THE CLOËTTA PRIZE 2022
IS AWARDED TO

PROFESSOR

ANNETTE OXENIUS

BORN IN 1968 IN USTER, SWITZERLAND
INSTITUTE OF MICROBIOLOGY
ETH ZURICH

FOR HER OUTSTANDING CONTRIBUTIONS
TO THE UNRAVELLING OF IMPORTANT MECHANISMS
OF ANTIVIRAL CELLULAR IMMUNITY

GENEVA, 25TH NOVEMBER 2022

IN THE NAME OF THE FOUNDATION BOARD:

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ANNETTE OXENIUS

CURRICULUM VITAE

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Personal Information

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Date of birth:	November 10, 1968
Nationality:	Swiss
URL for website:	http://www.micro.biol.ethz.ch/research/oxenius.html
Marital status:	married
Children:	One daughter (born 7.9.2009)

Education

1993	Diploma in Biochemistry, University of Zurich, Switzerland
1997	Dr. sc. nat ETH, Swiss Federal Institute of Technology, Zürich, Switzerland

Current Postion

2012–	Full Professor of Immunology at the Institute of Microbiology, ETH Zurich

Previous Positions

1997–1998	Postdoctoral fellow, Institute for Experimental Immunology, University Hospital Zurich
1999–2002	Postdoctoral fellow (funded by EMBO, the «Schweizerische Stiftung für medizinisch-biologische

	Stipendien» and the Novartis Foundation) at the Nuffield Department of Medicine, John Radcliffe Hospital in Oxford, UK.
2002–2007	Assistant Professor for Immunology at the Institute of Microbiology, ETHZ, Zürich, Switzerland
2007–2012	Associate Professor of Immunology at the Institute of Microbiology, ETHZ, Zürich, Switzerland

Fellowships and Awards

Diploma thesis:	«Semesterprämie» of the philosophical faculty II of the University of Zürich
Diploma exam:	Summa cum laude
PhD thesis:	Silver medal of the ETH Zürich
1999–2000	EMBO long-term fellowship
2001–2002	SNF Postdoctoral Fellowship from the Swiss National Science Foundation
2002	«Förderpreis» of the Swiss Society of Microbiology
2006	«Robert-Koch-Förderpreis» of the Robert-Koch-Gesellschaft
2006	EMBO Young Investigator Award
2017	ETH «Goldene Eule» Award for teaching

Supervision of Graduate Students and Postdoctoral Fellows

2002–	Direct supervision of 9 Postdocs, 37 PhD students, 47 Master students Institute of Microbiology, ETHZ, Zürich, Switzerland

Important Contributions to Careers Of Scientists

Two former members of my laboratory are currently holding professorial positions at academic institutions in Switzerland. Many former members of my laboratory have pursued postdoctoral careers and are either

still on this trajectory or have secured positions in industry with either a focus in research and development or in management / marketing.

Teaching Activities

2002–	Lectures in biology and in immunology at ETHZ, Zürich, Switzerland
2002–	Master courses and practical courses in immunology at ETHZ, Zürich, Switzerland
2017–	Basic biology lectures for medical curriculum and D-HEST at ETHZ, Zürich, Switzerland

Organisation of Scientific Meetings

2002–	Yearly organizer of the «Wolfsberg meeting» (scientific exchange of all immunology PhD students in Switzerland)
2006	President of the organizing committee of the annual meeting of the Swiss Society for Allergology and Immunology (SGAI) 2006 in Zurich
2010	Member of the organizing committee «DC2010», Lugano, Switzerland
2012	Member of the organizing committee of the European Congress of Immunology, Glasgow, 2012
2017	Co-organizer of SystIms (Systems Biology of Adaptive Immunity) conference, Monte Verità, Ascona, Switzerland, 2017
2021	Member of the scientific advisory board of the annual congress of the Swiss Society of Allergology and Immunology (Zurich)

Institutional Responsibilities

2020–	Chair Department of Biology, ETHZ
2019–2020	Vice Chair Department of Biology, ETHZ

2016–2018	Deputy Head of the Institute of Microbiology, ETHZ, Zürich, Switzerland
2016–2020	Member of the «Doktoratsausschuss» ETHZ
2013–2021	Member and head (since 2017) of sub commission «Life Sciences» of the ETHZ Research Commission
2013–2019	Director of the Microbiology and Immunology (MIM) PhD program Zurich
2013–2016	Head of the Institute of Microbiology, ETHZ, Zürich, Switzerland

Memberships of Scientific Societies

2013–2019	Member of the steering committee of the Swiss Society of Allergology and Immunology (SGAI)
2014–2019	Member of the steering committee «Verein Forschung für Leben»
2014–2016	Member of the American Association of Immunologists

Outreach

- Member of the advisory board of the Swiss National Scientific COVID-19 Task Force
- Member of the «Verein Forschung für Leben»
- Participation at the Science City activities (public information about research activities at ETHZ)
- Participation at «ETH Unterwegs» (information event for CH high schools)
- Participation at the «national future days» (information and hand-on event for CH pupils interested in MINT topics)

Publication Record

- > 200 peer-reviewed publications, 4 book chapters
- H-index: 70 (June 16, 2022, Google Scholar)
- Total citations: 18 460 (June 16, 2022, Google Scholar)

SELECTED PUBLICATIONS

Torti, N., S. M. Walton, T. Brocker, T. Rulicke, and **A. Oxenius.** 2011. Non-hematopoietic cells in lymph nodes drive memory CD8 T cell inflation during murine cytomegalovirus infection. *PLoS Pathog* 7: e1002313.

Borsa, M., I. Barnstorf, N. S. Baumann, K. Pallmer, A. Yermanos, F. Grabnitz, N. Barandun, A. Hausmann, I. Sandu, Y. Barral, and **A. Oxenius.** 2019. Modulation of asymmetric cell division as a mechanism to boost CD8(+) T cell memory. *Sci Immunol* 4.

Crouse, J., G. Bedenikovic, M. Wiesel, M. Ibberson, I. Xenarios, D. Von Laer, U. Kalinke, E. Vivier, S. Jonjic, and **A. Oxenius.** 2014. Type I interferons protect T cells against NK cell attack mediated by the activating receptor NCR1. *Immunity* 40: 961–973.

Frebel, H., V. Nindl, R. A. Schuepbach, T. Braunschweiler, K. Richter, J. Vogel, C. A. Wagner, D. Loffing-Cueni, M. Kurrer, B. Ludewig, and **A. Oxenius.** 2012. Programmed death 1 protects from fatal circulatory failure during systemic virus infection of mice. *J Exp Med* 209: 2485–2499.

Sandu, I., D. Cerletti, N. Oetiker, M. Borsa, F. Wagen, I. Spadafora, S. P. M. Welten, U. Stolz, **A. Oxenius,** and M. Claassen. 2020. Landscape of Exhausted Virus-Specific CD8 T Cells in Chronic LCMV Infection. *Cell Rep* 32: 108078.

Sandu, I., D. Cerletti, M. Claassen, and **A. Oxenius.** 2020. Exhausted CD8(+) T cells exhibit low and strongly inhibited TCR signaling during chronic LCMV infection. *Nat Commun* 11: 4454.

Greczmiel, U., N. J. Krautler, A. Pedrioli, I. Bartsch, P. Agnellini, G. Bedenikovic, J. Harker, K. Richter, and **A. Oxenius.** 2017. Sustained T follicular helper cell response is essential for control of chronic viral infection. *Sci Immunol* 2.

