., Tauro, S., McDonald, D., Osman, H., Assenmacher, M., Billingham, L., Steward, C., Crawley, C.*, et al.* (2005). Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers. J Exp Med *202*, 379-386.

Croft, M. (2003). Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? Nat Rev Immunol *3*, 609-620.

D'Souza, W. N., and Hedrick, S. M. (2006). Cutting edge: latecomer CD8 T cells are imprinted with a unique differentiation program. J Immunol *177*, 777-781.

Dooms, H., Wolslegel, K., Lin, P., and Abbas, A. K. (2007). Interleukin-2 enhances CD4+ T cell memory by promoting the generation of IL-7Ralpha-expressing cells. J Exp Med *204*, 547-557.

Dudley, M. E., Wunderlich, J., Nishimura, M. I., Yu, D., Yang, J. C., Topalian, S. L., Schwartzentruber, D. J., Hwu, P., Marincola, F. M., Sherry, R.*, et al.* (2001). Adoptive transfer of cloned melanoma-reactive T lymphocytes for the treatment of patients with metastatic melanoma. J Immunother *24*, 363-373.

Dudley, M. E., Wunderlich, J. R., Robbins, P. F., Yang, J. C., Hwu, P., Schwartzentruber, D. J., Topalian, S. L., Sherry, R., Restifo, N. P., Hubicki, A. M.*, et al.* (2002). Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science *298*, 850-854.

Dustin, M. L., and Chan, A. C. (2000). Signaling takes shape in the immune system. Cell *103*, 283-294.

Ernst, B., Lee, D. S., Chang, J. M., Sprent, J., and Surh, C. D. (1999). The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. Immunity *11*, 173-181.

Ferlazzo, G., Tsang, M. L., Moretta, L., Melioli, G., Steinman, R. M., and Munz, C. (2002). Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. J Exp Med *195*, 343-351.

Fontenot, J. D., and Rudensky, A. Y. (2005). A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. Nat Immunol *6*, 331-337.

Garcia, Z., Pradelli, E., Celli, S., Beuneu, H., Simon, A., and Bousso, P. (2007). Competition for antigen determines the stability of T cell-dendritic cell interactions during clonal expansion. Proc Natl Acad Sci U S A *104*, 4553-4558.

Gattinoni, L., Powell, D. J., Jr., Rosenberg, S. A., and Restifo, N. P. (2006). Adoptive immunotherapy for cancer: building on success. Nat Rev Immunol *6*, 383-393.

Geginat, J., Lanzavecchia, A., and Sallusto, F. (2003). Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines. Blood *101*, 4260-4266.

Gett, A. V., Sallusto, F., Lanzavecchia, A., and Geginat, J. (2003). T cell fitness determined by signal strength. Nat Immunol *4*, 355-360.

Goldrath, A. W., Bogatzki, L. Y., and Bevan, M. J. (2000). Naive T cells transiently acquire a memory-like phenotype during homeostasis-driven proliferation. J Exp Med *192*, 557-564.

Goldrath, A. W., Sivakumar, P. V., Glaccum, M., Kennedy, M. K., Bevan, M. J., Benoist, C., Mathis, D., and Butz, E. A. (2002). Cytokine requirements for acute and Basal homeostatic proliferation of naive and memory CD8+ T cells. J Exp Med *195*, 1515-1522.

Guarda, G., Hons, M., Soriano, S. F., Huang, A. Y., Polley, R., Martín-Fontecha, A., Stein, J. V., Germain, R. N., Lanzavecchia, A., and Sallusto, F. (2007). L-selectin-negative CCR7(-) effector and memory CD8(+) T cells enter reactive lymph nodes and kill dendritic cells. Nat Immunol *8*, 743-752.

Hamann, D., Baars, P. A., Rep, M. H., Hooibrink, B., Kerkhof-Garde, S. R., Klein, M. R., and van Lier, R. A. (1997). Phenotypic and functional separation of memory and effector human CD8+ T cells. J Exp Med *186*, 1407-1418.

