Essential and complementary function of dendritic cell subsets during immunotherapy

Sabrina Dähling

ORCID ID:

0000-0003-2304-1860

from Hagenow, Germany

Submitted in total fulfilment of the requirements of the joint degree of

Doctor of Philosophy (PhD)

of

The Medical Faculty

The Rheinische Friedrich-Wilhelms-Universität Bonn

and

The Department of Microbiology and Immunology

The University of Melbourne

Bonn/Melbourne, 2020

Performed and approved by the Medical Faculty of The Rheinische Friedrich-Wilhelms-Universität Bonn and The University of Melbourne

1. Supervisor: Prof. Dr. Natalio Garbi
2. Supervisor: Prof. Dr. Sammy Bedoui

Date of submission: September 2019

Date of the oral examination: February 2020

Institute of Experimental Immunology, Bonn

Director: Prof. Dr. Christian Kurts

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Abbreviations

A

ACK	Ammonium Chlorid Kalium
Arm	Armstrong
APC	Antigen-presenting cell
AP-1	Activator protein 1
αDG	Alpha-dystroglycan

B

Batf3	Basic leucine zipper transcription factor ATF-like 3
BSA	Bovine serum albumin
Blimp-1	B lymphocyte-induced maturation protein-1
BM	Bone marrow

C

CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
cDC	Conventional dendritic cell
CD62L	CD62 L-selectin
CLP	Common lymphoid progenitor
CLR	C-lectin type receptor
Cl13	Clone 13
CMP	Common myeloid progenitor
CTL	Cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte-associated protein 4
CTV	Cell trace violet
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor

D

DAMP	Damage-associated molecular patter

DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
DTR	Diphtheria toxin receptor
DTX	Diphtheria toxin

E

EDTA	Ethylenediaminetetraacetate
et al.	et alii; and others

F

FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
Flt3L	Fms-related tyrosine kinase 3 ligand
FOXO1	Forkhead Box O1
FRC	fibroblastic reticular cells

G

g	Gram or gravity accerelation
GFP	green fluorescent protein
GM-CSF	Granulocyte–macrophage colony-stimulating factor
gp	Glycoprotein

H

h	Hours
HBSS	Hanks balanced salt solution
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HEV	High endothelial vessels
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus

I

ICAM	Intracellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon regulatory factor
ITIM	Immunoreceptor tyrosine-based inhibition motif
ITSM	Immunoreceptor tyrosine-based switch motif
IU	Infectious unit
i.p.	Intraperitoneal
i.v.	Intravenous

J

K

KLRG1	killer cell lectin-like receptor G1

L

l	Litre
LCMV	Lymphocytic choriomeningitis virus
LFA-1	Leukocyte function-associated antigen 1
LN	Lymph node
LPS	Lipopolysaccharide

M

M	Molar
m	Milli
mAb	Monoclonal antibody

MALT	Mucosa-associated lymphoid tissue
MDA-5	Melanoma differentiation-associated protein 5
MDSC	Myeloid-derived suppressor cells
MFI	median fluorescence intensity
MHC	Major histocompatibility complex
min	Minutes
MPEC	Memory precursor effector cell
MyD88	Myeloid differentiation primary response gene 88

N

NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NK cell	Natural killer cell
NLR	NOD-like receptor
NMS	Normalized mouse serum
NOD	Nucleotide-binding oligomerization domain
NP	Nucleoprotein

O

OT-I	Ovalbumin-specific T cells, restricted to MHCI
OVA	Ovalbumin

P

p	P-value
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death 1 ligand 1
pDC	Plasmacytoid dendritic cell
PFA	Paraformaldehyde
pH	Potentia Hydrogenii
PLP	P-buffer L-lysin Paraformaldehyd

PRR	Pattern recognition receptor
P14	LCMV (gp33)-specific $CD8^+$ T cells, restricted to MHCI
p.i.	Post infection
P/S	Penicillin and streptavidin

Q

R

RIG-I	Retinoic acid Inducible Gene I
RLR	RIG-I-like receptor
RNA	Ribonucleotide acid
rpm	Rounds per minute
RT	Room temperature

S

s	Seconds
SEM	Standard error of the mean
SHP	Src homology region 2 domain-containing phosphatase
SIV	Simian immunodeficiency virus
SLEC	Short-lived effector T cell
S1P	Sphingosine-1-phosphate
S1PR1	Sphingosine-1-phosphate receptor
ssRNA	single stranded RNA

T

TCF-1	T cell factor 1
TCR	T cell receptor
T_{FH}	Follicular helper T cell
Th cell	T helper cell
Tim3	T cell immunoglobulin and mucin domain 3
TLR	Toll-like receptor
TNF	Tumour necrosis factor

	TOX	Thymocyte selection-associated HMG-box protein
	Treg	Regulatory T cell
U		
	U	Unit
V		
	v/v	Volume percent
W		
	WT	Wildtype
X		
	XCL1	C chemokine ligand 1
	XCR1	C chemokine receptor 1
Y		
	YFP	yellow fluorescent protein

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Abstract

T lymphocytes are critical components of the adaptive immune system that protects us against a variety of infections. However, during chronic infections such as with HIV, HCV and HBV as well as cancers, T cells become functionally impaired in order to limit immunopathology. This in turn allows these infections to persist or tumours to grow and spread. Scientific research over the last decades lead to the development of checkpoint inhibitors which can reinvigorate exhausted $CD8^+$ T cells. This enables them to fight persisting infections and to eliminate even advanced tumours. Based on this success, checkpoint immunotherapy has revolutionized cancer treatment. With the identification of the memory-like TCF-1^+ subset of exhausted $CD8^+$ T cells that responds to checkpoint immunotherapy, predictions for clinical responses can be further improved. Yet, despite the enormous success and routine application, a deeper mechanistic understanding of checkpoint immunotherapy is required in order to help patients in whom this therapy failed.

In this study, we elucidate critical cellular interaction partners of exhausted $CD8^+$ T cells during anti-PD-L1 treatment. Using the murine chronic LCMV model, we found that DC with their key function in T cell activation are pivotal for a successful therapy demonstrated by the expansion of virus-specific $CD8^+$ T cells and control of viral load. Notably, different DC subsets represent complementary roles in this context. The cross-presenting subset of $XCR1^+$ DC are critical to maintain the population of memory-like TCF-1^+ exhausted $CD8^+$ T cells while the remaining DC promote proliferation of such. Data presented in this study indicate that a complex network of signalling molecules delivered by DC subsets is involved in this process. Performing detailed transcriptional analysis of memory-like TCF-1^+ cells, we deciphered that this cell pool represents a heterogenous population and our results imply that the different subsets reside in different areas of secondary lymphoid organs. Investigation of the microanatomy of the $CD8^+$ T cell – DC interaction revealed three distinct areas of the spleen where communication of the cellular subsets occurs.

Taken together, we elucidated a complex interplay between exhausted $CD8^+$ T cells and DC during chronic viral infection and anti-PD-L1 treatment. This communication impacts on the transcriptional and functional level of exhausted $CD8^+$ T cells as well as on their localization in secondary lymphoid organs. Overall, our data identified critical cellular interaction partners and their specific anatomical localization that is essential for the successful reinvigoration of exhausted $CD8^+$ T cells during checkpoint immunotherapy.

Declaration

The work that is presented in this thesis was conducted at The Rheinische Friedrich-Wilhelms-Universität Bonn and The University of Melbourne in the laboratories of Professor Wolfgang Kastenmüller (Institute of Experimental Immunology, Bonn) and Professor Sammy Bedoui (Peter Doherty Institute, Melbourne). The research work was funded by the Bo&MeRanG Graduate School.

This is to certify that

(i) the thesis comprises only my original work towards the PhD except where indicated in the Preface,

(ii) due acknowledgement has been made in the text to all other material used,

(iii) the thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Sabrina Dähling

Preface

My contribution to the experiments within each chapter was as follows:

Chapter 3: 90 %

Chapter 4: 90 %

Chapter 5: 75 %

I acknowledge the important contributions of others to experiments presented herein:

Chapter 3: Anika Grafen, Dr. Lena Collenburg

Chapter 4: Anika Grafen, Dr. Lena Collenburg

Chapter 5: Konrad Knöpper, Annika Peters, Dr. Lena Collenburg

Acknowledgement

I would like to thank everyone who supported me during my PhD by assisting me with experiments, sharing their knowledge and discussing ideas or just getting a coffee.

First and foremost, I would like to thank my supervisors Wolfgang Kastenmüller and Sammy Bedoui. Both of you are amazing scientists and I am very grateful you have a good sense of humour as well. I read this once and think it fits perfectly: 'I am grateful for the wisdom of older people who have enriched my world.'
Wolfgang, from the very beginning you taught me to work independently and at the same time I knew I could ask any question any time. I learned from you how to communicate my research and to look at it from different perspectives. Your support really encouraged me and your passion for science is contagious.
Sammy, thank you for such a warm welcome in your lab and especially for always listening. Whenever I struggled, you helped me to find a solution. You have inspired me and I learned so much during my time in Melbourne.
To both of you: a very big thank you for supervising me as a Bo&MeRanG PhD student and for always supporting me even when I was 16000 km away.

Prof. Dr. Natalio Garbi, thank you for being the first supervisor of this thesis and for all your help during my time in Bonn and afterwards. Your ideas and input really advanced this project. Prof. Dr. Eicke Latz, thank you for joining my PhD committee in Bonn.

Further I want to thank Prof. Dr. Axel Kallies and Prof. Dr. Thomas Gebhardt for being part of my PhD committee in Melbourne and for your great support during my time there. Your advice, help and feedback were always highly appreciated.

This brings me to thanking every former and current member of the laboratories of Wolfgang and Sammy. First, all members of the AG Kastenmüller in Bonn

and Würzburg: Anna, Anika, Annika, Johannes, Karl, Kathrin, Konrad, Lena, Lennart, Marco, Meike, Milas, Sandra, Sarah and Steffi. All of you have been super helpful throughout the whole time and I am very grateful to work with you guys. Special thanks to Anika and Lena who helped me so much with experiments and continued the project in Germany while I was in Melbourne. Without you this project wouldn't be what it is now. Konrad, without your help I still wouldn't understand sequencing data. Thank you for teaching me.
To all members of the Bedoui and Gebhardt group: Annabell, Elise, Emma, Evangeline, Johanna, Katharina, Maike, Marie, Nathan and Paul. You made me feel welcome from the very first day and I learned so much from you. Special thanks to Paul: you have been such a good teacher. Thank you for always taking time to help me in the lab, discuss results and ideas. It's been a lot of fun.
I also want to thank all members of the Kallies group. Without your help I wouldn't have been able to perform many of the experiments in Melbourne. Especially Daniel, thanks for all your support, for answering my questions and sharing your knowledge.
To all of you: thanks for listening, laughing and having one or the other beer.

I also want to thank everyone organizing and taking part in the Bo&MeRanG program. Especially Lucie and Marie, I really appreciate all your effort throughout this first phase of the program.

Special thanks to all my friends who are always supportive, fun and simply just really cool people. I am very happy to have you!

Most importantly: my family. Mama, Papa, Nicole, Mandy, Jan, Mike, Jannis, Annalena, Marie und Johanna – ihr seid die Besten und ich bin unglaublich dankbar euch zu haben. Dank eurer Unterstützung habe ich nun sogar eine Doktorarbeit geschrieben. Die Sicherheit zu wissen, dass ihr immer für mich da seid, auch wenn ich am anderen Ende der Welt bin, gibt mir Kraft und Mut. Dafür danke ich euch von ganzem Herzen.

List of Publications

Brewitz, A., S. Eickhoff, **S. Dahling**, T. Quast, S. Bedoui, R. A. Kroczek, C. Kurts, N. Garbi, W. Barchet, M. Iannacone, F. Klauschen, W. Kolanus, T. Kaisho, M. Colonna, R. N. Germain and W. Kastenmuller (2017). "CD8+ T Cells Orchestrate pDC-XCR1+ Dendritic Cell Spatial and Functional Cooperativity to Optimize Priming." **Immunity** 46(2): 205-219.

Bachem, A., C. Makhlouf, K. J. Binger, D. P. de Souza, D. Tull, K. Hochheiser, P. G. Whitney, D. Fernandez-Ruiz, **S. Dahling**, W. Kastenmuller, J. Jonsson, E. Gressier, A. M. Lew, C. Perdomo, A. Kupz, W. Figgett, F. Mackay, M. Oleshansky, B. E. Russ, I. A. Parish, A. Kallies, M. J. McConville, S. J. Turner, T. Gebhardt and S. Bedoui (2019). "Microbiota-Derived Short-Chain Fatty Acids Promote the Memory Potential of Antigen-Activated CD8(+) T Cells." **Immunity** 51(2): 285-297 e285.

1. Introduction

The immune system protects the body from many different pathogens, such as bacteria, viruses, fungi, protozoa and helminths and is an important defence system against cancer. To protect the host from disease, this highly complex network of cells, signalling and effector molecules communicates on many different levels. The underlying processes, called the immune response, need to be tightly regulated.

Physical barriers such as skin and mucosa hinder pathogens from invading the body. For example, the skin protects the body from infection with features such as low pH, chemicals and a distinct microbial flora, that prevents pathogens from entering (Park and Lee 2017).

In case this first line of defence does not prevent pathogens from entering, the innate immune system can quickly recognize these invaders as well as tissue injury. The innate arm can control the infection to a certain extent but importantly also activates the adaptive immune system. T and B cells of this arm detect foreign antigen in a highly specific manner. Upon proliferation, these antigen-specific cells gain effector functions and produce antibodies to clear the infection. In addition, memory T and B cells are generated and deliver long-term protection from reinfection with the same pathogen (Ahmed and Gray 1996). The innate and the adaptive immune system represent two highly intertwined systems, which can together fully overcome an infection.

Nevertheless, some pathogens have developed strategies to evade these immune responses and are thus able to persist. A constant and highly activated immune response can severely harm the host and cause immunopathology. Therefore, regulatory elements are of great importance to ensure both: control of the infection and control of the immunological response.

1.1 The innate immune system

The innate immune response is an overall term for the rapid reaction of certain cells, antimicrobial enzymes and peptides as well as the complement system to the invasion of pathogens. Cells of the innate immune system are leukocytes, such as NK cells, mast cells, eosinophils and basophils, as well as phagocytic cells such as macrophages, monocytes, neutrophils and dendritic cells. Some of these cells reside in different tissues whereas others circulate through the body and are recruited to the tissue upon an infection (Iwasaki and Medzhitov 2010). In comparison to the adaptive immune response, the innate arm of the immune response is evolutionary older and exists in vertebrates as well as invertebrates and plants (Buchmann 2014).

Recognition of foreign and potentially pathogenic material is performed in a non-antigen-specific manner. Immune cells are able to recognize conserved molecular patterns, which are absent in the host, and are only expressed by pathogens. Such motifs are for example part of the cell wall and membrane of bacteria like lipoteichoic acid of gram-positive and lipopolysaccharides (LPS) of gram-negative bacteria. Importantly, the immune system also recognizes material which is found outside of its usual compartments. For example, viruses can be recognized by their nucleic acids. If DNA or RNA is detected in the cytoplasm or endosomal compartments, signalling pathways of the innate immune response are initiated (Mogensen 2009). Apart from these so-called pathogen-associated molecular patterns (PAMPs) (Janeway 1989) there are also damage-associated molecular patterns (DAMPs). Also called alarmins, these endogenous molecules are released by stressed cells or cells which undergo necroptosis (Matzinger 1994). PAMPs and DAMPs are recognized by pattern recognition receptors (PRRs) (Akira, Takeda *et al.* 2001, Janeway and Medzhitov 2002). A plethora of different PRRs have been identified to be active in the context of infection and pathogen recognition. These receptors can be distinguished by their cellular localization. Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) are expressed on the cell surface or in endosomal compartments (Zelensky and Gready 2005, Kaisho and Akira 2006). Retinoic

acid inducible gene I (RIG-I) -like receptors (RLRs) and nucleotide-binding oligomerization domain-containing (NOD) -like receptors (NLRs) sense foreign patterns in the cytosol (Takeuchi and Akira 2010). These innate sensing mechanisms are positioned at specific locations to optimally detect pathogens and distinguish the foreign material from the hosts own one by specific motifs and by their existence in an unusual location.

Signal transmission of these receptors depends on certain adaptor proteins with enzymatic activity, which are often shared between different PRRs. For example, all TLRs but TLR3, signal via the myeloid differentiation primary response gene 88 (MyD88) (Takeda and Akira 2004). MyD88 signalling induces the activation of other adaptor proteins (IRAK, TRAF6) which can lead to the activation of important transcription factors like nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB), activator protein 1 (AP-1) and interferon regulatory factor 3 and 7 (IRF3/7). Thereby, transcription of pro-inflammatory cytokines (e.g. IL-6, IL-1β, TNF, IL-12, IFN I) and co-stimulatory molecules is induced which are crucial for the control of infections (Theofilopoulos, Baccala *et al.* 2005, Kawai and Akira 2006). Cytoplasmic PRRs, like RIG-I and melanoma differentiation-associated protein 5 (MDA-5), both belonging to the RLR family and sensing viral nucleotides, also signal via adaptor proteins which induce the transcription of pro-inflammatory cytokines via NF-κB as well as IRF3 and IRF7 (Yoneyama, Kikuchi *et al.* 2004). This very rapid innate immune response can induce direct killing of pathogens and infected cells but importantly also enables the activation of the adaptive immune system.

1.2 The T cell response as part of the adaptive immune system

Invertebrates and plants solely rely on the protection by the innate immune response. However, in vertebrates a second arm of the immune system, the adaptive one, evolved. Hallmarks of the adaptive immune system are a highly specific recognition of pathogens and the ability to generate memory which protects from reinfection with the same pathogen. Full activation of the adaptive

immune system requires more time compared to the innate one. Nevertheless, this response is highly essential to combat infections.

The adaptive immune response is divided into the humoral and the cellular arm consisting of B lymphocytes (B cells) and T lymphocytes (T cells), respectively. Both cell types arise from the common lymphoid progenitor (CLP). In this study, we focused on T cell responses during chronic viral infection and will therefore focus on this cellular element of the adaptive immune response.

T cells do not recognize pathogens directly, but antigens which are processed and presented by antigen-presenting cells (APC). This recognition is enabled by the T cell receptors (TCR), which are genetically recombined receptors of different specificities (Garcia and Adams 2005). The cellular adaptive immune response is represented by $CD4^+$ and $CD8^+$ T cells, named after the expression of the co-receptors CD4 and CD8, which support TCR signalling. In comparison to B cells, CLPs which leave the bone marrow and enter the thymus via the blood, become T cell precursors (Pui, Allman *et al.* 1999, Wilson, MacDonald *et al.* 2001). These developing T cells do not yet express the co-receptors CD4 and CD8 and are therefore termed double negative (DN). DN thymocytes undergo β chain rearrangement and start expressing CD4 and CD8 (double positive (DP)) (Aifantis, Feinberg *et al.* 1999). Rearrangement of the α chain loci further enables TCR diversity. To ensure that TCR signalling is functional, these double positive cells undergo positive selection in the cortex. Epithelial cells present self-antigen via MHCI and MHCII molecules and only cells with a high affinity and therefore functional TCR engagement will survive (Jameson, Hogquist *et al.* 1995, Kisielow and von Boehmer 1995). This is followed by negative selection in the medulla with mainly DC presenting self-antigen. In contrast to the first selection step, T cells that recognize peptide with high affinity at this stage will be eliminated. This is a critical step during T cell development to generate self-tolerant T cells, which only recognize foreign antigen. Surviving T cells become MHCI or MHCII restricted by downregulation of either of the two co-receptors CD4 and CD8, respectively (Starr, Jameson *et al.* 2003). These mature $CD4^+$ and $CD8^+$ T cells exit the thymus and circulate in the blood.

Naïve T cells circulate through the body and are able to enter and exit secondary lymphoid organs like lymph nodes, spleen and mucosa-associated lymphoid tissue (MALT). By doing so, these T cells are able to constantly scan the body for foreign antigen. For this recirculation, certain homing receptors that enable migration from the blood into lymphoid tissue are expressed (von Andrian and Mempel 2003). L-selectin (CD62L) is expressed by naïve T cells and binding to its ligands GlyCAM-1 and MadCAM-1 on high endothelial vessels (HEVs) mediates transient attachment and is described as a rolling process. For activation-dependent arrest of the T cells, CCR7-CCL19/21 signalling is important. CCR7 is expressed on naïve T cells and CCL19 and CCL21 by endothelial cells. Signalling via this axis leads to the activation of leukocyte function-associated antigen 1 (LFA-1), an integrin which can in turn bind to intracellular adhesion molecule 1 (ICAM1) and ICAM2. This interaction finally enables transmigration of the cells into the lymphoid organ (von Andrian and Mempel 2003).
In the T cell zone, cells are able to quickly migrate and scan for their cognate antigen. This migration is supported by fibroblastic reticular cells (FRC) that are directly associated with HEVs and form a stromal network which helps guiding cells to their destinations (Bajenoff, Egen *et al.* 2006). If no antigen is recognized, cells exit the lymphoid organ. Upon signalling, CCR7 is internalized. In addition, egress is supported by an increasing gradient of lipid messenger sphingosine-1-phosphate (S1P) towards the efferent lymph. Binding of S1P to sphingosine-1-phosphate receptor (S1PR1), promotes exit of T cells into the lymph to re-enter the circulation (Matloubian, Lo *et al.* 2004, Pham, Baluk *et al.* 2010).

In the secondary lymphoid tissues, antigen-presenting cells (APC) like macrophages and dendritic cells (DC) present antigen via major histocompatibility complex (MHC) molecules. There are two different classes of MHC molecules – MHC class I and II. MHC I molecules are present on every nucleated cell, while MHC II molecules are primarily expressed by specific cells with phagocytic activity like macrophages and DC. Typically, endogenous

antigen from intracellular pathogens is processed into peptide fragments and presented with MHC I complexes (Vyas, Van der Veen *et al.* 2008). In contrast, exogenous material is internalized, processed and presented via MHC II complexes (Swain 1983).

T cells become activated once they recognize cognate antigen. $CD4^+$ T cells recognize antigen presented in complex with MHC II molecules (Gay, Maddon *et al.* 1987). This interaction is stabilized by the co-receptor CD4. In contrast, $CD8^+$ T cells become activated by TCR engagement with antigen presented by MHC I molecules. Again, the co-receptor (CD8) stabilizes this interaction (Rudolph, Stanfield *et al.* 2006). However, TCR engagement itself does not mount full activation of T cells. Without further signals, cells become anergic and subsequently die (Wells 2009).

Three different signals are described to be critical for complete T cell activation (Lenschow, Walunas *et al.* 1996, van Stipdonk, Lemmens *et al.* 2001, Curtsinger and Mescher 2010). TCR engagement is referred to as signal 1. Signal 2 is provided by co-stimulatory signalling pathways like CD80/CD86-CD28, CD40L-CD40 and ICOSL-ICOS (Greenwald, Freeman *et al.* 2005, Watts 2005). The third signal, that T cells need to receive during activation, is delivered via cytokines like IL-12, IFN I and to some extend IFNγ produced by APCs, innate lymphocytes and NK cells (Curtsinger, Lins *et al.* 2003, Curtsinger and Mescher 2010). In addition, IL-2 plays an important role for the proliferation and survival of activated T cells. Via autocrine production and the expression of the high affinity IL-2 receptor (IL2Ra, CD25), T cells boost their own response (Lanzavecchia and Sallusto 2001).

Activated T cells proliferate and remain in the lymphoid tissue for several days due to the expression of CD69. This activation marker causes internalization of S1PR1 which hinders cells from egress from secondary-lymphoid tissues (Cebrian, Yague *et al.* 1988). After proliferation, T cells are able to egress by downregulation of CD69 and subsequent S1PR1 signalling (Shiow, Rosen *et al.* 2006).

Depending on the infection, cytokines (signal 3) determine the differentiation of $CD4^+$ T cells into different subsets – Th1, Th2, Th17, T_{FH}, Treg cells. Differentiation into Type 1 helper cells (Th1) is induced by IFNγ and IL-12 (Grogan, Mohrs *et al.* 2001, Mullen, High *et al.* 2001). Th1 cells are able to activate infected macrophages via IFN-γ to promote microbial killing of intracellular pathogens and therefore resemble most of the $CD4^+$ T cells during viral infections (Schroder, Hertzog *et al.* 2004, Zhu and Paul 2008). On the other hand, IL-4 promotes differentiation of $CD4^+$ T cells into Type 2 helper cells (Th2) (Grogan, Mohrs *et al.* 2001). Th2 responses enable the recruitment of eosinophils, basophils and mast cells to fight extracellular parasites. T helper 17 cells (Th17) cells are activated by combined signalling of TGF-β and IL-6 (Park, Li *et al.* 2005). These cells release high amounts of IL-17, an important interleukin for the recruitment of neutrophils playing a role during infection with extracellular bacteria and helminths (Harrington, Hatton *et al.* 2005). IL-6 itself, without additional TGF-β signalling, rather promotes the differentiation into follicular helper T cells (T_{FH}), which are able to migrate to B cell follicles to optimally activate B cells and thereby the humoral arm of adaptive immunity (Reinhardt, Liang *et al.* 2009). On the other hand, TGF-β signalling by itself rather induces the generation of regulatory T cells (Treg). These cells play an important role during infections and autoimmunity as they regulate the immune response on various levels by the production of anti-inflammatory cytokines like IL-10 and TGF-β (Zheng, Wang *et al.* 2004).

$CD4^+$ T cells have been shown to play an essential role for optimal priming of $CD8^+$ T cells in various infection models. During HSV-1 infection, $CD4^+$ T cells were essential for optimal priming of $CD8^+$ T cells by DC (Greyer, Whitney *et al.* 2016). During chronic LCMV infection, $CD4^+$ T cells are crucial to control viral load as depletion of such prolongs viral persistence in various organs (Matloubian, Concepcion *et al.* 1994, Zajac, Blattman *et al.* 1998). Similar, during HCV infection, antigen-specific $CD4^+$ T cells highly impact the $CD8^+$ T cell response and disease outcome (Smyk-Pearson, Tester *et al.* 2008). However, $CD4^+$ helper T cells are not able to directly interact with $CD8^+$ T cells.

Our group could describe that a specific DC subset serves as a platform where helper signals get exchanged (Eickhoff, Brewitz *et al.* 2015).

Activated $CD8^+$ T cells become so-called cytotoxic T cells (CTL) and play a supreme role during viral infections (Haring, Badovinac *et al.* 2006). As mentioned above, three signals are essential for optimal priming (Butz and Bevan 1998, Curtsinger, Lins *et al.* 2003). Cytokines that impact and support $CD8^+$ T cell activation are for example IFN I, IFN-γ and IL-12 (Cousens, Peterson *et al.* 1999, Mescher, Curtsinger *et al.* 2006). IFN I cytokines are essential to mount a full $CD8^+$ T cell response upon LCMV infection (Keppler, Rosenits *et al.* 2012). Additionally, also IFN-γ boosts the expansion of $CD8^+$ T cells during LCMV infection (Badovinac, Tvinnereim *et al.* 2000). During VSV infection, IL-12 and IFN I signalling are critical (Keppler, Rosenits *et al.* 2012). Fully activated $CD8^+$ T cells release granules containing cytotoxic effector molecules like perforin and granzymes. Perforin is a cytolytic protein which forms pores into the target cell plasma membrane. Loss of membrane potential in combination with the ability of granzymes to enter the cell induce apoptosis in targeted cells. Granzymes are serine proteases which through activation of caspases initiate DNA degradation (Trapani and Smyth 2002). Importantly, the $CD8^+$ T cell pool displays a heterogeneous group of cells with some exhibiting stronger effector functions than others. The two main subsets are short-lived effector cells (SLECs) and memory-precursor effector cells (MPECs). Whereas most of the activated $CD8^+$ T cells during the peak of an immune response are SLECs, which are limited in their capacity to proliferate, some MPECs develop into long-lived memory cells. The $CD8^+$ T cell response to viral infections and the impact of certain cytokines will be discussed in more detail in chapter 1.4.

Overall, the adaptive immune response enables specific and highly regulated elimination of various pathogens with life-long protection. Nevertheless, for its full activation, the adaptive immune response relies on an intact innate immune system and professional cells that enable communication between these two.

1.3 Conventional dendritic cells

Dendritic cells (DC) play a superior role for the immune system as they represent a crucial link between the innate and adaptive arm. These professional antigen-presenting cells (APC) are most effective at stimulating naïve T cells (Steinman and Witmer 1978, Inaba and Steinman 1984). Discovered by Steinman and Cohn in 1973, DC were named after the Greek word for tree (dendron) due to their morphological features (Steinman and Cohn 1973).

DC derive from common myeloid progenitors (CMPs) in the BM, which further differentiate into macrophage-dendritic cell progenitors (MDPs) that give rise to common dendritic cell progenitors (CDPs). From these CDPs, pre-conventional DC (pre-cDC) or pre-plasmacytoid DC (pre-pDC) arise (Naik, Sathe *et al.* 2007, Liu, Victora *et al.* 2009). These pre-DC exit the BM and circulate in the blood from where they can migrate into secondary- and non-lymphoid tissues and differentiate into cDC and pDC (Dalod, Chelbi *et al.* 2014).

pDC are described to have a comparatively low potential to present antigen and rather act as important IFN I producers (Rodrigues, Alberti-Servera *et al.* 2018). In contrast, cDC are highly professional APCs. There are two distinct subgroups of cDC, migratory and tissue-resident DC, which can be further divided into cDC1 and cDC2. Notably, cDC1 have been shown to be critical for the activation of $CD8^+$ T cells whereas cDC2 promote the activation of $CD4^+$ T cells (Dudziak, Kamphorst *et al.* 2007). It is described that differentiation into either of the subsets takes place in the tissue. However, studies suggest that pre-cDC1 and pre-cDC2 already exist in the BM (Schlitzer, Sivakamasundari *et al.* 2015). Certain cytokines impact the generation and development of DC (Schmid, Kingston *et al.* 2010). Fms-like tyrosine kinase 3 ligand (Flt3L), produced by stromal, endothelial and activated T cells, is essential for DC development during steady state (Karsunky, Merad *et al.* 2003, Waskow, Liu *et al.* 2008). Especially cDC1 depend on Flt3 signalling (Ginhoux, Liu *et al.* 2009). Additionally, certain transcription factors were shown to drive differentiation of pre-DC into the different subsets. Whereas Batf3, Nfil3, Id2, and Irf8 support

cDC1 generation (Hildner, Edelson *et al.* 2008, Jackson, Hu *et al.* 2011, Kashiwada, Pham *et al.* 2011, Sichien, Scott *et al.* 2016), Irf4- and Zeb2-dependent pathways are able to control differentiation of cDC2 (Suzuki, Honma *et al.* 2004, Tamura, Tailor *et al.* 2005, Scott, Soen *et al.* 2016). DC have a short half-life of only 3-5 days and therefore need to be constantly replenished from the BM (Liu, Waskow *et al.* 2007).

Migratory DC sample foreign antigen in the periphery and subsequently migrate in a CCR7-dependent manner to the T cell zones of the draining lymph node (LN), where they present the antigen to naïve T cells. These DC are not found in the spleen (Liu and Nussenzweig 2010).

Resident DC are $MHCII^+$ and $CD11c^{hi}$ and can be distinguished by the expression of CD8 (cDC1) and CD11b (cDC2). Most of the $CD8^+$ cDC1 specifically express the chemokine receptor XCR1 (Dorner, Dorner *et al.* 2009, Bachem, Hartung *et al.* 2012). This chemokine receptor can bind its ligand XCL1, expressed by NK and activated $CD8^+$ T cells, and enables migration of $XCR1^+$ DC towards the source of XCL1. Our group revealed an essential role of the XCL1-XCR1 axis during acute viral infections (Brewitz, Eickhoff *et al.* 2017). However, the role of this signalling pathway during chronic infections remains unclear. Another feature of $XCR1^+$ DC, as well as their migratory counterpart the $CD103^+$ DC, is the ability of cross-presentation (Dudziak, Kamphorst *et al.* 2007, Bedoui, Whitney *et al.* 2009). As mentioned above, antigen from intracellular pathogens is typically presented with MHC I complexes (Vyas, Van der Veen *et al.* 2008) whereas exogenous material is internalized, processed and presented via MHC II complexes (Swain 1983). However, $XCR1^+$ DC are able to take up exogenous material and present it via MHC I to stimulate $CD8^+$ T cells (Bevan 1976). Therefore, this unique feature plays an important role for $CD8^+$ T cell responses during infection with intracellular pathogens such as viruses (Rock 2003, Joffre, Segura *et al.* 2012). Additionally, cytokine production by $XCR1^+$ DC was shown to strongly affect the T cell response. For example, IL-12 produced by DC regulates the differentiation of T cells (Hochrein, Shortman *et al.* 2001). Furthermore, DC-derived IL-15 is essential for

optimal priming of $CD8^+$ T cells during HSV-1 infection (Greyer, Whitney *et al.* 2016).

$CD11b^+$ DC (cDC2), in contrast to $XCR1^+$ DC, preferentially activate $CD4^+$ T cells by presentation of antigen in complex with MHC II molecules (Dudziak, Kamphorst *et al.* 2007). These DC do not produce IL-12 but instead shape T cell responses by secretion of IL-6 and IL-23 (Persson, Uronen-Hansson *et al.* 2013, Schlitzer, McGovern *et al.* 2013).

DC not only present antigen to T cells (signal 1) and secrete cytokines that drive T cell responses (signal 3), but also deliver important co-stimulatory molecules (signal 2). Under inflammatory conditions, DC upregulate CD40, CD80 and CD86 (Guermonprez, Valladeau *et al.* 2002, Belz and Nutt 2012). CD80 and CD86, for example, are the ligands for CD28, which is expressed on T cells. CD28 signalling supports TCR signalling and thereby T cell activation (Shahinian, Pfeffer *et al.* 1993).

The different DC subsets do not only deliver necessary signals, but are also optimally positioned in secondary lymphoid organs for rapid uptake of antigen and priming of T cells (Mildner and Jung 2014). In the spleen, $XCR1^+$ DC are mainly located in the T cell zones of the white pulp as well as the marginal zone. Few of these DC reside in the red pulp (Pulendran, Lingappa *et al.* 1997, Steinman, Pack *et al.* 1997). In contrast, $CD11b^+$ DC populate the bridging channels (Gatto, Wood *et al.* 2013). Therefore, position of DC subsets highly correlates with the T cell subsets they preferentially prime and exhibits another prerequisite for optimal activation of the adaptive immune response (Calabro, Liu *et al.* 2016).

1.4 T cell responses to acute and chronic viral infections

As described above, viral infection is rapidly detected by the innate immune response. PAMPs are recognized by PRRs expressed on various cell types. Cells of the innate immune system induce pro-inflammatory processes by cytokine and chemokine production and activate the adaptive arm of the immune response.

The T cell response during acute viral infections is initiated by a strong expansion phase to generate a cytotoxic CD8⁺ T cell response with which the infection can be cleared. This is followed by a rapid contraction of the cells to prevent an overrepresentation and dominance by CD8⁺ T cells specific for one pathogen (Badovinac and Harty 2006). Cells that survive will remain as memory CD8⁺ T cells which can quickly eliminate pathogens upon reinfection (Figure 1.1, left).