Krautler, N. J., A. Yermanos, A. Pedrioli, S. P. M. Welten, D. Lorge, U. Greczmiel, I. Bartsch, J. Scheuermann, J. D. Kiefer, K. Eyer, U. Menzel, V. Greiff, D. Neri, T. Stadler, S. T. Reddy, and **A. Oxenius.** 2020. Quantitative and Qualitative Analysis of Humoral Immunity Reveals Continued and Personalized Evolution in Chronic Viral Infection. *Cell Rep* 30: 997–1012 e1016.

Welten, S. P. M., A. Yermanos, N. S. Baumann, F. Wagen, N. Oetiker, I. Sandu, A. Pedrioli, J. D. Oduro, S. T. Reddy, L. Cicin-Sain, W. Held, and **A. Oxenius.** 2020. Tcf1(+) cells are required to maintain the inflationary T cell pool upon MCMV infection. *Nat Commun* 11: 2295.

Thom, J. T., T. C. Weber, S. M. Walton, N. Torti, and **A. Oxenius.** 2015. The Salivary Gland Acts as a Sink for Tissue-Resident Memory CD8(+) T Cells, Facilitating Protection from Local Cytomegalovirus Infection. *Cell Rep* 13: 1125–1136.

REGULATION OF ADAPTIVE IMMUNITY IN VIRAL INFECTIONS

Annette Oxenius
Institute of Microbiology, ETHZ

Summary

The mammalian immune system has evolved a plethora of cells and mechanisms that allow the detection and control of bacterial, fungal and viral infections. To reach this goal, immune cells need to communicate with each other, and their function needs to be tightly controlled. Furthermore, immune cells must recognize an enormous variety of different pathogens (i. e. epitopes of proteins or carbohydrates) and the type of immune response needs to be tailored to the invading pathogen, meaning that different types of effector functions need to be invoked for instance in case of viral or helminth infections. All these parameters depend on enormous diversity of immune cells with respect to their specificities, effector functions and longevity. How this diversity is generated and how immune responses are regulated in face of viral infections was – and is – at the center of research in my group at the ETH Zurich. We are particularly interested in understanding the regulation and differentiation of T and B lymphocytes in the context of acute or chronic viral infections.

Introduction

The immune system is a complex ensemble of diverse white blood cells (leukocytes) that are resident in tissues and patrol the body with the aim of detecting tissue injury invoked for instance by invasion of microorganisms such as bacteria and viruses. While cells of the innate immune system are responsible to sense invading microorganisms and respond to this sensing by inducing inflammation leading to the recruitment of circulating innate immune cells, cells of the adaptive immune system (T and B lymphocytes) are equipped with highly diverse receptors that can recognize antigens of microbes in a very specific manner. Activation of T and

B cells leads to their clonal expansion and differentiation into effector cells, with B cells secreting microbe-specific antibodies and T cells exerting effector functions such as cytokine secretion and cytotoxicity. In the context of viral infections, cytotoxicity exerted by CD8 T lymphocytes is of particular relevance, as it allows the detection and destruction of virus-infected cells, thereby contributing to termination of viral replication. After resolution of an infection, most activated effector cells die, but a considerable fraction of antigen-specific T and B cells is maintained long-term as memory cells. These memory cells can be quickly reactivated following a secondary infection, leading to enhanced control, a process known as immunological memory.

How diversity between activated T cells is generated and how such powerful and potentially dangerous adaptive immune responses are regulated, is at the center of our research interest.

Generation of diversity in activated T cells

T lymphocytes express T cell receptors (TCRs) conveying antigen specificity. T cell activation requires the integration of three signals: TCR engagement with peptide-presenting major histocompatibility (MHC) complexes on antigen presenting cells (APCs) (signal 1), engagement of costimulatory receptors (signal 2) and sensing of differentiation inducing cytokines (signal 3). The combination of these three signals leads to proliferation (clonal expansion) and acquisition of effector functions (differentiation). From this differentiation process, a heterogeneous population of T cells emerges with respect to their phenotype, function, longevity, localization, and their transcriptional, metabolic and epigenetic profile. Such generation of diversity fulfils the physiological requirements for short-term effective control of an infection and the establishment of long-lived memory, providing rapid protection in case of re-infection.

Both short-lived effector cells and long-lived memory cells can arise from a single activated antigen specific CD8 T cell (2–4), indicative that fate is not pre-determined in a single CD8 T cell. Divergent fate is not an exclusive feature of CD8 T cells, as activated B cells also generate effector progeny (antibody secreting cells) and memory cells (5).

The establishment of heterogeneity in activated CD8 T cells occurs early after priming, in the most extreme case already after the first mitosis in a process termed asymmetric cell division (ACD). Despite ACD being a conserved mechanism in biology to generate daughter cells with different fates, its role in T cell differentiation is still debated. In activated CD8 T cells, ACD relies primarily on the establishment of a polarization axis with the immune synapse (IS, the interaction surface between an antigen presenting cell and the T cell, where T cell receptor (TCR) engagement occurs) as an anchor point. Establishment of a stable IS and strong TCR triggering is required for ACD to occur (6, 7). Such polarization leads to the establishment of different layers of asymmetry (Fig. 1). Activated CD8 T cells exhibit polarization of membrane proteins towards the IS-proximal pole, including the TCR, CD8, co-stimulatory molecules, integrins and cytokine receptors (6, 7). Due to unequal proteasome activity between the two daughter cells upon mitosis, fate-determining transcription factors such as T-bet are asymmetrically partitioned into the APC-proximal daughter cell already in the first division after activation (8). Furthermore, unequal nutrient sensing establishes metabolism disparity between sibling lymphocytes (9, 10). As asymmetric distribution

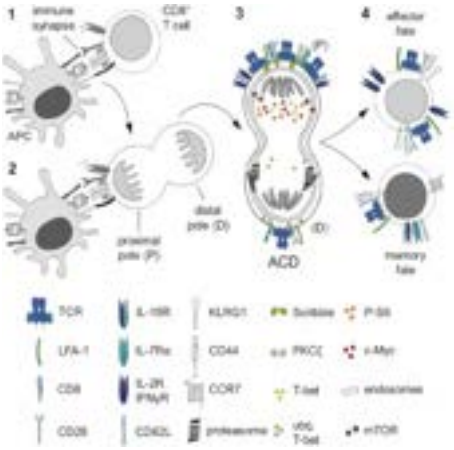

Figure 1. Asymmetric cell division as a mechanism that contributes to CD8 T cell differentiation. *Naïve CD8 T cells can recognize their cognate antigen presented by MHC-I molecules. The interface between the APC and the engaged T cell is termed immunological synapse (IS) (1). When TCR triggering is accompanied by co-stimulatory signals in an inflammatory environment, CD8 T cell activation is followed by polarization and asymmetric segregation of fate determinants (2–3). ACD leads to two daughter cells with unequal potential fates: the IS-proximal daughter is destined to become an effector cell, while the IS-distal daughter is committed with a memory fate. Courtesy Dr. Mariana Borsa.*

is maintained during mitosis – even after disengagement of antigen presenting cells (APCs) and T cells – two daughter cells emerge that differ in phenotype, transcription factor composition, metabolic status, and transcriptional profile (6–10). This leads to an early bifurcation of potential fates, where the IS-proximal daughter is destined to become an effector cell, while the IS-distal daughter is committed to a memory fate (11). However, up to now, the contribution of ACD for the generation of diversity is largely limited to the description of asymmetric partitioning of a variety of cellular constituents and signaling pathways, and formal proof for the importance of this partitioning mechanism for subset diversification is still lacking.