Hamilton, S. E., Wolkers, M. C., Schoenberger, S. P., and Jameson, S. C. (2006). The generation of protective memory-like CD8+ T cells during homeostatic proliferation requires CD4+ T cells. Nat Immunol *7*, 475-481.

Hammarlund, E., Lewis, M. W., Hansen, S. G., Strelow, L. I., Nelson, J. A., Sexton, G. J., Hanifin, J. M., and Slifka, M. K. (2003). Duration of antiviral immunity after smallpox vaccination. Nat Med *9*, 1131-1137.

Hand, T. W., Morre, M., and Kaech, S. M. (2007). Expression of IL-7 receptor alpha is necessary but not sufficient for the formation of memory CD8 T cells during viral infection. Proc Natl Acad Sci U S A *104*, 11730-11735.

Harrington, L. E., Janowski, K. M., Oliver, J. R., Zajac, A. J., and Weaver, C. T. (2008). Memory CD4 T cells emerge from effector T-cell progenitors. Nature *452*, 356-360.

Harty, J. T., and Badovinac, V. P. (2008). Shaping and reshaping CD8+ T-cell memory. Nat Rev Immunol *8*, 107-119.

Hataye, J., Moon, J. J., Khoruts, A., Reilly, C., and Jenkins, M. K. (2006). Naive and memory CD4+ T cell survival controlled by clonal abundance. Science *312*, 114-116.

Heit, A., Busch, D. H., Wagner, H., and Schmitz, F. (2008). Vaccine protocols for enhanced immunogenicity of exogenous antigens. Int J Med Microbiol *298*, 27-32.

Hendriks, J., Gravestein, L. A., Tesselaar, K., van Lier, R. A., Schumacher, T. N., and Borst, J. (2000). CD27 is required for generation and long-term maintenance of T cell immunity. Nat Immunol *1*, 433-440.

Heslop, H. E., and Rooney, C. M. (1997). Adoptive cellular immunotherapy for EBV lymphoproliferative disease. Immunol Rev *157*, 217-222.

Hikono, H., Kohlmeier, J. E., Takamura, S., Wittmer, S. T., Roberts, A. D., and Woodland, D. L. (2007). Activation phenotype, rather than central- or effector-memory phenotype, predicts the recall efficacy of memory CD8+ T cells. J Exp Med.

Hogan, R. J., Usherwood, E. J., Zhong, W., Roberts, A. A., Dutton, R. W., Harmsen, A. G., and Woodland, D. L. (2001). Activated antigen-specific CD8+ T cells persist in the lungs following recovery from respiratory virus infections. J Immunol *166*, 1813-1822.

Homann, D., Teyton, L., and Oldstone, M. B. (2001). Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. Nat Med *7*, 913-919.

Huster, K. M., Busch, V., Schiemann, M., Linkemann, K., Kerksiek, K. M., Wagner, H., and Busch, D. H. (2004). Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8+ memory T cell subsets. Proc Natl Acad Sci USA *101*, 5610-5615.

Huster, K. M., Koffler, M., Stemberger, C., Schiemann, M., Wagner, H., and Busch, D. H. (2006a). Unidirectional development of CD8+ central memory T cells into protective Listeria-specific effector memory T cells. Eur J Immunol *36*, 1453-1464.

Huster, K. M., Stemberger, C., and Busch, D. H. (2006b). Protective immunity towards intracellular pathogens. Curr Opin Immunol *18*, 458-464.

Jacob, J., and Baltimore, D. (1999). Modelling T-cell memory by genetic marking of memory T cells in vivo. Nature *399*, 593-597.

Janeway, C. A., Jr., and Medzhitov, R. (2002). Innate immune recognition. Annu Rev Immunol *20*, 197-216.

Janeway, C. A., Travers, P., and Walport, M. (2005). Immunobiology: the immune System in health and Disease, 6th edition edn, Garland Science Publishing).