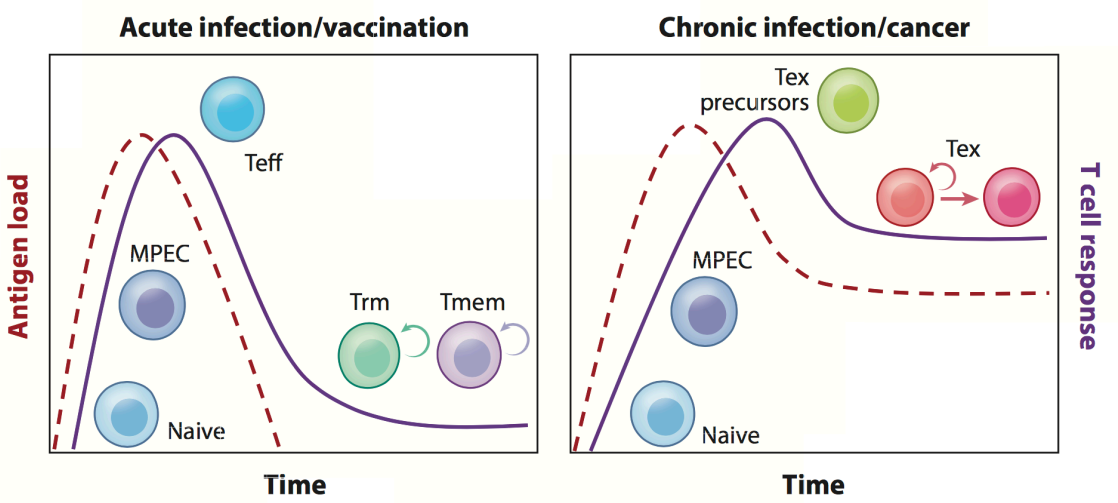


Figure 1.1: CD8⁺ T cell responses to acute and chronic infections
Upon an acute infection (left) naive CD8⁺ T cells expand and differentiate into memory precursor T cells (MPECS) and effector T cells (Teff). The infection can be controlled and cleared whereupon most of the effector T cells die during the contraction phase. Memory CD8⁺ T cells are able to survive and provide long-term protection from reinfection with the same pathogen. In comparison to acute infections, which can be resolved, chronic infections are marked by the persistence of antigen (right). To prevent immunopathology, CD8⁺ T cells become exhausted (Tex) and have a very limited potential to control the infection. Therefore, antigen load stays high and a fully functional memory cell pool cannot be established (McLane, Abdel-Hakeem et al. 2019).

In detail, APCs and especially DC prime and activate CD8⁺ T cells in secondary lymphoid organs by the presentation of antigen via MHC I complexes as well as by delivery of co-stimulatory signals and cytokines (van Stipdonk, Lemmens *et al.* 2001, Chopin, Allan *et al.* 2012). Following the activation by DC, antigen-

specific $CD8^+$ T cells proliferate in a so-called expansion phase and exit the lymphoid organs to fight the infection (Williams and Bevan 2007). Infected cells can be killed by cytotoxic T cells with the production and release of perforin and granzymes, which together cause apoptosis of target cells (Kaech and Cui 2012).

Activated $CD8^+$ T cells display a heterogeneous group of cells with most of them being short-lived effector cells (SLECs), distinguished by the expression of killer cell lectin-like receptor subfamily G member 1 (KLRG1) (Robbins, Terrizzi *et al.* 2003). These cells are terminally differentiated as they cannot proliferate after secondary contact with antigen and will die by apoptosis after clearance of the infection during the contraction phase. Besides SLECs, also long-lived cells exist which will give rise to the memory cell compartment and are therefore termed memory precursor effector cells (MPECs). This population expresses high levels of survival receptors such as IL7Rα (CD127) (Joshi and Kaech 2008). There are various models that explain how these different $CD8^+$ T cell subsets emerge. Nevertheless, a complete understanding of this process is still missing. The $CD8^+$ T cell response peaks around day 7 post infection. As the infection is cleared by this day, approximately 95 % of the effector $CD8^+$ T cells (SLECs) will die (Badovinac and Harty 2006). In contrast, MPECs are able to survive. The generation of memory cells, which are maintained in an antigen-independent manner, highly depends on certain transcription factors. For example, the interplay between EOMES and T-bet seems to influence this formation. T-bet expression favours the development of SLECs under pro-inflammatory conditions whereas stronger EOMES expression during weaker inflammatory responses drives MPECs (Joshi, Cui *et al.* 2007, D'Cruz, Rubinstein *et al.* 2009, Kaech and Cui 2012). Additionally, Id2 supports the formation of memory cells (Cannarile, Lind *et al.* 2006). Cytokines have been shown to play a pivotal role for effector and memory development as well. In this context, IL-7 and IL-15 signalling is essential for complete development of a memory cell pool (Schluns and Lefrancois 2003, Ma, Koka *et al.* 2006, Boyman, Purton *et al.* 2007). In summary, the T cell response during acute viral infections

is initiated by a strong expansion phase which leads to clearance of the infection, followed by a contraction phase and memory formation.

In contrast to an acute infection, chronic infections such as with HIV, HBV or HCV are not cleared by the immune system. These viruses evolved strategies to evade the immune response and the infection becomes chronic. With the continuous stimulation of the immune system caused by persisting antigen, the host is at high risk to be harmed by immunopathology rather than direct effects of the pathogen. An ongoing and excessive immune response needs to be prevented. Therefore, the immune system dampens itself with a highly complex and regulated network of inhibitors to a level where immunopathology is kept to a minimum (Cornberg, Kenney *et al.* 2013). However, in this attenuated state, the immune system is not able to clear the infection (Figure 1.1, right).

In the past decades, numerous research labs focused on the T cell response during chronic viral infections and most of the current understanding was achieved with studies using the lymphocytic choriomeningitis virus clone 13 (LCMV Cl13) infection model (Chapter 1.5). Initial $CD8^+$ T cell responses were described to be comparable during chronic and acute infection (Wherry 2011). However, recent studies show that expression of the exhaustion-driving HMG-box transcription factor (TOX) is significantly increased already at day four post infection with the chronic LCMV strain clone 13 (Khan, Giles *et al.* 2019). Other studies describe further differences at the transcriptional level of $CD8^+$ T cells eight days post infection, when comparing acute and chronic viral infections (Scott-Browne, Lopez-Moyado *et al.* 2016, Man, Gabriel *et al.* 2017). Early studies with LCMV Cl13 revealed that $CD8^+$ T cells enter a state of hyporesponsiveness, also called exhaustion (Zajac, Blattman *et al.* 1998) (Chapter 1.6). Associated with the expression of certain inhibitory receptors, T cell responses are downregulated and loss of effector functions appears in a progressive manner (Wherry 2011). Nevertheless, it remains unclear when exactly $CD8^+$ T cells enter this phase of exhaustion. Effector cells during the early phase of chronic infection express activation markers such as CD69, comparable to effector cells during acute infection (Wherry, Ha *et al.* 2007). Some $CD8^+$ T cells also keep in vivo killing activity, indicating that these cells

are not completely impaired (Fuller, Khanolkar *et al.* 2004, Agnellini, Wolint *et al.* 2007). However, the infection cannot be cleared and $CD8^+$ T cells that largely lost their functional activity can be detected as well (Fuller and Zajac 2003, Wherry, Blattman *et al.* 2003). During some chronic infections, extreme exhaustion ending in deletion of antigen-specific $CD8^+$ T cells has also been reported (Battegay, Moskophidis *et al.* 1994, Fuller, Khanolkar *et al.* 2004). One of the hallmarks of the adaptive immune system is the formation of memory cells. During chronic infection, however, $CD8^+$ T cells fail to differentiate into this cell type and the memory compartment is described to be absent (Wherry, Barber *et al.* 2004, Shin and Wherry 2007). Still, exhausted $CD8^+$ T cells display a very heterogeneous population and recent studies have shown that two main subpopulations can be described. Terminally exhausted $CD8^+$ T cells can be distinguished by the expression of Tim3, while memory-like exhausted $CD8^+$ T cells express TCF-1. These memory-like cells harbor some features of memory cells as well as stem cells and possess the ability of self-renewal as well as differentiation into terminally exhausted cells (Im, Hashimoto *et al.* 2016).

Overall, T cell responses during chronic infections differ substantially from responses during acute infections. T cells become exhausted, viral replication outcompetes control mechanisms and T cells fail to differentiate into memory cells (Figure 1.1). The underlying mechanisms of T cell exhaustion will be discussed in chapter 1.6. Nevertheless, there are many aspects of the immune response during chronic viral infection which remain elusive such as early factors which drive exhaustion of cells and the extent to which expansion and contraction of $CD8^+$ T cells contribute to this.

1.5 Infection model of lymphocytic choriomeningitis virus

In 1933, the American virologist Charles Armstrong isolated and described the lymphocytic choriomeningitis virus (LCMV) during an encephalitis epidemic (Muckenfuss 1934). Although LCMV was subsequently revealed to not be the cause of this epidemic, Armstrong's studies laid the foundation for many

immunological discoveries using LCMV as an infection model. Today, LCMV is one of the best-studied infection models in the fields of virology and immunology.

LCMV belongs to the family of Arenaviridae and mainly infects rodents (Childs, Glass *et al.* 1992). This enveloped virus contains two segments of negative single-stranded RNA coding for three structural proteins, the nucleoprotein (NP) and the glycoproteins GP-1 and GP-2, as well as the viral polymerase (L) and a small zinc finger motif protein (Z) (Lee, Novella *et al.* 2000, Lee, Perez *et al.* 2002). LCMV infects cells by binding to alpha-dystroglycan (αDG) and consequent receptor-mediated endocytosis (Cao, Henry *et al.* 1998, Buchmeier 2007). αDG is a surface receptor of various cell types with a high expression in developing tissues and plays a role for the assembly of basement membranes by linking the extracellular matrix to the actin cytoskeleton (Ervasti and Campbell 1991, Durbeej, Henry *et al.* 1998, Winder 2001). Virions are internalized upon binding to the receptor into vesicles and acidification of these vesicles leads to the fusion of the viral and vesicle membranes, hence allowing viral replication in the cytosol (Borrow and Oldstone 1994, Quirin, Eschli *et al.* 2008). Transmission of LCMV from mice to humans is rare and usually leads to asymptomatic and self-limiting infection (Emonet, Retornaz *et al.* 2007). However, in severe cases, aseptic meningitis and encephalitis have been reported (Barton and Hyndman 2000, Peters 2006).

There are two main features of the murine LCMV infection model which make it well suitable to study the immune system. First, LCMV is a non-cytolytic virus. Therefore, pathogenesis is solely caused by the immune response and allows the investigation of the innate and adaptive system on many different levels. Second, different strains of LCMV lead to acute and chronic infections which can be directly compared as T cells specific for the same epitope can be studied. The broadly used strains Armstrong (Arm) and Clone 13 (Cl13) differ in only three amino acids which induce a change in the GP and L proteins resulting in higher affinity to the entry receptor αDG (Matloubian, Kolhekar *et al.* 1993, Sullivan, Emonet *et al.* 2011). Therefore, infection with the Cl13 strain leads to a persistent infection with higher viral replication whereas an infection

with the Arm strain can be cleared by the immune system (Mims and Wainwright 1968, McChesney and Oldstone 1987).

Immunological research using the LCMV infection model greatly improved the understanding of immune responses. Conceptual discoveries made with this model are for example the presentation of foreign antigen to T cells by MHC complexes (Zinkernagel and Doherty 1974, Doherty and Zinkernagel 1975). Furthermore, formation and maintenance of immunological memory as part of the adaptive immune response were described (Lau, Jamieson *et al.* 1994, Murali-Krishna, Lau *et al.* 1999). Also, the phenomenon of T cell exhaustion as a state of hyporesponsiveness was observed and described using the LCMV model and led to a better understanding of exhaustion in other chronic infections as well as cancer (Moskophidis, Lechner *et al.* 1993, Gallimore, Glithero *et al.* 1998, Zajac, Blattman *et al.* 1998).

The innate immune response to LCMV infection is based on the recognition by endosomal and cytoplasmic PRRs. As LCMV infects cells through endocytosis, the endosomal TLR7 plays a crucial role for the initial recognition of the virus (Walsh, Teijaro *et al.* 2012). Additionally, signalling via RIG-I-like receptors (RLR), which detect viral RNA in the cytosol, is also critical to induce production of IFN I cytokines in response to LCMV infection (Clingan, Ostrow *et al.* 2012).

Cytokines generated by the innate immune response effectively activate cells of the adaptive arm of the immune system. It has been shown that $CD4^+$ T cells are not required for viral clearance during acute infection (Matloubian, Concepcion *et al.* 1994). In contrast, during chronic LCMV infection depletion of $CD4^+$ T cells leads to increased viral load and further perpetuates viral persistence in serum and spleen (Matloubian, Concepcion *et al.* 1994, Blackburn, Shin *et al.* 2009). $CD4^+$ T cell help during chronic infection was shown to support $CD8^+$ T cell as well as B cell responses (Battegay, Moskophidis *et al.* 1994, Ciurea, Hunziker *et al.* 2001). Partly, $CD4^+$ T cell interact with antigen-presenting cells and enable optimal co-stimulatory signalling via CD40-CD40L to induce cytotoxic T cell responses (Schoenberger, Toes *et al.* 1998). Additionally, $CD4^+$ T cells support cytotoxic T cell function by

the production of IL-21 (Elsaesser, Sauer *et al.* 2009, Frohlich, Kisielow *et al.* 2009, Yi, Du *et al.* 2009).

Killing of infected cells is dependent on cytotoxic functions of $CD8^+$ T cells. Depletion of these T cells during acute LCMV infection prevents clearance of the infection (Buchmeier, Welsh *et al.* 1980, Matloubian, Concepcion *et al.* 1994). These findings are mirrored by studies with simian immunodeficiency virus (SIV). Depletion of $CD8^+$ T cells during the course of infection leads to increased viral loads and persistence (Schmitz, Kuroda *et al.* 1999, Gaufin, Ribeiro *et al.* 2010). Overall, clearance of LCMV infection highly depends on the strain and route of infection. Perforin-mediated as well as IFNγ-mediated cytotoxicity were shown to be pivotal for the control of acute LCMV infection (Kagi, Ledermann *et al.* 1994, Walsh, Matloubian *et al.* 1994, Nansen, Jensen *et al.* 1999, Ou, Zhou *et al.* 2001). However, during chronic infection perforin plays a two-sided role. It mediates control of the infection and at the same time regulates the expansion of antigen-specific $CD4^+$ and $CD8^+$ T cells in order to avoid an overt cytokine production (Matloubian, Suresh *et al.* 1999).

The present study focuses on chronic infection with the LCMV Cl13 strain. Whereas the acute Arm strain mediates strong expansion of T cells, is cleared within eight days of infection and leads to the formation of memory cells that protect from reinfection with the same virus, Cl13 infection persists and causes T cell exhaustion (Ahmed, Salmi *et al.* 1984). LCMV Cl13 causes high viremia in blood, spleen and liver for up to three months and persists in kidney, brain and salivary glands (Wherry, Blattman *et al.* 2003). However, infection with this chronic LCMV strain prevents lethal immunopathology by partial paralysis of the immune response. Especially the phenomenon of T cell exhaustion described during chronic LCMV infection served as a model to understand immune responses during other chronic infection such as HIV and HCV as well as in the context of cancer. With this knowledge new therapies, targeting the reactivation of exhausted T cells, have been developed and revolutionized cancer treatment.

1.6 $CD8^+$ T cell exhaustion during chronic infection and cancer

The phenomenon of T cell exhaustion was first described by Gallimore *et al.* and Zajac *et al.* in 1998 by studying the cellular adaptive immune response during chronic LCMV infection (Gallimore, Glithero *et al.* 1998, Zajac, Blattman *et al.* 1998). As described in chapter 1.4, T cell exhaustion arises during chronic infections and cancer with persistent antigen and is characterized by a hyporesponsiveness of T cells. Exhaustion can be described as downregulation of immune responses, with the aim to prevent severe immunopathology and therefore presents a regulatory element that protects the host. At the same time, it prevents clearance of infection or eradication of tumour cells and thereby contributes to chronicity, which in turn further supports exhaustion. The exact underlying mechanisms which drive T cell exhaustion are still not fully understood, but immunological research of the last decades shed more light into the complexity of T cell responses and exhaustion, which also occurs during human chronic infections such as with HIV, HBV and HCV as well as during cancer (Virgin, Wherry *et al.* 2009).

Exhausted $CD8^+$ T cells are distinct from effector and memory $CD8^+$ T cells, which arise during acute infections, on the transcriptional, epigenetic and metabolic level. The exhausted or dysfunctional state of T cells is described by a progressive loss of certain effector functions. The ability to produce IL-2 is one of the first functions which is lost during the development of $CD8^+$ T cell exhaustion. This is followed by the loss of TNF production, cytotoxic functions and proliferative capacity. More severely exhausted $CD8^+$ T cells also show defects in IFNγ production (Wherry, Blattman *et al.* 2003, Fuller, Khanolkar *et al.* 2004). In the final stage of exhaustion, $CD8^+$ T cells can be physically deleted (Battegay, Moskophidis *et al.* 1994, Wherry, Blattman *et al.* 2003, Fuller, Khanolkar *et al.* 2004) (Figure 1.2). Interestingly, during chronic LCMV infection, $CD8^+$ T cells specific for certain epitopes are more prone to this physical deletion than others. Gp33- and gp276-specific $CD8^+$ T cells are dysfunctional but are preserved during the course of chronic infection (Moskophidis, Lechner *et al.* 1993, Wherry, Blattman *et al.* 2003, Fuller, Khanolkar *et al.* 2004).

However, why some exhausted cells are deleted whereas others can survive remains to be clarified.

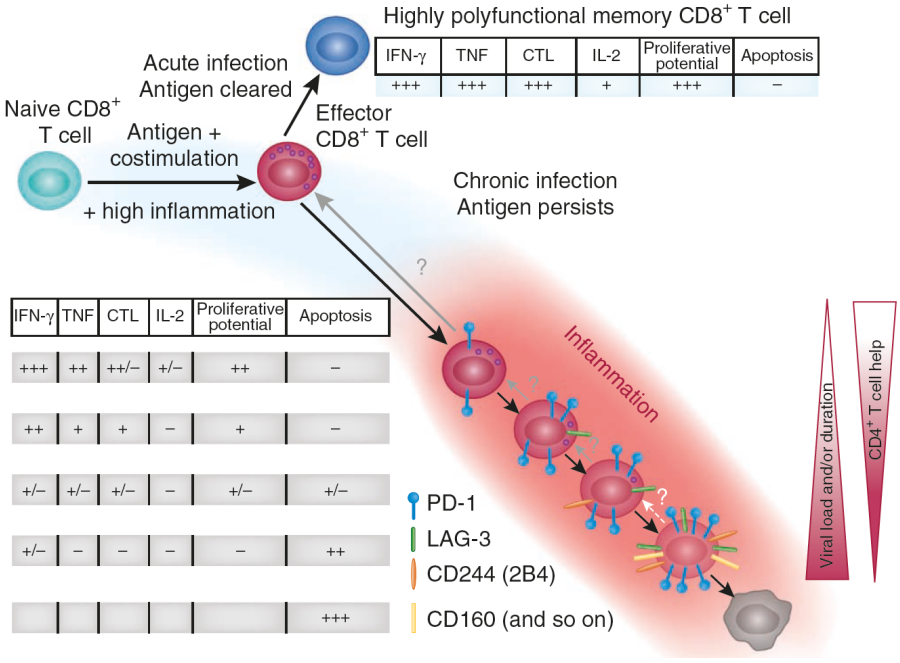


Figure 1.2: Progressive loss of effector functions during T cell exhaustion
Naïve CD8⁺ T cells are activated by antigen presentation and co-stimulation and are able to differentiate into effector and memory precursor cells during acute infection. The viral infection can be cleared and highly functional memory cells are generated. These memory cells display a strong proliferative capacity and can rapidly produce cytokines and exert cytotoxic functions upon reinfection with the same pathogen. In contrast, during chronic infections antigen persists and the CD8⁺ T cells become exhausted. This exhaustion follows a hierarchical pattern, starting with the loss of IL-2 production, followed by TNF and finally IFNγ production. Simultaneously, exhausted CD8⁺ T cells lose the ability to proliferate and perform cytotoxic functions. This dysfunctional phenotype is partly caused by a strong co-expression of various inhibitory receptors like PD-1, Lag-3 and 2B4. Furthermore, terminally exhausted CD8⁺ T cells are prone to undergo apoptosis. Adopted from (Wherry 2011).

As described, exhausted CD8⁺ T cells lose many effector functions. Nevertheless, some effector functions are not completely abolished and the infection can be controlled to a certain extent. These findings are supported by studies where CD8⁺ T cells were depleted during the course of chronic

infection. For example, depletion of $CD8^+$ T cells during infection with simian immunodeficiency virus (SIV) lead to an increase of viral load in untreated animals as well as animals under antiretroviral therapy (Jin, Bauer *et al.* 1999, Schmitz, Kuroda *et al.* 1999, Cartwright, Spicer *et al.* 2016). Furthermore, exhausted T cells have also been shown to be essential for the content of tumour growth and spread (Fridman, Pages *et al.* 2012, Li, van der Leun *et al.* 2019).

In addition, it is important to mention, that there are two different types of exhausted $CD8^+$ T cells. Most of them display an effector, but terminally differentiated phenotype and can be identified by the expression of Tim3. A smaller subset of TCF-1^+ exhausted $CD8^+$ T cells displays a memory-like phenotype and plays a crucial function as this subset can not only undergo self-renewal but also gives rise to the $Tim3^+$ subset. A more detailed overview of the differences between memory-like exhausted and terminally exhausted $CD8^+$ T cells as well as factors which influence the generation and maintenance of both will be discussed below.

First, an important question that is still not fully answered is how and when T cell exhaustion develops post infection. Studies using the chronic LCMV model describe that the persistence of antigen drives exhaustion (Wherry, Blattman *et al.* 2003). This factor has very likely a strong impact as normal effector and memory cells similar to the ones generated during acute infections were detected when antigen was removed early during the course of a chronic infection (Angelosanto, Blackburn *et al.* 2012, Ahn, Youngblood *et al.* 2016). In contrast, when antigen was removed at a later time point, the $CD8^+$ T cells retained their exhausted phenotype and generation of normal effector and memory cells was impeded (Utzschneider, Legat *et al.* 2013, Wieland, Kemming *et al.* 2017). Additionally, studies show that the severity of exhaustion correlates with antigen load (Wherry, Blattman *et al.* 2003, Blackburn, Shin *et al.* 2009). For example, the chronic LCMV strain Cl13 exhibits a stronger affinity to the entry receptor αDG than the acute LCMV strain Arm. This was shown to affect viral tropism, as it enables LCMV Cl13 to infect DC and replicate faster compared to LCMV Arm (Kunz, Sevilla *et al.* 2001). The high load of antigen

during chronic infections and as a consequence continuous TCR signalling could be the main driver of T cell exhaustion. Transcriptional changes which are connected to T cell exhaustion have been described already four days post infection with LCMV Cl13 (Khan, Giles *et al.* 2019). Therefore, factors like viral tropism and antigen presentation might introduce transcriptional programs triggering the start of exhaustion. Further on, antigen persistence and a complex network of intrinsic (expression of inhibitory receptors, transcriptional and epigenetic changes) and extrinsic factors (cytokines, lack of $CD4^+$ T cell help and regulatory suppressor cells) sustains the exhausted phenotype later on during chronic infection (Wherry and Kurachi 2015). Again, all of these elements are upregulated to protect the host from an overwhelming immune response and immunopathology, but at the same time sustains chronicity of the infection (Speiser, Utzschneider *et al.* 2014).

One of the hallmarks of T cell exhaustion is the expression of high levels of various inhibitory receptors such as PD-1, CTLA-4, Lag-3, 2B4, TIGIT and Tim-3 (Jones, Ndhlovu *et al.* 2008, Blackburn, Shin *et al.* 2009, Golden-Mason, Palmer *et al.* 2009, Jin, Anderson *et al.* 2010). Many of these inhibitory receptors are expressed during acute infections where they also display inhibitory functions (Blackburn, Shin *et al.* 2009, Cook and Whitmire 2016, Ahn, Araki *et al.* 2018). They play an important role for the regulation of T cell responses and prevent T cell hyperactivation as well as subsequent immunopathology and are therefore termed immune checkpoints. However, during acute infection, expression levels of these inhibitory receptors are lower and downregulated with the control of viral infection (Ahn, Araki *et al.* 2018). Additionally, sustained co-expression of various inhibitory receptors can only be detected during chronic infections (Blackburn, Shin *et al.* 2009). One of the most relevant inhibitory receptors during chronic infection is programmed cell death protein 1 (PD-1). Its signalling pathway and the possible blockade by checkpoint immunotherapy will be discussed in the chapters 1.7 and 1.8. Exhausted $CD8^+$ T cells express up to seven inhibitory receptors simultaneously which share some overlapping as well as non-overlapping pathways of inhibition (Blackburn, Shin *et al.* 2009). Many inhibitory receptors, such as PD-1,

2B4 and TIGIT, express an immunoreceptor tyrosine-based inhibition motif (ITIM) in their cytoplasmic domain. This intracellular signalling leads to the recruitment of phosphatases which inhibit TCR and co-stimulatory signalling pathways (Hannier, Tournier *et al.* 1998, Hui, Cheung *et al.* 2017, McLane, Abdel-Hakeem *et al.* 2019). Lag3, in contrast, functions through a KIEELE motif and inhibits cell cycle progression (Workman, Dugger *et al.* 2002). Tim3 also executes its inhibitory function by non-ITIM intracellular motifs, which can interfere with intracellular TCR signalling (Anderson, Joller *et al.* 2016). CTLA-4 binds the ligands of the co-stimulatory receptor CD28: CD80 and CD86. Therefore, it competes with CD28 and can decrease co-stimulatory signalling (Walker and Sansom 2011). Some of the precise mechanisms of inhibitory signalling pathways are still not fully understood, but the simultaneous expression of various checkpoint receptors dampens T cell activation on various levels and blockade of more than one inhibitory receptor mostly showed synergistic effects for checkpoint immunotherapy (Chapter 1.8).

In addition to the upregulation of inhibitory receptors, T cell exhaustion is linked to specific transcriptional changes. Recent studies have revealed a key role for the transcription factor TOX in promoting $CD8^+$ T cell exhaustion (Alfei, Kanev *et al.* 2019, Khan, Giles *et al.* 2019, Scott, Dundar *et al.* 2019, Seo, Chen *et al.* 2019, Yao, Sun *et al.* 2019). Already four days post infection with a virus causing a chronic infection enhanced TOX expression was detected (Khan, Giles *et al.* 2019). In the absence of TOX, $CD8^+$ T cells did not acquire an exhausted phenotype but rather differentiated into functional effector cells expressing KLRG1 (Alfei, Kanev *et al.* 2019). However, in this scenario, mice suffered from severe immunopathology. TOX induces the upregulation of inhibitory receptors and was shown to be crucial for the survival of the memory-like TCF-1^+ exhausted $CD8^+$ T cell population (Alfei, Kanev *et al.* 2019). Additionally, it also impacts the differentiation of cells on the epigenetic level as it has been described to promote chromatin remodelling (Khan, Giles *et al.* 2019). Therefore, TOX seems to play a key role in the formation of T cell exhaustion. Moreover, other transcription factors have been described to drive exhaustion. Already early post infection $CD8^+$ T cells from chronically infected

animals expressed higher levels of Irf-4, Batf and Nfat-1 (Man, Gabriel *et al.* 2017). These transcription factors have been described to promote T cell exhaustion by upregulation of inhibitory receptors, they suppress T cell expansion and can also lead to metabolic reprogramming (Martinez, Pereira *et al.* 2015, Scott-Browne, Lopez-Moyado *et al.* 2016).

Exhausted $CD8^+$ T cells also display a unique phenotype regarding their epigenetic landscape. Epigenetic effects such as DNA methylation and histone modification have been described and studied in the context of acute and chronic infections by comparing effector and memory $CD8^+$ T cells with exhausted $CD8^+$ T cells. These studies revealed that there is a great number of differentially accessible chromatin regions in exhausted $CD8^+$ T cells (Sen, Kaminski *et al.* 2016). One locus studied more intensively is the *Pdcd1* locus which encodes for PD-1. During acute infection, this locus was shown to be demethylated during the effector phase, allowing transcription of the gene. Upon viral clearance and memory formation, the locus was remethylated, preventing *Pdcd1* transcription (Bally, Austin *et al.* 2016). However, during chronic infection, the *Pdcd1* locus was demethylated throughout the course of infection and even post checkpoint immunotherapy, when viral load was decreased (Pauken, Sammons *et al.* 2016). The constant demethylation of the *Pdcd1* locus was also detected in $CD8^+$ T cells during HIV infection (Youngblood, Oestreich *et al.* 2011). To which extent a full reinvigoration of exhausted $CD8^+$ T cells can be maintained after immunotherapy therefore needs to be evaluated.

All these T cell-intrinsic factors can induce and maintain exhaustion, but the cellular state is additionally highly influenced by extrinsic factors. As viral loads are high and persist during chronic infections, a plethora of cytokines, both pro-inflammatory and anti-inflammatory, have been described to play a role for $CD8^+$ T cell exhaustion. However, the expression of certain cytokine receptors and signalling pathways are again different to the ones seen in bona fide effector and memory $CD8^+$ T cells (Wherry, Ha *et al.* 2007, Wherry and Kurachi 2015). Some cytokines have been described to be critical for the maintenance

of functions exerted to a limited extent by exhausted $CD8^+$ T cells. Among them are IL-2 and IL-21. IL-2 drives the expansion of $CD8^+$ T cells during acute infections. Likewise, IL-2 signalling has been shown to be important for the physical maintenance of antigen-specific cells during chronic LCMV infection (Bachmann, Wolint *et al.* 2007). Similar to IL-2, IL-21 has also been described to sustain functionality of exhausted $CD8^+$ T cells (Elsaesser, Sauer *et al.* 2009, Frohlich, Kisielow *et al.* 2009, Yi, Du *et al.* 2009). Importantly, the two receptors for IL-2 and IL-21 remain expressed on exhausted $CD8^+$ T cells, albeit to a lesser extent than on $CD8^+$ T cells during acute infections (Ingram, Yi *et al.* 2011, Beltra, Bourbonnais *et al.* 2016).

In contrast to these cytokines which support $CD8^+$ T cell functions, other cytokines, like IL-10 and TGF-β, further drive exhaustion and inhibit effector functions. During chronic LCMV infection as well as during human chronic viral infections, IL-10 levels are increased and have been associated with suppressive capacities regarding T cell activation and proliferation (Brooks, Trifilo *et al.* 2006, Wilson and Brooks 2011, Wherry and Kurachi 2015). During chronic LCMV infection, $CD4^+$ T cells were described to be a critical source of IL-10. This was put in context with the viral tropism of the chronic LCMV Cl13 strain, which unlike the acute strain LCMV Arm is able to infect DC (Baca Jones, Filippi *et al.* 2014). Smith *et al.* showed that IL-10 signalling negatively impacts TCR signalling and thereby increased the threshold for $CD8^+$ T cell activation (Smith, Boukhaled *et al.* 2018).

Compared to cytokines which either oppress or support $CD8^+$ T cell exhaustion, the IFN I cytokines IFNα and IFNβ have a more complex role during chronic infections. Early during the infection, IFNα/β signalling plays a supportive role for effector $CD8^+$ T cells and therefore enhances viral control as an important signal 3 cytokine (Teijaro, Ng *et al.* 2013, Wilson, Yamada *et al.* 2013). However, ongoing IFN I signalling can induce IL-10 as well as PD-L1 expression and drive $CD8^+$ T cell exhaustion (Ivashkiv and Donlin 2014). Such a dual role, where ongoing IFN I signalling has deleterious rather than beneficial effects, is described during chronic LCMV as well as SIV and HIV infection (Dagenais-Lussier, Loucif *et al.* 2017). Some of these cytokines have been

tested as therapeutic targets mostly in combination with checkpoint immunotherapy, which will be discussed in more detail in chapter 1.8.

Besides soluble mediators, cellular interaction partners with their specific surface molecules are able to strongly impact $CD8^+$ T cell responses and exhaustion during chronic infections. An important driver of $CD8^+$ T cell exhaustion is missing help from $CD4^+$ T cells. During acute infections, $CD4^+$ T cells potently support $CD8^+$ T cell responses by optimizing the interaction with DCs (Ridge, Di Rosa *et al.* 1998, Eickhoff, Brewitz *et al.* 2015). In this study, we mainly focus on $CD8^+$ T cell exhaustion. Nevertheless, it must be mentioned that exhaustion is also described for $CD4^+$ T cells (Oxenius, Zinkernagel *et al.* 1998, Brooks, Teyton *et al.* 2005). Similar transcriptional changes caused by chronic infections have been detected for $CD4^+$ and $CD8^+$ T cells (Crawford, Angelosanto *et al.* 2014). Missing $CD4^+$ T cell help in the context of chronic LCMV infection as well as during HIV infection and cancer leads to a higher viral burden and promotes $CD8^+$ T cell exhaustion (Matloubian, Concepcion *et al.* 1994, Kalams, Buchbinder *et al.* 1999, Borst, Ahrends *et al.* 2018). Additionally, as described earlier, $CD4^+$ T cells can impact the $CD8^+$ T cell exhaustion by the production of cytokines such as IL-21 and IL-10. Moreover, $CD4^+$ T cell help for B cells by T_{FH} cells supports the generation of neutralizing and non-neutralizing antibodies, which in turn control viral titers and therefore influence persistence (Planz, Ehl *et al.* 1997, Richter and Oxenius 2013, Greczmiel, Krautler *et al.* 2017). In contrast to $CD4^+$ T cells which support $CD8^+$ T cell activation, also immunosuppressive regulatory $CD4^+$ T cells (Treg cells) play a role during chronic infections. However, it remains unclear if Treg cells are able to directly drive T cell exhaustion or contribute to an immunosuppressive environment by the production of TGF-β and IL-10 (Veiga-Parga, Sehrawat *et al.* 2013, Wherry and Kurachi 2015). Besides Treg cells, myeloid-derived suppressor cells (MDSC) expanded during chronic LCMV infection and the depletion of these innate immune cells improved the activation of $CD8^+$ T cells. MDSC were also correlated with the progression of human HCV infection (Cai, Qin *et al.* 2013).

The plethora of different players of T cell exhaustion (inhibitory receptors, transcriptional and epigenetic regulation, soluble mediators and cellular interaction partner) reflects that $CD8^+$ T cell exhaustion is regulated on numerous levels. This complex network of different factors which impact the state of exhausted $CD8^+$ T cells is still not fully understood and certain elements may vary during different chronic infections and cancer.