Immune control of viral infections

Viral infections can lead to distinct outcomes, ranging from acute resolved to persistent infections. Acute infections are generally resolved by the innate and adaptive immune system in 8 to 10 days after infection in a process that often relies on the production of pro-inflammatory cytokines (e. g. type I interferons, IFNγ, IL-12), cytotoxic activity of antigen-specific CD8 T cells, or production of virus-neutralizing antibodies. At the peak of expansion, the majority of CD8 T cells present an effector phenotype, and only cells committed to a memory fate survive the contraction phase. Upon reinfection, the pool of memory cells can generate new effector cells, leading to a robust immune response and faster viral clearance in comparison to the first antigen encounter (Fig. 2A, upper panel). If analyzed at higher resolution, early progenies of activated CD8 T cells can be distinguished into short-lived effector cells (SLECs) and memory precursor effector cells (MPECs) at about 7 days post infection. In a scenario of antigen re-encounter, the progenies of MPECs, which survived and formed the memory pool, are responsible to mount the recall immune response (Fig. 2A, lower panel) (12–15).

Persistent infections can be divided into latent or active chronic infections. In latent persistent infections, most prominently caused by the Herpesviridae family, control of lytic virus replication presents distinct kinetics in different organs. Complete eradication of the virus, however, is never achieved, as herpes viruses can enter a state of latency, in which

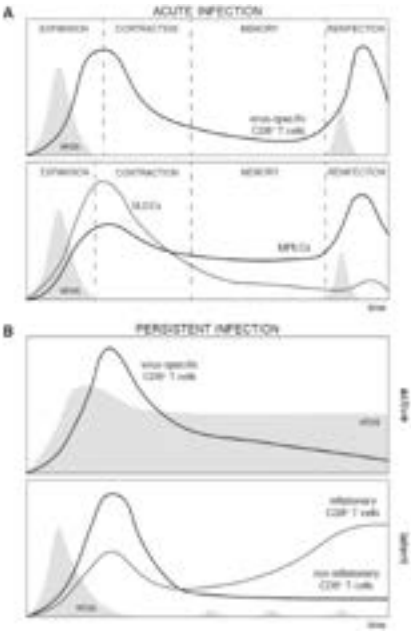

Figure 2. CD8 T cell responses upon acute and persistent infections. *(A) Acute resolved infections lead to robust proliferation of CD8 T cells and are cleared after 8–10 days. After viral clearance, contraction of the CD8 T cell population results in the maintenance of a memory pool. At early stages after infection, short-lived effector cells (SLECs) and memory precursor effector cells (MPECs) can be distinguished. Upon re-infection, the progenies of MPECs, which survived and formed the memory pool, are responsible to mount the recall immune response. (B) Persistent viral infections can be classified in active or latent ones. Active persistent infections are marked by continuous viral replication, and reduction in the pool of virus-specific cells. Latent infections are characterized by intermittent bursts of viral reactivation, and different proliferation kinetics in inflationary and non-inflationary CD8 T cells. Courtesy Dr. Mariana Borsa.*

viral genomes persist in form of episomal DNA with no or limited transcription and translation of viral gene products. Sporadic and local reactivation events, however, can take place in response to various cellular stress responses. In immunocompetent hosts, such reactivation events are quickly controlled by the adaptive immune system. In case of cytomegalovirus (CMV) infection, CD8 T cells follow distinct proliferation and maintenance kinetics, depending on the antigen they are specific for, and are distinguished into "non-inflators" and "inflationary" cells. The "non-inflators" exhibit an expansion and contraction kinetics similar to the one found in acute infections, while inflationary cells show a continuous increase and eventual settlement at high frequency and numbers during the period of viral latency (Fig. 2B, lower panel) (16–18). The inflationary response kinetics results from (continuous) sensing of viral reactivation events (19).

Active persistent virus infections are commonly induced by non- or poorly cytopathic viruses. Viral replication can be maintained for long periods

or is even never controlled. After the peak of CD8 T cell expansion, continuous exposure to high amounts of antigen results in numeric reduction of virus-specific cells (18) and also to CD8 T cell dysfunction, referred to as exhaustion, characterized by impaired proliferation, maintenance and execution of specific effector functions (Fig. 2B, upper panel) (20–23).

Lymphocytic choriomeningitis virus (LCMV) infection is a well-established model to study the regulation of CD8 T cell differentiation (24–27). Depending on the LCMV strain and the inoculation dose, differences in the viral tropism and replication kinetics determine the outcome of the infection (28–30) (Fig. 2A and B). Furthermore, the existence of LCMV-specific TCR transgenic mouse strains, such as the P14 mouse, that contains CD8 T cells which specifically recognize the gp_{33-41} peptide from the LCMV glycoprotein (31), allow cell intrinsic and extrinsic factors to be dissected using adoptive transfer experiments.

Regulation of adaptive immunity

Generation of diversity: Role of asymmetric cell division (ACD) in fate determination

Asymmetric partitioning of fate-determinants is a mechanism that contributes to T cell diversification. However, it was unclear whether the ability of T cells to divide asymmetrically is influenced by their differentiation state, as well as if enforcing asymmetric cell division rates would have an impact on T cell diversification. Using the murine LCMV infection model, we established a correlation between cell stemness and the ability of CD8 T cells to undergo asymmetric cell division (ACD). Transient mTOR inhibition proved to increase ACD rates in naïve and memory cells, and to install this ability in exhausted CD8 T cells. Functionally, enforced ACD correlated with increased memory potential, leading to more efficient recall response and viral control upon secondary LCMV infection. Moreover, transient mTOR inhibition also increased ACD rates in human CD8 T cells. Transcriptional profiling revealed that progenies emerging from enforced ACD exhibited more pronounced early memory signatures, which functionally endowed these cells with better survival in absence of antigen exposure and more robust homing to secondary lymphoid organs, providing critical access to survival niches. Our data

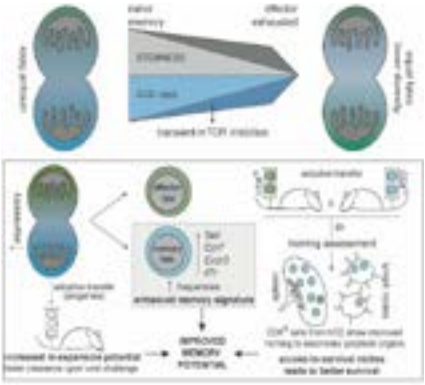

Figure 3 Asymmetric cell division (ACD) contributes to T cell differentiation. *ACD is established in the context of antigen presentation by an APC where the proximal daughter (CD8^{hi}) preferentially adopts an effector fate, and the distal daughter (CD8^{lo}) preferentially gives rise to a memory cell. CD8 T cells with stemness can divide asymmetrically, while terminally differentiated cells lack this feature. Transient mTOR inhibition increases ACD rates in activated CD8 T cells. Progenies generated by enhanced asymmetric cell division show (1) better re-expansion potential upon adoptive transfer followed by cognate antigen challenge, (2) memory-potential daughter cells (CD8^{lo}) with strengthened memory gene signature, and (3) improved survival and homing to secondary lymphoid organs. Courtesy Dr. Mariana Borsa.*

provide important insights into how ACD can improve long-term survival and function of T cells and opens new perspectives for vaccination and adoptive T cell transfer therapies (Figure 3) (32).