Janssen, E. M., Droin, N. M., Lemmens, E. E., Pinkoski, M. J., Bensinger, S. J., Ehst, B. D., Griffith, T. S., Green, D. R., and Schoenberger, S. P. (2005). CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. Nature *434*, 88-93.

Janssen, E. M., Lemmens, E. E., Wolfe, T., Christen, U., von Herrath, M. G., and Schoenberger, S. P. (2003). CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. Nature *421*, 852-856.

Joshi, N. S., Cui, W., Chandele, A., Lee, H. K., Urso, D. R., Hagman, J., Gapin, L., and Kaech, S. M. (2007). Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. Immunity *27*, 281-295.

Jung, S., Unutmaz, D., Wong, P., Sano, G., De los Santos, K., Sparwasser, T., Wu, S., Vuthoori, S., Ko, K., Zavala, F.*, et al.* (2002). In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. Immunity *17*, 211-220.

Jung, T., Schauer, U., Heusser, C., Neumann, C., and Rieger, C. (1993). Detection of intracellular cytokines by flow cytometry. J Immunol Methods *159*, 197-207.

Kaech, S. M., and Ahmed, R. (2001). Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. Nat Immunol *2*, 415-422.

Kaech, S. M., Hemby, S., Kersh, E., and Ahmed, R. (2002). Molecular and functional profiling of memory CD8 T cell differentiation. Cell *111*, 837-851.

Kaech, S. M., Tan, J. T., Wherry, E. J., Konieczny, B. T., Surh, C. D., and Ahmed, R. (2003). Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nat Immunol *4*, 1191-1198.

Kaech, S. M., and Wherry, E. J. (2007). Heterogeneity and Cell-Fate Decisions in Effector and Memory CD8(+) T Cell Differentiation during Viral Infection. Immunity *27*, 393-405.

Kamimura, D., and Bevan, M. J. (2007). Naive CD8+ T cells differentiate into protective memory-like cells after IL-2 anti IL-2 complex treatment in vivo. J Exp Med *204*, 1803-1812.

Kearney, E. R., Pape, K. A., Loh, D. Y., and Jenkins, M. K. (1994). Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. Immunity *1*, 327-339.

Kieper, W. C., and Jameson, S. C. (1999). Homeostatic expansion and phenotypic conversion of naive T cells in response to self peptide/MHC ligands. Proc Natl Acad Sci U S A *96*, 13306-13311.

Knabel, M., Franz, T. J., Schiemann, M., Wulf, A., Villmow, B., Schmidt, B., Bernhard, H., Wagner, H., and Busch, D. H. (2002). Reversible MHC multimer staining for functional isolation of T-cell populations and effective adoptive transfer. Nat Med *8*, 631-637.

Knoblich, J. A. (2008). Mechanisms of asymmetric stem cell division. Cell *132*, 583-597.

Kolumam, G. A., Thomas, S., Thompson, L. J., Sprent, J., and Murali-Krishna, K. (2005). Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. J Exp Med *202*, 637-650.

Lanzavecchia, A., and Sallusto, F. (2000). Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. Science *290*, 92-97.

Lanzavecchia, A., and Sallusto, F. (2002). Progressive differentiation and selection of the fittest in the immune response. Nat Rev Immunol *2*, 982-987.

Lanzavecchia, A., and Sallusto, F. (2005). Understanding the generation and function of memory T cell subsets. Curr Opin Immunol *17*, 326-332.

Lau, L. L., Jamieson, B. D., Somasundaram, T., and Ahmed, R. (1994). Cytotoxic T-cell memory without antigen. Nature *369*, 648-652.

Lauvau, G., Vijh, S., Kong, P., Horng, T., Kerksiek, K., Serbina, N., Tuma, R. A., and Pamer, E. G. (2001). Priming of memory but not effector CD8 T cells by a killed bacterial vaccine. Science *294*, 1735-1739.

Lipscomb, M. F., and Masten, B. J. (2002). Dendritic cells: immune regulators in health and disease. Physiol Rev *82*, 97-130.