As mentioned above, all antigen-specific cells display an exhausted phenotype during chronic infections and generation of bona fide memory cells is lacking. However, this pool of exhausted $CD8^+$ T cells is heterogeneous and immunological research of recent years lead to a better understanding of its subpopulations. Interestingly, a small group of exhausted $CD8^+$ T cells shares features with memory cells. These cells are therefore termed memory-like exhausted $CD8^+$ T cells. The second and bigger group of exhausted $CD8^+$ T cells displays effector functions but at the same time is more exhausted. These cells have been described as terminally exhausted $CD8^+$ T cells (He, Hou *et al.* 2016, Im, Hashimoto *et al.* 2016, Leong, Chen *et al.* 2016, Utzschneider, Charmoy *et al.* 2016). One of the first studies describing different subpopulations of exhausted $CD8^+$ T cells was published by Blackburn *et al.* in 2008. By blocking the PD-1/PD-L1 signalling pathway during chronic LCMV infection, they showed that only a subset, the later termed memory-like cells, responded to therapy (Blackburn, Shin *et al.* 2008). Phenotypically, the subsets were distinguished by the expression of PD-1 and CD44. The subset, which responded to immune checkpoint therapy expressed lower levels of PD-1 and higher levels of CD44 and therefore displayed a less exhausted phenotype. Their terminally exhausted counterpart expressed higher levels of PD-1 and lower levels of CD44 (Blackburn, Shin *et al.* 2008). Since 2016, these two subsets have been described in more detail with transcriptional, epigenetic and phenotypic differences as well as differences in their localization throughout different organs. In detail, to date memory-like exhausted $CD8^+$ T cells can be best distinguished from terminally exhausted $CD8^+$ T cells by the expression of the transcription factor T cell factor 1 (TCF-1), whereas their counterpart

expresses the surface receptor T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) (Im, Hashimoto *et al.* 2016, Wu, Ji *et al.* 2016). These two populations will be termed TCF-1$^+$ and Tim3$^+$ from now on. TCF-1 is also expressed by naïve and memory CD8$^+$ T cells (Zhou, Yu *et al.* 2010). Exhausted TCF-1$^+$ cells are able to perform self-renewal and additionally give rise to the Tim3$^+$ subset (Figure 1.3), which expresses higher levels of effector molecules such as perforin and granzymes (Im, Hashimoto *et al.* 2016). This subset expresses lower levels of certain inhibitory receptors such as PD-1 and largely lack the expression of Tim3 and 2B4 (Im, Hashimoto *et al.* 2016). Another marker for this subset is the chemokine receptor CXCR5 (Im, Hashimoto *et al.* 2016). However, there TCF-1$^+$ cells which are CXCR5$^-$. Therefore, distinguishing these cells by TCF-1 might be more accurate.

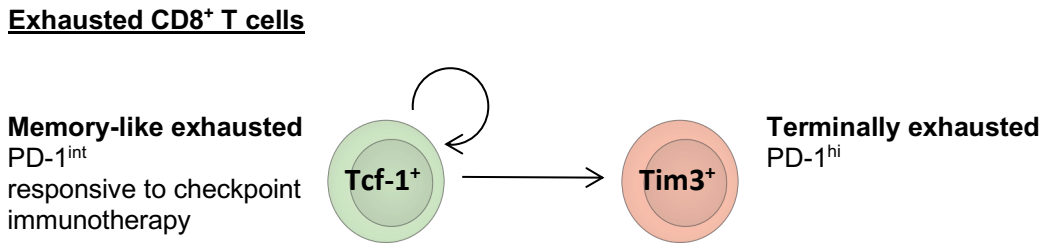


Figure 1.3: Exhausted CD8$^+$ T cells during chronic viral infection
Memory-like exhausted (TCF-1$^+$) and terminally exhausted (Tim3$^+$) CD8$^+$ T cells develop during chronic LCMV infection. TCF-1$^+$ cells express lower levels of PD-1 compared to Tim3$^+$ cells and are able to respond to checkpoint immunotherapy. These memory-like cells are able to self-renew and give rise to the terminally exhausted subset of CD8$^+$ T cells. Adopted from (Philip and Schietinger 2019).

Certain transcriptional differences have been described to drive the differentiation into TCF-1$^+$ or Tim3$^+$ cells. The transcription factor B lymphocyte-induced maturation protein-1 (Blimp-1) presents a strong regulator of the differentiation of exhausted CD8$^+$ T cells as it downregulates TCF-1 expression (Shin, Blackburn *et al.* 2009, Leong, Chen *et al.* 2016). In contrast, the transcription factor Forkhead Box O1 (FOXO1) induces TCF-1 expression and

thereby promotes the memory-like subpopulation (Staron, Gray *et al.* 2014, Utzschneider, Delpoux *et al.* 2018). As the TCF-1$^+$ population gives rise to the Tim3$^+$ population which present important effector functions, mice deficient of FOXO1 were not able to control the infection to the extent that WT mice did (Staron, Gray *et al.* 2014, Utzschneider, Delpoux *et al.* 2018). Additionally, the transcriptional regulators Id2 and Id3 drive differentiation of exhausted CD8$^+$ T cells. Important for the generation of memory cells, Id3 is also expressed by memory-like TCF-1$^+$ cells. In contrast, Id2 is expressed by terminally exhausted Tim3$^+$ cells (Im, Hashimoto *et al.* 2016, Leong, Chen *et al.* 2016). Interestingly, the transcription factors have been described to impact the expression of CXCR5 (Leong, Chen *et al.* 2016). To date, there are only few studies investigating the impact of the localization of the generation and maintenance of the two subsets. In contrast to Tim3$^+$ cells, TCF-1$^+$ cells have been detected only in lymphoid tissues. In the spleen, they mainly reside in the T cell zone (Im, Hashimoto *et al.* 2016). These cells also express the co-stimulatory receptor CD28, which was shown to be essential for the reinvigoration by checkpoint immunotherapy (Kamphorst, Wieland *et al.* 2017). Importantly, the subpopulation of TCF-1$^+$ and CXCR5$^+$ memory-like exhausted CD8$^+$ T cells have also been described in various tumour models and also there displays the population of exhausted CD8$^+$ T cells which are able to respond to immune checkpoint inhibition (Siddiqui, Schaeuble *et al.* 2019).

In summary, CD8$^+$ T cells undergo several states of exhaustion during chronic infections and cancer. This process is initiated to protect the host from immune-related pathology and regulated on various levels by intrinsic and extrinsic factors. However, a subset of exhausted CD8$^+$ T cells maintains certain functions similar to bona fide memory CD8$^+$ T cells and can be targeted by checkpoint immunotherapy to reinvigorate CD8$^+$ T cell responses and decrease viral or tumour burden.

1.6.1 The PD-1 signalling pathway

Programmed cell death 1 (PD-1) is one of the most studied inhibitory receptors which drives T cell exhaustion during chronic infection and cancer. In addition, targeting this signalling pathway with monoclonal antibodies, revolutionized cancer treatment. PD-1 is a transmembrane protein which belongs to the CD28 family of regulatory receptors (Schildberg, Klein *et al.* 2016). The extracellular part comprises a IgV domain and the cytoplasmic regions harbour ITIM and ITSM motifs, which are important phosphorylation sites during signalling (Arasanz, Gato-Canas *et al.* 2017). The ligands of PD-1 are PD-L1 and PD-L2 (Zhang, Schwartz *et al.* 2004). PD-L1 is expressed on various immune and non-immune cells and can be upregulated on infected cells as well as tumour cells. Here, several cytokines such as IFN I, IFNγ, TNFα and IL-10 have been reported to induce upregulation of PD-L1 (Eppihimer, Gunn *et al.* 2002). In contrast, PD-L2 expression has been described for DC, macrophages and germinal center B cells (Schildberg, Klein *et al.* 2016). There are also soluble forms of PD-L1 and PD-L2, however, their contribution to T cell exhaustion remains incompletely understood. In this study, we focus on PD-1 expression on $CD8^+$ T cells. However, it can also be expressed by $CD4^+$ T cells, NK cells, B cells, macrophages and DC (Nishimura, Agata *et al.* 1996, Petrovas, Casazza *et al.* 2006, Chang, Kim *et al.* 2008, Liu, Yu *et al.* 2009).

$CD8^+$ T cells upregulate PD-1 upon activation by TCR stimulation within 24 h and various cytokines, such as IFN I, IL-2, IL-7, IL-15 and IL-21, can further promote the expression (Kinter, Godbout *et al.* 2008, Terawaki, Chikuma *et al.* 2011, Schildberg, Klein *et al.* 2016). In contrast to the transient expression on activated effector $CD8^+$ T cells during acute infection, PD-1 expression becomes permanent during chronic infections and cancer. Due to persistence of high loads of antigen, PD-1 levels stay upregulated (Blattman, Wherry *et al.* 2009). Upon binding of its ligands PD-L1 or PD-L2, tyrosine residues of ITIM and ITSM motifs of the intracellular tail of PD-1 become phosphorylated (Freeman, Long *et al.* 2000, Schildberg, Klein *et al.* 2016). This results in the recruitment of the phosphatases SHP1 and SHP2 (Yokosuka, Takamatsu *et al.*

2012). These phosphatases are able to dephosphorylate various adaptor proteins of the intracellular domains of the TCR as well as the co-stimulatory receptor CD28 (Sheppard, Fitz *et al.* 2004, Hui, Cheung *et al.* 2017). ZAP70 and PI3K belong to the signalling cascade of TCR and CD28 receptors and are targets of SHP1 and SHP2. Thereby, T cell expansion and various functions are inhibited (Figure 1.4).

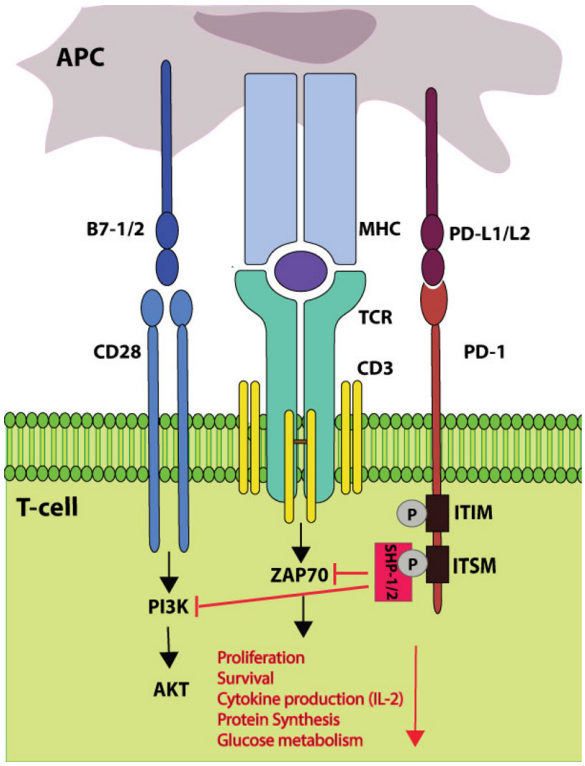


Figure 1.4: PD-1 signalling
Upon binding of its ligands PD-L1 or PD-L2, the intracellular ITIM and ITSM motifs of PD-1 are phosphorylated and recruit the phosphatases SHP-1 and SHP-2. Dephosphorylation of TCR and CD28 adaptor proteins ZAP70 and PI3K inhibits downstream signalling and results in a decrease of proliferation, cytokine production and cytotoxicity of the cells (Lee, Ahn et al. 2015).

Although PD-1 strongly impacts T cell exhaustion and has been shown to play a crucial role during various chronic infections and cancer, exhaustion does also develop in the absence of PD-1 (Odorizzi, Pauken *et al.* 2015). Ultimately, PD-$1^{-/-}$ $CD8^{+}$ T cells display a more exhausted phenotype than WT cells (Frebel,

Nindl *et al.* 2012, Odorizzi, Pauken *et al.* 2015). As mentioned earlier, strong TCR signalling drives exhaustion and the expression of various inhibitory receptors. As PD-1 signalling decreases activation of $CD8^+$ T cells, it also prevents ongoing strong TCR signalling.

Importantly, the state of T cell exhaustion is at least to some extent reversible and targeting PD-1 signalling showed great success during chronic infections and cancer. By the administration of monoclonal antibodies that either block PD-1 or PD-L1, signalling can be abrogated and the inhibitory function of this checkpoint receptor suppressed.

1.6.2 Checkpoint Immunotherapy: PD-1 and PD-L1 blockade

Through the detailed examination of $CD8^+$ T cell responses during chronic infection and cancer and the connection between functional paralysis and the expression of inhibitory receptors, new treatment strategies for the reinvigoration of exhausted $CD8^+$ T cells could be developed. The great success of checkpoint immunotherapy was awarded with the 2018 Nobel Prize in Physiology or Medicine to James P. Allison and Tasuku Honjo "for their discovery of cancer therapy by inhibition of negative immune regulation" (NobelPrize.org 2019).

In 2006, Barber *et al.* showed that inhibition of the PD-1/PD-L1 signalling pathway with monoclonal antibodies can reinvigorate exhausted $CD8^+$ T cells and decrease viral load in the chronic LCMV model (Barber, Wherry *et al.* 2006). This reinvigoration included expansion of $CD8^+$ T cells as well as increased frequencies of IFNγ- and TNFα-producing $CD8^+$ T cells, reflecting regained effector functions (Barber, Wherry *et al.* 2006). Yet, the reactivation of exhausted $CD8^+$ T cells was dependent on CD28 signalling, revealing an essential role for co-stimulatory signalling pathways in the context of checkpoint immunotherapy (Hui, Cheung *et al.* 2017, Kamphorst, Wieland *et al.* 2017). The necessity of CD28 signalling strongly points towards a critical role for specific cellular interaction partners that deliver these co-stimulatory ligands. However,

very little is known about the complex network of immune and non-immune cells which contribute to checkpoint immunotherapy.

To date, several immune checkpoint inhibitors blocking PD-1 and CTLA-4 signalling are used for cancer treatment (Rotte 2019). With these therapies melanoma, non-small cell lung carcinoma, urothelial carcinoma and other tumour entities can be successfully treated (Rotte 2019). Nevertheless, immune checkpoint inhibitors are not suitable for all types of cancer and chronic infections and in many patients severe side effects have been reported.

As described in chapter 1.6, a broad range of soluble and cellular components influence T cell exhaustion. All these different elements are possible therapeutical targets and a plethora of combinatorial treatment approaches are currently investigated in animal models as well as clinical trials.

Approaches either target inhibitory elements, such as inhibitory receptors and regulatory soluble and cellular mediators, as well as factors which maintain T cell functions during exhaustion such as co-stimulatory receptors and cytokines. For example, combined blockade of several inhibitory receptors has been shown to provoke synergistic effects. Anti-PD-1 together with anti-CTLA-4 treatment showed improved outcome over monotherapy for the treatment of melanoma (Larkin, Hodi *et al.* 2015). In contrast to treatment of certain cancers, CTLA-4 blockade during chronic infections such as LCMV, SIV and HIV was not sufficient to reinvigorate exhausted $CD8^+$ T cells and decrease viral load (Barber, Wherry *et al.* 2006, Kaufmann and Walker 2009). However, in combination with anti-PD1 treatment it showed synergistic effects, yet also increased side-effects (Kaufmann, Kavanagh *et al.* 2007). Similarly, co-treatment of anti-PD-1 with anti-Lag3 or anti-Tim3 lead to improved control of chronic viral infections and tumour burden (Blackburn, Shin *et al.* 2009, Jin, Anderson *et al.* 2010, Kassu, Marcus *et al.* 2010, Sakuishi, Apetoh *et al.* 2010). Next to inhibitory receptors, immune regulatory cells, such as Treg cells, enhance T cell exhaustion as depletion of such increased the number of antigen-specific $CD8^+$ T cells during chronic LCMV infection. Viral titers could be decreased significantly by the combination of Treg cell depletion and blockade of the PD-1 pathway (Penaloza-MacMaster, Kamphorst *et al.* 2014).

Notably, Treg cells can affect T cell exhaustion by the production of immunosuppressive cytokines such as IL-10 but also by the consumption of IL-2, which is a key cytokine important for survival and activation of $CD8^+$ T cells. Both cytokines have been investigated as therapeutical targets. Blockade of the inhibitory cytokine IL-10 by the administration of neutralizing antibodies early during chronic LCMV infection enabled reactivation of $CD8^+$ T cells and viral clearance (Ejrnaes, Filippi *et al.* 2006). Combined treatment of anti-IL10 with anti-PD-L1 during the chronic infection phase, enhanced control of the infection and restored $CD8^+$ T cell functions (Brooks, Ha *et al.* 2008). Beneficial effects have been also described for the administration of IL-2 during anti-PD-L1 treatment of chronic LCMV infection. The synergistic effects lead to an increase of antigen-specific $CD8^+$ T cells and a reduction of the viral load (West, Jin *et al.* 2013).

In general, an early start of treatment of chronic infections and cancer to restore T cell responses correlates with better therapy outcome. Again, pointing towards a progressive loss of effector functions and development of exhaustion. Additionally, targeting the reactivatable memory-like TCF-1^+ $CD8^+$ T cell pool could further improve therapy outcome. The frequency of TCF-1^+ cells already serves as a valuable biomarker for therapy success in melanoma patients (Sade-Feldman, Yizhak *et al.* 2018). Nevertheless, T cell exhaustion is a fine-tuned state in which immunopathology is repressed. By the reinvigoration of exhausted $CD8^+$ T cells with checkpoint immunotherapy, the risk of inducing productive inflammatory responses which harm the host increases. Indeed, during combined blockade of PD-1 and CTLA-4 severe immune-related adverse events such as colitis have been reported (Montfort, Colacios *et al.* 2019). Prophylactic treatment with TNF inhibitors not only showed success in mouse models but could also prevent immune-related enterocolitis in cancer patients treated with immune checkpoint inhibitors (Badran, Cohen *et al.* 2019, Perez-Ruiz, Minute *et al.* 2019).

Conclusively, immunotherapy with checkpoint inhibitors shows an enormous success and revolutionized cancer treatment. However, T cell exhaustion is regulated by a complex network of elements. A better understanding of soluble

and cellular interaction partners which drive T cell exhaustion and are essential to restore the response will greatly improve the development of new therapeutically approaches.

1.7 Thesis aims

Chronic viral infections and cancer exploit a state of hyporesponsiveness of reactive T cells that developed to protect the host from severe immunopathology. In this process, T cells adopt an exhausted phenotype and are functionally impaired which prevents them from clearing the infection or eradicating tumour cells (Hashimoto, Kamphorst *et al.* 2018). However, checkpoint inhibitor-based immunotherapies enable reactivation of exhausted T cells and are an enormous success for the treatment of various cancer types. The scientific field that addresses the underlying mechanism of PD-1/PD-L1 blockade and explores its efficacy in various experimental settings and human conditions has received much attention and is rapidly evolving. Experimental studies deciphered a subset of exhausted $CD8^+$ T cells representing a memory-like cell population that responds to therapy with checkpoint inhibitors (Im, Hashimoto *et al.* 2016, Utzschneider, Charmoy *et al.* 2016). Nevertheless, the exact underlying mechanisms of $CD8^+$ T cell reinvigoration are still not completely elucidated. Therefore, it is of great interest to better understand factors that impact on T cell exhaustion as well as checkpoint immunotherapy.
In this study, we aimed to illuminate critical cellular interaction partners, which influence the reinvigoration of exhausted $CD8^+$ T cells during anti-PD-L1 treatment. Specifically, the role of DC and certain subsets of these antigen-presenting cells was investigated in this context. Furthermore, we endeavoured to resolve signalling pathways that are involved in the cellular communication and how these impact on the activation and differentiation of exhausted $CD8^+$ T cells during anti-PD-L1 treatment. Focusing on the group of memory-like exhausted $CD8^+$ T cells, we asked the question if these cells represent a heterogenous population and aimed to examine the interplay with DC subsets in secondary lymphoid organs.

Overall, the aim of this study was to elucidate which DC subsets influence subsets of exhausted $CD8^+$ T cells during anti-PD-L1 treatment on a transcriptional and functional level as well as in the context of their splenic localization.

2. Materials and Methods

2.1 Materials

2.1.1 Mice

Mice were bred and maintained under specific pathogen-free conditions and in accordance to institutional animal guidelines in the animal facility (House of Experimental Therapy, HET) of the University of Bonn (Germany), the animal house facility of the Department of Microbiology and Immunology at the University of Melbourne, Peter Doherty Institute (Australia) and the animal facility of the Institute of Systems Immunology I, University of Würzburg (Germany). All animal experiments were approved by the animal ethics committees of the associated universities. C57BL/6J (B6) wildtype strains (H-2Kb) were purchased from Janvier (Le Genest-Saint-Isle, France). All mice are on the genetic background of C57BL/6J and were used at 8-12 weeks of age.

Mouse strain	**Publication and description**
C57BL/6J	C57BL/6J non-transgenic mice were used as wildtype controls (WT).
CD11c.DOG	B6.Cg-Tg(Itgax-DTR/OVA/EGFP)1Gjh/Crl. Mice of this strain express the model antigen ovalbumin fragment containing sequences encoding amino acids 140-386 and the human DTR under control of the *CD11c* promotor. (Hochweller, Striegler *et al.* 2008)
CD45.1 P14	CD45 is a pan leukocyte marker with two allelic variants. While C57BL/6J WT mice express CD45.2, CD45.1 mice

	express CD45.1. In addition, these mice have a transgenic TCR that recognizes the LCMV glycoprotein gp33-43 (gp33) epitope (KAVYNFATCGI) in the context of MHC I. (Shen, Saga *et al.* 1985, Pircher, Burki *et al.* 1989)
OT-I	Mice of this strain have a transgenic TCR that recognizes the $OVA_{257-264}$ peptide (SIINFEKL) in the context of MHC I. (Hogquist, Jameson *et al.* 1994)
P14	Mice of this strain have a transgenic TCR that recognizes the LCMV glycoprotein gp33-43 (gp33) epitope (KAVYNFATCGI) in the context of MHC I. (Pircher, Burki *et al.* 1989)
tdTomato	Mice of this strain express the fluorescent protein tdTomato under the ubiquitin promotor. (Kastenmuller, Brandes *et al.* 2013)
$XCR1^{+/DTRVenus}$	Mice of this strain express the human DTR and the fluorescent protein Venus under the control of the *Xcr1* promotor. Heterozygous mice still express XCR1 whereas homozygous mice are XCR1-deficient. (Yamazaki, Sugiyama *et al.* 2013)
$XCR1^{+/Venus}$	Mice of this strain express the fluorescent protein Venus under the control of the *Xcr1* promotor. Heterozygous animals still express XCR1 whereas homozygous animals are XCR1-deficient. (Yamazaki, Sugiyama *et al.* 2013)

2.1.2 Viruses and cells

The original stocks of lymphocytic choriomeningitis virus strain clone 13 (LCMV Cl13) were kindly provided by the laboratories of Dietmar Zehn (Division of Animal Physiology and Immunology, School of Life Sciences Weihenstephan, Technical University of Munich, Germany) and Hans Christian Probst (Institute for Immunology, University Medical Center Mainz, Germany).
The MC57G fibrosarcoma cell line, L929 murine fibroblast cell line and the VL-4 (rat αLCMV-NP) hybridoma cell line were also provided by the laboratory of Hans Christian Probst and subsequently kept in culture.

2.1.3 Equipment

Table 2.1: Equipment

Device	Name and Company
Autoclave	Belimed, Cologne, Germany
Balances	CP224S-0CE and CP2201, Sartorius, Göttingen, Germany
Bead mill homogenizer	Bead Ruptor Elite; OMNI International, Kennesaw, GA, USA
Cell sorter	Influx, Becton-Dickinson, Franklin Lakes, NJ, USA Aria II, Becton-Dickinson, Franklin Lakes, NJ, USA
Centrifuge	Multifuge 3 S-R Heraeus, Hanau, Germany Sprout mini centrifuge, Biozym, Hessisch Oldendorf, Germany Mikro 200R centrifuge, Hettich, Tuttlingen, Germany
Cryostat	CM 3050S, Leica, Hamburg, Germany

Flow cytometer	FACSCanto™ II, LSRFortessa™, FACSCelesta™, Becton-Dickinson, Franklin Lakes, NJ, USA
Freezer -20°C	Bosch, Munich, Germany
Freezer -80°C	Heraeus, Hanau, Germany
Ice machine	Icematic Scotsman®; Frimont Bettolinc, Pogliano, Italy
Incubator	HeraCell, Heraeus, Hanau, Germany
Infrared lamp	HP3616, Philips, Hamburg, Germany
Lasers	Tunable Chameleon laser, Coherent, Santa Clara, CA, USA
MACS cell separator	QuadroMACS, Miltenyi Biotec, Bergisch-Gladbach, Germany
Microscopes	Olympus CKX31, Olympus, Düsseldorf, Germany Primovert, Zeiss, Jena, Germany LSM 780, Zeiss, Jena, Germany LSM 710, Zeiss, Jena, Germany SP8, Leica, Wetzlar, Germany
Mouse cages	Tecniplast Smartflow, Hohenpeißenberg, Germany
Neubauer chamber	Assistent, Karl Hecht GmbH, Sondheim, Germany
Pipette boy	Integra Biosciences; Biebertal, Germany
Pipets	Research plus (10, 20, 200, 1000), Eppendorf, Hamburg, Germany
Preparation instruments	Bochem, Weilburg; Hammacher, Solingen; F.S.T., Heidelberg, Germany
Refrigerator 4°C	Bosch, Munich, Germany
Sieves, steel	Mechanical Workshop, University

	Hospital Bonn, Germany
Water bath	TW8, Julabo, Seelbach, Germany
Workbench (sterile)	HeraSafe, Heraeus, Hanau, Germany
Vortex Mixer	neolab, Heidelberg, Germany

2.1.4 Consumables and reagents

Table 2.2: Consumables

Consumables	Name and Company
Cell culture flask	(T25, T75, T150) Greiner bio-one, Frickenhausen, Germany
Cell strainer	BD Bioscience, USA
Cover slips	controlled thickness 0.17 ± 0.01 mm, CE, Assistent, Karl Hecht GmbH, Sondheim, Germany
Cryomolds	Tissue-Tek®, Sakura, Alphen aan den Rijn, Netherlands
Cryo tubes	Cryopure 1.6ml, Sarstedt, Nümbrecht, Germany
FACS tubes	Sarstedt, Nümbrecht, Germany
FALCON tubes	15 ml, Greiner bio-one, Solingen, Germany 50 ml, Sarstedt, Nümbrecht, Germany
Glass pipets	Greiner, Nürtingen, Germany
Injection needles	27G, 25G, 20G; BD Microlance, Becton, Dickinson and Company, Franklin Lakes, NJ, USA
Microscopy slides	Superfrost Plus, VWR, Darmstadt, Germany
Microtome blades	Feather, Osaka, Japan
Nylon gauze	Labomedic, Bonn, Germany

Pasteur pipettes	150 mm and 230 mm; Roth, Karlsruhe, Germany
Petri dishes	10 cm; Greiner bio-one, Solingen, Germany
pH value indicator stripes	stripes pH fix 4.5-10.0; Macherey-Nagel; Düren, Germany
Pipet tips	sterile pipette tips 0.2 ml/0.01 ml, VWR, Darmstadt, Germany Ultratip 1 ml, Greiner bio-one, Frickenhausen, Germany TipOne 0.2 ml, STARlab, Hamburg Pipette Tip 0.01 ml neutral, Sarstedt, Nümbrecht, Germany
Reaction tubes	microtubes (0.5 ml, 1.5 ml, 2 ml), Sarstedt, Nümbrecht, Germany
Sterile filter	AcroVac Filter Unit PES 0.2 µm Supor, Pall Life Sciences, Dreieich, Germany
Syringes	2 ml/5 ml BD Discardit II, Becton-Dickinson, Heidelberg, Germany
Tissue culture plates	TPP Tissue culture testplates (96, 24, 6 wells), Trasadingen, Switzerland

Table 2.3: Chemicals and reagents

Chemicals and Reagents	Name and Company
Bovine serum albumin (BSA)	Roth, Karlsruhe, Germany
Brefeldin A	Sigma Aldrich, Munich, Germany
Cell Trace Violet (CTV)	Life Technologies, Carlsbad, CA, USA
Collagenase (type 3)	Roche Diagnostics, Mannheim, Germany Worthington, Lakewood, NJ, USA

Dimethylsulfoxid (DMSO)	Roth, Karlsruhe, Germany
Diphtheria toxin (DTX)	Calbiochem, San Diego, CA, USA
Disodium phosphate (Na_2HPO_4)	AppliChem, Darmstadt, Germany
DNAse I	recombinant, Grade I, Roche, Mannheim, Germany
Embedding medium	Tissue-Tek® O.C.T.TM Compound, Sakura, Alphen aan den Rijn, Netherlands
Ethanol 70% (v/v)	Otto Fischar, Saarbrücken, Germany
Ethylene diamine tetraacetic acid (EDTA)	0.5M pH8.0, Applichem, Damstadt, Germany
Fcγ block	Privigen, CSL Behring, Marburg, Germany
Fetal bovine serum (FBS/FCS)	Good Filtrated Bovine Serum, PAN Biotech, Aidenbach, Germany
Fluoromount-G	ebioscience, San Diego, CA, USA
Formaldehyde	Roth, Karlsruhe, Germany
Gelatine from cold waterfish skin (GCWFS)	SigmaAldrich, Munich, Germany
Hank's balanced salt solution with indicator (HBSS)	Gibco, Thermo Fisher Scientific, Waltham, MA, USA
Heparin sodium (25000 IU)	Ratiopharm, Ulm, Germany
HEPES	Gibco, Thermo Fisher Scientific, Waltham, MA, USA
Hydrochloric acid (HCl)	Roth, Karlsruhe, Germany
Hydrophobic Barrier (PAP) Pen	ImmEdgeTM Pen (H-4000), Vector Laboratories, Burlingame, CA, USA
Isoflurane	AbbVie, Wiesbaden, Germany
L-Lysine	Sigma Aldrich, Munich, Germany
NaCl solution (0.9%)	Braun, Melsungen, Germany
Normal mouse serum (NMS)	Life Technologies, Carlsbad, CA, USA

Optiprep	Sigma Aldrich, Munich, Germany
Paraformaldehyde (PFA)	AppliChem, Darmstadt, Germany
Penicillin/Streptomycin/Glutamine	Gibco, Thermo Fisher Scientific, Waltham, MA, USA
Phosphate-buffered saline (PBS)	Biochrom AG, Berlin, Germany
RPMI 1640 medium	Invitrogen, Darmstadt, Germany
Sodium dihydrogen phosphate (NaH_2PO_4)	Merck, Darmstadt, Germany
Sodium hydroxide (NaOH)	Roth, Karlsruhe, Germany
Sucrose	Sigma Aldrich, Munich, Germany
Tris base	AppliChem, Darmstadt, Germany
Triton X-100	Promega, Madison, WI, USA
Trypan Blue (0.4 %)	Lonza, Cologne, Germany
Trypsin-EDTA (0.05 %)	Gibco, Thermo Fisher Scientific, Waltham, MA, USA
β-Mercaptoethanol (C_2H_6OS)	Sigma Aldrich, St. Louis, MO, USA

2.1.5 Antibodies

Table 2.4: Antibodies used for *in vivo* blockade of interactions

Antigen	Clone	Company
CD4	GK1.5	BioXcell, West Lebanon, NH, USA
CD70	FR70	BioXcell, West Lebanon, NH, USA
IL-15	AIO.3	BioXcell, West Lebanon, NH, USA
PD-L1	10F.9G2	BioXcell, West Lebanon, NH, USA

Table 2.5: Antibodies used for flow cytometry and immunofluorescence
Application: flow cytometry (F), immunofluorescence (IF)

Antigen	Application, Clone	Conjugate	Company
B220	IF, RA3-6B2	AF700	BioLegend
CD3ε	F, 145-2C11	BUV 395	BD Biosciences
CD4	F, RM4-5	BV786, PE-Cy7	BD Biosciences
CD8α	F, 53-6.7	BV786	BioLegend
	F, 53-6.7	PB, PE	BD Biosciences
CD11b	F, M1/70	BV711	BioLegend
CD11c	F, N418	PE-Cy7	eBioscience
CD19	F, 1D3	APC-Cy7	BD Biosciences
	F, 6D5	BV605	BioLegend
CD44	F, IM7	FITC, BV510	BD Biosciences
CD45.1	F, A20	PE-Cy7	eBioscience
	F, A20	FITC	BD Biosciences
CD62L	F, MEL-14	PE-Cy7	eBioscience
CD169	IF, 3D6.112	primary	BioRad
CD244	F, ebio244F4	FITC	LifeTechnologies
CXCR5	F, 2G8	biotinylated	BD Biosciences
CXCR6	F, SA051D1	PE	BioLegend
CX3CR1	F, SA01F11	BV786	BioLegend
F4/80	F, BM8	APC	eBioscience
	IF, BM8	BV421	BioLegend
GzmB	F, GB11	PE	Invitrogen
Ki-67	F, B56	BV510	BD Biosciences
MHCII	F, M5/114.15.2	AF700	eBioscience
PD-1	F, RMP1-30	APC	eBioscience
	F, 29F.1A12	PE	BioLegend
Tcf7	F, C63D69	PB	Cell signalling
Tim-3	F, RMT3-23	PE-Cy7	BioLegend
Va2	F, B20.1	PE-Cy7	BD Biosciences

XCR1	F, ZET	PE	BioLegend
Secondary antibodies	F, IF	AF-conjugated	Invitrogen
MHC I Tetramer gp33 H-2D(b) KAVYNFATC	F	PB, APC	NIH Tetramer Core Facility

2.1.6 Buffers, media and solutions

Prior to use, all stock solutions and buffers were sterile filtered or autoclaved and kept under sterile conditions afterwards.

Buffer, medium or solution	**Composition**
Erythrocyte lysis buffer (10x ACK)	1.5 M NH_4Cl 100 mM $KHCO_3$ 10 mM EDTA-Na_2 in distilled water (pH value 7.2)
Blocking buffer	1 % (v/v) FCS 1% (m/v) GCWFS 0.3 % (v/v) Triton-X 100 in 0.1 M Tris 1 % NMS added directly before use
Cell medium	8 % heat-inactivated FCS 50 µM β-Mercaptoethanol 4 mM L-Glutamin 100 U/ml Penicillin 100 µg/ml Streptomycin in RPMI 1640 medium
Digestion medium	7 mg/ml type 3 collagenase 1 mg/ml DNAse I (grade 2)

	in KDS-RPMI 2 % aliquots kept at -20 °C
FACS buffer	2 % (v/v) FCS 0.02 % (v/v) NaN_3 in 1x PBS
Fixation buffer (PLP)	2.12 mg $NaIO_4$ 3.75 ml P-buffer 3.75 ml L-Lysine 2.5 ml 4% PFA (pH value 7.4, adjusted with 10 M NaOH) preparation directly before use
HEPES/NaCl	2.2 g NaCl (0.88 % w/v) 0.596 g HEPES (10mM) 1.25 g BSA (0.5 %) 2.5 ml 0.1 M EDTA (pH 8) in 250 ml water
KDS-RPMI 2%	2 % heat-inactivated FCS 50 µM β-Mercaptoethanol 2 mM L-Glutamin 100 U/ml Penicillin 100 µg/ml Streptomycin in KDS-RPMI medium
L-Lysin solution	0.2 M L-Lysine in P-buffer
Na_2HPO_4 solution	35.6 g Na_2HPO_4 (M = 177.99 g/mol) in 1 l ddH_2O

NaH_2PO_4 solution	31.2 g NaH_2PO_4 (M = 156.01 g/mol) in 1 l ddH_2O
PBS	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4 in ddH_2O
P-buffer	40.5 % (v/v) 0.1 M Na_2HPO_4 9.5 % (v/v) NaH_2PO_4 in ddH_2O (pH value 7.4)
PFA solution	4 % (w/v) PFA in 1x PBS gradually heated (pH value 7.4)
Sucrose solution	30 % (w/v) Sucrose in P-buffer
T cell enrichment buffer	0.5 % (v/v) BSA 2 mM EDTA in 1x PBS
Tris buffer	1 M Tris base in distilled water (pH value 7.5, adjusted with 10 M HCl)
Triton-X 100 buffer	0.5% Triton-X 100 in 1x PBS

2.1.7 Kits

Table 2.6: Kits

Name	Company
CD8α T cell isolation kit, mouse	Miltenyi Biotec, Bergisch Gladbach, Germany
Cytofix/Cytoperm	BD Biosciences, Heidelberg, Germany
CellTracer Violet Cell Proliferation Kit	Invitrogen, Darmstadt, Germany
Foxp3 / Transcription Factor Staining Buffer Set	Invitrogen, Darmstadt, Germany
LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit	Invitrogen, Darmstadt, Germany

2.1.8 Software

Table 2.7: Software

Software	Company
BD FACS Diva 8.0.1	BD Biosciences, Heidelberg, Germany
EndNote X9	Thompson Reuters, Frankfurt a. Main, Germany
FlowJo V10.0.7	Tree star, Ashland, OR, USA
GraphPad Prism6	GraphPad Software, La Jolla, CA, USA
Imaris 8.2.1	Bitplane, Zurich, Switzerland
LasX Life Science	Leica, Wetzlar, Germany
Microsoft Office 2018	Microsoft, Redmond, WA, USA
RStudio	RStudio Inc., Boston, MA, USA
ZEN black and blue	Zeiss, Jena, Germany

2.2 Methods

2.2.1 Virus and cell culture

LCMV Clone 13 (Cl13) was produced in a L929 murine fibroblast cell line. Virus titer of freshly produced stocks as well as virus titer of spleens of infected mice were measured with a limiting dilution assay. For this assay, MC57G fibrosarcoma cells were used.