Ageing of the immune system, a multifaceted phenomenon also known as immunosenescence, reduces T cell diversity, as a result of thymic involution and antigen exposure history. This culminates in both inefficient immune responses and increased susceptibility to autoimmunity (33–35). In some cell types, such as haematopoietic stem cells (HSCs), functional deterioration observed during ageing has been linked to an impaired ability to undergo ACD (36). To address the question whether altered ACD rates might also be apparent in CD8 T cells during ageing and potentially linked to age-related impairment of CD8 T cell function, we compared ACD rates in CD8 T cells of young and aged mice. We found that ageing leads to an overall decline in the ability of CD8 T cells to undergo ACD, which was linked to impaired expansion and memory potential. Pharmacological enforcement of ACD restored the expansion and memory potential of naïve CD8 T cells from aged mice. Lower ACD rates were exclusively found in naïve CD8 T cells from aged animals, as "virtual" memory cells (CD8 T$_{VM}$), which gradually accumulate in aged individuals, retained the ability to undergo ACD and showed better re-expansion potential in adoptive transfer ex-

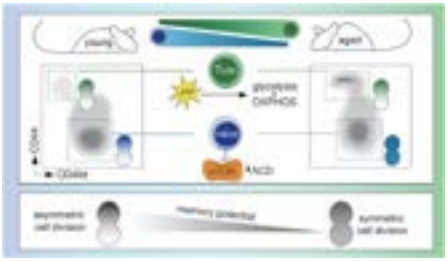

Figure 4 ACD and memory potential of CD8 T cells from young and aged mice. *The ability of naïve CD8 T cells to undergo ACD is impaired in ageing but can be rescued by transient mTOR inhibition. TVM cells can undergo ACD, irrespective of age. TVM cells might be an adaptation to poor naïve T cell immunity in ageing. Courtesy Dr. Mariana Borsa.*

periments compared to their naïve counterparts, providing additional evidence of a correlation between the ability to divide asymmetrically and memory potential (Figure 4) (32).

Fate of virus-specific CD8 T cells in absence of signal 3

Proper activation, expansion and differentiation of T cells is critical for the clearance of viral infections and this activation is dependent on three key signals; antigen presentation, co-stimulation and cytokine signaling. The importance of signal 3 cytokine signaling for sustained expansion, effector and memory cell differentiation has been demonstrated in various infection models (37–40), where the nature of the invading pathogen

Figure 5 T cells primed in absence of signal 3 become susceptible to NK cell mediated killing *T cells lacking the ability to directly sense type-I interferons (blue cells) are highly susceptible to NK cell meditated killing during LCMV infection, whereas T cells that receive type-I interferons (purple cells) are protected. The ability of activated T cells to receive signals through the type-I IFN receptor prevents the expression of ligands (sweat drops) for the activating NK cell receptor NCR-1, thereby being protected against cytolytic attack by NK cells (black). In contrast, activated T cells which are unable to sense type-I interferons are killed by NK cells in a perforin (stars) dependent manner, demonstrating an important immunoregulatory role of NK cells for "inappropriately" activated T cells. Courtesy Dr. Josh Crouse*

determines which cytokines serve as signal 3, the two most studied being IL-12 and type-I interferons (IFNs). T cell responses are critically dependent on type-I IFNs during LCMV infection, where the inability to directly sense type-I IFNs leads to dramatically curtailed expansion (37, 38) and altered differentiation of antiviral T cells (41). We investigated the cause(s) for the abortive expansion of T cells lacking the type-I IFN receptor ($IFNAR^{-/-}$) during acute LCMV infection. By performing a whole genome gene expression analysis, we found many molecules involved in cell death being differentially regulated in $IFNAR^{-/-}$ LCMV-specific CD8 T cells compared to their WT counterparts, amongst which were multiple NK cell activating and inhibitory ligands. *In vivo* depletion of NK cells revealed a key role for NK cells in the negative regulation of $IFNAR^{-/-}$ LCMV-specific T cells, with NK cell depletion during priming leading to a complete recovery of the early $IFNAR^{-/-}$ T cell expansion. We further found that NK cells selectively killed activated $IFNAR^{-/-}$ T cells in a perforin-dependent manner via engagement of NCR1 ligands being specifically up-regulated on $IFNAR^{-/-}$ T cells. Our data establish a mechanism whereby type-I IFN signalling on activated T cells is pivotal to protect them from NCR1-mediated NK cell attack (Figure 5) (42).

Regulation of adaptive immunity: chronic viral infection

Ineffective clearance by the host immune system is the cause for certain infections. Prominent viruses causing chronic infections are HIV, HCV and HBV in humans and LCMV in the mouse. The principal strategy employed by these viruses to establish and maintain persistence relies on "outpacing" the immune system. One major challenge actively replicating chronic infections impose on host immunity is the continued presence of viral antigens. In the attempt to control the infection to a certain level, while avoiding detrimental immunopathology, immune cell numbers and their function need to be tightly regulated. Furthermore, rapid viral mutations and pressure exerted by the immune system lead to the emergence of viral escape variants, presenting a continuously evolving spectrum of (new) antigenic determinants to the host's immune system.

Regulation of virus-specific CD8 T cells during chronic infection

A complex regulatory network adjusts the size and the function of adaptive immune responses during chronic infections. This regulation is particularly well understood for CD8 T cell responses which bear the potential to cause major immunopathological insult via direct cytotoxicity and pro-inflammatory cytokine production (21, 43). Regulation of virus-specific CD8 T cell immunity is characterized by reduced numbers and function of antiviral T cells, collectively termed T cell exhaustion (21, 43). As opposed to effector and memory CD8 T cells developing after acute infections, CD8 T cells are functionally compromised with respect to inflammatory cytokine production and responsiveness to homeostatic cytokines. Regulatory pathways inferring such dysfunction include sustained T cell receptor (TCR) stimulation, continued expression of co-inhibitory receptors, exposure to anti-inflammatory cytokines and control by regulatory T cells (Tregs) (21, 43, 44). Despite the fact that numeric and functional attenuation of virus-specific CD8 T cells supports viral persistence, it is a key regulatory mechanism to prevent overt immunopathology. Indeed, we could show that genetic absence of one key co-inhibitory receptor

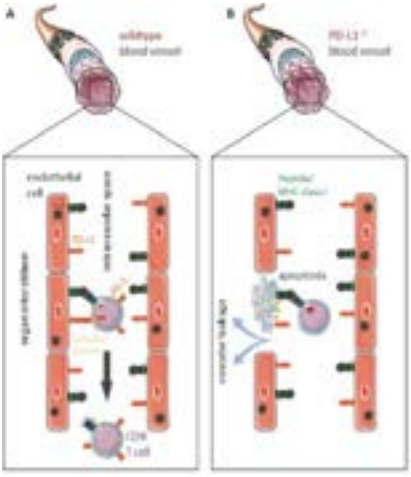

Figure 6 Lethal immunopathology induced by LCMV-specific CD8 T cells in absence of PD-1

(A) Primed anti-viral CD8 T cells are recruited to peripheral sites of inflammation during persistent LCMV infection. When interacting with LCMV-infected vascular endothelial cells, CD8 T cells are restrained from killing these cells via engagement of PD-1 (on CD8 T cells) and PD-L1 (on endothelial cells), thereby preventing vascular leakage.

(b) In PD-1 ko mice, anti-viral CD8 T cell cytotoxicity cannot be downregulated by endothelial PD-L1 expression. Therefore, CD8 T cells kill infected endothelial cells, compromising vascular integrity and increasing vascular leakage. P: Perforin; Ag: Antigen; TCR: T cell receptor. Courtesy Dr. Helge Frebel

(PD-1) on virus-specific CD8 T cells enhances functionality of these cells to the point that they induce lethal immunopathology (Figure 6) (45).

Differentiation of virus-specific CD8 T cells during chronic infection

In the context of chronic antigen exposure, CD8 T cells undergo a differentiation program that differs markedly from the one observed during acute resolved infection. Previous studies have analysed and inferred differentiation trajectories of virus-specific CD8 T cells using bulk or single cell transcriptomic profiling in various systems, including chronic LCMV infection (46–48). Asynchronicity in this process as well as different micro-environments that CD8 T cells experience result in a heterogeneous population of cells at a given time point of the infection. One sub-population of virus-specific T cells acquires a phenotype that shares properties with memory T cells from acute infection and is characterized by the expression of T cell Factor 1 (TCF1) (46, 49). In contrast to terminally exhausted or effector T cells, these cells retain proliferative activity and have better survival in the infected host (47). It is not yet fully understood how and when these different cell states arise during the course of the infection and which intermediate cell states precede these end states.