Liu, Y. J., Soumelis, V., Watanabe, N., Ito, T., Wang, Y. H., Malefyt Rde, W., Omori, M., Zhou, B., and Ziegler, S. F. (2007). TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. Annu Rev Immunol *25*, 193-219.

Lotz, M., Menard, S., and Hornef, M. (2007). Innate immune recognition on the intestinal mucosa. Int J Med Microbiol *297*, 379-392.

Manjunath, N., Shankar, P., Wan, J., Weninger, W., Crowley, M. A., Hieshima, K., Springer, T. A., Fan, X., Shen, H., Lieberman, J., and von Andrian, U. H. (2001). Effector differentiation is not prerequisite for generation of memory cytotoxic T lymphocytes. J Clin Invest *108*, 871-878.

Marzo, A. L., Klonowski, K. D., Le Bon, A., Borrow, P., Tough, D. F., and Lefrancois, L. (2005). Initial T cell frequency dictates memory CD8+ T cell lineage commitment. Nat Immunol *6*, 793-799.

Marzo, A. L., Yagita, H., and Lefrancois, L. (2007). Cutting edge: migration to nonlymphoid tissues results in functional conversion of central to effector memory CD8 T cells. J Immunol *179*, 36-40.

Masopust, D., Vezys, V., Marzo, A. L., and Lefrancois, L. (2001). Preferential localization of effector memory cells in nonlymphoid tissue. Science *291*, 2413-2417.

Mempel, T. R., Henrickson, S. E., and Von Andrian, U. H. (2004). T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. Nature *427*, 154-159.

Mercado, R., Vijh, S., Allen, S. E., Kerksiek, K., Pilip, I. M., and Pamer, E. G. (2000). Early programming of T cell populations responding to bacterial infection. J Immunol *165*, 6833-6839.

Mescher, M. F., Curtsinger, J. M., Agarwal, P., Casey, K. A., Gerner, M., Hammerbeck, C. D., Popescu, F., and Xiao, Z. (2006). Signals required for programming effector and memory development by CD8+ T cells. Immunol Rev *211*, 81-92.

Moon, J. J., Chu, H. H., Pepper, M., McSorley, S. J., Jameson, S. C., Kedl, R. M., and Jenkins, M. K. (2007). Naive CD4(+) T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. Immunity *27*, 203-213.

Moss, P., and Rickinson, A. (2005). Cellular immunotherapy for viral infection after HSC transplantation. Nat Rev Immunol *5*, 9-20.

Murali-Krishna, K., and Ahmed, R. (2000). Cutting edge: naive T cells masquerading as memory cells. J Immunol *165*, 1733-1737.

Murali-Krishna, K., Lau, L. L., Sambhara, S., Lemonnier, F., Altman, J., and Ahmed, R. (1999). Persistence of memory CD8 T cells in MHC class I-deficient mice. Science *286*, 1377-1381.

Murphy, K. M., and Reiner, S. L. (2002). The lineage decisions of helper T cells. Nat Rev Immunol *2*, 933-944.

Nesic, D., and Vukmanovic, S. (1998). MHC class I is required for peripheral accumulation of CD8+ thymic emigrants. J Immunol *160*, 3705-3712.

Neudorfer, J., Schmidt, B., Huster, K. M., Anderl, F., Schiemann, M., Holzapfel, G., Schmidt, T., Germeroth, L., Wagner, H., Peschel, C*., et al.* (2007). Reversible HLA multimers (Streptamers) for the isolation of human cytotoxic T lymphocytes functionally active against tumor- and virus-derived antigens. J Immunol Methods *320*, 119-131.

Neuenhahn, M., and Busch, D. H. (2007). Unique functions of splenic CD8alpha(+) dendritic cells during infection with intracellular pathogens. Immunol Lett.

Neuenhahn, M., Kerksiek, K. M., Nauerth, M., Suhre, M. H., Schiemann, M., Gebhardt, F. E., Stemberger, C., Panthel, K., Schroder, S., Chakraborty, T*., et al.* (2006). CD8alpha+ dendritic cells are required for efficient entry of Listeria monocytogenes into the spleen. Immunity *25*, 619-630.