2.2.1.1 Cell culture

L929 murine fibroblasts and MC57G fibrosarcoma cells

L929 murine fibroblasts and MC57G fibrosarcoma cells were kept at -80 °C in FCS with 10 % DMSO with one vial containing $1x10^6$ cells. Cells were briefly thawed in a water bath at 37 °C and immediately put on ice afterwards. The cell medium for L929 cells contains DMEM with 10 % FCS and 1 % P/S. Cell medium for MC57G cells contains DMEM with 10 % FCS, 1 % sodium pyruvate and 1 % P/S. After thawing, cells were resuspended in 30 ml cold cell medium and centrifuged for 5 min at 1600 rpm. After washing with 30 ml cold medium, cells were collected in 15 ml warm medium and transferred to a 75 cm^2 cell culture flask. Cells were cultured for two days at 37 °C, 5 % CO_2. Every third day, cells were splitted by aspirating the medium, washing the cells with 10 ml warm PBS and adding 1 ml Trypsin/EDTA. To dissociate the cell layer, cells were incubated for 4 min at 37 °C. By gently hitting the flask and rinsing with 9 ml warm cell medium, cells were detached from the flask. To split the cells 1:10, 1.5 ml of the cell suspension was left in the flask and 13.5 ml of fresh cell medium were added.

VL-4 hybridoma cells

For the culture of VL-4 hybridoma cells and production of anti-LCMV NP antibodies, cells were thawed as described above. The cell medium consists of IMDM with 10 % FCS, 1 % Glutamin, 1 % pyruvate and 1 % P/S. Cells were collected in a 50 ml tube, centrifuged and the pellet resuspended in 30 ml cell medium. Cells were transferred to a 75cm^2 flask. After two to three days of culture at 37 °C and 5 % CO_2, the supernatant containing anti-LCMV NP antibodies, was collected and stored in the fridge for the LCMV limiting dilution assay (Chapter 0). Cells were frozen at -80 °C in FCS containing 10 % DMSO at a cell concentration of 1x10^6 cells per vial.

2.2.1.2 Generation of LCMV stocks

For the generation of LCMV Cl13 stocks, L929 cells were seeded at 3x10^6 cells per 150cm^2 flask in DMEM 5 % FCS. Cells were kept in the incubator at 37 °C, 5 % CO_2 overnight. The following day, the cell medium was aspirated and 3x10^4 IU of LCMV Cl13 were added in a volume of 3 ml. For the infection of the cells, flasks were kept at room temperature (RT) for 30 min and gently rocked several times to prevent the cells from drying out. The supernatant was aspirated and 15 ml of fresh cell medium were added. Cells were kept at 37 °C, 5 % CO_2 for 48 h. After this incubation, the virus containing medium was harvested in 1 ml aliquots and immediately stored at -80 °C. 15 ml of fresh cell medium was added and virus-containing medium harvested again after 72 h post infection. Virus titer of newly prepared stocks was measured with a limiting dilution assay (Chapter 0).

2.2.1.3 LCMV limiting dilution assay

For the titration of viral stocks and the measurement of viral load in the spleen, a limiting dilution assay was performed. For this, MC57G cells were prepared at a concentration of 4x10^6 cells/ml in DMEM with 10 % FCS, 1 % sodium

pyruvate and 1 % P/S. 50 µl of the cell suspension were added into each well of a 96 well flat bottom plate. Immediately after, serial virus dilutions from $2x10^{-2}$ to $2x10^{-6}$ were added in a total volume of 50 µl per well and mixed gently with the cells. For each dilution, eight replicates were prepared. Medium that did not contain virus served as a negative control. The mixture of cells and virus was incubated at 37 °C, 5 % CO_2 for 72 h. After 48 h, 70 µl medium were carefully exchanged without touching the cell layer. Before staining, cells were washed twice with PBS and permeabilized and fixed using the BD Cytofix/Cytoperm kit. Therefor, 50 µl of the fixation/permeabilization solution were added to each well and cells were incubated for 30 min on ice. After washing twice with BD Perm/Wash buffer (1x), 50 µl of PBS with 10 % FCS were added to each well to block non-specific binding sites for 60 min at RT. Cells were washed again with BD Perm/Wash buffer and 50 µl of 1:20 VL-4 hybridoma cell line supernatant were added to each well for 60 min at RT. The VL-4 hybridoma cell supernatant contains antibodies which are specific for the LCMV nucleoprotein (NP). Thereby, infected cells can be detected. For visualisation of infected cells, these were washed twice with BD Perm/Wash buffer and a secondary fluorescently-labelled anti-rat AF488 antibody was added at a 1:500 concentration in a total volume of 50 µl to the cells. After incubation for 60 min at RT in the dark, cells were washed again with BD Perm/Wash buffer. Using an epifluorescent microscope wells that contain infected cells were counted and the virus titer was calculated.

2.2.2 Treatment of mice

Virus, cells and reagents were diluted in sterile PBS prior to experimental injections. For intraperitoneal (i.p.) injection, a total volume of 100-500 µl were injected into the peritoneal cavity of the mice. Intravenous (i.v.) injections were performed by injecting a total volume of 100-200 µl into the tail vein. To dilate the veins, mice were exposed to a heat lamp before an injection.

2.2.2.1 Infection with LCMV

Highly purified LCMV Cl13 was used for this study. Prior to usage, it was diluted in sterile PBS. Mice were warmed up in a clean cage using a heat lamp and challenged with an infectious dose of $2x10^6$ IU i.v. After injection, mice were transferred back into their original cages and monitored closely. To induce a chronic infection, mice were treated with anti-CD4 depleting antibody two days prior and on the day of infection (Chapter 0).

2.2.2.2 Submandibular bleeding

Submandibular bleeding was performed for the collection and analysis of blood lymphocytes. Therefor, mice were restrained with a neck scruff hold. A 23 G needle or 4/5mm lancet was used to gently puncture the anterior facial vein (submandibular). The required blood volume (less than 1 % of body weight) was collected by holding a tube underneath the puncture site. Mice were transferred back into their cage and monitored the following day.

2.2.2.3 Treatment with blocking and depletion antibodies

In vivo blocking (anti-CD70, anti-IL-15, anti-PD-L1) and depletion antibodies (anti-CD4) were diluted in sterile PBS prior to injection.

For immunotherapy of chronically infected mice, PD-L1 signalling was blocked using anti-PD-L1 mAb (aPD-L1, 10F.9G2). 200 μg aPD-L1 were injected i.p. in a total volume of 200 μl every three days. A maximum of five consecutive treatments were given to the mice.

To block the CD70-CD27 signalling pathway, 300 μg anti-CD70 mAb (aCD70, FR70) were i.p. injected in a total volume of 200 μl every three days. A maximum of three consecutive treatments were given to the mice.

To block the IL-15/IL-15Rα signalling pathway, 200 µg anti-IL-15 mAb (aIL-15, AIO.3) were i.p. injected in a total volume of 200 µl every three days. A maximum of three consecutive treatments were given to the mice.
For the depletion of $CD4^+$ cells two days before and on the day of LCMV Cl13 infection, mice received i.p. injections of 300 µg anti-CD4 mAb (aCD4, GK1.5) in a total volume of 500 µl. $CD4^+$ cells opsonized by this antibody are recognized and depleted by macrophages.

2.2.2.4 Depletion of cells with DTX

CD11c.DOG and $XCR1^{+/DTRVenus}$ transgenic mice and their WT littermates were used for cell depletion experiments. These two transgenic mouse strains express a diphtheria toxin receptor (DTR) under the *CD11c* or *XCR1* promotor. By treating these mice with diphtheria toxin (DTX), $CD11c^+$ cells and $XCR1^+$ DC can be depleted, respectively. WT mice do not express the DTR and are thereby protected from the effects of DTX. Prior to injection, DTX was diluted in sterile PBS. Injections were performed i.p. with a total volume of 200 µl.
For the depletion of $CD11c^+$ cells, CD11c.DOG mice received 250 ng DTX i.p. every second day with five treatments in total unless indicated otherwise.
To deplete $XCR1^+$ DC, $XCR1^{+/DTRVenus}$ mice received one i.p. injection of 500 ng DTX followed by four i.p. injections of 250 ng DTX every second day unless indicated otherwise.

2.2.3 Isolation of cells

2.2.3.1 Isolation of lymphocytes from the blood

To isolate lymphocytes from blood, 100 µl heparin (1:100) were transferred to a tube and blood was collected by submandibular bleeding (Chapter 0). 200 µl of the sample were transferred into one well of a 96 well round bottom plate and centrifuged for 2 min at 1600 rpm. The supernatant was discarded and 200 µl

ACK lysis buffer were added for 4 min at RT to lyse erythrocytes. After another centrifugation step, cells were washed with 100 µl PBS. Afterwards, cells were used for flow cytometry staining (Chapter 0).

2.2.3.2 Isolation of lymphocytes from organs

For the isolation of lymphocytes from organs, spleens and lymph nodes were harvested into PBS on ice. Immediately afterwards, organs were homogenized through a metal sieve strainer into a 50 ml falcon tube. The cell strainer was rinsed twice with 10 ml PBS. The cell suspension was centrifuged at 1600 rpm for 7 min at 4 °C. After centrifugation, the supernatant was discarded and the cell pellet resuspended in 2 ml ACK buffer and incubated for 4 min at RT to lyse erythrocytes. To stop lysis, PBS was added and the cell suspension was filtered using nylon gauze. After centrifugation (1600 rpm, 7 min, 4 °C), the cell pellet was resuspended in 5 ml cell medium.

2.2.3.3 Enrichment of T cells

To enrich $CD4^+$ and $CD8^+$ T cells from mouse spleen and lymph nodes, single cell suspensions were prepared as described (Chapter 0). The EasySep™ mouse $CD8^+$ and $CD4^+$ T cell isolation kits were used for negative selection of T cells. Cells were resuspended in T cell enrichment buffer (PBS containing 0.5 % BSA, 2 mM EDTA). Isolation was performed according to manufacturer's instructions. To exclude pre-activated T cells, a biotinylated anti-CD44 antibody (1:10,000) was added to the antibody cocktail.

2.2.3.4 Isolation and enrichment of dendritic dells

For the isolation of dendritic cells (DC), spleens were isolated and collected in 6 ml KDS-RPMI 2 % each. Spleens were transferred to a petri dish and vigorously dissociated with a scalpel. The emulsified tissue was transferred back into the tube with KDS-RPMI 2 % medium. 1 ml of digestion medium

(collagenase/DNase) was added and the suspension pipetted up and down for 20 min with a pasteur pipette. 700 µl 0.1 M EDTA were added and pipetted for 5 min. Afterwards, the cell suspension was filtered through a 40 µm mesh and the sample collected by centrifugation at 1500 rpm for 5 min at 4 °C.

To isolate DC from this cell suspension, an Optiprep™ density gradient centrifugation was performed. All reagents were used at RT. A 17 % Optiprep solution was prepared with HBSS (5.66 ml Optiprep solution + 14.33 ml HBSS with indicator) and a 12 % Optiprep solution was prepared with HEPES/NaCl buffer (4.04 ml Optiprep + 15.96 ml HEPES/NaCl). The cell pellet was resuspended in 3 ml of the 17 % Optiprep solution and transferred to a 15 ml tube. This was carefully overlayed with 3 ml of the 12 % Optiprep solution and finally, another overlay was performed with 3 ml HBSS. By centrifugation with minimal acceleration and without brake for 15 min at 600 x g at 20 °C, cells can be separated by their density. DC can be found in the first layer of cells. The second layer contains lymphocytes and erythrocytes.

To further isolate different DC populations, cells were stained with DC marker (Chapter 0) and sorted using a BD Influx cell sorter. Isolated $CD11b^+$ and $XCR1^+$ DC were used for analysis by flow cytometry (Chapter 0) and *ex vivo* antigen presentation assay (Chapter 0).

2.2.3.5 Determination of cell numbers

To determine cell numbers of cultured and isolated cells, cell suspensions were diluted 1:10 with trypan blue solution to stain dead cells. 10 µl of the cell suspension were added to a Neubauer counting chamber and unstained viable cells in four large squares were counted. Cell numbers were calculated using the following formula:

$$\frac{\text{cells}}{\text{ml}} = \left(\frac{\text{cell count}}{4}\right) \text{x } 10\ (\text{dilution factor}) \text{x } 10{,}000\ (\text{chamber factor})$$

2.2.4 Cell labelling

Cell Trace Violet labelling

To monitor proliferation, cells were labelled using the Cell Trace Violet (CTV) Cell Proliferation kit. With every cell division the dye is diluted. Therefore, cells will show different CTV intensities depending on how often they divided which can be measured by flow cytometry. For the labelling, cells were diluted to $1x10^6$ cells/ml in PBS. 1 µl CTV was added to each ml of the cell suspension and mixed thoroughly. Cells were incubated in a water bath for 20 min at 37 °C and gently mixed in between. Thereafter, cell medium (five times the volume of the cell suspension) was added and cells were incubated in the water bath for another 5 min. Subsequently, cells were centrifuged at 1600 rpm for 7 min, resuspended in cell medium to a final concentration of $5x10^5$ cells/ml and used for the *ex vivo* antigen presentation assay (Chapter 0).

2.2.5 Adoptive T cell transfer

Isolated, enriched and labelled cells (Chapter 0 and 0) were transferred into recipient mice at cell concentrations varying from $1x10^6$ to $2.5x10^6$ (stated for each experiment). For this, cells were resuspended in sterile PBS and injected into the tail vein of mice in a total volume of 200 µl.

2.2.6 *Ex vivo* antigen presentation assay

Antigen presenting cells (APC), are able to present antigen via major histocompatibility complexes (MHC) to T cells. T cells are able to recognize the presented antigen with their T cell receptor (TCR) and together with the help of other signals are able to proliferate. To investigate the potential of antigen presentation by DC (and other APC), DC were co-cultured with T cells from transgenic mice expressing a specific TCR. These T cells will only proliferate if

the APC presents antigen. Thereby, the proliferation of the T cells can be used as a readout for antigen presentation.

In detail, APC (mainly DC and different subpopulations) were isolated and enriched from spleens (Chapter 0). 1:2 dilutions starting from $5x10^4$ DC per well to $3.125x10^3$ DC per well were prepared in a total volume of 100 µl/well in a V-bottom 96 well plate.

For the co-culture with transgenic cells, LCMV-specific $CD8^+$ T cells from P14 transgenic mice (spleen and lymph nodes) were isolated (Chapter 0) and enriched (Chapter 0). P14 cells were labelled with CTV (Chapter 0) and $5x10^4$ cells were added to each well containing different concentrations of DC. Samples were prepared in duplicates. As a negative control, CTV-labelled P14 cells were co-cultured with naive DC. P14 cells with gp33 peptide (1:1000) as well as P14 cells co-cultured with gp33 peptide-pulsed DC (Chapter 0) served as positive controls. Cells were gently mixed and incubated for 65 h at 37 °C, 5 % CO_2. Afterwards, cells were stained with different surface markers to detect the proliferation and activation of P14 cells by flow cytometry (Chapter 0).

2.2.7 Preparation of gp33 peptide-pulsed and OVA-loaded DC

DC were isolated and enriched as described above (Chapter 0).

To peptide-pulse DC with gp33 peptide, $5x10^5$ cells were resuspended in 1 ml PBS and 1 µl gp33 peptide was added. Cells and peptide were mixed thoroughly and incubated in a water bath for 30 min at 37 °C. Thereafter, cells were washed three times with cell medium and used as a positive control for the *ex vivo* antigen presentation assay (Chapter 0).

For the preparation of ovalbumin (OVA)-loaded DC, 10 mg OVA were dissolved in 1 ml HBSS and mixed thoroughly. DC isolated from one spleen were resuspended in 1 ml OVA solution and incubated in the water bath for 10 min at 37 °C. After the incubation, cells were washed three times with cell medium. OVA-loaded DC were co-cultured with OT-I cells for the *ex vivo* antigen presentation assay (Chapter 0).

2.2.8 Flow Cytometry

2.2.8.1 Cell surface staining

Single cell suspensions were prepared as described above (Chapter 0). $2x10^6$ cells were transferred to one well of a 96 well plate. After centrifugation at 1600 rpm for 2 min, the cell pellet was resuspended and stained in 50 µl of a staining solution consisting of FACS buffer, Fc block (1:66) and antibodies (Chapter 0) for 30 min at 4 °C in the dark. Subsequently, cells were washed twice, collected in 150 µl FACS buffer and transferred to FACS tubes containing 150 µl FACS buffer. Samples were acquired immediately using an LSRFortessa™, FACSCanto™ II or FACSCelesta™ with FACSDiva™ software. FlowJo™ software was used for the analysis.

2.2.8.2 Tetramer staining

To stain LCMV-specific $CD8^+$ T cells, a H-2Db gp33 tetramer was used. For optimal staining, the tetramer was added 1:100 to the surface stain cocktail and staining was performed for 1 h at RT in the dark. Cells were washed twice with FACS buffer and either directly used for acquisition or for subsequent intracellular staining (Chapter 0).

2.2.8.3 Intracellular cytokine and transcription factor staining

Intracellular staining was performed post staining of surface markers and tetramer staining as described above (Chapters 0 and 0). For intracellular cytokine and chemokine staining, cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit which enables the permeabilization of the cell membrane. For this, cells were resuspended in 100 µl fixation/permeabilization solution and incubated for 20 min at 4 °C in the dark. For the staining of transcription factors, not only the cell membrane but also the nuclear membrane needs to be permeabilized. To achieve this, the Foxp3 staining kit was used.

Cells were resuspended and incubated in 100 µl fixation/permeabilization solution for 20 min at 4 °C in the dark. After fixation and permeabilization, cells were washed twice with BD Perm/Wash buffer and stained intracellularly/intranuclearly in 50 µl of staining solution consisting of Perm/Wash buffer and antibodies (Chapter 0) for 30 min at 4 °C in the dark. After washing twice, cells were kept in Perm/Wash buffer until acquisition at an LSRFortessa™, FACSCanto™ II or FACSCelesta™ with FACSDiva™ software. FlowJo™ software was used for the analysis.

2.2.9 Confocal microscopy

2.2.9.1 Fixation and embedding of spleen samples

To analyze the localization of certain immune cells in the spleen of chronically infected and differently treated animals, confocal microscopy was performed. For this, spleens were isolated and immediately fixed in 500 µl of freshly prepared PLP buffer for 12 h at 4 °C. Samples were washed twice with 500 µl P-buffer and afterwards dehydrated in 500 µl 30 % (v/v) sucrose at 4 °C overnight. Finally, samples were embedded in Tissue-Tek and stored at -80 °C for cryo cutting and subsequent immunofluorescence staining.

2.2.9.2 Immunofluorescence staining

Serial 30 µm sections were cut on a CM3050S cryostat and adhered to Superfrost Plus object slides. Frozen sections were rehydrated with 0.1 M Tris buffer for 10 min at RT, followed by permeabilization and blocking with blocking buffer containing 1 % normalized mouse serum (NMS) for at least 30 min at RT. Staining with fluorescently labelled antibodies was performed in blocking buffer (with 1 % NMS) at 4 °C overnight. Afterwards, sections were washed with 0.1 M Tris buffer and mounted with Fluoromount-G. Serial spleen sections were visually inspected using epifluorescent light microscopy. Several representative sections from different spleen areas were acquired using a Zeiss LSM 710

confocal microscope with ZEN acquisition control software or a Leica SP8 confocal microscope with LasX software for detailed analysis.

2.2.10 Single-cell RNA sequencing and analysis

Single-cell RNA sequencing was performed with the help of the Helmholtz Institute for RNA-based Infection Research in Würzburg, the Core Unit Systems Medicine of the University of Würzburg and the Helmholtz Centre for Infection Research in Braunschweig.
Splenic lymphocytes were isolated and stained as described in 2.2.3.2 and 2.2.8.1. Cells were sorted using a FACS Aria II cell sorter. Sorted cells were counted and droplet-based sequencing performed using the 10x Genomics kit. The counted reads were analysed using the Seurat R toolkit (version 3.0). Cells with a percentage above 5 % of mitochondrial genes were excluded. Further on, cells with less than 200 or more than 2000 genes were removed as outliers. A log transformation was performed on the counts and variable genes were selected based on average expression and dispersion. For dimensional reduction, principal component analysis was performed using variable genes. Cluster were calculated with statistical relevant principle components and plotted with UMAP. Based on the expression of the known macrophage marker *Lyz2*, one cluster containing 86 cells was excluded from downstream analysis. Clustering, UMAP plotting and significant differentially expressed genes were recalculated after removal of this cluster.

2.2.11 Statistical Analysis

Raw data sets were analysed using Microsoft Excel and GraphPad Prism 6 software. Prior to statistical analysis, Gaussian (normal) distribution of data sets was tested with D'Agostino-Pearson omnibus normality test and Shapiro-Wilk normality test. Student's t-test (two-tailed) and Mann-Whitney test were used for the statistical analysis of differences between two groups with normal and non-normal distribution, respectively. To determine statistical differences between

more than two groups, a repeated-measurements one-way ANOVA was used with the Dunnett post-test for parametric data sets and the Kruskal-Wallis test for non-parametric data sets. Stars indicate significances (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). Error bars indicate the standard error of the mean (SEM).

3. Dendritic cells play an essential role for anti-PD-L1 therapy

3.1 Introduction

During chronic infections as seen with HIV, HCV and HBV or the establishment of cancer, the immune system is unable to clear the infection or eradicate malignant cells. Under such conditions, T cells typically adapt an exhausted phenotype that is associated with the permanent presence of antigen (Pauken and Wherry 2015). An important hallmark of $CD8^+$ T cell exhaustion is the expression of inhibitory receptors, which are upregulated due to ongoing TCR signalling (Blackburn, Shin *et al.* 2009). This inhibitory signalling causes a strong impairment of proliferation and cytokine production (Wherry and Kurachi 2015). A great improvement for cancer therapy and treatment of chronic infections has been the development of blocking antibodies that prevent inhibitory signalling by binding directly to the inhibitory receptor or its ligand. Thereby, exhausted T cells can be reinvigorated and regain proliferative capacity and effector functions (Barber, Wherry *et al.* 2006). The effect of so-called immunotherapy was especially promising when treating with antibodies that block PD-1 (Programmed cell death protein 1) signalling.

PD-1 is a surface protein belonging to the CD28/CTLA-4 family and plays an important role in the regulation of T cell responses (Sharpe, Wherry *et al.* 2007). Upon binding of its ligand PD-L1, PD-1 signalling leads to the inhibition of TCR and costimulatory receptor signalling (Sharpe, Wherry *et al.* 2007). During acute infections, this regulatory signalling inhibits effector functions, such as granzyme B production, and overall impedes fast viral clearance (Ahn, Araki *et al.* 2018). Early during chronic infections, PD-1 signalling plays an important role in preventing immunopathology (Ahn, Araki *et al.* 2018). However, it also leads to the exhaustion of T cells and therefore the persistence of pathogens as described above (Blackburn, Shin *et al.* 2009). In recent years, many studies have focused on exhaustion and immunotherapy in the context of chronic infections and cancer. It is well established that only a subset of exhausted $CD8^+$ T cells can be reinvigorated (Barber, Wherry *et al.* 2006). These cells

display a memory-like phenotype and can be distinguished from terminally exhausted $CD8^+$ T cells by the expression of the transcription factor TCF-1 (Utzschneider, Charmoy *et al.* 2016). In addition, there are also studies which revealed that blocking more than one inhibitory receptor can have a cumulative effect (McLane, Abdel-Hakeem *et al.* 2019). Nevertheless, the beneficial effects of immunotherapy are still limited to certain cancer types and PD-1 blockade can provoke various and partly severe side effects (Sharma and Allison 2015, Wu, Gu *et al.* 2019).

Therefore, we aimed to investigate immunotherapy during chronic viral infection in more detail. To this end, we made use of the chronic strain of lymphocytic choriomeningitis virus clone 13 (LCMV Cl13). This widely used and well characterized infection model played an instrumental role for the understanding of T cell exhaustion (Zehn and Wherry 2015). Furthermore, it has been the first model with which successful immunotherapy using anti-PD-1 antibodies has been described (Barber, Wherry *et al.* 2006). We first examined the kinetics of immunotherapy with blocking the ligand of PD-1, PD-L1, by varying the duration of treatment. In addition, the role of dendritic cells (DC) during immunotherapy was investigated. DC are important antigen-presenting cells (APC) and are highly efficient in activating naïve T cells (Villadangos and Schnorrer 2007, Segura and Villadangos 2009). The aim of this work was to clarify if DC are essential for successful immunotherapy. We further aimed at elucidation whether different DC subsets have non-redundant function and if antigen presentation is critical in this context. Deciphering these open questions could help to better understand the effects of PD-L1 blockade and therefore improve therapy outcome or may help top limit adverse effects.

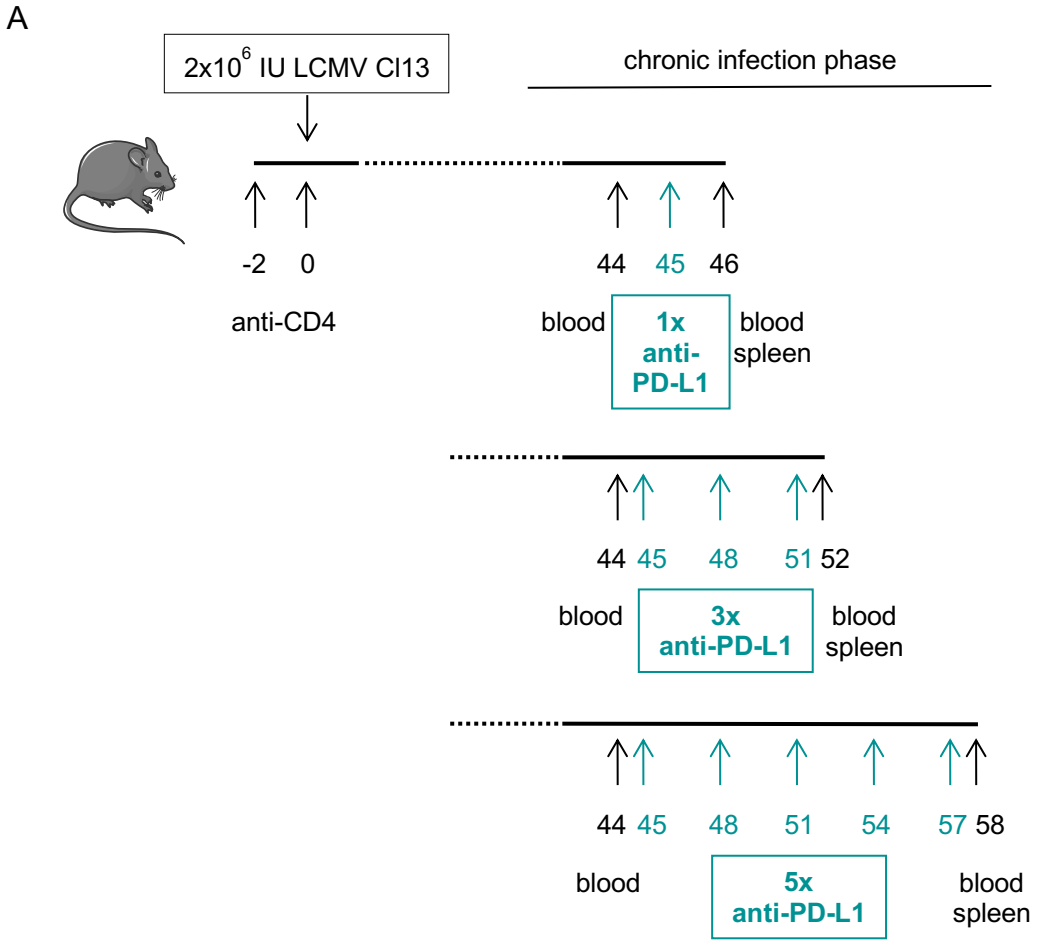
3.2 Results

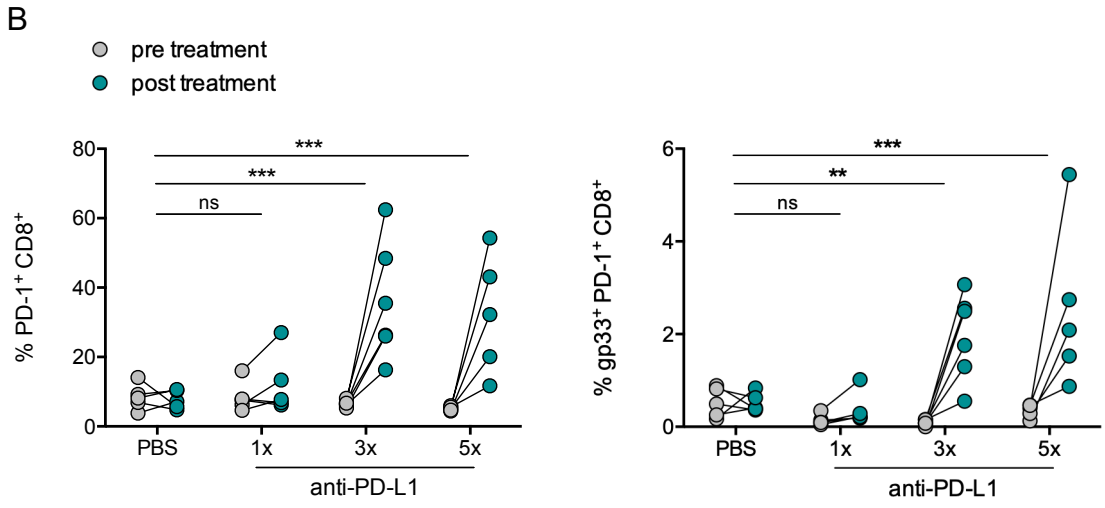
3.2.1 Kinetics of PD-L1 blockade during chronic LCMV infection

To examine the effects of PD-L1 blockade during chronic infection, the LCMV (Cl13) infection model was used. An i.v. infection with $2x10^6$ IU of the chronic LCMV strain clone 13 (Cl13) in combination with the depletion of $CD4^+$ cells, leads to a persistent infection (Blackburn, Shin *et al.* 2009). The immune system is not able to clear the infection and the antigen-specific $CD8^+$ T cells become exhausted (Moskophidis, Lechner *et al.* 1993, Gallimore, Glithero *et al.* 1998, Zajac, Blattman *et al.* 1998). To reactivate the exhausted $CD8^+$ T cells during chronic LCMV infection, mice were treated with 200 µg anti-PD-L1 antibody i.p. (Clone 10F.9G2). This monoclonal antibody binds to PD-L1 and thereby blocks the interaction with the inhibitory receptor PD-1 on exhausted $CD8^+$ T cells. To date, the response kinetics of anti-PD-L1 therapy are not fully understood. To investigate the response in more detail, C57BL/6J (WT B6) mice received one, three or five injections of anti-PD-L1. Isolation and analysis of the spleen by flow cytometry was performed 24 h after anti-PD-L1 treatment. In addition, the therapeutic effect was directly tracked by paired analysis of blood samples taken before and after the treatment (Figure 3.1A).

LCMV-specific $CD8^+$ T cells from chronically infected mice display a distinct phenotype compared to memory LCMV-specific $CD8^+$ T cells generated during an acute infection (Wherry, Ha *et al.* 2007). These exhausted $CD8^+$ T cells can be easily distinguished by their expression of PD-1. Whereas memory LCMV-specific cells are negative for PD-1, exhausted $CD8^+$ T cells express PD-1 among other co-inhibitory receptors (Blackburn, Shin *et al.* 2009). For this work, LCMV-specific $CD8^+$ T cells were stained with PD-1 and gp33 tetramer. PD-1 is upregulated on $CD8^+$ T cells after antigen encounter as it is directly connected to TCR signalling (Oestreich, Yoon *et al.* 2008). Thereby, antigen-experienced cells can be defined as $PD\text{-}1^+$. The pool of $PD\text{-}1^+$ $CD8^+$ T cells encompasses cells specific for different epitopes of LCMV. The immunodominant LCMV epitope in C57BL/6 mice is the glycoprotein 33-41 (gp33) (Murali-Krishna,

Altman *et al.* 1998). Analysis of these cells was performed using a gp33 peptide-MHC tetramer. The effect of PD-L1 blockade was investigated by comparing the frequencies of LCMV-specific $CD8^+$ T cells ($PD\text{-}1^+$ and $gp33^+PD\text{-}1^+$) in the blood prior to and after the treatment. In control mice, which received PBS as well as in mice treated once with anti-PD-L1, the frequency of the cells did not change (Figure 3.1B). By contrast, mice that received three injections of anti-PD-L1 harbored significantly more antigen-experienced ($PD\text{-}1^+$) and gp33 $tetramer^+$ cells after the treatment. A similar strong expansion of LCMV-specific $CD8^+$ T cells was observed in mice that received five anti-PD-L1 injections (Figure 3.1B).