Recent advances in sequencing technologies enable to profile individual cells on a genome-wide transcriptional level using single-cell RNA sequencing (scRNAseq). This technology allows capturing the transcriptional heterogeneity of multiple cell populations and to computationally infer orders of cell states traversed during dynamic processes such as T cell differentiation in chronic infection. When analyzing scRNAseq data-sets, cells are treated as points in transcriptome space based on their expression profile. Dimensionality reduction techniques like t-SNE (50) and UMAP (51) construct two-dimensional representations for analysis and interpretation of the high dimensional single-cell expression data. Pseudotime and lineage inference methods aim at constructing likely transitions between cell states (52). In addition, directionality information is available for trajectory inference via RNA velocity analysis. RNA velocity (53) considers additional information about the ratio of unspliced to spliced mRNA in transcript data, which serves as a measure to

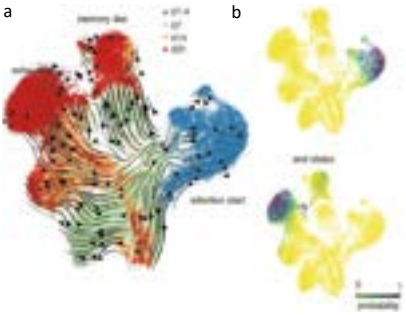

Figure 7 RNA velocity analysis.
Stream plot visualizing likely transitions between cells inferred from RNA velocity (a).
The stationary distribution of the backward and the forward transition matrix, respectively, indicate start and end cell states (b).
From Cerletti et al., (1).

determine the stage (early, intermediate, late) of individual gene expressions and allows to predict the future expression state and hence to better infer the directionality towards their neighbours in the high-dimensional transcriptional space. We conducted scRNAseq measurements at multiple time-points, ranging from the beginning of chronic LCMV infection until manifestation of exhaustion three weeks after infection. We included information from RNA velocity analysis to perform simulation-based trajectory inference of differentiation events leading to the different terminal CD8 T cell states observed in chronic LCMV infection. This analysis allowed us to construct faithful lineage trajectories towards the two endpoints of differentiation, namely a terminally exhausted and a TCF1$^+$ cell population. We identified a potential branching point in the initially shared trajectories and validated our findings using adoptive transfer experiments of cells positioned before or after the branching point. (Figure 7) (1).

Virus-specific phenotypes during chronic infection are shaped by tissues of residence

Exhaustion is a gradual, continuous process which, amongst other factors, is triggered by persistent T cell receptor (TCR) stimulation, the degree of exhaustion depending on the TCR signalling strength, antigen abundance, and affinity (54–56). As a result, the pool of exhausted CD8 T cells is a heterogeneous population. Some studies suggested that the phenotype of exhausted virus-specific CD8 T cells depends on the tissue location (56, 57), but most transcriptional analyses are not informative

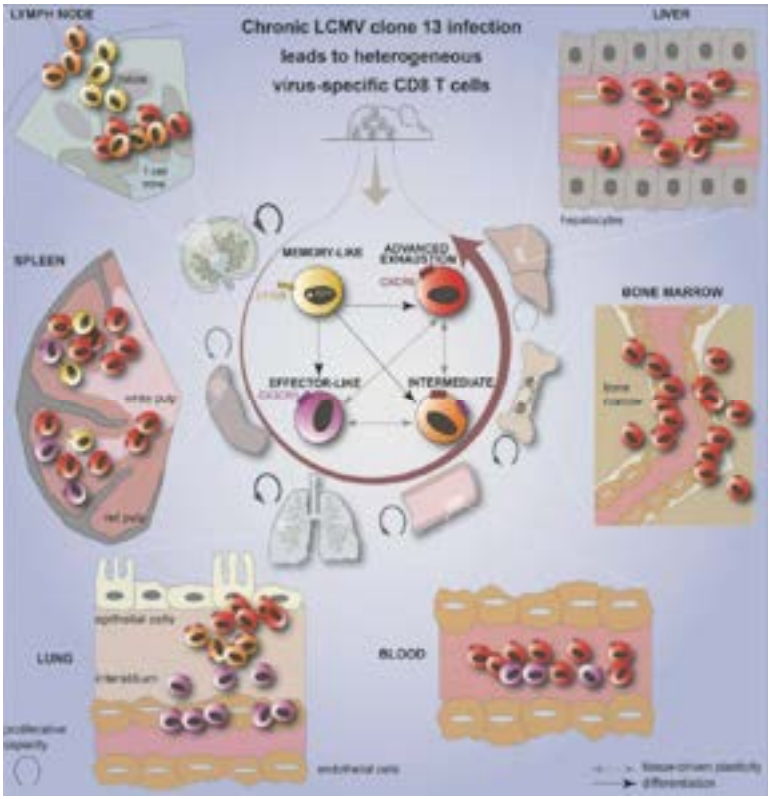

Figure 8 Exhausted virus-specific cells are plastic and heterogeneous

A hallmark of chronic infections is the presence of exhausted CD8 T cells, characterized by a distinct transcriptional program compared to functional effector or memory cells, co-expression of multiple inhibitory receptors, and impaired effector function. Single-cell RNA sequencing of virus-specific CD8 T cells isolated from six different tissues during established LCMV infection revealed that exhausted cells are heterogeneous, adopt organ-specific transcriptomic profiles and can be divided into five main functional subpopulations: advanced exhaustion (red), effector-like (purple), intermediate (orange), memory-like (yellow), and proliferating (circular arrows). In vivo antibody labelling showed that cells belonging to these subpopulations are differentially positioned in these tissues, with effector-like and intermediate phenotype cells being close to the vasculature and memory-like and more exhausted cells residing deeper in the tissue. Additionally, adoptive transfer experiments showed that phenotype of virus-specific CD8 T cells is largely plastic and shaped by the microenvironment. Courtesy Dr. Ioana Sandu.

about inter-tissue heterogeneity of LCMV-specific CD8 T cells during chronic infection since most studies focused on cells isolated from the spleen (22, 46, 58). Two major subpopulations of exhausted CD8 T cells were described in secondary lymphoid tissues: a less exhausted TCF1^{hi} T-bet^{hi} PD-1^{lo}, more functional population, termed memory-like, and a more terminally exhausted population TCF1^{neg} PD-1^{hi} EOMES^{hi} CD39^{hi} (46, 59, 60). Additionally, single-cell RNA sequencing (scRNAseq) of exhausted CD8 T cells isolated from the spleen revealed four distinct subsets: effector-like, proliferating, memory-like TCF1^{hi}, and terminally exhausted PD-1^{hi} CD39^{hi} (48, 49, 61). However, the extent of heterogeneity has not been resolved with respect to an unbiased selection of cell markers, as well as for other tissues than secondary lymphoid organs.

We evaluated the heterogeneity of single-cell transcriptomes of virus-specific CD8 T cells isolated from six different tissues (spleen, lymph nodes (LN), bone marrow (BM), lung, liver, and blood) in mice with chronic LCMV infection. Overall, the population of virus-specific CD8 T cells could be classified into five functional phenotypes (memory-like, proliferating, effector-like, intermediate, and advanced state of exhaustion), based on distinct transcriptional profiles regarding T cell activation and inhibition, chemokine and interleukin receptor, and transcription factor expression. Cells with these functional phenotypes were represented at different frequencies in specific tissues, resulting in tissue-specific phenotype transcription profiles, most apparent in those tissues where the population of virus-specific CD8 T cells was predominantly composed of cells with a single functional phenotype. Adoptive transfer experiments showed that these phenotypes are plastic, suggesting that the tissue microenvironment has a major impact in shaping the phenotype and function of virus-specific CD8 T cells during chronic infection (Figure 8) (62).