Northrop, J. K., Thomas, R. M., Wells, A. D., and Shen, H. (2006). Epigenetic remodeling of the IL-2 and IFN-gamma loci in memory CD8 T cells is influenced by CD4 T cells. J Immunol *177*, 1062-1069.

Obst, R., van Santen, H. M., Mathis, D., and Benoist, C. (2005). Antigen persistence is required throughout the expansion phase of a CD4(+) T cell response. J Exp Med *201*, 1555-1565.

Oldenhove, G., de Heusch, M., Urbain-Vansanten, G., Urbain, J., Maliszewski, C., Leo, O., and Moser, M. (2003). CD4+ CD25+ regulatory T cells control T helper cell type 1 responses to foreign antigens induced by mature dendritic cells in vivo. J Exp Med *198*, 259-266.

Opferman, J. T., Ober, B. T., and Ashton-Rickardt, P. G. (1999). Linear differentiation of cytotoxic effectors into memory T lymphocytes. Science *283*, 1745-1748.

Pamer, E., and Cresswell, P. (1998). Mechanisms of MHC class I--restricted antigen processing. Annu Rev Immunol *16*, 323-358.

Pamer, E. G. (2004). Immune responses to Listeria monocytogenes. Nat Rev Immunol *4*, 812-823.

Pantaleo, G., and Koup, R. A. (2004). Correlates of immune protection in HIV-1 infection: what we know, what we don't know, what we should know. Nat Med *10*, 806-810.

Park, J. H., Yu, Q., Erman, B., Appelbaum, J. S., Montoya-Durango, D., Grimes, H. L., and Singer, A. (2004). Suppression of IL7Ralpha transcription by IL-7 and other prosurvival cytokines: a novel mechanism for maximizing IL-7-dependent T cell survival. Immunity *21*, 289-302.

Pope, C., Kim, S. K., Marzo, A., Masopust, D., Williams, K., Jiang, J., Shen, H., and Lefrancois, L. (2001). Organ-specific regulation of the CD8 T cell response to Listeria monocytogenes infection. J Immunol *166*, 3402-3409.

Prlic, M., Hernandez-Hoyos, G., and Bevan, M. J. (2006). Duration of the initial TCR stimulus controls the magnitude but not functionality of the CD8+ T cell response. J Exp Med *203*, 2135-2143.

Prlic, M., Lefrancois, L., and Jameson, S. C. (2002). Multiple choices: regulation of memory CD8 T cell generation and homeostasis by interleukin (IL)-7 and IL-15. J Exp Med *195*, F49-52.

Pulendran, B., and Ahmed, R. (2006). Translating innate immunity into immunological memory: implications for vaccine development. Cell *124*, 849-863.

Reiner, S. L., Sallusto, F., and Lanzavecchia, A. (2007). Division of labor with a workforce of one: challenges in specifying effector and memory T cell fate. Science *317*, 622-625.

Reinhardt, R. L., Khoruts, A., Merica, R., Zell, T., and Jenkins, M. K. (2001). Visualizing the generation of memory CD4 T cells in the whole body. Nature *410*, 101-105.

Riddell, S. R., and Greenberg, P. D. (1995). Principles for adoptive T cell therapy of human viral diseases. Annu Rev Immunol *13*, 545-586.

Riddell, S. R., Watanabe, K. S., Goodrich, J. M., Li, C. R., Agha, M. E., and Greenberg, P. D. (1992). Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. Science *257*, 238-241.

Ridge, J. P., Di Rosa, F., and Matzinger, P. (1998). A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature *393*, 474-478.

Sakaguchi, S. (2005). Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. Nat Immunol *6*, 345-352.

Sakaguchi, S., and Powrie, F. (2007). Emerging challenges in regulatory T cell function and biology. Science *317*, 627-629.