A
2x10^6 IU LCMV Cl13
chronic infection phase
-2
0
anti-CD4
44
45
46
blood
1x anti-PD-L1
blood
spleen
44
45
48
51
52
blood
3x anti-PD-L1
blood
spleen
44
45
48
51
54
57
58
blood
5x anti-PD-L1
blood
spleen


B
pre treatment
post treatment
% PD-1+ CD8+
80
60
40
20
0
***
***
ns
PBS
1x
3x
5x
anti-PD-L1
% gp33+ PD-1+ CD8+
6
4
2
0
***
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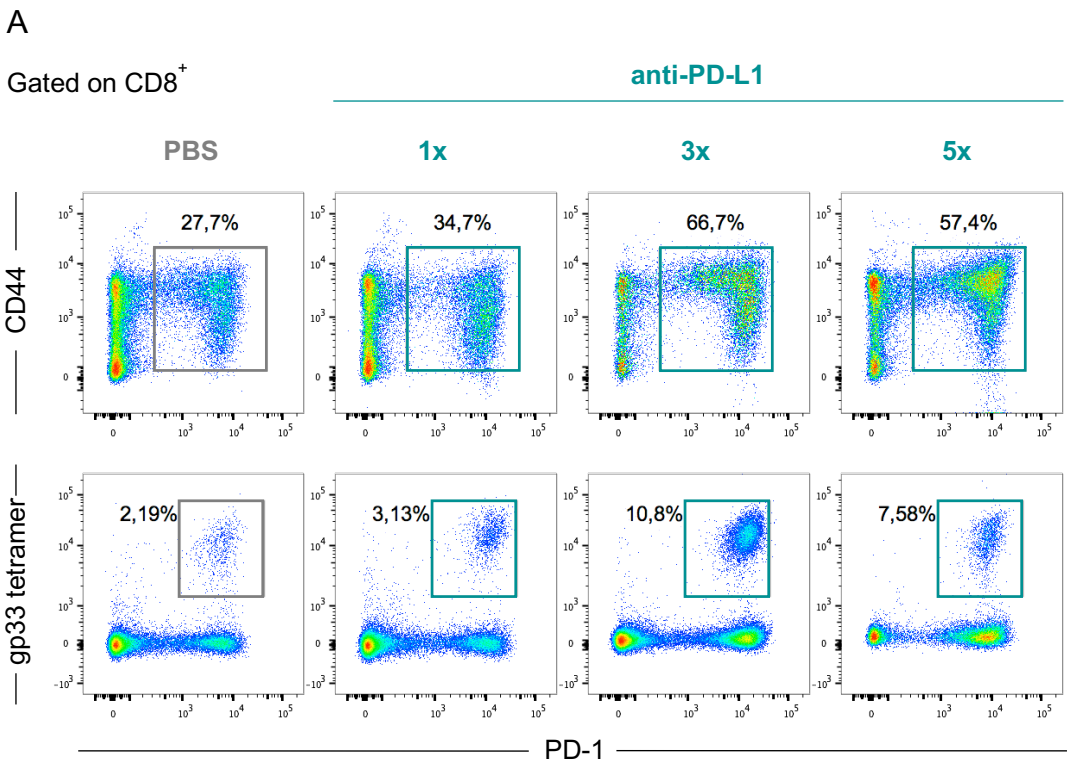
ns
PBS
1x
3x
5x
anti-PD-L1

Figure 3.1: Mouse model of chronic LCMV infection and PD-L1 blockade. **(A)** Adult male mice (8-12 weeks old) received 300 µg anti-CD4 (GK1.5) i.p. on d-2 and d0 of infection to deplete $CD4^+$ cells. Mice were i.v. infected with $2x10^6$ IU LCMV Cl13 on d0. Blood was taken prior to the start of anti-PD-L1 treatment (d44). On d45, mice received 200 µg anti-PD-L1 blocking antibody (10F.9G2) i.p. once, three or five times every three days. 24 h post anti-PD-L1 administration, blood and spleen were analysed. **(B)** Paired frequencies of antigen-experienced ($PD\text{-}1^+$) and gp33 $tetramer^+$ LCMV-specific $CD8^+$ T cells from the blood before and after anti-PD-L1 treatment. Data are representative of three independent experiments (n = 5 mice/group). Comparison between groups was calculated using a 2-way ANOVA * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

For the analysis of LCMV-specific $CD8^+$ T cells in the spleen of chronically infected mice that received PD-L1 blockade, lymphocytes were isolated and stained with PD-1 and gp33 tetramer. Representative flow cytometry plots show the frequencies of $PD\text{-}1^+$ and $gp33^+$ $PD\text{-}1^+$ $CD8^+$ T cells (Figure 3.2A). Quantitative analysis of the data displayed no change in frequency and absolute number of the cells after one injection of anti-PD-L1 (Figure 3.2B). However, after three treatments with anti-PD-L1, a two- to three-fold increase of $PD\text{-}1^+$ and $gp33^+$ in frequency and a five- to ten-fold increase in absolute numbers was found. Notably, prolonged treatment did not further augment the expansion of LCMV-specific $CD8^+$ T cells. Rather, after five treatments with anti-PD-L1 the frequencies and absolute numbers of LCMV-specific cells were slightly lower than after three treatments (Figure 3.2B). These data reveal that the expansion of exhausted $CD8^+$ T cells during immunotherapy peaks with three treatments. Further injections sustained $CD8^+$ T cell activation, yet did not further increase the number of antigen-specific $CD8^+$ T cells.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anti-PD-L1
PBS
1x
3x
5x
CD44
gp33 tetramer
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34,7%
66,7%
57,4%
2,19%
3,13%
10,8%
7,58%
PD-1

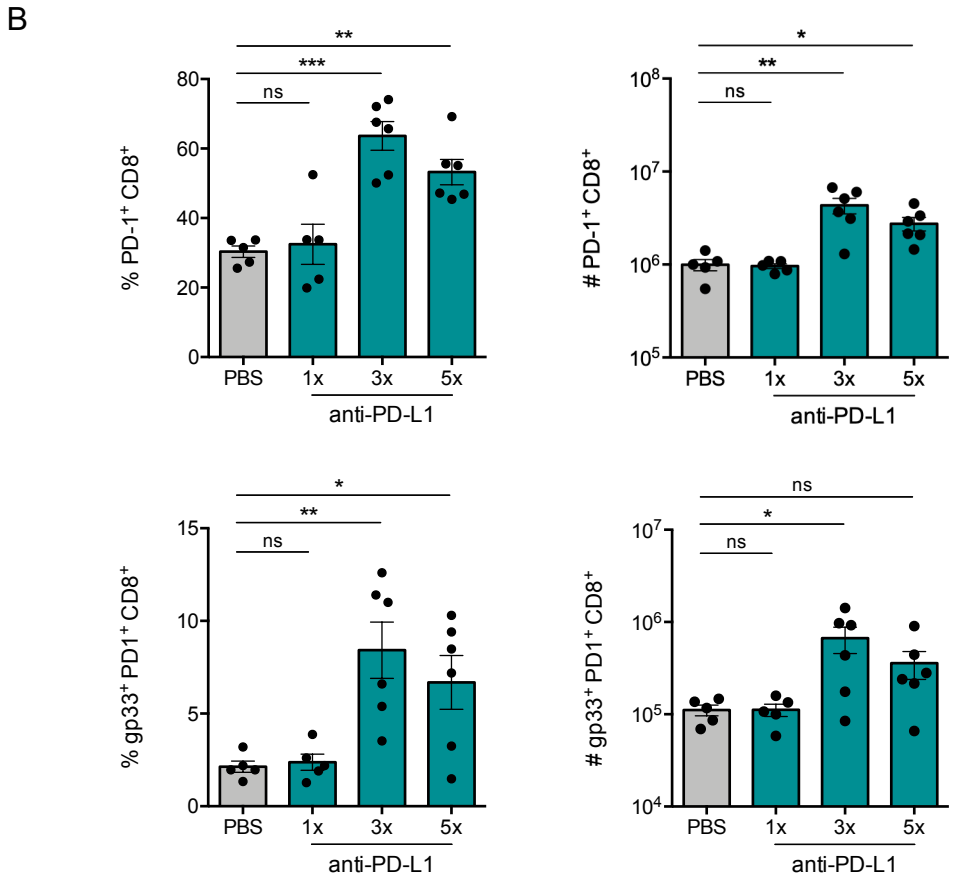
B
% PD-1+ CD8+
# PD-1+ CD8+
% gp33+ PD1+ CD8+
# gp33+ PD1+ CD8+
ns
*
**
***
PBS
1x
3x
5x
anti-PD-L1

Figure 3.2: Splenic LCMV-specific CD8$^+$ T cells proliferate upon PD-L1 blockade. Chronically infected mice were treated one, three or five times with 200 µg anti-PD-L1. 24 h post treatment, splenic CD8$^+$ T cells were isolated and analysed by flow cytometry. **(A)** Representative flow cytometry plots of antigen-experienced (PD-1$^+$) and gp33$^+$ LCMV-specific CD8$^+$ T cells after anti-PD-L1 treatment. **(B)** Frequency and absolute number of antigen-experienced (PD-1$^+$) and gp33$^+$ LCMV-specific CD8$^+$ T cells after anti-PD-L1 treatment. Data are representative of three independent experiments (n = 5 mice/group). Error bars indicate the mean $\pm$ SEM. For statistical analysis a one-way ANOVA was used. * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

To further investigate the proliferation of exhausted cells after anti-PD-L1 treatment, splenic gp33$^+$ PD-1$^+$ CD8$^+$ T cells were stained for Ki67. Ki67 is a protein which is not present during cellular quiescence but is expressed during all phases of cell division (Scholzen and Gerdes 2000). Therefore, the presence of Ki67 within cells correlates with a state of active cell division. As the previous data revealed an increase of LCMV-specific CD8$^+$ T cells, we speculated that these cells would also express Ki67 after treatment with anti-PD-L1. Representative histograms show the frequency and intensity of Ki67 expression (Figure 3.3A). Indeed, quantitative analysis revealed that the pattern of expansion of LCMV-specific cells is reflected by Ki67 expression. 24 h after one anti-PD-L1 injection, no change in frequency and absolute number of Ki67$^+$ gp33$^+$ cells occurred (Figure 3.3B). In comparison to PBS treatment or after one injection of anti-PD-L1, after three treatments the frequency of Ki67-expressing cells doubled and the absolute number increased up to 10-fold. As described above, fewer cells expanded after five compared to three anti-PD-L1 treatments. This was also reflected in the frequency and absolute number of Ki67$^+$ gp33$^+$ cells. Compared to untreated mice, mice which received five anti-PD-L1 injections did not harbor significantly more Ki67$^+$ cells, again, indicating no advance of five over three anti-PD-L1 treatments (Figure 3.3B).

Additionally, the splenic viral titer was measured and is depicted as infectious units per gram spleen. The viral load was significantly decreased in mice that received three anti-PD-L1 treatments compared to PBS-treated animals (Figure 3.3C).

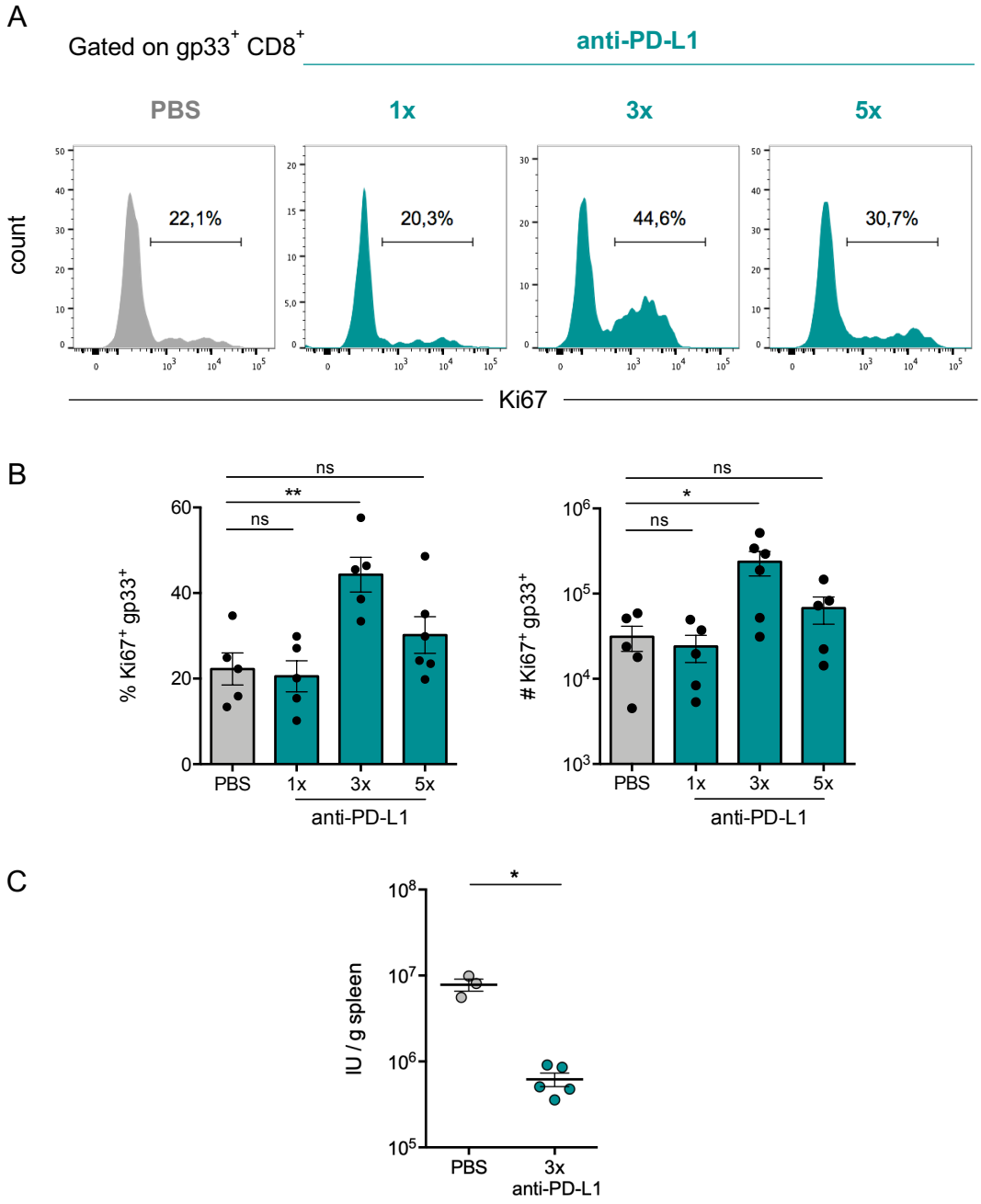

Figure 3.3: PD-L1 blockade-induced proliferation peaks after three treatments. Chronically infected mice were treated one, three or five times with 200 µg anti-PD-L1. 24 h post treatment, splenic $CD8^+$ T cells were isolated and analysed by flow cytometry. **(A)** Representative histograms of $gp33^+$ LCMV-specific $CD8^+$ T cells after anti-PD-L1 treatment showing frequency of $Ki67^+$ proliferating cells. **(B)** Frequency and absolute number of $Ki67^+$ $gp33^+$ $CD8^+$ T cells after anti-PD-L1 treatment. **(C)** Viral load depicted as infectious units (IU) per g spleen of PBS-treated and 3x anti-PD-L1-treated animals. Data are representative of three independent experiments (n = 5 mice/group). Error bars indicate the mean ± SEM. For statistical analysis a one-way ANOVA was used. * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

As an additional readout for the reactivation of exhausted $CD8^+$ T cells during anti-PD-L1 treatment, the expression of PD-1 can be used, as it directly correlates with T cell stimulation (Agata, Kawasaki *et al.* 1996). Upon TCR engagement, PD-1 is rapidly expressed with NFATc1 (nuclear factor of activated T cells c1) being one of the driving transcription factors (Oestreich, Yoon *et al.* 2008). During chronic infections, persistent antigen presentation supports the high expression of PD-1. Given that anti-PD-L1 treatment upregulates TCR signalling, a further increase in PD-1 expression is anticipated under this condition. To test this notion, the median fluorescence intensity (MFI) of LCMV-specific cells ($PD\text{-}1^+$ and $gp33^+$) was measured after anti-PD-L1 treatment. As shown above, 24 h after a single anti-PD-L1 injection, exhausted $CD8^+$ T cells did not change in number or Ki67 expression. However, a significant increase of PD-1 expression was detected on antigen-experienced $CD8^+$ T cells ($PD\text{-}1^+$) (Figure 3.4A). This was also seen when specifically analysing $gp33^+$ $CD8^+$ T cells (Figure 3.4B). The increased PD-1 expression reveals that cells are already activated by a single anti-PD-L1 injection. PD-1 expression was further increased after three anti-PD-L1 treatments. However, it was significantly decreased after five anti-PD-L1 injections. Here, the PD-1 expression level of $gp33^+$ cells decreased to similar levels as in untreated animals (Figure 3.4A and B). These data are in line with our previous interpretation that anti-PD-L1 therapy reaches a plateau after the third injection. The T cell response peaks after three injections and surprisingly decreases with ongoing treatment. The reason for these findings remains to be elucidated.

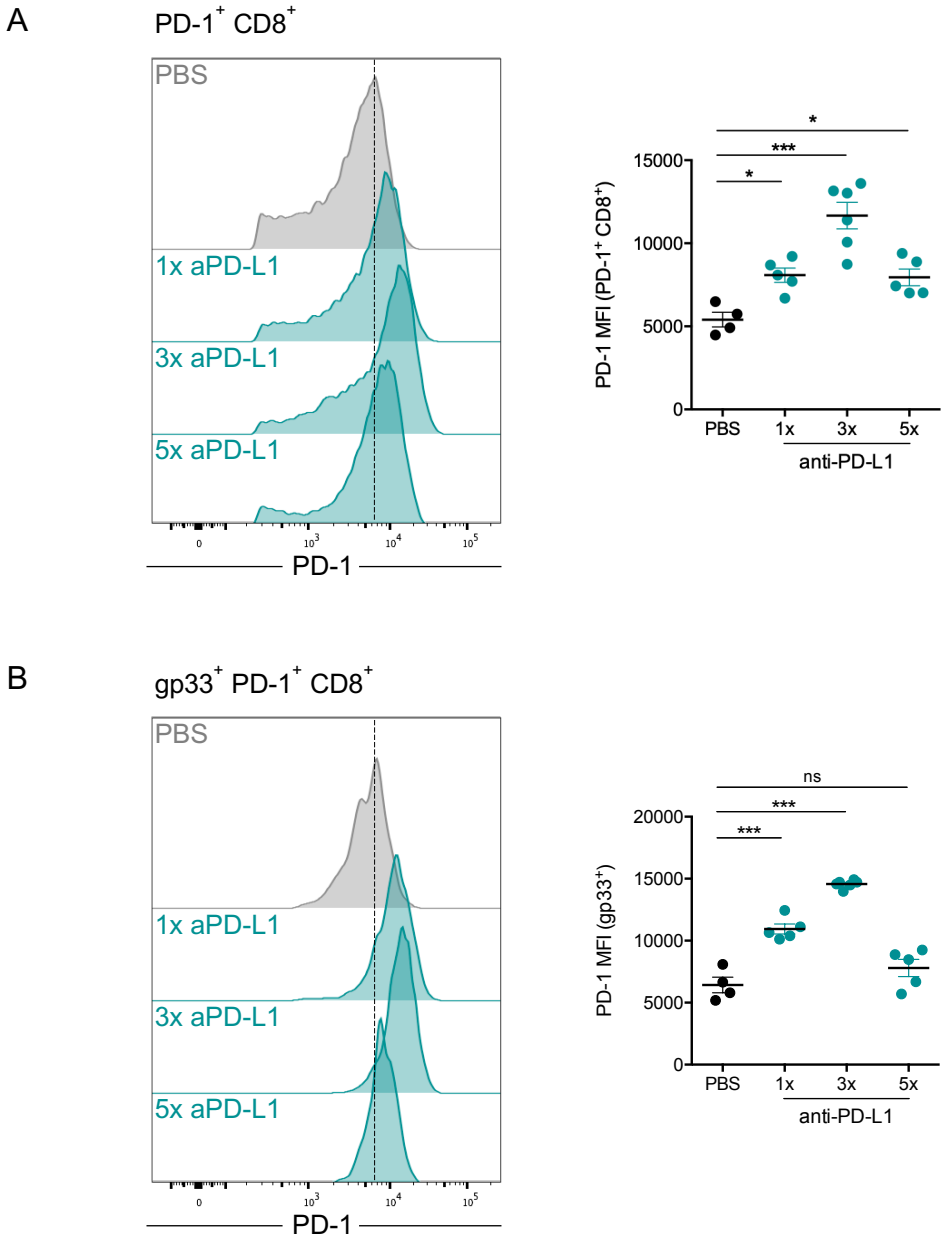

Figure 3.4: PD-1 expression peaks after three anti-PD-L1 treatments. Chronically infected mice were treated one, three or five times with 200 µg anti-PD-L1. 24 h post treatment, splenic $CD8^+$ T cells were isolated and analysed by flow cytometry. **(A)** Left: representative histograms show PD-1 expression of $PD\text{-}1^+$ cells after anti-PD-L1 treatment, right: quantitative analysis of median fluorescence intensity (MFI) of $PD\text{-}1^+$ cells. **(B)** Left: representative histograms show PD-1 expression of $gp33^+$ cells after anti-PD-L1 treatment, right: quantitative analysis of PD-1 MFI of $gp33^+$ cells. Data are representative of three independent experiments (n = 5 mice/group). Error bars indicate the mean ± SEM. For statistical analysis a one-way ANOVA was used. * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Exhausted CD8$^+$ T cells are described to consist of two main subsets of cells, which differ on a transcriptional, metabolic and functional level (Im, Hashimoto *et al.* 2016, Utzschneider, Charmoy *et al.* 2016, Man, Gabriel *et al.* 2017). Most importantly, only one of the subsets responds to immunotherapy with proliferation and consecutive reinvigoration. To differentiate the two subsets by flow cytometry as well as for quantitative analysis, Tim3 and TCF-1 were used as markers (Figure 3.5A). The Tim3$^+$ subset represents cells, which express higher levels of co-inhibitory receptors and are therefore considered to be more exhausted and functionally impaired. These cells display a terminally differentiated phenotype (Paley, Kroy *et al.* 2012). In comparison, the TCF-1$^+$ subset expresses lower levels of PD-1 and other co-inhibitory receptors and harbors memory-like features (Im, Hashimoto *et al.* 2016). This smaller subset of exhausted CD8$^+$ T cells is able to respond to immunotherapy when either PD-1 or PD-L1 is blocked (Im, Hashimoto *et al.* 2016, Leong, Chen *et al.* 2016, Utzschneider, Charmoy *et al.* 2016, Siddiqui, Schaeuble *et al.* 2019). Memory-like TCF-1$^+$ cells undergo self-renewal but also give rise to Tim3$^+$ cells with heightened effector capacity.

After one or three treatments with anti-PD-L1, no change in frequency of TCF-1$^+$ or Tim3$^+$ gp33$^+$ cells occurred (Figure 3.5B and C, left). However, after five injections, a strong shift towards the Tim3$^+$ subset was observed. The TCF-1$^+$ cell pool decreased to more than half of its frequency when compared to untreated samples whereas the Tim3$^+$ cell pool increased and constituted 95 % of the LCMV-specific CD8$^+$ T cell population. Importantly, as shown in figure 3.2B, the absolute number of gp33$^+$ PD-1$^+$ CD8$^+$ T cells drastically increased especially after three treatments. At this point, both subsets, TCF-1$^+$ and Tim3$^+$, equally increased in absolute cell numbers (Figure 3.5B and C, right). However, after five treatments, the TCF-1$^+$ cell pool was reduced to the level of untreated mice, whereas the Tim3$^+$ cells were as many as after three injections.

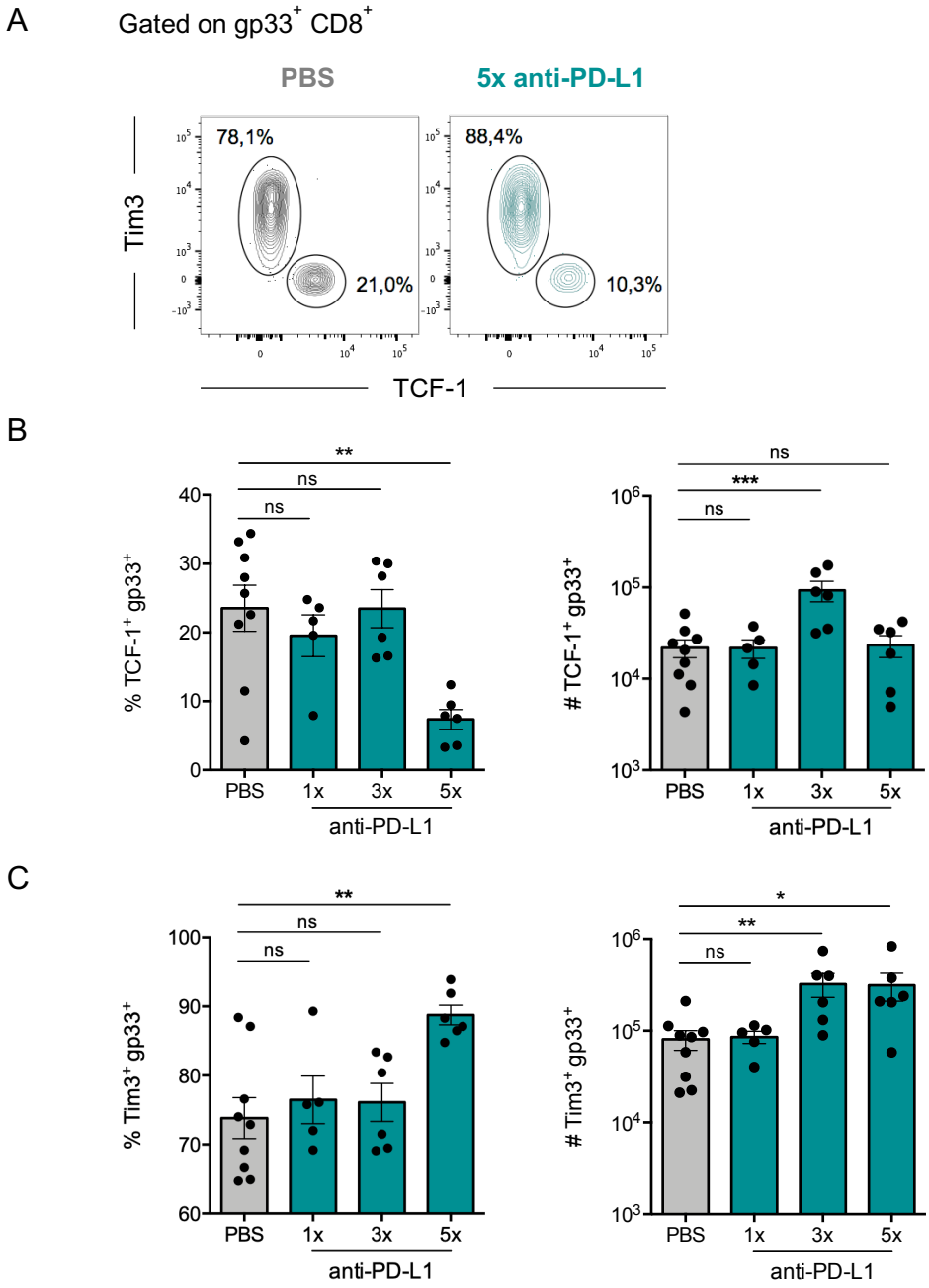

Figure 3.5: Distinct subsets of LCMV-specific CD8⁺ T cells respond differentially to PD-L1 blockade. Chronically infected mice were treated one, three or five times with 200 µg anti-PD-L1. 24 h post treatment, splenic CD8⁺ T cells were isolated and analysed by flow cytometry. **(A)** Representative flow cytometry plots showing the two subpopulations (TCF-1⁺ and Tim3⁺) of LCMV-specific CD8⁺ T cells with and without PD-L1 blockade. **(B)** Frequency and absolute number of TCF-1⁺ gp33⁺ CD8⁺ T cells after anti-PD-L1 treatment. **(C)** Frequency and absolute number of Tim3⁺ gp33⁺ CD8⁺ T cells. Data are representative of three independent experiments (n = 5 mice/group). Error bars indicate the mean ± SEM. For statistical analysis a one-way ANOVA was used. * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

In summary, 24 h after a single treatment with 200 µg anti-PD-L1 antibody, LCMV-specific CD8$^+$ T cells did not expand in blood and spleen and did not differ in phenotype. However, the increased expression of PD-1 that correlates with TCR engagement indicates that exhausted CD8 T cells respond to the first injection, but have not yet entered cell cycle. In contrast to that, after three treatments with anti-PD-L1, a significantly higher number of LCMV-specific cells was detected in blood and spleen of chronically infected mice. Additionally, mice treated three times harbored an increased number of proliferating Ki67$^+$ cells. Interestingly, this increase in cell number was detected for both subsets, TCF-1$^+$ and Tim3$^+$ exhausted T cells. However unexpectedly, after five anti-PD-L1 injections, the expansion of cells did not further increase. Compared to after three injections, there were less PD-1$^+$ and gp33$^+$ cells and fewer of them were proliferating (Ki67$^+$). The expression of PD-1 was drastically decreased after five treatments. Importantly, after five treatments, a strong shift towards the terminally differentiated Tim3$^+$ phenotype occurred. The memory-like TCF-1$^+$ cells were significantly reduced. The kinetic analysis of anti-PD-L1 therapy unveiled a limit of cell proliferation, which is reached between three and five treatments. In addition to that, the number of TCF-1$^+$ cells first increases during immunotherapy but is reduced with ongoing treatment. It is important to clarify which factors enable proliferation and which possibly prevent further expansion as well as influence the differentiation of the cells during PD-L1 blockade. As the PD-1 expression level of LCMV-specific CD8$^+$ T cells after five anti-PD-L1 treatments falls back to levels detected before treatment, it is very likely that TCR engagement and signalling is decreased as well. In principle, these results can be explained by both T cell-intrinsic factors as well as potential extrinsic factors like changes in antigen presentation, activation of suppressive lymphoid or myeloid cell populations, immunomodulatory cytokines or changes in localization that alter preferred cellular interaction partners. To approach these various possibilities, we first aimed to identify the critical cellular players that activate exhausted CD8$^+$ T cells during checkpoint immunotherapy.

3.2.2 CD11c$^+$ cells are critical for successful immunotherapy

In 2017, Kamphorst *et al.* and Hui *et al.* revealed an important prerequisite for proliferation of exhausted CD8$^+$ T cells upon immunotherapy: co-stimulatory signalling via the CD28 receptor (Hui, Cheung *et al.* 2017, Kamphorst, Wieland *et al.* 2017). The ligands for CD28, CD80 and CD86, are primarily expressed by antigen-presenting cells (APC) (Lenschow, Walunas *et al.* 1996). During infections the priming and expansion of antigen-specific T cells is highly dependent on the presentation of antigen by these cells (Parish and Kaech 2009). The most professional antigen-presenting cells are dendritic cells (DC) (Chopin, Allan *et al.* 2012). Equipped with various pathogen-sensing receptors and superior in up-take, processing and presentation of antigen via MHCI and MHCII, DC are most efficient in stimulating naïve T cells. Furthermore, by the expression of co-stimulatory molecules, such as CD80 and CD86, DC deliver additional signals to induce an optimal T cell response (Larsen, Ritchie *et al.* 1992, Inaba, Witmer-Pack *et al.* 1994). Therefore, we hypothesized that DC also play an important role during immunotherapy. Whereas PD-L1 blockade prevents inhibitory signalling by PD-1, DC can further stimulate the T cells with the presentation of antigen and the delivery of co-stimulatory signals.

To investigate this hypothesis, we made use of the CD11c.DOG mouse line. Unlike CD11c DTR (Jung, Unutmaz *et al.* 2002) or zDC DTR mice (Meredith, Liu *et al.* 2012), the CD11c.DOG line does not require to perform bone marrow chimera for continued depletion without severe side-effects. DC express high levels of the transmembrane protein CD11c, also known as Integrin alpha X (Belz and Nutt 2012). This mouse line expresses a diphtheria toxin receptor (DTR) under the *CD11c* promotor. Therefore, treatment of these mice with diphtheria toxin (DTX), leads to the depletion of CD11c$^+$ cells (Hochweller, Striegler *et al.* 2008). WT mice do not express the DTR and are thereby protected from the effects of DTX.

CD11c.DOG mice were infected with 2x10^6 IU LCMV Cl13 in combination with CD4$^+$ cell depletion. During the chronic infection phase, mice were treated with 250 ng DTX (five treatments every two day) and 200 µg anti-PD-L1 (four

treatments every three days). To investigate how the absence of $CD11c^+$ cells impacts on the response to anti-PD-L1 therapy, a second group that only received anti-PD-L1 and no DTX served as a control. 24 h after the last anti-PD-L1 injection, LCMV-specific $CD8^+$ T cells from blood and spleen were analysed by flow cytometry. To directly track the effect in each animal, blood was taken before and after the treatment (Figure 3.6A). Representative flow cytometry plots show the successful depletion of $CD11c^{hi}$ $MHCII^{hi}$ DC in CD11c.DOG mice when DTX was administered (Figure 3.6B).

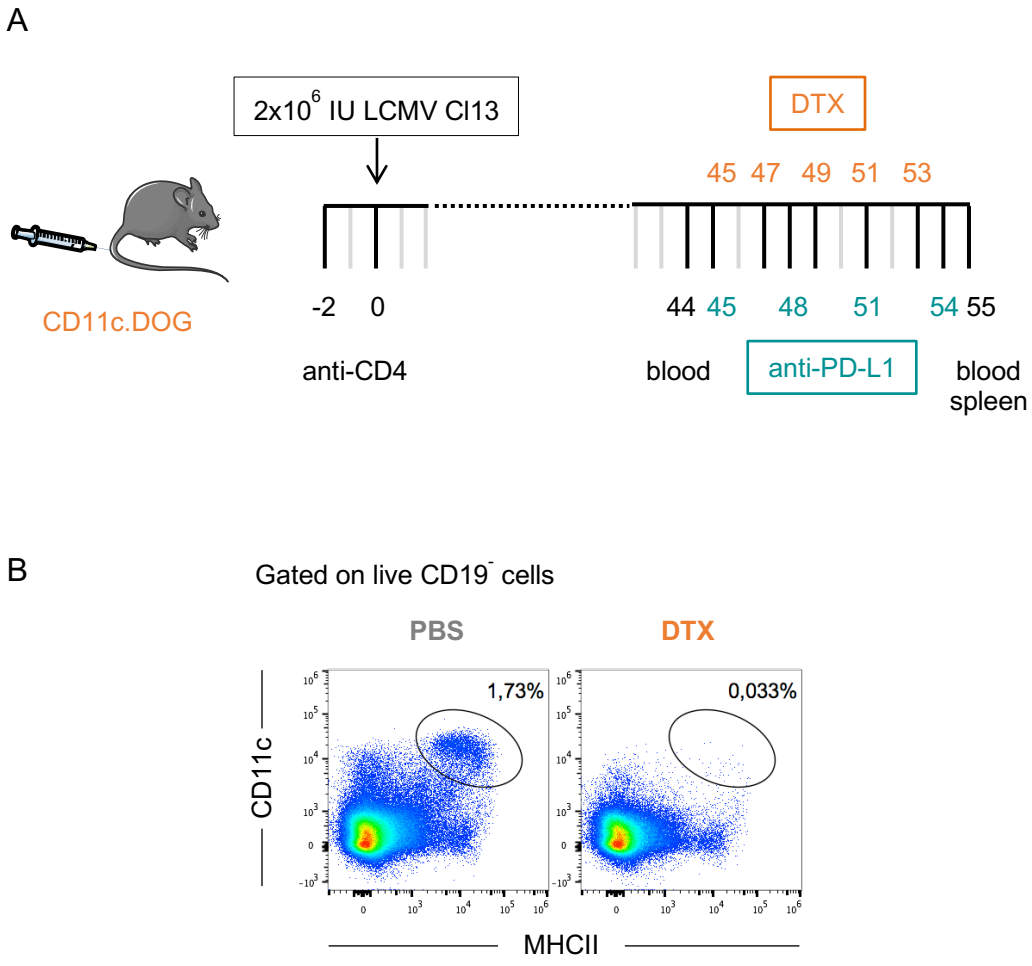

Figure 3.6: Mouse model of CD11c$^+$ cell depletion and PD-L1 blockade during chronic LCMV infection. (A) Adult male CD11c.DOG mice received 300 µg anti-CD4 i.p. on d-2 and d0 of infection. Mice were i.v. infected with 2x10^6 IU LCMV Cl13 on d0. Blood was taken prior to treatment (d44). From d45 onwards, mice received 200 µg anti-PD-L1 blocking antibody i.p., four times every three days. To deplete CD11c$^+$ cells, mice received 250 ng DTX i.p. from d45 onwards five times every second day. 24 h after the last anti-PD-L1 injection, blood and spleen were isolated for flow cytometry analysis. **(B)** Representative flow cytometry plots showing the depletion of CD11c$^+$ MHCII$^+$ DC in CD11c.DOG mice after treatment with DTX.