In vivo TCR stimulation is strongly attenuated during chronic viral infection

An important feature of exhausted CD8 T cells is the co-expression of multiple co-inhibitory receptors (such as PD-1, CTLA-4, LAG-3, TIGIT, CD39, TIM-3), which dampen T cell activation (20, 60, 63–68) by various mechanisms. These include limiting co-stimulation by receptor com-

petition (CTLA-4, TIGIT(68)), direct inhibition of signal transduction downstream of T cell receptor (TCR) engagement by limiting the phosphorylation of signaling molecules such as CD3, ZAP70 and PCK (PD-1(69), TIM-3(70)), restraining metabolic changes (71, 72), changes at the transcriptional level (PD-1(73)), interfering with proliferation (LAG-3(74)), or suppressing inflammatory cues (CD39(60)). Significant effort has been invested to enhance the effector functions of exhausted cells; indeed, checkpoint inhibitors, targeting various of the above mentioned co-inhibitory receptors, are very efficient in improving CD8 T cell numbers and effector function of exhausted CD8 T cells in both cancer and chronic infections (67). The phenotypic and functional landscape of exhausted cells is very diverse (48, 49, 61, 75), translating into differential responsiveness to checkpoint inhibition. A specific subset of non-terminally exhausted cells, termed memory-like and characterized by expression of TCF1 and SLAMF6 (46), was shown to replenish the pool of terminally exhausted cells and to respond to checkpoint blockade by proliferation and differentiation into more effector-like and eventually terminally exhausted cells (46, 75, 76). Despite the compelling evidence that CD8 T cell function is impaired in chronic LCMV infection and that continued exposure to antigen significantly contributes to exhaustion (54), there is little insight into how much TCR signaling is actually ongoing in exhausted CD8 T cells *in vivo* during established chronic infection.

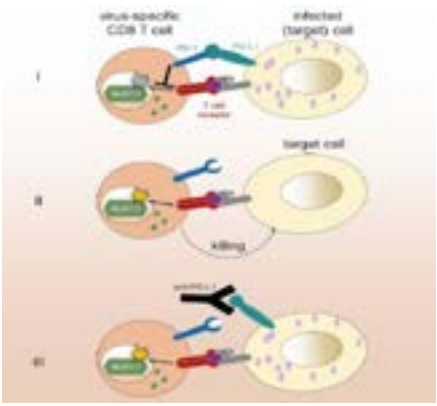

Figure 9 TCR signalling in CD8 T cells during chronic LCMV infection

TCR signalling in LCMV-specific CD8 T cells (measured by NUR77 expression) and cytotoxic potential of exhausted CD8 T cells are strongly inhibited in vivo during chronic infection (I). Absence of inhibitory ligands (II) or checkpoint blockade (III) lead to increased signalling. Courtesy Dr. Ioana Sandu.

We characterized *in vivo* TCR signaling in virus-specific CD8 T cells in the setting of chronic LCMV infection. To this end, we used virus-specific TCR transgenic CD8 T cells expressing the *Nr4a1*-GFP reporter as proxy for TCR signaling in adoptive transfer experiments. We show that, despite abundant availability of antigen in form of peptide-MHC class I complexes, there is very limited TCR signaling ongoing in exhausted CD8 T cells during chronic infection, evidenced by low expression of the GFP reporter and by RNAseq analysis of TCR signaling associated genes. We observed enhanced TCR signaling after *in vivo* blocking of PD-1/PD-L1 interaction or *in vivo* exposure of exhausted CD8 T cells to antigen on naïve target cells, which express little / no ligands for co-inhibitory receptors. This observation indicates that the engagement of co-inhibitory receptors, such as PD-1, exerts a pronounced inhibition of TCR signaling *in vivo* (Figure 9) (77).

CD4 T cell and B cell immunity during chronic viral infection

In contrast to CD8 T cell immunity, less is currently known about the regulation of CD4 and B cell immunity during chronic viral infections. Recent reports indicate that CD4 T cells are not generally down-modulated on an overall functional level, but rather differentiate into follicular T helper cells (T_{FH}) during chronic LCMV infection (78, 79) and HIV infection (80, 81). This deviation from a typical pro-inflammatory Th1 response to a subset of T helper cells which is chiefly involved in regulating B cell responses in germinal centres of secondary lymphoid organs, suggests that the host attempts to capitalize on humoral instead of cellular immunity. This might constitute a means by which the host tries to reach an optimal equilibrium between virus control and avoidance of immunopathology. Even though this is a very attractive hypothesis, it remains unclear, however, whether such emphasis on T_{FH} cell differentiation during viral chronicity is indeed linked to optimizing antibody (ab) responses. Ab responses are involved in (relative) immune control of chronic infections, illustrated by the failure of B cell-, antibody- or CD4 T cell-deficient hosts to eventually control LCMV infection (82–85), by the selection of ab escape variants in LCMV and HIV infection (86–88) and by the ability of LCMV-specific abs (neutralizing as well as non-neutralizing) to prevent viral chronicity (89–92).

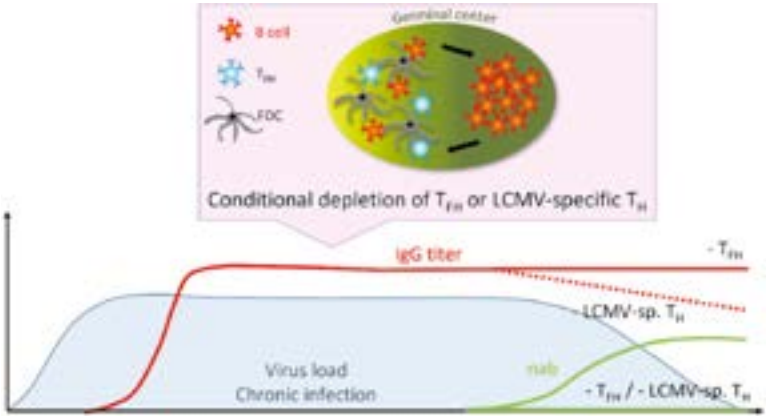

Figure 10 Conditional depletion of T_{FH} cells during established chronic LCMV infection prevents the emergence of LCMV neutralizing antibodies. *Experimental depletion of T_{FH} cells during established chronic LCMV infection has no impact on the overall LCMV-specific IgG titers but abolishes the emergence of LCMV-neutralizing antibodies. Experimental depletion of all LCMV-specific CD4 T cells during established chronic LCMV infection abolished the emergence of LCMV-neutralizing antibodies and lead to a reduction of overall LCMV-specific IgG titers.*

We have therefore addressed the physiological relevance of sustained T_{FH} activity during chronic viral infection. Using chronic LCMV infection in conjunction with an engineered *in vivo* system in which T_{FH} cells can be conditionally ablated, we have shown that sustained activity of virus-specific T_{FH} cells is pivotal for the late emergence of neutralizing LCMV-specific antibodies that keep viral titers in check and ultimately allow mice to clear the established chronic infection in absence of overt immunopathology (Figure 10) (93).

Dynamics of the LCMV-specific antibody response

Chronic viral infections are often characterized by the late emergence of neutralizing abs (nabs), i.e. abs that have the capacity to directly curtail viral replication by interfering with viral attachment to or fusion with the host cell *in vitro* (87, 94). This slow development of potent nabs favours viral persistence (85, 95). Furthermore, mutational diversification of the circulating virus and selection of variants which escape recognition by

present antibodies challenges B (and T) cell immunity. In contrast to T cells, B cells are, in principle, equipped to cope with such antigenic variation via the germinal center reaction. After an antigenic challenge antigen-specific B cells enter germinal centers and in a sequence of events involving somatic hypermutation (SHM) of their immunoglobulin genes and selection of cells expressing higher affinity B cell receptors (BCRs), the affinity of circulating antibodies increases over time in a process termed “affinity maturation” (96).