Sallusto, F., Lenig, D., Forster, R., Lipp, M., and Lanzavecchia, A. (1999). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature *401*, 708-712.

Sarkar, S., Kalia, V., Haining, W. N., Konieczny, B. T., Subramaniam, S., and Ahmed, R. (2008). Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. J Exp Med.

Schiemann, M., Busch, V., Linkemann, K., Huster, K. M., and Busch, D. H. (2003). Differences in maintenance of CD8+ and CD4+ bacteria-specific effector-memory T cell populations. Eur J Immunol *33*, 2875-2885.

Schluns, K. S., Kieper, W. C., Jameson, S. C., and Lefrancois, L. (2000). Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. Nat Immunol *1*, 426-432.

Schoenberger, S. P., Toes, R. E., van der Voort, E. I., Offringa, R., and Melief, C. J. (1998). T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature *393*, 480-483.

Schuler, T., Hammerling, G. J., and Arnold, B. (2004). Cutting edge: IL-7-dependent homeostatic proliferation of CD8+ T cells in neonatal mice allows the generation of long-lived natural memory T cells. J Immunol *172*, 15-19.

Schwartz, R. H. (2003). T cell anergy. Annu Rev Immunol *21*, 305-334.

Seder, R. A., and Ahmed, R. (2003). Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. Nat Immunol *4*, 835-842.

Seder, R. A., Darrah, P. A., and Roederer, M. (2008). T-cell quality in memory and protection: implications for vaccine design. Nat Rev Immunol *8*, 247-258.

Shedlock, D. J., and Shen, H. (2003). Requirement for CD4 T cell help in generating functional CD8 T cell memory. Science *300*, 337-339.

Shin, H., Blackburn, S. D., Blattman, J. N., and Wherry, E. J. (2007). Viral antigen and extensive division maintain virus-specific CD8 T cells during chronic infection. J Exp Med *204*, 941-949.

Stemberger, C., Neuenhahn, M., Buchholz, V. R., and Busch, D. H. (2007). Origin of CD8+ Effector and Memory T Cell Subsets. Cell Mol Immunol *4*, 399-405.

Stockinger, B., and Veldhoen, M. (2007). Differentiation and function of Th17 T cells. Curr Opin Immunol *19*, 281-286.

Stoll, S., Delon, J., Brotz, T. M., and Germain, R. N. (2002). Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. Science *296*, 1873-1876.

Sun, J. C., and Bevan, M. J. (2003). Defective CD8 T cell memory following acute infection without CD4 T cell help. Science *300*, 339-342.

Sun, J. C., Lehar, S. M., and Bevan, M. J. (2006). Augmented IL-7 signaling during viral infection drives greater expansion of effector T cells but does not enhance memory. J Immunol *177*, 4458-4463.

Sun, J. C., Williams, M. A., and Bevan, M. J. (2004). CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. Nat Immunol *5*, 927-933.

Tan, J. T., Ernst, B., Kieper, W. C., LeRoy, E., Sprent, J., and Surh, C. D. (2002). Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells. J Exp Med *195*, 1523-1532.

Tang, Q., and Bluestone, J. A. (2008). The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. Nat Immunol *9*, 239-244.

Taub, D. D., and Longo, D. L. (2005). Insights into thymic aging and regeneration. Immunol Rev *205*, 72-93.

Tewari, K., Walent, J., Svaren, J., Zamoyska, R., and Suresh, M. (2006). Differential requirement for Lck during primary and memory CD8+ T cell responses. Proc Natl Acad Sci U S A *103*, 16388-16393.

Tuma, R. A., and Pamer, E. G. (2002). Homeostasis of naive, effector and memory CD8 T cells. Curr Opin Immunol *14*, 348-353.