The comparison of antigen-experienced (PD-1$^+$) and gp33 tetramer$^+$ LCMV-specific T cells in the blood of mice before and after the treatment, revealed that cells did not expand by anti-PD-L1 treatment in mice which were depleted of CD11c$^+$ cells (Figure 3.7A). A similar result was obtained when analyzing the spleens of these mice. The frequency and absolute number of PD-1$^+$ as well as gp33$^+$ CD8$^+$ T cells were significantly decreased in mice that did not harbor CD11c$^+$ cells as compared to CD11c$^+$ cell proficient animals (Figure 3.7B). In addition, the fraction of CD8$^+$ T cells expressing the proliferation marker Ki67 was also slightly decreased in DTX-treated mice (Figure 3.7C).

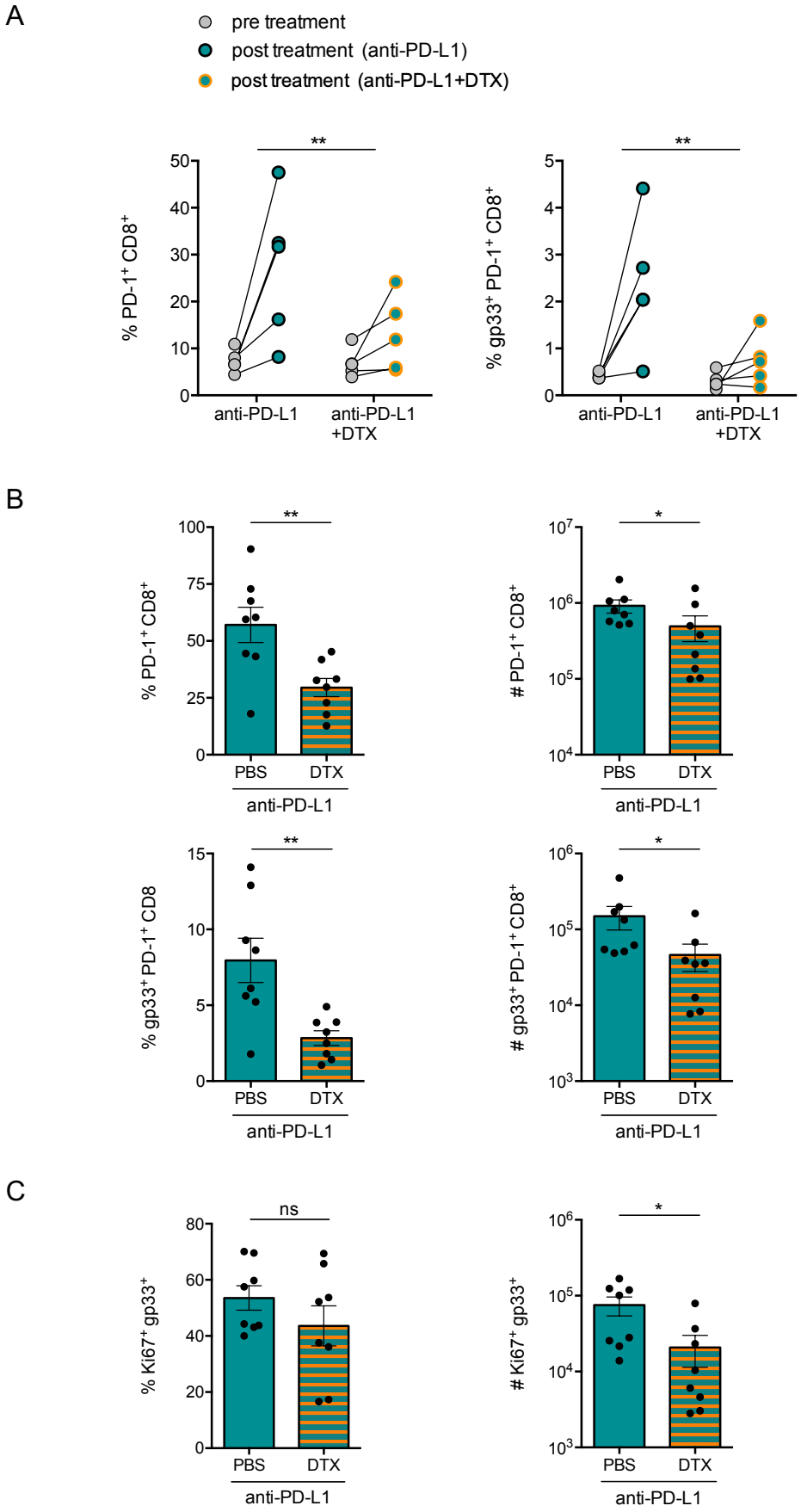
A
pre treatment
post treatment (anti-PD-L1)
post treatment (anti-PD-L1+DTX)
% PD-1+ CD8+
% gp33+ PD-1+ CD8+
anti-PD-L1
anti-PD-L1 +DTX
B
% PD-1+ CD8+
# PD-1+ CD8+
% gp33+ PD-1+ CD8
# gp33+ PD-1+ CD8+
PBS
DTX
anti-PD-L1
C
% Ki67+ gp33+
# Ki67+ gp33+
ns

Figure 3.7: PD-L1 blockade-induced expansion of CD8$^+$ T cells is dependent on CD11c$^+$ cells. (A) Paired frequencies of antigen-experienced LCMV-specific (PD-1$^+$) and gp33$^+$ CD8$^+$ T cells in the blood prior to and post anti-PD-L1 treatment in the presence and absence of CD11c$^+$ cells **(B)** Frequency and absolute number of splenic PD-1$^+$ and gp33 $^+$ CD8$^+$ T cells, as well as proliferating Ki67$^+$ gp33$^+$ cells **(C)**, post anti-PD-L1 treatment with and without CD11c$^+$ cell depletion. Data were pooled from two independent experiments (n = 4 mice/group). Error bars indicate the mean $\pm$ SEM. Comparison between groups was calculated using the unpaired Student‘s t-test. * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Activated $CD8^+$ T cells express CD11c to a certain extent and are therefore possibly targeted by DTX treatment (Vinay and Kwon 2010). To check if the decreased number of LCMV-specific $CD8^+$ T cells is caused by the depletion of such, we adoptively transferred $CD8^+$ T cells from transgenic P14 mice. These mice harbor $CD8^+$ T cells, which express a transgenic TCR specific for gp33. The cells do not express a DTR and are therefore not depleted upon DTX treatment. On the day of infection, CD11c.DOG mice received $2x10^3$ P14 cells and were treated as described previously (Figure 3.8A). The response of P14 cells to PD-L1 blockade in the presence or absence of $CD11c^+$ cells was assessed by flow cytometry. Clearly, the representative flow cytometry plots (Figure 3.8B) as well as the quantitative analysis (Figure 3.8C) show that P14 cells expanded to a significantly lower extend in DTX-treated mice. This finding fully reflects the effect seen with endogenous $CD8^+$ T cells and confirms that the expansion of antigen-specific $CD8^+$ T cells during PD-L1 blockade is dependent on $CD11c^+$ cells.

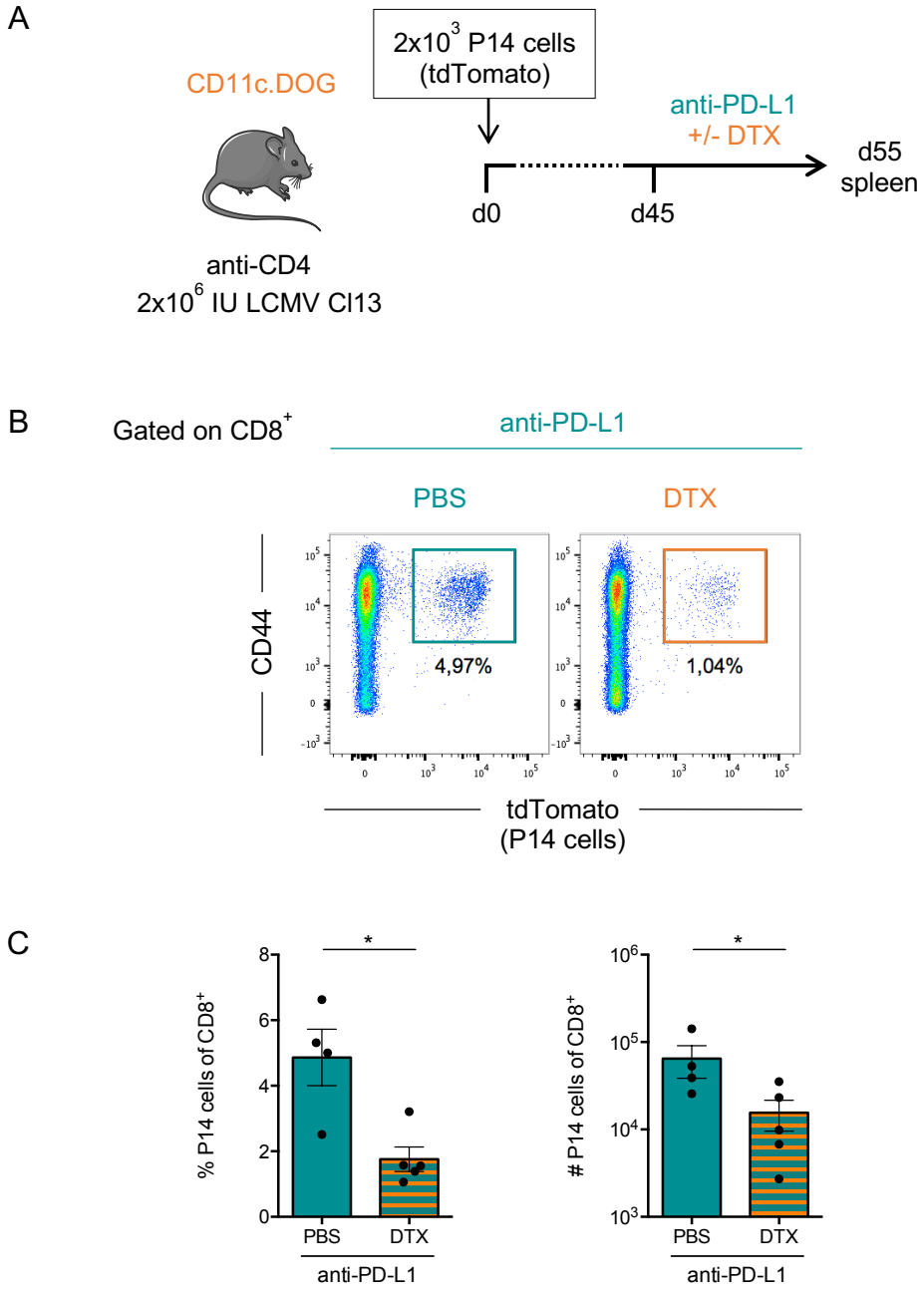

Figure 3.8: Impaired expansion of LCMV-specific CD8⁺ T cells is not caused by the depletion of CD11c⁺ CD8⁺ T cells. (A) CD11c.DOG mice received $2x10^3$ transgenic LCMV-specific P14 cells and were infected with LCMV Cl13. During the chronic infection phase mice were treated with anti-PD-L1 in combination with DTX to deplete CD11c⁺ cells. **(B)** Representative flow cytometry plots of P14 cells from spleens of mice with and without CD11c⁺ cells. **(C)** Frequency and absolute number of splenic P14 cells after anti-PD-L1 in combination with DTX treatment. Error bars indicate the mean ± SEM. Comparison between groups was calculated using the unpaired Student's t-test. * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

As described earlier, the two subsets of memory-like (TCF-1$^+$) and terminally exhausted (Tim3$^+$) CD8$^+$ T cells, respond differently to PD-L1 blockade. We investigated if the presence or absence of CD11c$^+$ cells has an impact on the differentiation of exhausted CD8$^+$ T cells. Here, we found similar frequencies of TCF-1$^+$ and Tim3$^+$ cells in both groups (Figure 3.9A). As the absolute number of LCMV-specific cells was lower in mice that received DTX, the absolute cell numbers of TCF-1$^+$ as well as Tim3$^+$ cells were decreased in these mice to the same level as in PBS-treated controls.

Surprisingly, cells from DTX-treated mice were still able to produce GzmB as an important effector molecule (Figure 3.9B). However, measuring the viral titer in spleens of anti-PD-L1-treated mice in combination with DTX revealed that these mice carried a higher viral load (Figure 3.9C). This points to a situation where the cells were not sufficiently reinvigorated to exert their effector functions and eliminate infected cells.

Up to this point, CD11c$^+$ cells were depleted only in combination with immunotherapy. The question arises if DC are also critical to control the viral load during chronic infection in the absence of immunotherapy. To investigate this, CD11c.DOG mice were infected with LCMV Cl13 and CD11c$^+$ cells were depleted during the chronic infection phase with DTX (Figure 3.10A). Here, the frequency and number of antigen-specific cells (PD-1$^+$ and gp33$^+$) were only slightly decreased in DTX-treated mice (Figure 3.10B). In line with the previous data, CD11c$^+$ cell depletion itself did not affect the differentiation of LCMV-specific CD8$^+$ T cells regarding their expression of TCF-1 and Tim3 (Figure 3.10C). Additionally, splenic viral titers were not significantly changed in mice that did not harbor CD11c$^+$ cells. Pointing towards a critical role of CD11c$^+$ DC in the context of anti-PD-L1 treatment.

Collectively, these experiments show the importance of DC for the proliferation of exhausted CD8$^+$ T cells upon immunotherapy. The mere blockade of the inhibitory PD-1 pathway is not sufficient to reinvigorate these cells. Professional antigen presentation as well as the delivery of co-stimulatory signals by DC seem to play an essential and maybe previously underestimated role.

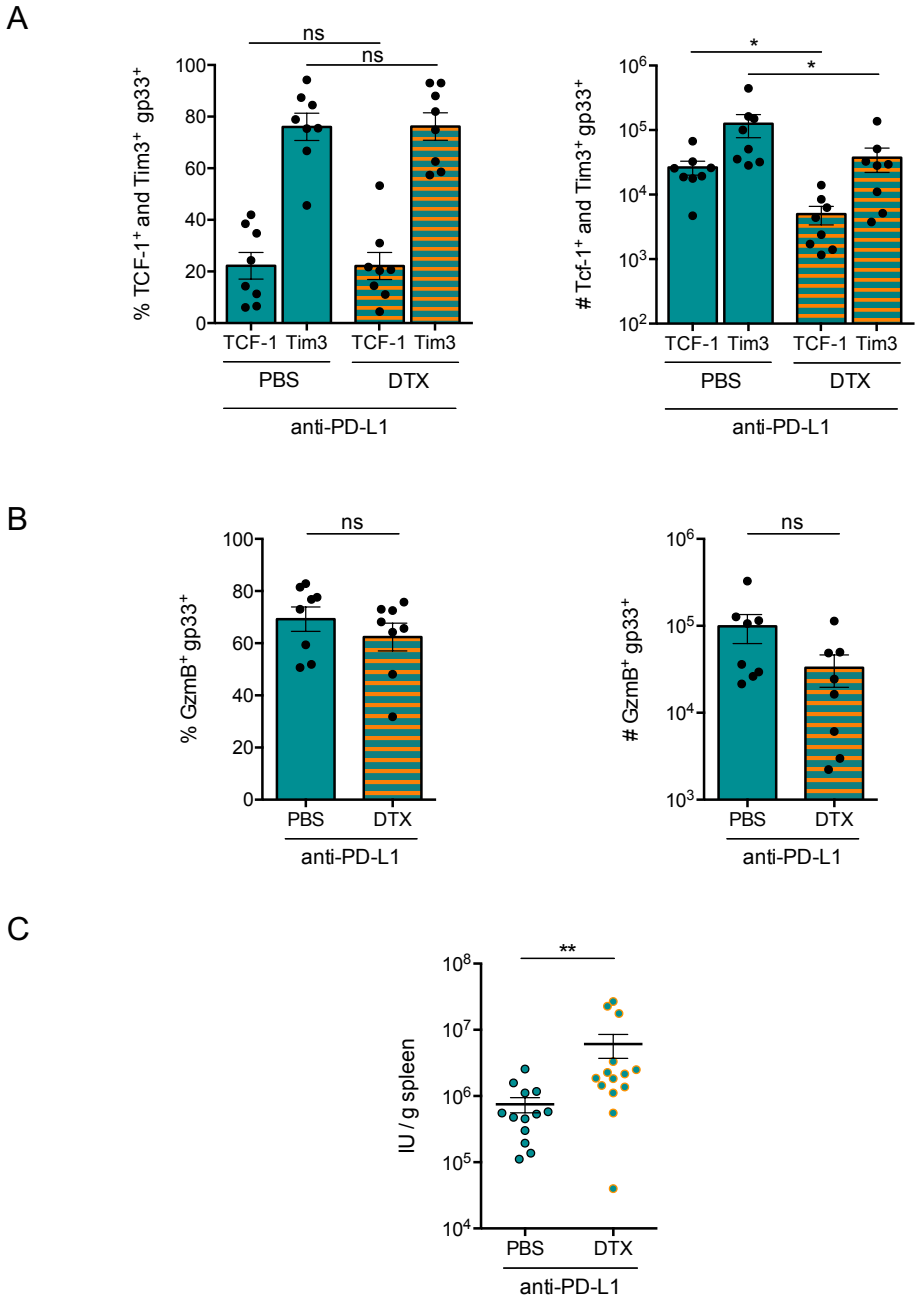

Figure 3.9: Viral load but not differentiation and GzmB production are affected by CD11c$^+$ cell depletion during PD-L1 blockade. (A) Frequency and absolute number of TCF-1$^+$ and Tim3$^+$ gp33$^+$ CD8$^+$ T cells after anti-PD-L1 treatment with and without CD11c$^+$ cell depletion. **(B)** Frequency and absolute number of GzmB$^+$ gp33$^+$ cells. **(C)** Viral load depicted as infectious units (IU) per g spleen of anti-PD-L1-treated animals with and without CD11c$^+$ cell depletion. Data are pooled from two independent experiments (n = 4 mice/group). Error bars indicate the mean ± SEM. Comparison between groups was calculated using the unpaired Student's t-test. * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

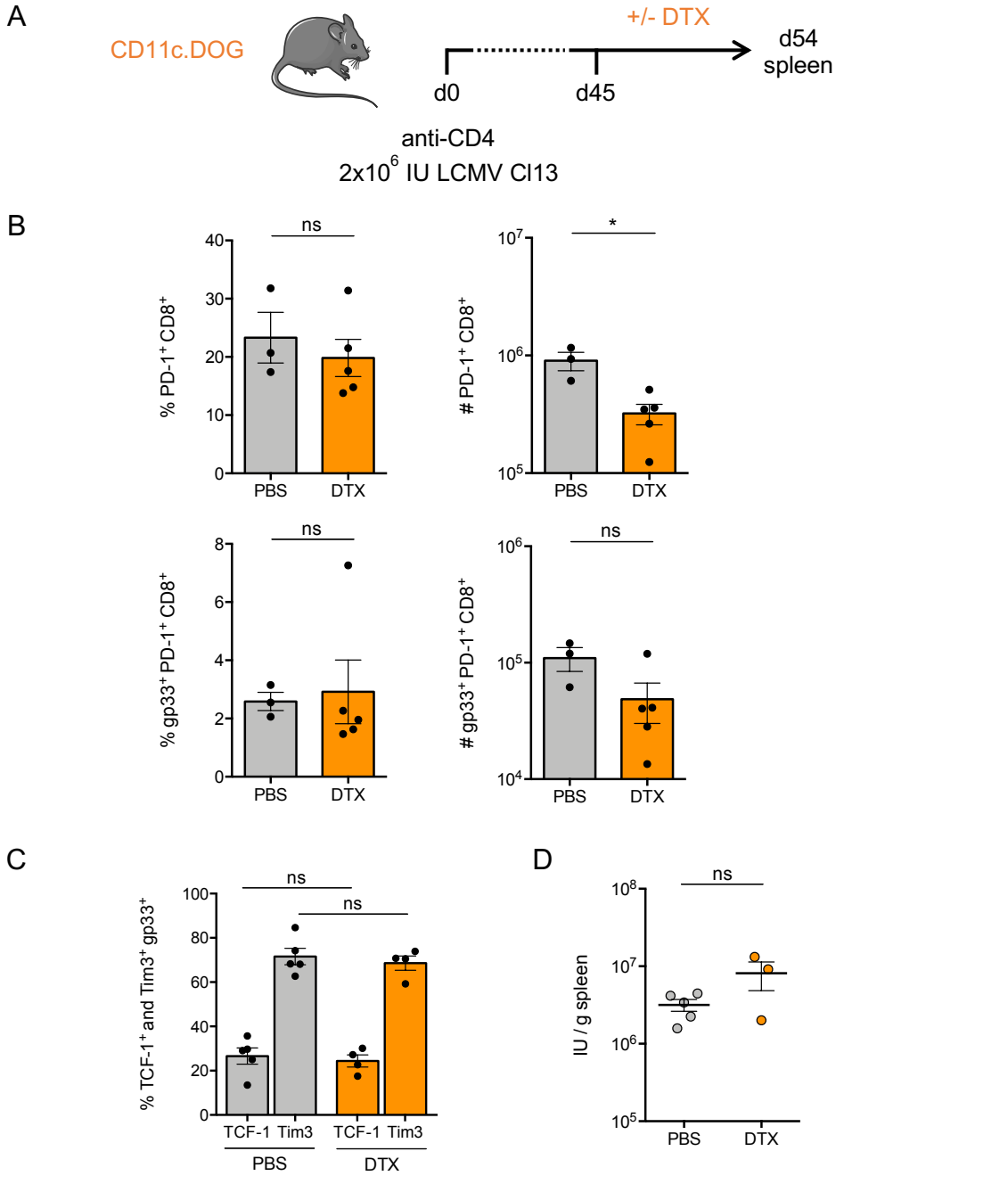

Figure 3.10: Depletion of CD11c$^+$ cells increases viral titer without affecting the proliferation and differentiation of LCMV-specific CD8$^+$ T cells. (A) CD11c.DOG mice received 300 µg anti-CD4 i.p. on d-2 and d0 and were i.v. infected with 2x10^6 IU LCMV Cl13 on d0. From d45 onwards, mice received 250 ng DTX i.p. every second day. On d54, spleens were isolated and analysed. **(B)** Frequency and absolute number of PD-1$^+$ and gp33$^+$ CD8$^+$ T cells. **(C)** Frequency of TCF-1$^+$ and Tim3$^+$ gp33$^+$ CD8$^+$ T cells with and without depletion of CD11c$^+$ cells. **(D)** Splenic viral load of mice harboring CD11c$^+$ cells or not. Error bars indicate the mean ± SEM. Comparison between groups was calculated using the unpaired Student's t-test. * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.2.3 Decreasing viral antigen presentation by DC during immunotherapy

As shown in the previous chapter, DC play an important role for the reinvigoration of exhausted $CD8^+$ T cells during immunotherapy. Subsequent questions are how DC support the reactivation of these cells and why PD-L1 blockade reaches a limit between three and five treatments. For the full activation of naive $CD8^+$ T cells, DC play a supreme role as they deliver all of the three required signals – antigen presentation, co-stimulatory signals and cytokines (van Stipdonk, Lemmens *et al.* 2001, Chopin, Allan *et al.* 2012). We hypothesized, that in the context of $CD11c^+$ depletion and PD-L1 blockade, exhausted $CD8^+$ T cells are not able to proliferate as antigen presentation together with co-stimulation is missing. Without proper TCR stimulation and co-stimulatory signals, the expansion of exhausted T cells cannot take place even though the inhibitory effects of PD-1 signalling are blocked.

To investigate the level of antigen presentation by APC from chronically infected mice, we made use of an *ex vivo* antigen presentation assay. For this, APC from spleens of chronically infected mice were isolated by digestion and density gradient centrifugation (Figure 3.11A). To investigate which APC subsets present antigen, we co-cultured them with transgenic P14 cells that are specific for LCMV glycoprotein. Prior to the co-culture with APC, P14 cells were labelled with Cell Trace Violet (CTV). This dye enables the analysis of proliferation by flow cytometry with dye dilution. Each new generation of divided cells is marked by a 50 % decreased dye intensity. APC and P14 cells were co-cultured for 65 h at 37 °C with 5 % CO_2 (Figure 3.11B). During this time, P14 cells divide if antigen is presented, TCR is activated and co-stimulatory signals are transmitted. As a positive control, isolated splenic APC from d3 post infection were used. Flow cytometry analysis shows that antigen presentation by APC from chronically infected mice was significantly lower compared to antigen presentation by APC from d3 post infection (Figure 3.11C).

To further elucidate which APC present antigen during the chronic infection phase, cells were sorted by FACS into B cells, DC ($XCR1^+$ DC (cDC1) and $CD11b^+$ DC (cDC2)) and remaining cells, which are $MHCII^{mid\text{-}low}$ and among

others contain macrophages. Again, an *ex vivo* antigen presentation assay with CTV-labelled P14 cells was performed. The assay clearly revealed that during chronic infection, DC were the dominant antigen-presenting cells, whereas B cells and macrophages were not able to stimulate P14 cells *ex vivo*. Notably, co-culture with cDC2 ($CD11b^+$ DC) lead to a stronger proliferation of P14 cells compared to cDC1 ($XCR1^+$ DC) (Figure 3.11D).

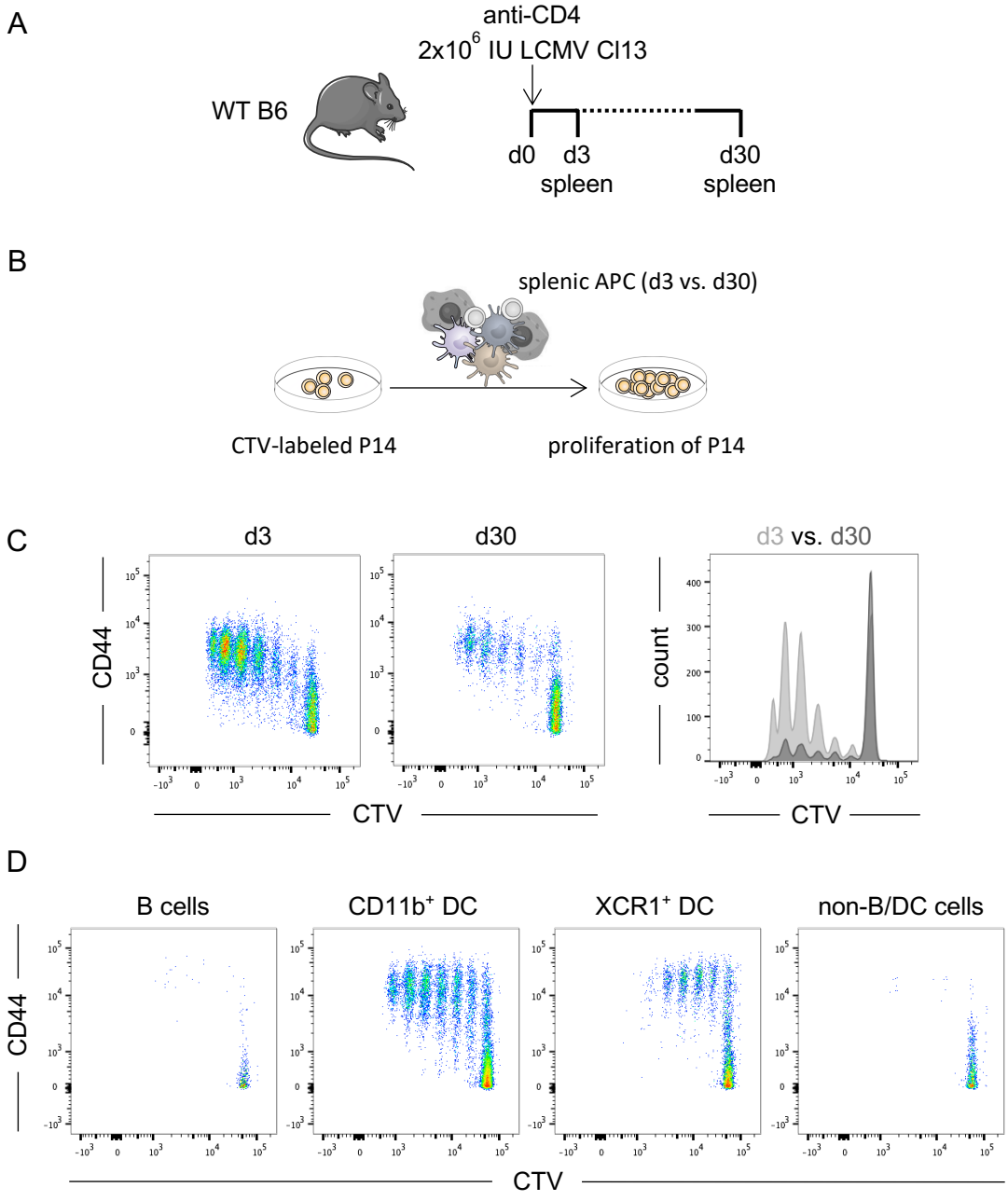

Figure 3.11: DC present antigen during chronic infection phase. (A) Adult male WT B6 mice received 300 µg anti-CD4 i.p. on d-2 and d0 of infection and were i.v. infected with $2x10^6$ IU LCMV Cl13 on d0. Spleens were isolated and APC enriched by digestion and density gradient centrifugation. **(B)** In an *ex vivo* co-culture assay $5x10^4$ APC were co-cultured with $5x10^4$ CTV-labelled P14 cells for 65 h at 37 °C, 5 % CO_2. Proliferation of P14 cells was determined by flow cytometry. **(C)** Representative flow cytometry plots showing P14 cell proliferation after co-culture with APC isolated at d3 and d30 post infection. **(D)** Representative flow cytometry plots showing P14 cell proliferation after co-culture with sorted B cells, $CD11b^+$ DC, $XCR1^+$ DC and non-B/DC cells (mainly macrophages) from chronically infected mice (d30).

An interesting question that remains is whether antigen presentation by DC changes in the context of immunotherapy. In order to address this question, chronically infected mice were treated one, three or five times with 200 µg anti-PD-L1. 24 h post treatment, spleens were isolated (Figure 3.1A). Untreated mice served as a control. $CD11b^+$ DC and $XCR1^+$ DC were enriched and used for the above described co-culture assay with CTV-labelled P14 cells. In general, a 1:1 co-culture of DC and P14 cells induced the strongest proliferation (Figure 3.12). The number of divided P14 cells decreased with each serial 1:2 dilution of DC. As observed before, co-culture with $CD11b^+$ DC provoked a higher P14 expansion. $CD11b^+$ and $XCR1^+$ DC from mice treated once with anti-PD-L1 did not stimulate the expansion of P14 cells to the same extent as DC from untreated mice (Figure 3.12). Unexpectedly, this effect was more pronounced when mice were treated three or five times with anti-PD-L1. $CD11b^+$ and $XCR1^+$ DC isolated from mice that received three or five injections were not able to significantly induce proliferation in P14 cells. Even at the highest concentration of DC (1:1 co-culture with P14), P14 cells did not divide (Figure 3.12). These findings reveal that the capacity of DC to activate exhausted $CD8^+$ T cells declines during the course of immunotherapy. Notably, B cells and macrophages were not able to drive the proliferation of P14 cells *ex vivo* in untreated nor anti-PD-L1-treated mice (data not shown). This is in line with our *in vivo* DC depletion experiments shown above, indicating that DC are the only APC able to drive antigen-specific $CD8^+$ T cell proliferation during immunotherapy of chronically infected mice.

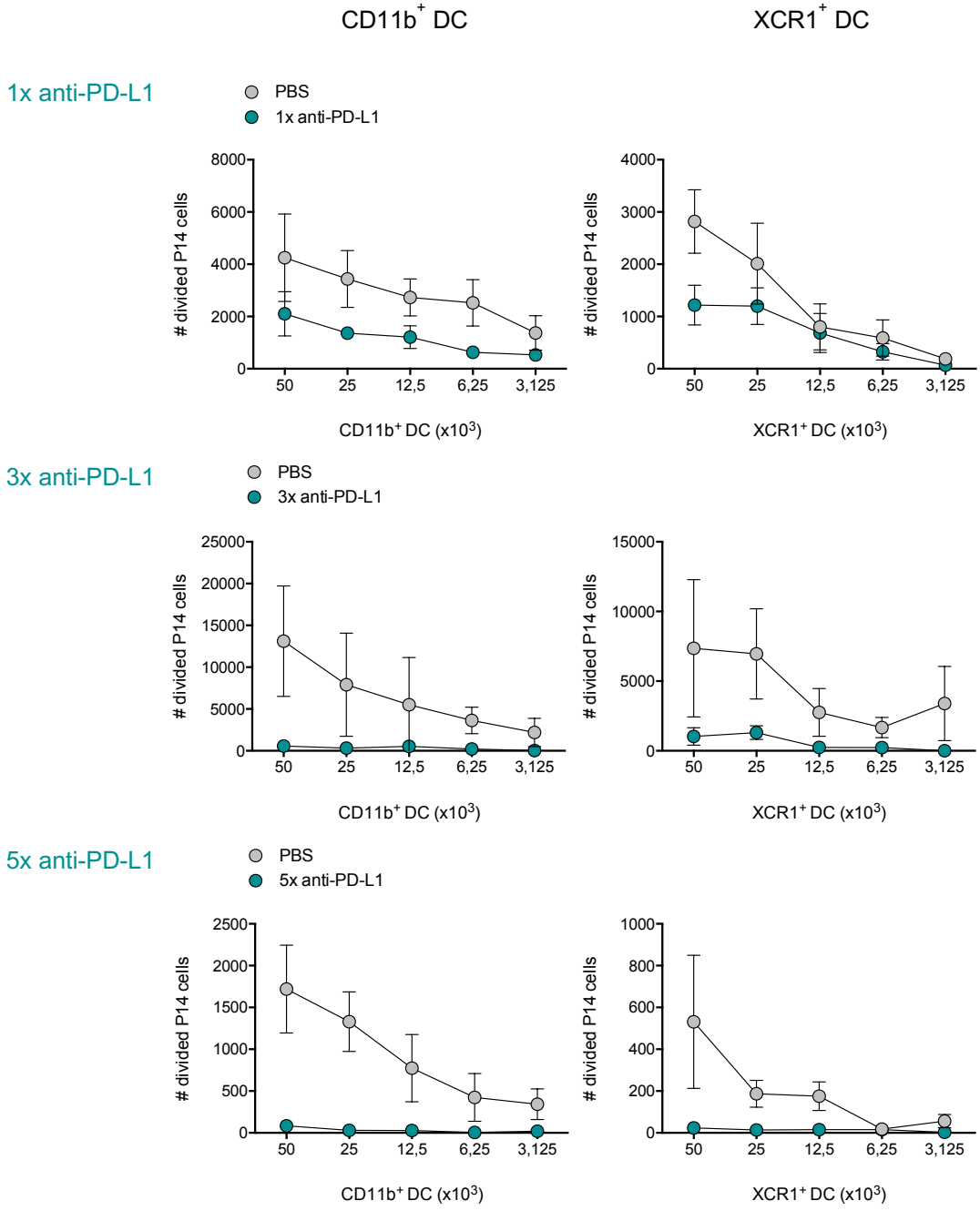

Figure 3.12: Antigen presentation by DC from anti-PD-L1-treated mice is highly decreased. WT mice received anti-CD4 i.p. on d-2 and d0 and were i.v. infected with $2x10^6$ IU LCMV Cl13. On d45 mice received 200 µg anti-PD-L1 i.p. one, three or five times every three days. 24 h post anti-PD-L1 application, spleens were isolated and $CD11b^+$ and $XCR1^+$ DC sorted by FACS. In an *ex vivo* co-culture assay $5x10^4$ DC (and serial 1:2 dilutions) were co-cultured with $5x10^4$ CTV-labelled P14 cells for 65 h at 37 °C. Proliferation of P14 cells was determined by flow cytometry. Graphs depict the number of divided P14 cells after co-culture with DC. Data are representative of three independent experiments (n = 5 mice/group). Error bars indicate the mean ± SEM.