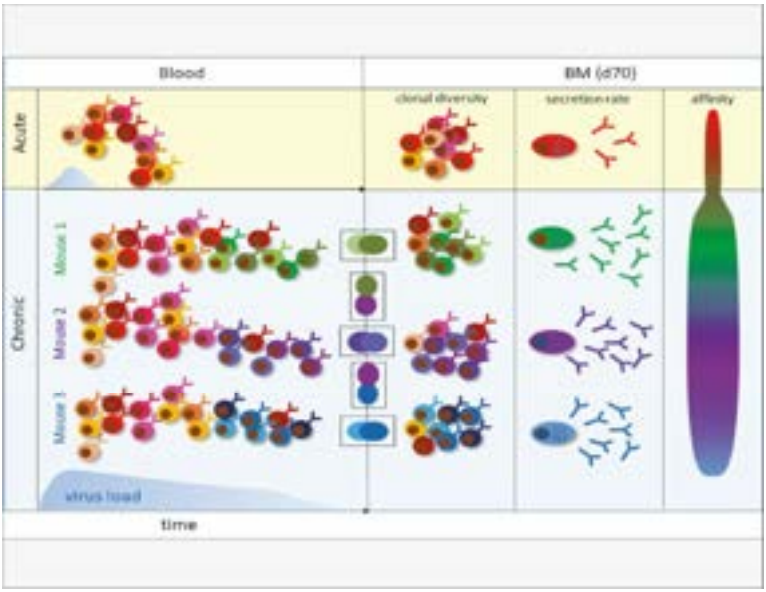

Figure 11 Evolution of antibody repertoires and functional properties of LCMV-specific antibodies. *Acute and chronic LCMV infection induce massive clonal expansion of multiple ab-producing clones (B cells) that converge in their composition during early stages of infection (red / orange / pink B cells). During later stages of infection, the initially induced B cell repertoires vanishes from the circulation in acutely infected mice (yellow background), while it is preserved long-term in individuals being chronically infected (blue background). However, these repertoires continuously evolve to adapt a personalized repertoire (indicated by green, purple and blue colour in individual mice) in chronically infected mice, selecting cells with higher secretion rates of high affinity LCMV-specific antibodies. Courtesy D. Nike Kräutler.*

Consistent with human chronic viral infections such as HIV or HCV, LCMV-neutralizing abs are only detectable late during chronic infection (87), and in addition to T cells (97, 98), LCMV-specific abs can exert critical immune pressure to select for viral escape mutants (86, 91). We used the model of chronic LCMV infection to study the molecular and functional evolution of the LCMV-specific IgG response over the course of the chronic infection. Longitudinal systems-immunological analyses of total IgG antibody repertoire evolution revealed different patterns during acute and chronic LCMV infection. Whereas early during the response, repertoire expansion and composition was remarkably similar in acute and chronic infection, later repertoire maturation and diversification was only sustained during chronic infection in a highly individual manner. Analyses of IgG genealogies support a scenario in which new clones are continuously recruited into the overall antibody response during chronic infection, and less a scenario with sustained clonal maturation, indicating that with time, the overall IgG repertoire diversifies rather than focusses on a few specific clones. On a functional level, high affinity LCMV-specific IgG antibodies were already observed early during acute and chronic infection, supporting the notion of diversification being more dominant than affinity maturation in the evolution of antibody responses during chronic viral infection (Figure 11) (99).

Regulation of adaptive immunity: latent viral infection

CD8 T cell response during cytomegalovirus infection

Cytomegalovirus (CMV) is a β-herpesvirus that is universally present in the world's population, though, this infection is largely asymptomatic in healthy individuals. CMV infection results first in lytic replication of the virus which is controlled by CMV-specific T cells, followed by establishment of viral latency. CMV can sporadically reactivate in latently infected cells. These reactivation events are quickly detected and controlled by CMV-specific T cells. Upon human CMV (HCMV) and murine CMV (MCMV) infection, an atypical CD8 T cell response is initiated, characterized by the accumulation of a subset of CMV-specific CD8 T cells exhibiting an effector-like phenotype in blood and peripheral tissues, a process termed "memory inflation" (100–103). During viral latency the CD8

T cell response is dominated by these inflationary T cells which are restricted to a few epitopes (104). Their activated effector memory phenotype is suggestive of repetitive antigen encounter, and indeed one major factor driving T cell inflation is the recurrent presentation of viral antigens in cells undergoing sporadic viral reactivation (19, 105–107).

Due to the large numbers of effector-like T cells in peripheral tissues, the use of CMV-based vectors has gained interest for vaccination purposes, and it has been shown that CMV-based vaccine vectors can provide protection against heterologous viral and tumour challenges (108–114). As the success of CMV-based vaccines is based on the induction of large populations of effector-like CD8 T cells in peripheral tissues (101, 108, 109, 115), it is important to delineate the factors that maintain this population at high numbers.

Although the half-life of inflationary T cells in mice is estimated to be around 6–8 weeks in circulation and 10–12 weeks in the periphery (17, 116), the peripheral pool of inflationary T cell reaches high numbers and stabilizes at high frequencies. This implies that there is continuous replenishment of the peripheral effector cell pool (117). A small subset of the inflationary MCMV-specific T cell population, enriched in the lymph nodes (LNs), has a non-activated central memory phenotype (19), judged by high expression of the LN homing receptor CD62L and enhanced proliferation capacity (118) and the expression of the transcription factor T cell factor 1 (Tcf1, encoded by *Tcf7*) (119). We hypothesized that $Tcf1^+$ cells are critical for the maintenance of the inflationary T cell pool by fueling the population of peripheral effector-like T cells. Indeed, we showed that the inflationary T cell population contains a small subset of cells expressing the transcription factor Tcf1. These $Tcf1^+$ cells resembled central memory T cells and were proliferation competent. Upon sensing viral reactivation events, $Tcf1^+$ cells feed into the pool of peripheral $Tcf1^-$ cells and depletion of $Tcf1^+$ cells impaired memory inflation. TCR repertoires of $Tcf1^+$ and $Tcf1^-$ populations largely overlapped, with the $Tcf1^+$ population showing higher clonal diversity. These data show that $Tcf1^+$ cells are necessary for sustaining the inflationary T cell response, and upholding this subset is likely critical for the success of CMV-based vaccination approaches (Figure 12) (119).

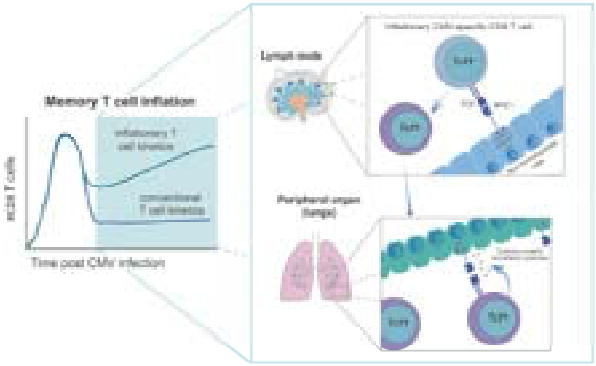

Figure 12 Tcf1⁺ cells are critical in maintaining the inflationary T cell pool
Memory T cell inflation refers to the accumulation of functional effector like T cells in the blood and peripheral tissues and is most commonly described upon for CMV infection. Within the CMV-specific inflationary T cell pool, a small subset of cells expresses the transcription factor Tcf1. These Tcf1⁺ cells are enriched in the lymph nodes and are proliferation competent. Upon sensing sporadic viral reactivation events, most likely in the lymph nodes, Tcf1⁺ CD8 T cells respond by proliferation and give rise to a pool of Tcf1⁻ cells that migrate into the periphery and have the ability to locally eliminate infected target cells, and are essential for the stable maintenance of the inflationary CD8 T cells pool. Courtesy Dr. Suzanne Welten.