Unsoeld, H., and Pircher, H. (2005). Complex memory T-cell phenotypes revealed by coexpression of CD62L and CCR7. J Virol *79*, 4510-4513.

van Faassen, H., Saldanha, M., Gilbertson, D., Dudani, R., Krishnan, L., and Sad, S. (2005). Reducing the stimulation of CD8+ T cells during infection with intracellular bacteria promotes differentiation primarily into a central (CD62LhighCD44high) subset. J Immunol *174*, 5341-5350.

van Stipdonk, M. J., Lemmens, E. E., and Schoenberger, S. P. (2001). Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. Nat Immunol *2*, 423-429.

Vieira, P. L., Christensen, J. R., Minaee, S., O'Neill, E. J., Barrat, F. J., Boonstra, A., Barthlott, T., Stockinger, B., Wraith, D. C., and O'Garra, A. (2004). IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. J Immunol *172*, 5986-5993.

Vinuesa, C. G., Tangye, S. G., Moser, B., and Mackay, C. R. (2005). Follicular B helper T cells in antibody responses and autoimmunity. Nat Rev Immunol *5*, 853-865.

Viret, C., Wong, F. S., and Janeway, C. A., Jr. (1999). Designing and maintaining the mature TCR repertoire: the continuum of self-peptide:self-MHC complex recognition. Immunity *10*, 559-568.

Walter, E. A., Greenberg, P. D., Gilbert, M. J., Finch, R. J., Watanabe, K. S., Thomas, E. D., and Riddell, S. R. (1995). Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N Engl J Med *333*, 1038-1044.

Weaver, C. T., Hatton, R. D., Mangan, P. R., and Harrington, L. E. (2007). IL-17 family cytokines and the expanding diversity of effector T cell lineages. Annu Rev Immunol *25*, 821-852.

Wherry, E. J., Teichgraber, V., Becker, T. C., Masopust, D., Kaech, S. M., Antia, R., von Andrian, U. H., and Ahmed, R. (2003). Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat Immunol *4*, 225-234.

Whitmire, J. K., Tan, J. T., and Whitton, J. L. (2005). Interferon-gamma acts directly on CD8+ T cells to increase their abundance during virus infection. J Exp Med *201*, 1053-1059.

Williams, M. A., and Bevan, M. J. (2006). Effector and Memory CTL Differentiation. Annu Rev Immunol.

Williams, M. A., Holmes, B. J., Sun, J. C., and Bevan, M. J. (2006a). Developing and maintaining protective CD8+ memory T cells. Immunol Rev *211*, 146-153.

Williams, M. A., Tyznik, A. J., and Bevan, M. J. (2006b). Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. Nature *441*, 890-893.

Wong, P., Lara-Tejero, M., Ploss, A., Leiner, I., and Pamer, E. G. (2004). Rapid development of T cell memory. J Immunol *172*, 7239-7245.

Wong, P., and Pamer, E. G. (2003). Feedback regulation of pathogen-specific T cell priming. Immunity *18*, 499-511.

Yang, J., Huck, S. P., McHugh, R. S., Hermans, I. F., and Ronchese, F. (2006). Perforin-dependent elimination of dendritic cells regulates the expansion of antigen-specific CD8+ T cells in vivo. Proc Natl Acad Sci U S A *103*, 147-152.

Yee, C., Thompson, J. A., Byrd, D., Riddell, S. R., Roche, P., Celis, E., and Greenberg, P. D. (2002). Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. Proc Natl Acad Sci U S A *99*, 16168-16173.

Yeh, J. H., Sidhu, S. S., and Chan, A. C. (2008). Regulation of a late phase of T cell polarity and effector functions by Crtam. Cell *132*, 846-859.

Zeng, R., Spolski, R., Finkelstein, S. E., Oh, S., Kovanen, P. E., Hinrichs, C. S., Pise-Masison, C. A., Radonovich, M. F., Brady, J. N., Restifo, N. P.*, et al.* (2005). Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function. J Exp Med *201*, 139-148.

Zhang, M., Byrne, S., Liu, N., Wang, Y., Oxenius, A., and Ashton-Rickardt, P. G. (2007). Differential survival of cytotoxic T cells and memory cell precursors. J Immunol *178*, 3483-3491.