In order to address whether DC isolated from anti-PD-L1-treated animals have an overall impaired capacity to stimulate antigen-specific $CD8^+$ T cells, we added exogenous antigen. Specifically, DC were loaded with an unrelated protein, the model antigen ovalbumin (OVA), and co-cultured with $CD8^+$ T cells, that express a specific transgenic TCR for this antigen (OT-I T cells). In detail, DC from mice that received one, three or five anti-PD-L1 injections, were enriched and sorted for $CD11b^+$ and $XCR1^+$ DC. These DC were loaded with OVA and subsequently co-cultured with OT-I T cells. Only if the DC are able to process the full OVA protein and present it via MHCI to OT-I cells, these cells will proliferate. OT-I cells were labelled with CTV to track their proliferation by flow cytometry. As displayed in representative histograms, $CD11b^+$ and $XCR1^+$ DC were clearly able to process and present OVA (Figure 3.13). OT-I T cells strongly proliferated during co-culture with DC from anti-PD-L1-treated mice.

These data show that DC fully retain their capacity to stimulate antigen-specific $CD8^+$ T cells during PD-L1 blockade in chronically infected animals. However, these DC do not seem to present viral antigen to a level that is sufficient to drive the proliferation of exhausted $CD8^+$ T cells over the course of treatment. These results fit well to the proliferation data obtained after several rounds of anti-PD-L1 therapy, showing that the positive effects of immunotherapy reach a limit over time.

In summary, we conclude that DC are the critical cellular element that underlie successful immunotherapy. Additionally, the level of viral antigen presentation by DC determines and limits the effect of immunotherapy. Interestingly, both DC subsets $XCR1^+$ DC (cDC1) and to a higher level $CD11b^+$ DC (cDC2) presented viral antigen and were able to drive $CD8^+$ T cell proliferation *ex vivo*. Therefore, we next aimed to address whether these two distinct DC subsets have a similar or differential role during checkpoint immunotherapy *in vivo*.

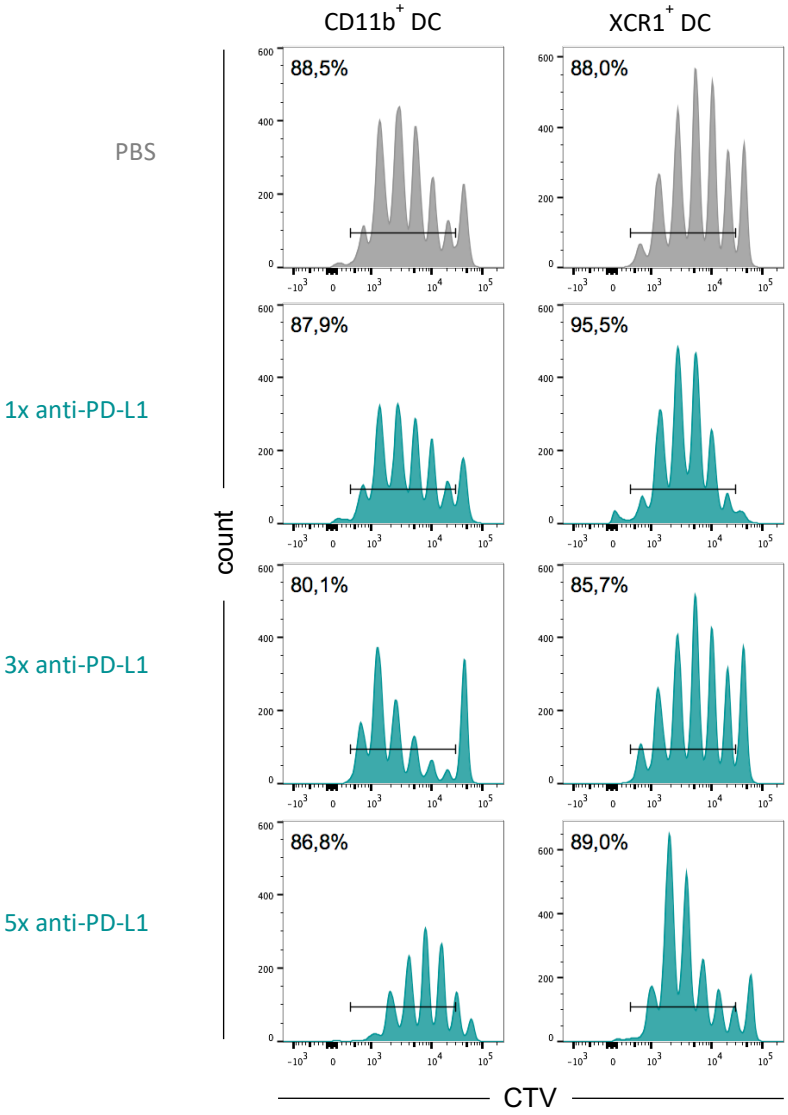

Figure 3.13: DC from anti-PD-L1-treated mice are able to process and present antigen. WT B6 mice were infected with LCMV Cl13. DC from untreated mice and mice that received one, three or five anti-PD-L1 injections during the chronic infection phase were isolated. Sorted $CD11b^+$ and $XCR1^+$ DC were loaded with OVA. In an *ex vivo* co-culture assay $5x10^4$ OVA-loaded DC were co-cultured with $5x10^4$ CTV-labelled OT-I cells for 65 h at 37 °C. Proliferation of OT-I cells was determined by flow cytometry. Representative histograms depict CTV expression of proliferated OT-I T cells. Data are representative of two independent experiments (n = 5 mice/group).

3.3 Discussion

In 2006, Barber *et. al* described the reinvigoration of exhausted $CD8^+$ T cells with anti-PD-1/PD-L1 treatment during chronic LCMV infection (Barber, Wherry *et al.* 2006). Only eight years later, pembrolizumab, the first anti-human PD-1 blocking antibody, was approved for the treatment of metastatic melanoma by the US Food and Drug Administration (FDA). To date, there are several different checkpoint inhibitors on the market and many more are currently tested in clinical trials. The cornerstone of this immunological breakthrough, the discovery of inhibitory receptors, has been rewarded with the 2018 Noble Prize in Physiology or Medicine for James P. Allison and Tasuku Honjo (NobelPrize.org 2019). Despite the enormous success of checkpoint immunotherapy, the exact underlying mechanisms are not fully understood, and treatment is limited to certain cancer types (Wu 2019). Little is reported on the kinetics of anti-PD-L1 treatment and the cellular interaction partners involved in $CD8^+$ T cell reinvigoration.

In this chapter, we investigated the $CD8^+$ T cell response during chronic LCMV infection and anti-PD-L1 therapy and found that DC play a critical role for the reinvigoration of exhausted $CD8^+$ T cells and control of viral load. During chronic viral infections, such as with LCMV and HIV, DC have been described to be partly dysfunctional (Ng, Sullivan *et al.* 2011, Ng, Snell *et al.* 2013). Paralysed DC downregulate MHC I molecules, co-stimulatory molecules (CD40, CD80, CD86) and show a decreased production of inflammatory cytokines (Sevilla, McGavern *et al.* 2004). In addition, DC can induce the secretion of immunosuppressive cytokines, such as IL-10, which further drive exhaustion of T cells during chronic infection (Baca Jones, Filippi *et al.* 2014). Therefore, T cell activation by DC might be strongly impaired as in addition exhausted $CD8^+$ T cells express high levels of various inhibitory receptors which block TCR and co-stimulatory signalling (Blackburn, Shin *et al.* 2009). Depletion of DC during the chronic infection phase without anti-PD-L1 treatment did not affect $CD8^+$ T cell responses or viral load. These data are in line with findings obtained during the acute LCMV infection where depletion of DC did not impact the expansion

and function of T cells (Hilpert, Sitte *et al.* 2016). Likely, other APCs are able to stimulate and activate T cells. In addition, non-hematopoietic cells have been shown to present antigen and drive T cell exhaustion during chronic LCMV infection (Mueller and Ahmed 2009). In contrast to this, DC play an important role for the reactivation of exhausted $CD8^+$ T cells during checkpoint immunotherapy. In line with previous reports, we have shown that DC, $XCR1^+$ DC as well as $CD11b^+$ DC, still present antigen during chronic LCMV infection. $CD11b^+$ DC are directly infected and can directly present antigen to $CD8^+$ T cells (Baca Jones, Filippi *et al.* 2014). $XCR1^+$ DC might not be infected but are able to cross-present antigen and thereby activate $CD8^+$ T cells (Belz, Shortman *et al.* 2005). Notably, in this study we made use of the CD11c.DOG mouse line which allows depletion of $CD11c^+$ cells without the need to generate mixed bone marrow chimera like other mouse lines which enable depletion of DC (Hochweller, Striegler *et al.* 2008). However, CD11c can be expressed by other cells than DC, which are also be depleted in our model. These cells are for example red pulp macrophages and marginal zone macrophages, which are also antigen-presenting cells, that can activate $CD8^+$ T cells. Therefore, we cannot exclude a contribution of these cells for the reactivation of exhausted $CD8^+$ T cells during checkpoint immunotherapy. However, there are other studies, which support the important role of DC during checkpoint immunotherapy. A study by Kamphorst *et al.* described that co-stimulatory CD28 signalling is critical to reinvigorate the $CD8^+$ T cell response with PD-L1 blockade (Kamphorst, Wieland *et al.* 2017). The ligands for CD28, CD80 and CD86, can be delivered by APCs, especially DC. This already led them to the hypothesis that DC participate in the activation of exhausted $CD8^+$ T cells (Kamphorst, Wieland *et al.* 2017). In addition, studies on checkpoint immunotherapy in the context of cancer also revealed a role for DC. During anti-PD-1 therapy, IL-12-producing DC supported the reactivation of exhausted $CD8^+$ T cells and reduced of tumour burden (Garris, Arlauckas *et al.* 2018). Interestingly, IFNγ, produced by $CD8^+$ T cells upon anti-PD-1 administration, induced IL-12 production by DC. These findings hint to a feed-back scenario where DC themselves need to be stimulated to then support $CD8^+$ T cells.

Another study further supports this notion as TLR4-stimulation during anti-PD-1 treatment during chronic viral infection highly improved $CD8^+$ T cell responses by an increased expression of CD80 and CD86 on DC (Wang, Chung *et al.* 2019). Here, the improved co-stimulatory activity of DC resulted in a significantly increased expansion and activation of exhausted $CD8^+$ T cells and subsequently in a better control of the viral load compared to anti-PD-L1 treatment alone (Wang, Chung *et al.* 2019). Our findings combined with other studies therefore show the necessity of DC-$CD8^+$ T cell communication for successful anti-PD-L1 treatment.

Additionally, we investigated the $CD8^+$ T cell response after treating one, three or five times with anti-PD-L1. Notably, most studies investigating anti-PD-1/anti-PD-L1 therapy during chronic LCMV infection, used a standard treatment of 200 µg anti-PD-1/anti-PD-L1 i.p. every three days with five injections in total (Barber, Wherry *et al.* 2006, Blackburn, Shin *et al.* 2009, Im, Hashimoto *et al.* 2016, Utzschneider, Charmoy *et al.* 2016). 24 h after a single injection of anti-PD-L1, expansion nor phenotypical changes of antigen-specific $CD8^+$ T cells could be detected. During acute viral infections, antigen-specific $CD8^+$ T cells are able to divide within 24 h (Murali-Krishna, Altman *et al.* 1998). Notably, during chronic infection, the $CD8^+$ T cells are exhausted and divide less efficiently. It is possible that the decreased proliferative capacity has not been overcome post a single anti-PD-L1 treatment. We also cannot exclude that the dose of antibodies administered with a single injection is sufficient to reach the T cell zones of secondary lymphoid organs where reactivatable exhausted $CD8^+$ T cells predominantly reside. However, as an increase in the expression of PD-1, reflecting an increase in TCR signalling, was detected after a single anti-PD-L1 injection, the exhausted cells already displayed a state of increased activation. In other words, we do see evidence for an effect of PD-L1 blockade already after one injection. However, the strongest effect of PD-L1 blockade was observed after three injections. Surprisingly, T cell responses were not further elevated with ongoing treatment but rather reached a plateau or declined slightly. T cell intrinsic as well as extrinsic factors could be the basis for this

observation. One possible reason could be that with increased TCR signalling, inhibitory receptors are even further upregulated and dampen the activation of the $CD8^+$ T cells. This hypothesis can be supported by various studies demonstrating that co-treatment of anti-PD-1/anti-PD-L1 with antibodies blocking other inhibitory receptor signalling pathways (CTLA-4, Lag3, Tim3, TIGIT) showed a synergistic and increased effect over monotherapy (Hashimoto, Kamphorst *et al.* 2018, McLane, Abdel-Hakeem *et al.* 2019). Additionally, it has been described that the exhausted state of $CD8^+$ T cells is imprinted by epigenetic changes. During T cell exhaustion by chronic viral infection and cancer, de novo DNA methylation mediated by the DNA methyltransferase Dnmt3a has been shown to restrict T cell responses and expansion (Ghoneim, Fan *et al.* 2017). This unique epigenetic signature of exhausted $CD8^+$ T cells also limited the expansion of $CD8^+$ T cells upon anti-PD-1 treatment. The effect could be reversed by the usage of a demethylating agent prior to checkpoint immunotherapy (Ghoneim, Fan *et al.* 2017). Furthermore, extrinsic factors like regulatory cells could play a role in dampening the $CD8^+$ T cell response upon anti-PD-L1 treatment. Indeed, a role for Treg cells during checkpoint immunotherapy has recently been reported during treatment of advanced gastric cancer patients with anti-PD-1. Cancer progression was detected for a fraction of patients and could be set in context with increased numbers of Treg cells (Kamada, Togashi *et al.* 2019).

However, simultaneously to the plateau of the $CD8^+$ T cells response, we observed a strong decrease of viral antigen presentation by $XCR1^+$ DC and $CD11b^+$ DC during the course of anti-PD-L1 treatment. As we found that DC are essential for the successful reinvigoration of exhausted $CD8^+$ T cells, we hypothesize that this decreased antigen presentation limits activation of exhausted $CD8^+$ T cells. As a consequence, a prolonged anti-PD-L1 treatment is ineffective at least with regards to $CD8^+$ T cell expansion. This points to an overall scenario in which checkpoint immunotherapy is self-limiting. The question that remains is why antigen presentation is negatively impacted during ongoing anit-PD-L1 treatment. As DC were still able to present ovalbumin antigen, an impaired functionality seems highly unlikely. During acute infections

and tumour development, APCs which initially activate $CD8^+$ T cells become themselves targets of the cytotoxic response and get eliminated (Hermans, Ritchie *et al.* 2000, Ritchie, Hermans *et al.* 2000, Ludewig, Bonilla *et al.* 2001). This regulatory feedback mechanism is important to limit an overwhelming inflammatory response by antigen-specific T cells during acute viral and bacterial infections. We hypothesize that during early anti-PD-L1 treatment exhausted $CD8^+$ T cells regain their ability to receive TCR signalling as the inhibitory PD-1 signalling is blocked. This TCR as well as co-stimulatory signalling is delivered by DC. However, the reactivation of $CD8^+$ T cells also restores their cytotoxic capacity. As DC can be infected with LCMV Cl13 or cross-present antigen (Belz, Shortman *et al.* 2005, Baca Jones, Filippi *et al.* 2014), these antigen-presenting cells are likely targets of reactivated $CD8^+$ T cells. DC have a high turnover and are constantly replenished (Liu and Nussenzweig 2010). Therefore, DC extracted after three and five treatments with anti-PD-L1 seem to be uninfected or at least do not present viral antigen. A possible explanation for this finding is that antigen-presenting DC have been removed by cytotoxic T cells and been replaced by newly generated DC from the bone marrow. In line with these observations is the finding that $CD8^+$ T cells expressed significantly lower levels of PD-1 after five anti-PD-L1 treatments compared to three injections, again, pointing towards reduced TCR engagement. However, measuring the splenic viral titer after three injections of anti-PD-L1, showed that the infection is not completely cleared. Therefore, it remains unclear where antigen persists and why the newly arriving DC do not have efficient access to this antigen. Mueller *et al.* revealed that the stromal network of lymphoid organs, namely fibroblastic reticular cells (FRC), are targeted by LCMV Cl13 (Mueller, Matloubian *et al.* 2007). In a second study, they further described that non-hematopoietic cells present antigen during chronic infection and that this antigen presentation contributes to exhaustion (Mueller, Matloubian *et al.* 2007). It is possible that the virus persists in these non-hematopoietic cells, which developed further mechanisms to protect themselves, and therefore the organ architecture, from being attacked by $CD8^+$ T cells. If reactivated $CD8^+$ T cells can quickly regain cytotoxic functions and

target DC, they hence target an important cellular interaction partner, which is critical for further reinvigoration of the exhausted $CD8^+$ T cells. Further studies investigating if DC are indeed eliminated by reactivated $CD8^+$ T cells and where exactly antigen persists in the presence of continuous anti-PD-L1 treatment will help to understand this complex network of cellular interaction during chronic infection and checkpoint immunotherapy. Additionally, it is important to understand where exhausted $CD8^+$ T cells and DC in lymphoid tissues interact and whether certain DC subsets impact the $CD8^+$ T cell response differentially. In other words, we need to elucidate the microanatomy of checkpoint immunotherapy and define critical cellular interactions in the tissue context. These issues will be addressed in the following chapters.

4. Dendritic cell subsets exhibit a differential role for T cell proliferation and differentiation during checkpoint therapy

4.1 Introduction

In the previous chapter, I discussed the essential role of DC during checkpoint immunotherapy. During the chronic infection phase, viral antigen is presented by both XCR1$^+$ DC (cDC1) and CD11b$^+$ DC (cDC2) subsets. We hypothesized that viral antigen presentation together with co-stimulatory signals delivered by conventional DC (cDC) are essential for the expansion of exhausted CD8$^+$ T cells in the context of checkpoint immunotherapy. In animals that were depleted of cDC during checkpoint immunotherapy, CD8$^+$ T cells failed to expand despite the blockade of inhibitory signalling via the PD-1 pathway.

Importantly, cDC display a heterogeneous cell population consisting of lymphoid tissue-resident and migratory DC. The group of lymphoid tissue-resident DC can be further divided into XCR1$^+$ DC (cDC1) and CD11b$^+$ DC (cDC2). Both subsets differ in their localization and function. XCR1$^+$ DC excel at stimulating CD8$^+$ T cells whereas CD11b$^+$ DC preferentially activate CD4$^+$ T cells (Dudziak, Kamphorst *et al.* 2007). In the spleen, most XCR1$^+$ DC reside centrally in the T cell zone of the white pulp and fewer in the red pulp. CD11b$^+$ DC are located at the bridging channels of the marginal zone (Gerner, Kastenmuller *et al.* 2012, Gatto, Wood *et al.* 2013). In addition, XCR1$^+$ DC, as well as their migratory counterpart of CD103$^+$ DC in peripheral tissues and lymph nodes, are specialized in cross-presenting viral antigen (Bevan 1976, Gurka, Hartung *et al.* 2015). Therefore, these DC are able to present viral antigen via MHCI to CD8$^+$ T cells without the necessity of being directly infected. The exact mechanism of this process is still incompletely understood, yet it allows XCR1$^+$ DC to activate antiviral CD8$^+$ T cells without being under the control of an intracellular pathogen (Gutierrez-Martinez, Planes *et al.* 2015). Functionally, this DC subset has been shown to be essential to mount an adaptive cytolytic CD8$^+$ T cell response and is required to clear infections such as HSV-1 and *L. monocytogenes* (Bedoui, Whitney *et al.* 2009, Jirmo, Nagel *et*

al. 2009, Alexandre, Ghilas *et al.* 2016). Optimal T cell responses against tumour antigens also depend on cross-presentation executed by XCR1$^+$ DC (Sanchez-Paulete, Teijeira *et al.* 2017). Many studies investigating the role of cross-presenting XCR1$^+$ DC during infection were performed with cytolytic viruses. Here, infected cells are killed and antigen is released. Under such conditions, XCR1$^+$ DC can efficiently take up viral antigen and cross-present it via MHCI. Notably, also after acute infection with LCMV Armstrong, a non-cytolytic virus, cross-presentation by XCR1$^+$ DC was reported (Belz, Shortman *et al.* 2005). Additionally, XCR1$^+$ DC have been shown to present a unique cellular platform that mediates CD4$^+$ T cell help to CD8$^+$ T cells. Therefore, mature XCR1$^+$ DC are able to deliver necessary co-stimulatory signals which support the activation of CD8$^+$ T cells (Eickhoff, Brewitz *et al.* 2015, Hor, Whitney *et al.* 2015).

As we revealed an essential role for DC during chronic infection and immunotherapy, we aimed to investigate this in more detail by focusing on the subset of XCR1$^+$ DC with its well-described non-redundant function in CD8$^+$ T cell activation. To this end, we made use of the XCR1 Venus DTR mouse line that allows depletion of XCR1$^+$ DC by administration of DTX. By comparing these results with the previous findings looking at CD11c$^+$ cell depletion, we can decipher the contribution of different DC subsets for the reinvigoration of exhausted CD8$^+$ T cells during PD-L1 blockade.

Additionally, we aimed to determine key signalling molecules delivered by XCR1$^+$ DC that could impact the T cell response during immunotherapy. For the optimal activation of naïve CD8$^+$ T cells, three signals delivered by APC are of great importance: TCR stimulation, co-stimulation and cytokines (van Stipdonk, Lemmens *et al.* 2001). Our data reveal that antigen presentation and hence TCR stimulation is of importance during immunotherapy as we observed a decrease in CD8$^+$ T cell expansion when antigen presentation by DC was highly reduced with ongoing anti-PD-L1 treatment. The importance of co-stimulatory signalling during reactivation of exhausted CD8$^+$ T cells was described by Kamphorst *et. al*. (Kamphorst, Wieland *et al.* 2017). Their study revealed that co-stimulation by CD28 is essential during immunotherapy. In addition, there is

evidence that $CD4^+$ T cell help is required to contain chronic infection and to support the response to immunotherapy (Aubert, Kamphorst *et al.* 2011). The co-stimulatory signalling axis of CD27-CD70 plays an important role in this context: mature DC, which received $CD4^+$ T cell help, upregulate CD70. CD70 binds to the co-stimulatory receptor CD27, which is expressed by $CD8^+$ T cells. In the context of influenza infection, it was shown that $CD8^+$ T cells can thereby be optimally primed (Belz, Bedoui *et al.* 2007). To examine if CD27-CD70 signalling is also required during checkpoint immunotherapy, we blocked CD70 with monoclonal antibodies *in vivo*.

Moreover, we addressed the role of IL-15 signalling during anti-PD-L1 blockade. IL-15 is described as an important cytokine being critical for the maintenance of memory $CD8^+$ T cells upon acute infections (Schluns and Lefrancois 2003, Oh, Perera *et al.* 2008). IL-15 signalling is primarily mediated by transpresentation. In detail, IL-15 binds to the IL-15Rα on the same cell and this IL-15:IL-15Rα complex can in turn bind the complex of IL-2Rβ and the common gamma chain on $CD8^+$ T cells (Dubois, Mariner *et al.* 2002). Notably, a supreme role of IL-15 produced by $XCR1^+$ DC was described during HSV-1 infection in the context of $CD4^+$ T cell help (Greyer, Whitney *et al.* 2016). By blocking of IL-15 signalling *in vivo* using anti-IL-15 blocking antibodies, we examined the impact of this signalling pathway in the context of chronic LCMV infection and PD-L1 blockade. In this chapter, we therefore aimed to elucidate differential roles for certain DC subsets and key signalling molecules, which further support the T cell response during checkpoint immunotherapy.

4.2 Results

4.2.1 XCR1$^+$ DC are required to preserve the TCF-1$^+$ CD8$^+$ T cell compartment during immunotherapy

The findings presented in chapter 3 revealed that DC are essential for the proliferation of exhausted CD8$^+$ T cells during anti-PD-L1 blockade. As tissue-resident DC of the spleen encompass two distinct populations, XCR1$^+$ DC (cDC1) and CD11b$^+$ DC (cDC2), we aimed to unravel if these subsets differ in their ability to support CD8$^+$ T cell reinvigoration. With specific depletion of XCR1$^+$ DC, we deciphered the role of this DC subset in more detail to better understand its contribution during anti-PD-L1 treatment.

For this approach, XCR1 DTR mice were used. These mice harbour a DTR cassette under the *Xcr1* promoter. By the administration of DTX, XCR1$^+$ cells can be efficiently depleted. XCR1 DTR mice were treated with anti-CD4 on d-2 and d0 and infected with 2x10^6 IU LCMV Cl13. In the chronic infection phase, mice were treated with 250 ng DTX (five treatments every two days) and 200 µg anti-PD-L1 (four treatments every three days). 24 h after the last anti-PD-L1 injection, blood and spleen were isolated and analysed by flow cytometry. A direct comparison of LCMV-specific CD8$^+$ T cells in the blood before and after the treatment was performed (Figure 4.1A). Representative flow cytometry plots display the successful depletion of XCR1$^+$ DC in DTX-treated mice (Figure 4.1B). Notably, the overall frequency of CD11c^{hi} MHCII$^+$ DC was not affected.

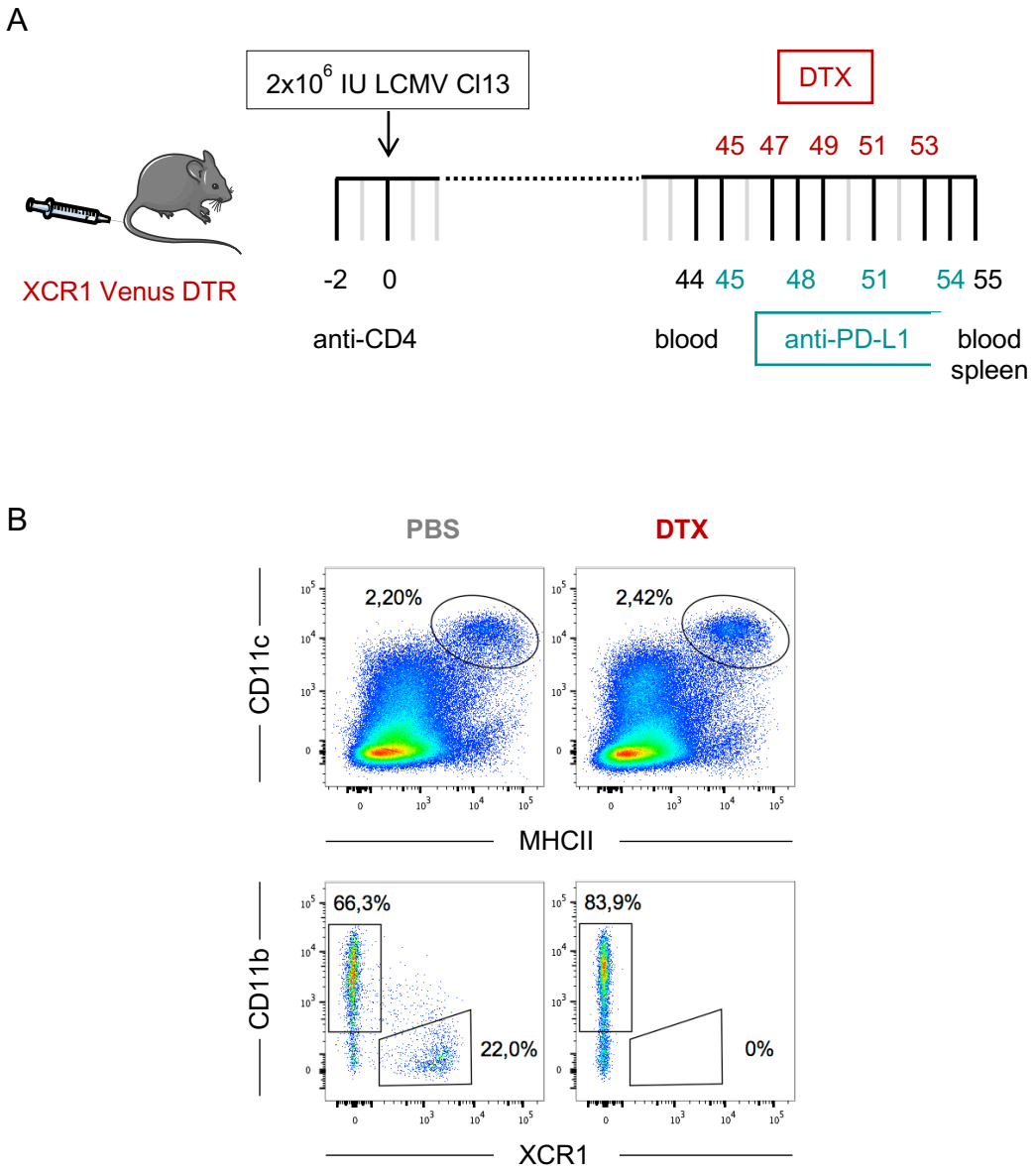

Figure 4.1: Mouse model of XCR1⁺ DC depletion and PD-L1 blockade during chronic LCMV infection. (A) Adult male XCR1 Venus DTR mice received 300 µg anti-CD4 i.p. on d-2 and d0 of infection and were i.v. infected with $2x10^6$ IU LCMV Cl13 on d0. Blood was taken prior to treatment (d44). From d45 onwards, mice received 200 µg anti-PD-L1 blocking antibody i.p. four times every three days. To deplete XCR1⁺ DC, mice received 250 ng DTX i.p. from d45 onwards five times every second day. 24 h after the last anti-PD-L1 injection, blood and spleen were isolated for analysis. **(B)** Representative flow cytometry plots showing the depletion of XCR1⁺ DC in DTX-treated mice.

Comparing the frequency of LCMV-specific $CD8^+$ T cells ($PD\text{-}1^+$ and $gp33^+$) in the blood before and after treatment revealed that the absence of $XCR1^+$ DC did not affect $CD8^+$ T cell expansion (Figure 4.2A). Exhausted $CD8^+$ T cells were still able to respond to PD-L1 blockade when $XCR1^+$ DC were absent. Likewise, no significant differences regarding LCMV-specific $CD8^+$ T cells could be detected in the spleen. Mice that received anti-PD-L1 and mice which were treated with anti-PD-L1 and DTX, exhibited similar frequencies and absolute cell numbers of LCMV-specific $CD8^+$ T cells ($PD\text{-}1^+$ and $gp33^+$) (Figure 4.2B). In contrast to mice depleted of all DC during immunotherapy (Chapter 3, figure 3.7B), the depletion of $XCR1^+$ DC during immunotherapy did not affect the expansion of exhausted T cells. Consistent with this finding, no difference in the frequency and number of $Ki67^+$ proliferating $gp33^+$ cells were observed (Figure 4.2C). Compared to PBS-treated mice (Chapter 3, figure 3.3), both groups displayed elevated levels of $Ki67^+$ $gp33^+$ $CD8^+$ T cells. Unexpectedly, these data point towards a redundant role of $XCR1^+$ DC regarding the expansion of exhausted $CD8^+$ T cells during anti-PD-L1 treatment.