Early studies have established the concept of circulatory immune surveillance with memory T cells being in constant exchange with either lymphoid or non-lymphoid tissues via the circulation. More recently it was demonstrated that a proportion of peripheral memory T cells is non-migratory and resides at the site of previous pathogen encounter (120, 121). These tissue resident memory T cells (T_{RM}) are readily positioned at barrier tissues prone to pathogenic invasion or reactivation and are superior to circulating T_{EM} cells in protecting against local secondary infections, positioning T_{RM} cells as critical guards of peripheral immunity.

Secretory glands pose an attractive target tissue for viruses to persist and exploit mucosal secretions as vehicles for dissemination. Human cytomegalovirus (HCMV) transmission is fostered by prolonged shedding from infected mucosae such as the salivary glands (SGs), which support chronic viral replication for months after virus is controlled in all other organs. Experimental infection of mice with murine CMV (MCMV) has

revealed that virus-mediated MHC class I downregulation renders the SG uniquely resistant to CD8 T cell mediated virus control (122). Instead, CD4 T cells are required to cease virus replication during primary CMV infection (123, 124).

We analyzed MCMV-specific CD8 and CD4 memory T cells in the SG with respect to their migratory potential, their maintenance, and their protective capacity upon localized pathogen encounter. We demonstrated an exquisite ability of the SG to induce CD4 and CD8 T_{RM} populations that are excluded from the circulation. While CD8 T_{RM} induction was completely independent of cognate antigen, CD4 T_{RM} generation was dependent on the presence of local antigen. CD103 expression in CD8 T cells depended on TGFβ, supported tissue retention, and coincided with localization of CD8 T cells to epithelial structures of glandular ducts, while $CD103^-$ CD8 T cells and CD4 T cells preferentially localized outside epithelial duct structures. Functionally, using intraglandular infection, we demonstrated that MCMV-specific CD8 T_{RM} cells conferred local protective immunity, owing to initial virus replication in non-epithelial cells that are refractory towards complete MCMV-mediated MHC class I downregulation. Therefore, our findings established a role for MCMV-specific CD8 T cells in the control of localized virus replication in the SG, thus likely contributing to the containment of CMV transmission episodes (125).

CD4 T cell response during cytomegalovirus infection

Although primary MCMV infection is controlled in most visceral organs within one to two weeks, the salivary glands (SGs) are a peripheral glandular tissue where lytic viral replication is continuing for many weeks (126), facilitating horizontal transmission via saliva. While control of lytic MCMV replication in most tissues is mediated by MCMV-specific CD8 T cells, this is not the case for the SGs (127–129). MCMV-encoded MHC class I immune evasion genes are particularly potent in avoiding recognition of infected cells by cytotoxic CD8 T cells (130, 131), as deletion of these immune evasion genes restores CD8 T cell recognition of MCMV harbouring cells, consequently leading to CD8 T cell-mediated immune control of MCMV infection in the SGs (124). Therefore, under

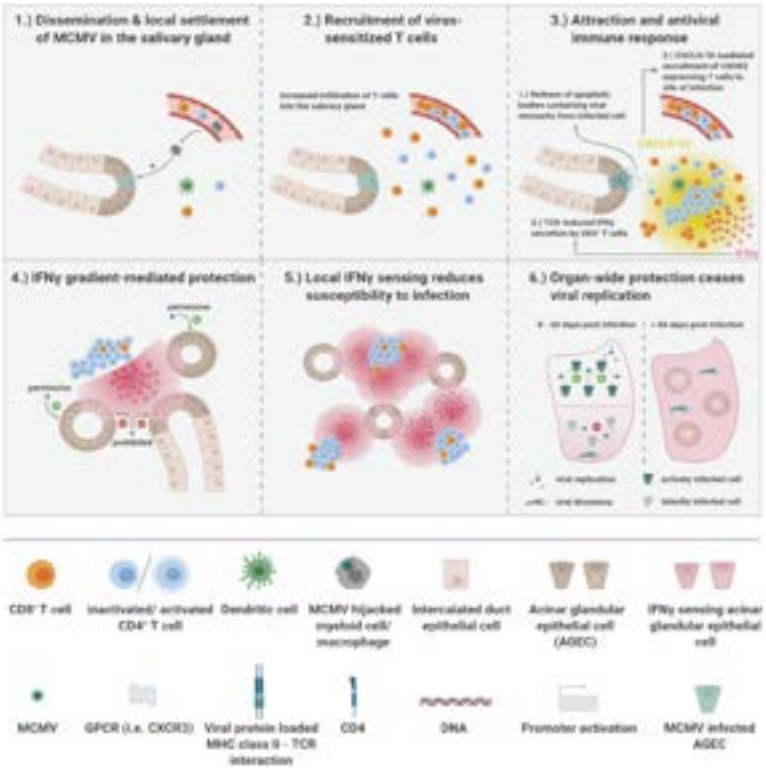

Figure 13 CD4 T cell mediated control of MCMV infection in the salivary gland.
MCMV infection of epithelial cells in the salivary gland precedes the infiltration of activated MCMV-specific T cells. Directly infected cells are not recognized by MCMV-specific CD4 or CD8 T cells. Instead, apoptotic bodies from previously infected cells are taken up by local phagocytes and processed peptides of MCMV proteins are presented to CD4 T cells. This leads to local IFNγ production. MCMV-specific CD4 T cells accumulate at these sites of antigen presentation and the cumulative IFNγ production produces sufficient IFNγ to protect the tissue within a limited perimeter. Accumulation of such protected sites eventually leads to organ-wide control of MCMV replication. Courtesy Dr. Josua Oderbolz.

normal circumstances, control of MCMV infection in the SGs completely relies on CD4 T cells that exert their protective effector functions primarily through the secretion of the pro-inflammatory cytokines interferon gamma (IFNγ) *and* tumor necrosis factor alpha (TNFα) (123, 132). We

had shown that sensing of CD4 T cell-produced IFNγ by non-hematopoietic cells in the SGs is required for eventual control of lytic viral replication (124). However, the question why and how long-lasting productive virus infection is maintained in the SG in face of marked infiltration of functional MCMV-specific CD4 T cells early upon infection (125, 133) remains open. One important aspect that has so far not received much attention is information about micro-anatomical conditions and constraints in the SGs during MCMV infection. This includes spatial information about infection foci, distribution of infiltrating virus-specific CD4 T cells, sites of antigen recognition and IFNγ production, and the range of IFNγ sensing. We used advanced microscopy methods to visualize key components of the antiviral immune response with high spatiotemporal resolution. By combining previous knowledge with our experimental data, we further generated a mathematical model that simulates the CD4 T cell-mediated immune control of MCMV infected SGs. We propose a scenario in which MCMV antigens in the SGs are sensed by virus-specific CD4 T cells only in a delayed and indirect manner, after remnants of previously infected cells have been engulfed by local antigen-presenting cells (APCs). This leads to locally confined IFNγ secretion, affording protection only in this restricted area. However, non-protected areas of the SGs continue to be permissive for infection and replication, evidenced by long-term maintenance of high viral loads in the SGs. Eventual control occurs if local IFNγ-concentrations are sufficiently effective to allow accumulation of protected sites, and thus restriction of viral spread. (Figure 13) (134).

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