Zhang, Y., Joe, G., Hexner, E., Zhu, J., and Emerson, S. G. (2005). Host-reactive CD8+ memory stem cells in graft-versus-host disease. Nat Med *11*, 1299-1305.

Ziegler, S. F. (2006). FOXP3: of mice and men. Annu Rev Immunol *24*, 209-226.

11 ACKNOWLEDGMENTS

An dieser Stelle möchte ich die Gelegenheit wahrnehmen all den Leuten zu danken, die mich während meiner Doktorarbeit praktisch und geistig unterstützt haben, und ohne die ein Gelingen der Arbeit nicht möglich gewesen wäre.

Großer Dank geht an meinen Arbeitsgruppenleiter Prof. Dirk Busch, der mich stets vorbildlich während meiner Arbeit betreut hat und für mich ein großartiger Lehrer war. Auch für die Möglichkeit an diesem renommierten Institut zu forschen und meine Erfolge auf internationalen Kongressen vorzustellen, möchte ich mich bei Prof. Busch und nicht zuletzt bei unserem Institutsleiter Prof. Herman Wagner erkenntlich zeigen. Des Weiteren danke ich Prof. Alfons Gierl für die Betreuung meiner Dissertation sowie Prof. Siegfried Scherer für den Vorsitz der Prüfungskommission.

Insbesondere gilt mein Dank der großartigen Arbeitsgruppe Busch. Die freundschaftliche, kommunikative und wissenschaftlich fundierte Atmosphäre im Labor war bei zähen und schwierigen Versuchen über all die Jahre eine essentielle Quelle, um neue Kraft zu schöpfen und hilfreiche Anregungen zu erhalten. Zunächst möchte ich Dr. Friedemann Gebhardt, Veit Buchholz und Dr. Michael Neuenhahn erwähnen mit denen es immer ein Genuss war schwierige Ergebnisse umfassend zu diskutieren und auch unsere geliebten Mäuse zu versorgen. Des Weiteren wären ohne die exzellenten Sortfähigkeiten und IT-Kenntnisse von Dr. Matthias Schiemann viele Experimente nicht durchzuführen gewesen. Für erheiternde Diskussionen und große Unterstützung möchte ich mich ganz besonders bei Dr. Katharina Huster bedanken. Im Speziellen möchte ich natürlich noch ganz herzlich bei Magdalena Nauerth, Dr. Anja Kriegeskorte, Florian Anderl und Dr. Robert Knall dafür bedanken, daß sie mit mir das Doktorandendasein gemeistert haben, daß sie immer ein offenes Ohr hatten, immer für einen Spaß zu haben waren und vor Allem aber für die zahlreichen nervenaufreibenden Kickerspiele.

Ein extra großes Dankeschön geht an Anna Hochholzer, Katleen Götsch und Martina Koffler für Ihre exzellente technische- und freundschaftliche Unterstützung. Vielen Dank Martina für deine enorme Hilfe!

Darüber hinaus bin ich sehr dankbar für die fruchtbaren Kooperationen und Diskussionen mit Dr. Wolfgang Kastenmüller und Dr. Georg Gasteiger. Nicht nur die stets prompte Belieferung mit Reagenzien und technischem „Know-How“ sondern auch die zahlreichen abendlichen Fußballspiele werden mir in sehr schöner Erinnerung bleiben.

Nicht zuletzt möchte ich mich bei meinen privaten „Kooperationspartnern“ bedanken. Hier stehen natürlich meine Eltern an vorderster Stelle, auf die ich mich immer verlassen kann und die mich zu jeder Zeit unterstützten und mir viel Rückhalt geben. Daneben war für mich natürlich Elisabeth die größte Unterstützung die man sich vorstellen kann – nicht nur während des Endspurts – sondern auch wenn es einmal nicht so gut lief! Zu guter letzt geht natürlich auch ein ganz großes Danke an alle meine Freunde, die für die nötige Abwechslung und Zerstreuung außerhalb des Labors unersetzlich waren.