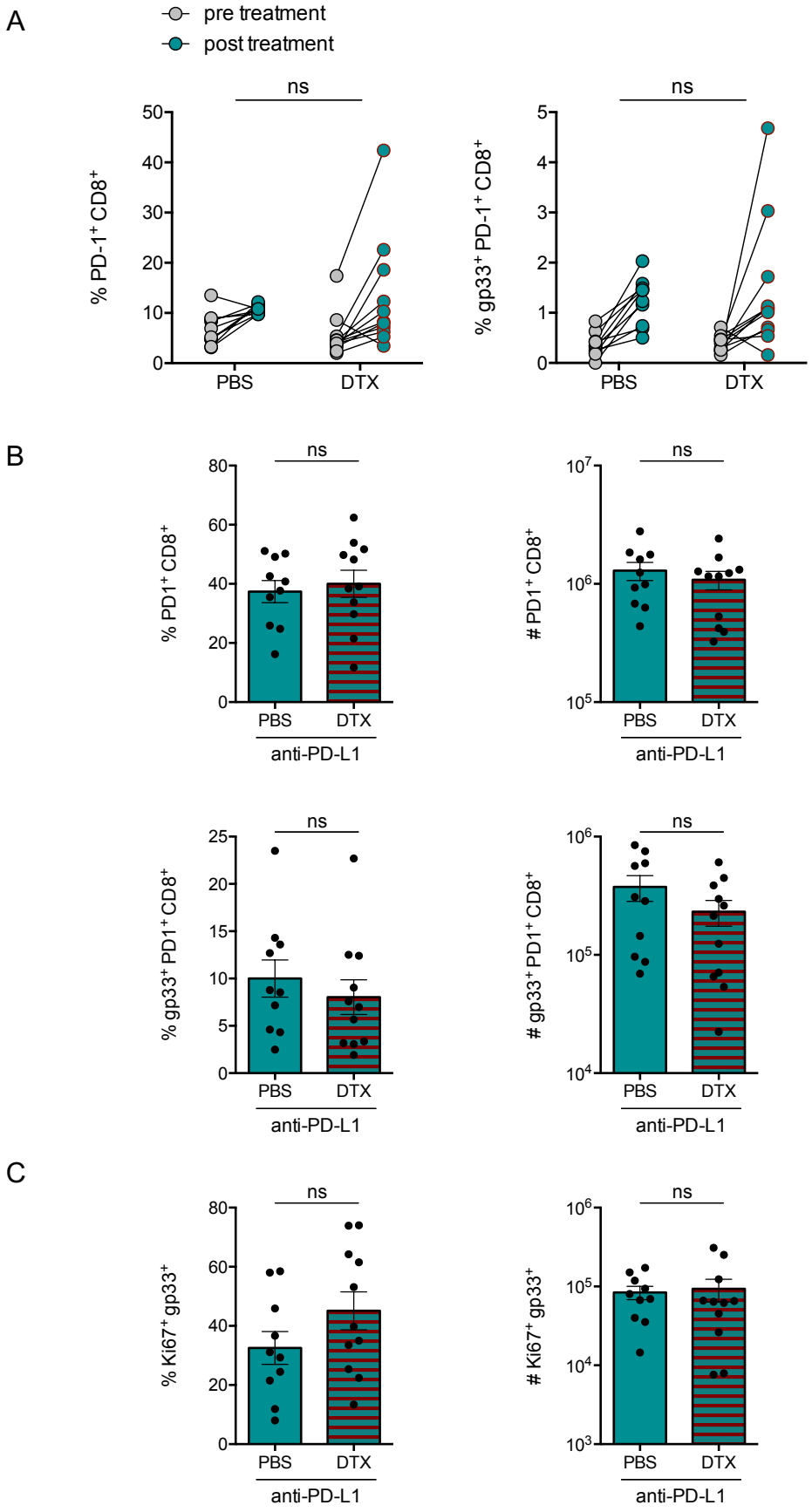
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Figure 4.2: XCR1$^+$ DC do not promote proliferation of exhausted CD8$^+$ T cells during PD-L1 blockade. (A) Paired frequencies of LCMV-specific PD-1$^+$ and gp33$^+$ CD8$^+$ T cells in the blood prior to and post anti-PD-L1 treatment in the presence and absence of XCR1$^+$ DC. **(B)** Frequency and absolute number of splenic PD-1$^+$ and gp33$^+$ CD8$^+$ T cells post anti-PD-L1 treatment with and without XCR1$^+$ DC depletion. **(C)** Frequency and absolute number of proliferating Ki67$^+$ gp33$^+$ T cells. Data are pooled from three independent experiments (n = 4 mice/group). Error bars indicate the mean $\pm$ SEM. Comparison between groups was calculated using the unpaired Student‘s t-test. * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

As previously described, there are two main subsets of exhausted $CD8^+$ T cells. The memory-like TCF-1^+ cells, which respond to immunotherapy by proliferation and functional reinvigoration and the terminally exhausted $Tim3^+$ cells (Utzschneider, Charmoy *et al.* 2016). TCF-1^+ cells give rise to $Tim3^+$ cells but are also able to self-renew (Im, Hashimoto *et al.* 2016). During PD-L1 blockade, a relative shift from TCF-1^+ cells to $Tim3^+$ cells was detected that is based on an absolute increase of the latter subset (Chapter 3, figure 3.5). Since T cell expansion was dependent on the presence of DC, there was no shift towards $Tim3^+$ cells when $CD11c^+$ DC where depleted (Chapter 3, figure 3.9A). In the context of $XCR1^+$ DC depletion during anti-PD-L1 treatment, the differentiation of $gp33^+$ cells was examined as well. Surprisingly, the frequency of TCF-1^+ cells was strongly reduced in mice that were depleted of $XCR1^+$ cells (Figure 4.3A). Importantly and in contrast to the relative loss of TCF-1^+ cells during anti-PD-L1 therapy alone, additional depletion of $XCR1^+$ DC induced an absolute loss of TCF-1^+ cells (Figure 4.3B). The absolute numbers of $Tim3^+$ T cells was similar between anti-PD-L1-treated mice in absence or presence of $XCR1^+$ DC. This argues for a model in which proliferation is driven by $XCR1^-$ DC, that is $CD11b^+$ DC, while maintenance of stemness or prevention of full differentiation towards a $Tim3^+$ phenotype during immunotherapy is regulated by $XCR1^+$ DC.

Besides the proliferation and differentiation of exhausted $CD8^+$ T cells after PD-L1 blockade in combination with $XCR1^+$ DC depletion, frequency and absolute number of GzmB-producing cells was measured. DTX-treated mice harboured the same number of GzmB-producing cells as mice that were treated with anti-PD-L1 alone (Figure 4.4A). Both groups showed an increase in $GzmB^+$ cells compared to completely untreated mice. Comparable to the expansion and proliferation of exhausted $CD8^+$ T cells upon PD-L1 blockade, $XCR1^+$ DC are not essential for the increase of $GzmB^+$ cells during therapy.

Next, we addressed whether the absolute loss of $TCF1^+$ $CD8^+$ T cells was associated with an altered viral control. To this end, we compared the viral titers in mice treated with PD-L1 blockade alone or in combination with $XCR1^+$ DC depletion. Indeed, mice that were depleted of $XCR1^+$ DC during PD-L1 blockade displayed an increased viral load compared to mice that received

anti-PD-L1 treatment alone (Figure 4.4B). This indicates that XCR1$^+$ DC not only maintain the numbers of the memory-like subpopulation of exhausted CD8$^+$ T cells (TCF1$^+$) but are functionally critical for the decrease of viral load during immunotherapy. In other words, they are essential for a successful immunotherapy although they are not the main drivers of CD8$^+$ T cell proliferation.

Whether these two findings (loss of the TCF1$^+$ cell compartment and reduced viral control in the absence of XCR1$^+$ DC) are directly connected, a consequence of each other or reflect different roles of XCR1$^+$ DC during immunotherapy, remains to be clarified.

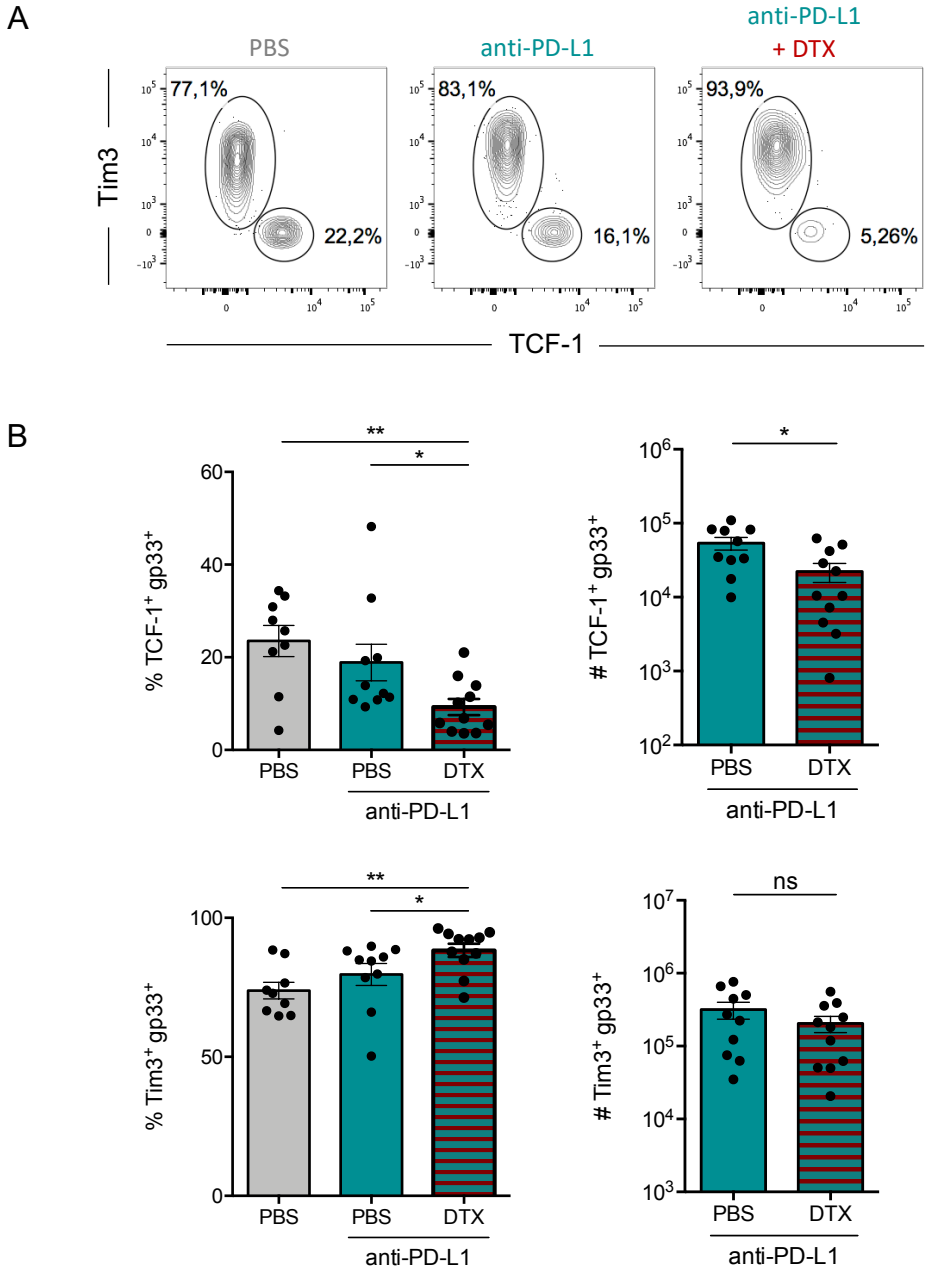


Figure 4.3: XCR1⁺ DC maintain memory-like cells during anti-PD-L1 therapy. XCR1 Venus DTR mice were chronically infected with 2x10^6 IU LCMV Cl13 in combination with CD4⁺ cell depletion. From d45 onwards, mice were left untreated, treated with anti-PD-L1 or anti-PD-L1 in combination with DTX to deplete XCR1⁺ DC. **(A)** Representative flow cytometry plots of TCF-1⁺ und Tim3⁺ subpopulations of exhausted gp33⁺ CD8⁺ T cells from differentially treated mice. **(B)** Frequency and absolute number of TCF-1⁺ and Tim3⁺ gp33⁺ CD8⁺ T cells after anti-PD-L1 treatment with and without XCR1⁺ cell depletion. Data are pooled from two independent experiments (n = 4 mice/group). Error bars indicate the mean ± SEM. Comparison between groups was calculated using the unpaired Student‘s t-test. * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

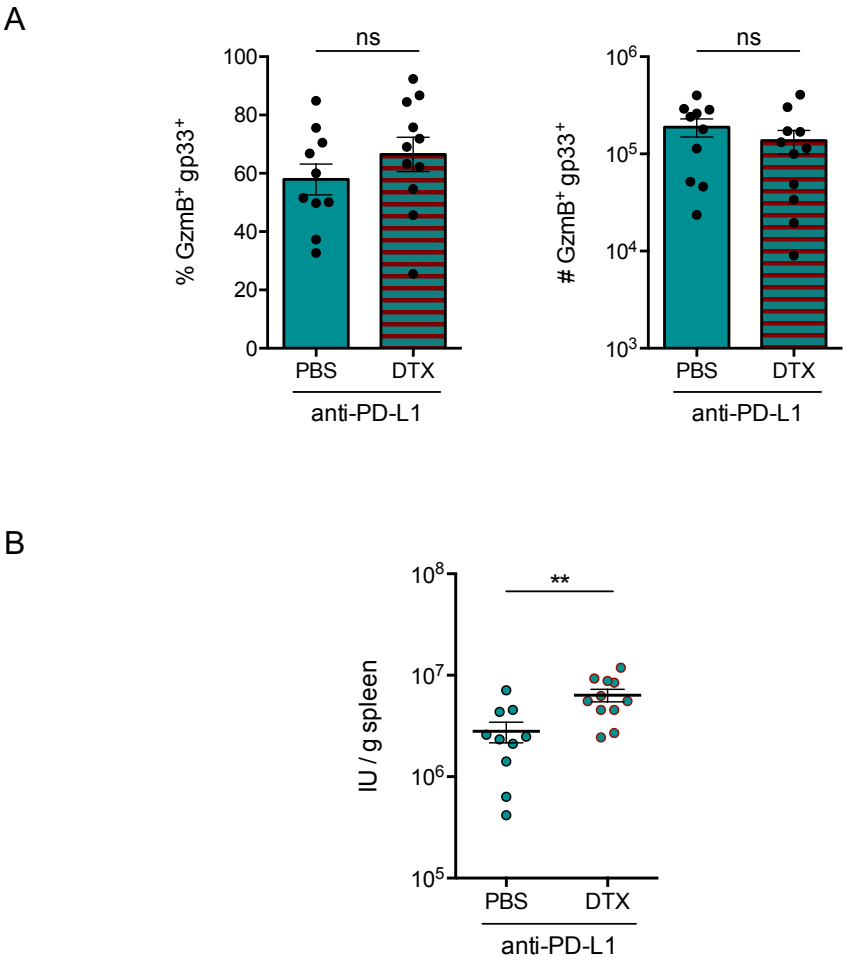


Figure 4.4: Increased viral load in chronically infected mice depleted of XCR1⁺ DC during immunotherapy despite intact GzmB production. (A) Frequency and absolute number of GzmB⁺ gp33⁺ CD8⁺ T cells from anti-PD-L1 treated mice in the presence or absence of XCR1⁺ DC. **(B)** Viral load depicted as infectious units (IU) per g spleen of anti-PD-L1 treated animals in combination with XCR1⁺ DC depletion. Data are pooled from three independent experiments (n = 3-4 mice/group). Error bars indicate the mean ± SEM. Comparison between groups was calculated using the unpaired Student‘s t-test. * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

So far, the role of XCR1$^+$ DC during immunotherapy was investigated. To uncover if XCR1$^+$ DC are critical for the maintenance of memory-like cells during chronic infection, XCR1$^+$ DC were depleted without simultaneous PD-L1 blockade. In detail, XCR1 Venus DTR mice received anti-CD4 and were infected with 2x10^6 IU LCMV Cl13. During the chronic infection phase, 250 ng DTX were administered five times every second day (Figure 4.5A). 24 h after the last DTX injection, spleens were isolated and analysed by flow cytometry. We detected a tendency towards less LCMV-specific PD-1$^+$ and gp33$^+$ cells in DTX-treated mice compared to untreated animals (Figure 4.5B). However, frequency and absolute number of these cells did not change significantly with XCR1$^+$ DC depletion. In addition, the depletion of XCR1$^+$ DC did not impact the differentiation of exhausted gp33$^+$ CD8$^+$ T cells regarding their expression of TCF-1 and Tim3 (Figure 4.5C). This is in contrast to the impact of XCR1$^+$ DC depletion during anti-PD-L1 treatment, where a significant shift from the TCF-1$^+$ to the Tim3$^+$ cell population was observed. Moreover, XCR1$^+$ DC depletion during the chronic infection phase did not significantly impact the viral load in the spleen (Figure 4.5D).

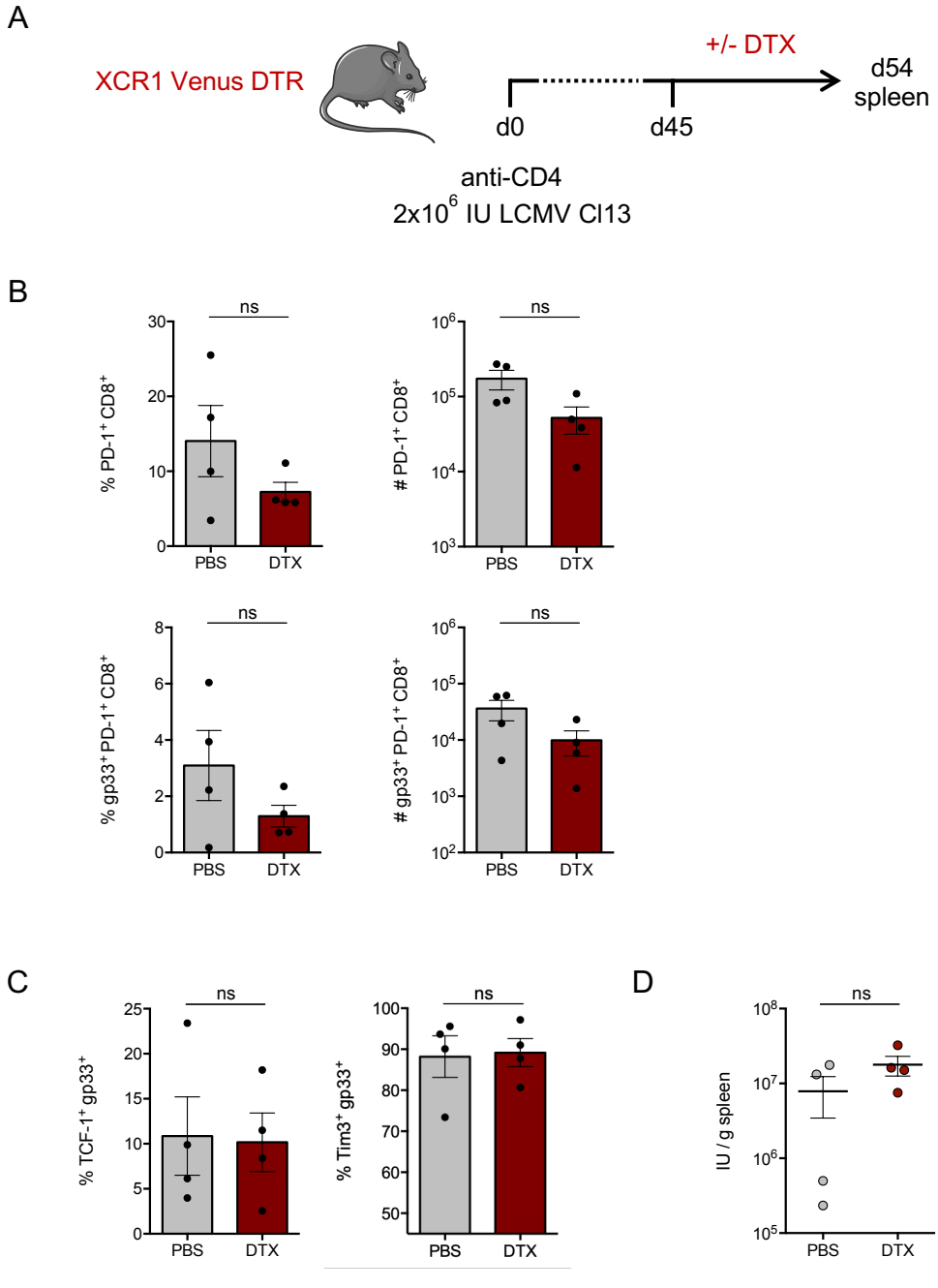


Figure 4.5: Depletion of XCR1⁺ DC during chronic LCMV infection. **(A)** XCR1 Venus DTR mice received anti-CD4 and were i.v. infected with $2x10^6$ IU LCMV Cl13. To deplete XCR1⁺ DC, mice received 250 ng DTX i.p. from d45 onwards five times every second day. 24 h after the last DTX injection, spleens were isolated for analysis. **(B)** Frequency and number of LCMV-specific CD8⁺ T cells (PD-1⁺ and gp33⁺) in the presence and absence of XCR1⁺ DC. **(C)** Frequency of TCF-1⁺ and Tim3⁺ gp33⁺ cells. **(D)** Splenic viral load of untreated and DTX-treated mice. Error bars indicate the mean ± SEM. Comparison between groups was calculated using the unpaired Student's t-test. * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.2.2 XCL1-XCR1 signalling is not required for the reinvigoration of exhausted CD8$^+$ T cells

As XCR1$^+$ DC play an important role for the maintenance of memory-like TCF-1$^+$ cells during PD-L1 blockade, we aimed to investigate the factors that enable the communication between these two cell types. Memory-like exhausted CD8$^+$ T cells are functionally and transcriptionally distinct from terminally exhausted cells (Im, Hashimoto *et al.* 2016, Utzschneider, Charmoy *et al.* 2016). One of the genes that is differentially expressed between the two exhausted cell types, is *XCL1* (Im, Hashimoto *et al.* 2016). XCL1 is a chemokine, which is expressed by NK cells and activated CD8$^+$ T cells (Dorner, Dorner *et al.* 2009). The exclusive receptor for XCL1 is XCR1, which is solely expressed by cDC1. Our group revealed that CD8$^+$ T cells attract XCR1$^+$ DC via the production of XCL1, thus enabling the orchestration of an ideal cellular microenvironment for optimal CD8$^+$ T cell priming (Brewitz, Eickhoff *et al.* 2017). The high expression of XCL1 by TCF-1$^+$ cells and the loss of these cells when XCR1$^+$ DC are depleted, points towards an important role of XCL1-XCR1 signalling during PD-L1 blockade. To investigate this notion, we made use of XCR1$^{-/-}$ mice. These mice still harbour cDC1 (XCR1$^+$ DC), but the DC do not express the XCR1 receptor. Thereby XCL1-XCR1 signalling is genetically abolished in these mice. The mice were chronically infected with LCMV Cl13 and treated with 200 µg anti-PD-L1 (five times, every second day) during the chronic infection phase. WT mice which still expressed XCR1, served as a control. Spleens were analysed 24 h after the last anti-PD-L1 injection (Figure 4.6A). Quantitative analysis of CD8$^+$ T cells from both groups revealed similar frequencies and absolute numbers of LCMV-specific cells (PD-1$^+$ and gp33$^+$) (Figure 4.6B). Furthermore, the differentiation of exhausted CD8$^+$ T cells was not affected by the expression of XCR1 on cDC1. Both groups harboured similar frequencies of TCF-1$^+$ and Tim3$^+$ cells (Figure 4.6C). Also, the splenic viral load was comparable in both groups (Figure 4.6D). These findings match the results obtained with XCL1$^{-/-}$ mice. Using the same experimental setup as above, we found that reinvigoration of exhausted CD8$^+$ T cells by anti-PD-L1

treatment was unaltered in the absence of XCL1 (data not shown). These results argue that the XCR1-XCL1 signalling axis is not essential in the context of chronic LCMV infection and anti-PD-L1 treatment. The question how XCR1$^+$ DC maintain memory-like cells during immunotherapy remains to be answered.

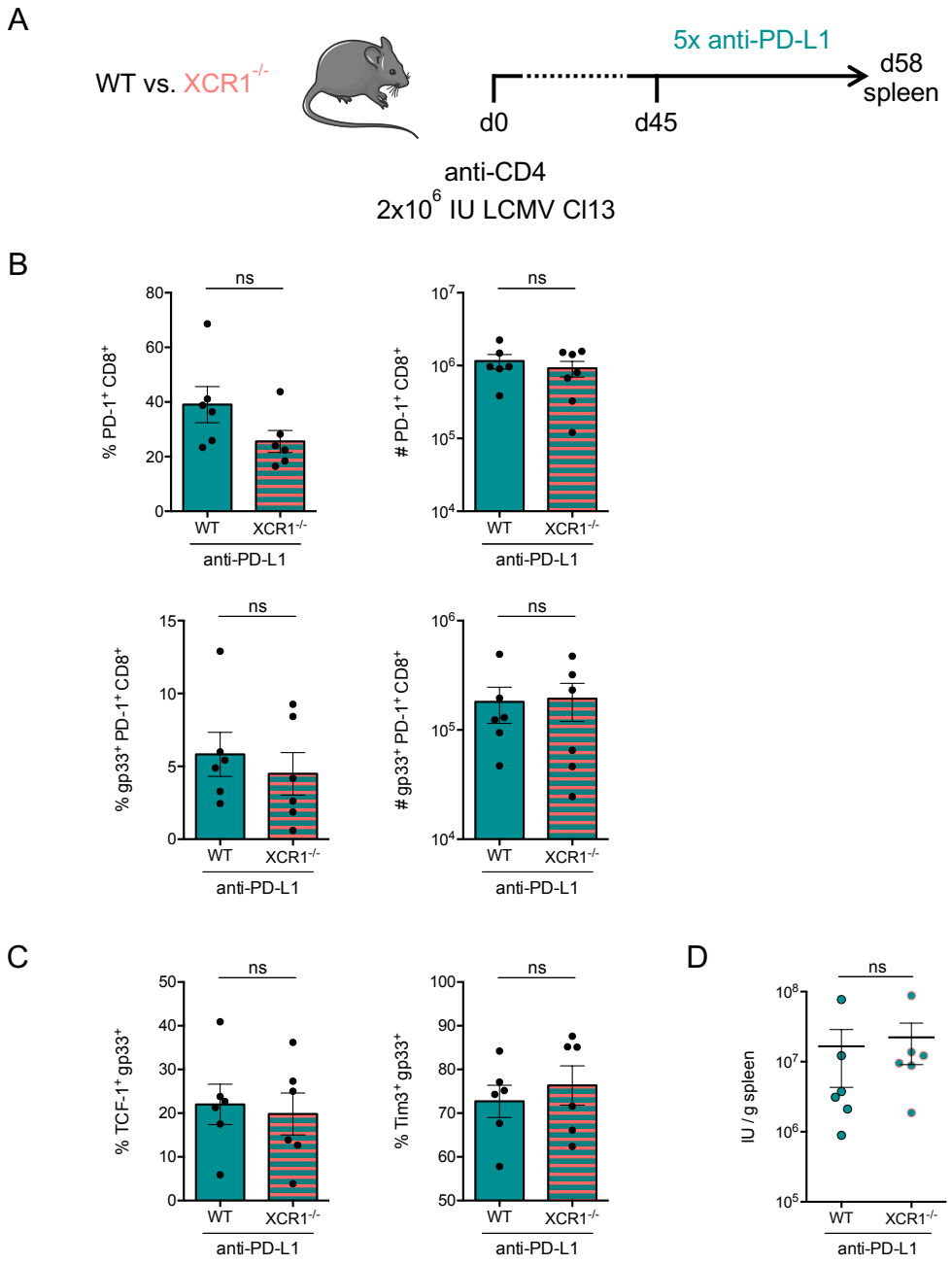


Figure 4.6: XCR1 receptor is not essential to maintain TCF-1⁺ cells during PD-L1 blockade. (A) WT and XCR1$^{-/-}$ mice received anti-CD4 and were i.v. infected with 2x10^6 IU LCMV Cl13. During the chronic infection phase mice were treated with five injections of 200 µg anti-PD-L1 i.p. 24 h after the last injection, spleens were isolated and analysed. **(B)** Frequency and number of LCMV-specific CD8⁺ T cells (PD-1⁺ and gp33⁺) in WT and XCR1$^{-/-}$ mice. **(C)** Frequency of TCF-1⁺ and Tim3⁺ gp33⁺ cells. **(D)** Splenic viral load of WT and XCR1$^{-/-}$ mice. Data are pooled from two independent experiments (n = 3 mice/group) Error bars indicate the mean ± SEM. Comparison between groups was calculated using the unpaired Student's t-test. * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.2.3 CD27-CD70 signalling is not critical for checkpoint immunotherapy during chronic viral infection

As shown above, DC subsets differently impact the proliferation and differentiation of exhausted $CD8^+$ T cells during anti-PD-L1 blockade. During the priming phase of $CD8^+$ T cells, $CD4^+$ T helper signals are at least in part mediated by CD27-CD70 interactions (Feau, Garcia *et al.* 2012). These helper signals are not directly transmitted to $CD8^+$ T cells but instead indirectly and exclusively via $XCR1^+$ DC (Eickhoff, Brewitz *et al.* 2015, Hor, Whitney *et al.* 2015). Therefore, we speculated that this pathway may also be critical during checkpoint immunotherapy and may be the basis for the impact of $XCR1^+$ DC on $CD8^+$ T cell differentiation that we described above. To examine if co-stimulatory signalling via the CD27-CD70 pathway influences reactivation of exhausted T cells during immunotherapy, we made use of an *in vivo* anti-CD70 blocking antibody (clone FR70). This monoclonal antibody binds to CD70 and thereby prevents its interaction with CD27. WT mice were chronically infected with $2x10^6$ IU LCMV Cl13 in combination with $CD4^+$ cell depletion. During the chronic infection phase, mice received 300 µg anti-CD70 i.p. on d45, d48 and d51. The control group received PBS. In addition, both groups were treated with 200 µg anti-PD-L1 on d46, d49 and d52. 24h after the last anti-PD-L1 injection, spleens were isolated and analysed (Figure 4.7A). Blocking of CD70 signalling during immunotherapy had no impact on the expansion of exhausted $CD8^+$ T cells. Both groups showed similar frequencies and absolute numbers of cells ($PD\text{-}1^+$ and $gp33^+$) (Figure 4.7B). Moreover, CD70 signalling did not affect differentiation of exhausted $CD8^+$ T cells during PD-L1 blockade. The frequency of $TCF\text{-}1^+$ and $Tim3^+$ $gp33^+$ $CD8^+$ T cells was comparable when CD70 signalling was blocked (Figure 4.7C). In summary, blocking of CD70 had no impact on the frequency, number and phenotype of $CD8^+$ T cells during immunotherapy. In line with these results, the splenic viral load was comparable in mice receiving anti-PD-L1 treatment with and without CD70 blockade (Figure 4.7D). Combining these results, we show that co-stimulatory signalling by the CD27-CD70 axis is not critical for successful immunotherapy during chronic LCMV infection.

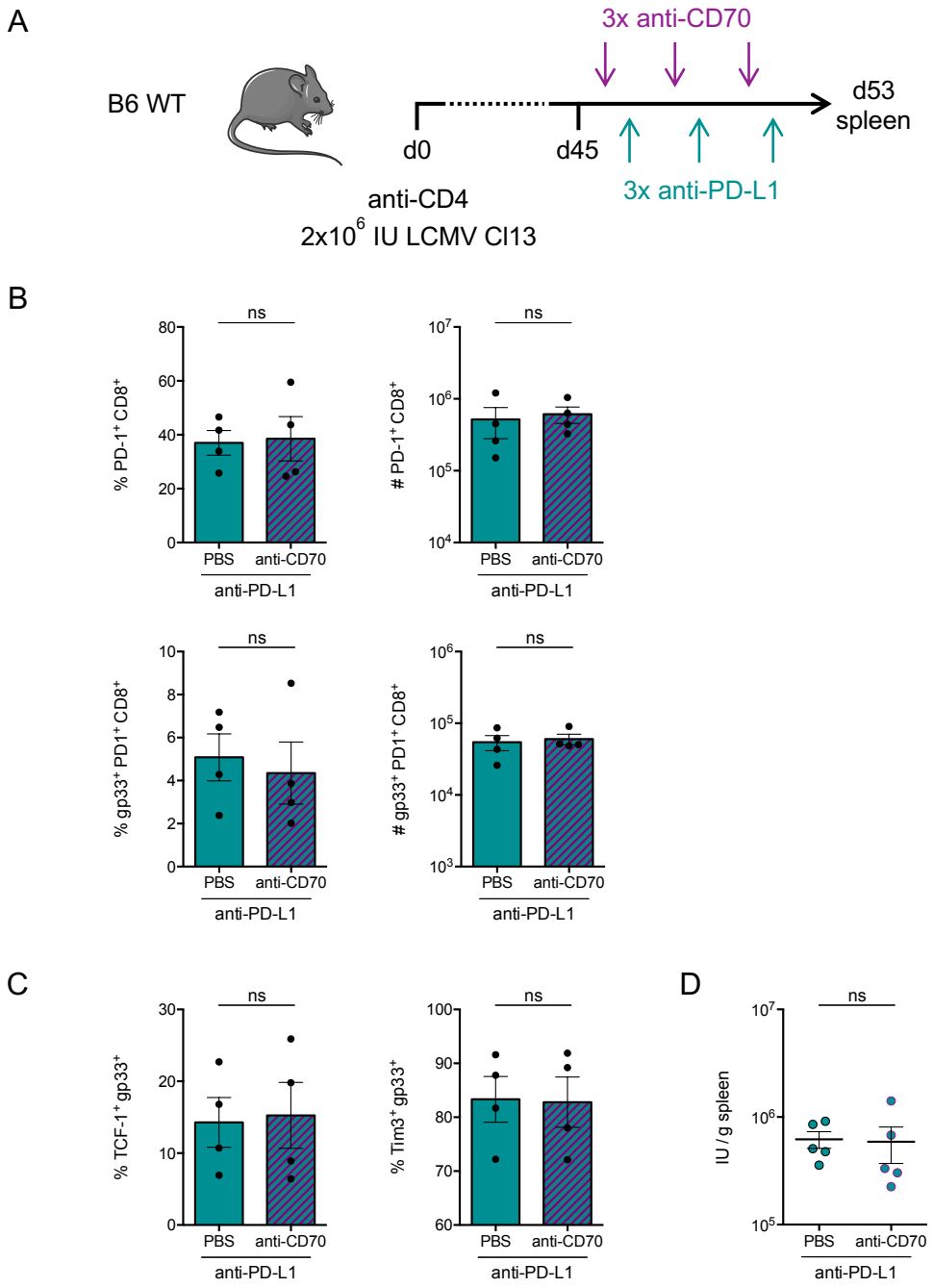


Figure 4.7: CD70 signalling is not critical during immunotherapy. (A) B6 WT mice received anti-CD4 and were i.v. infected with $2x10^6$ IU LCMV Cl13. During the chronic infection phase, mice were treated with three i.p. injections of 300 µg anti-CD70 and three injections of 200 µg anti-PD-L1. 24 h after the last injection, spleens were isolated and analysed. **(B)** Frequency and number of LCMV-specific $CD8^+$ T cells ($PD\text{-}1^+$ and $gp33^+$). **(C)** Frequency of $TCF\text{-}1^+$ and $Tim3^+$ $gp33^+$ cells. **(D)** Splenic viral load depicted as IU / g spleen. Data present one experiment (n = 4 mice/group). Error bars indicate the mean ± SEM. Comparison between groups was calculated using the unpaired Student's t-test. * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.2.4 IL-15 supports control of viral load during immunotherapy

IL-15 is described as an important cytokine, that promotes survival of T, B and NK cells and is shown to regulate the differentiation of memory T cells (Fehniger and Caligiuri 2001, Kokaji, Hockley *et al.* 2008). Given the prominent function of XCR1$^+$ DC to mediate IL-15 signalling (Greyer, Whitney *et al.* 2016), we speculated that IL-15 may be the molecular link between XCR1$^+$ DC and the maintenance of the memory-like TCF1$^+$ CD8$^+$ T cell population. To examine whether IL-15 plays an important role for the reactivation of exhausted CD8$^+$ T cells during immunotherapy, an anti-IL-15 blocking antibody (clone AIO.3) was administered during anti-PD-L1 therapy of chronically infected mice. In detail, B6 WT mice were infected with 2x10^6 IU LCMV Cl13 in combination with CD4$^+$ T cell depletion. During the chronic infection phase, mice received 200 µg anti-IL-15 i.p. on d45, d48 and d51 and 200 µg anti-PD-L1 i.p. on d46, d49 and d52. The control group received anti-PD-L1 treatment alone. 24 h after the final anti-PD-L1 injection, spleens were isolated and analysed (Figure 4.8A). The frequency of antigen-experienced CD8$^+$ T cells (PD-1$^+$) was significantly increased in mice in which IL-15 signalling was blocked (Figure 4.8B). However, this effect was less profound within the gp33$^+$-specific CD8$^+$ T cell pool. Importantly, the absolute cell number of LCMV-specific CD8$^+$ T cells did not differ when mice were treated with anti-IL-15 during immunotherapy (Figure 4.8B). Among gp33$^+$ CD8$^+$ T cells, the frequency of TCF-1$^+$ and Tim3$^+$ slightly shifted to the TCF-1$^+$ cell population in mice treated with anti-IL-15 and anti-PD-L1 (Figure 4.8C). In addition, anti-IL-15-treated mice exhibited a higher splenic viral load compared to mice that received only anti-PD-L1 (Figure 4.8D). Combining these results, the effects seen with IL-15 blockade do not recapitulate the results obtained in the context of XCR1$^+$ DC depletion. IL-15 signalling seems to play a complex role during immunotherapy with several cellular sources being involved and multiple cell populations (CD8$^+$ T cells and NK cells) receiving this signal. The absolute expansion of CD8$^+$ T cells seems to be unaltered by IL-15 blockade, yet functionally the blockade leads to an impaired control of chronic LCMV infection.

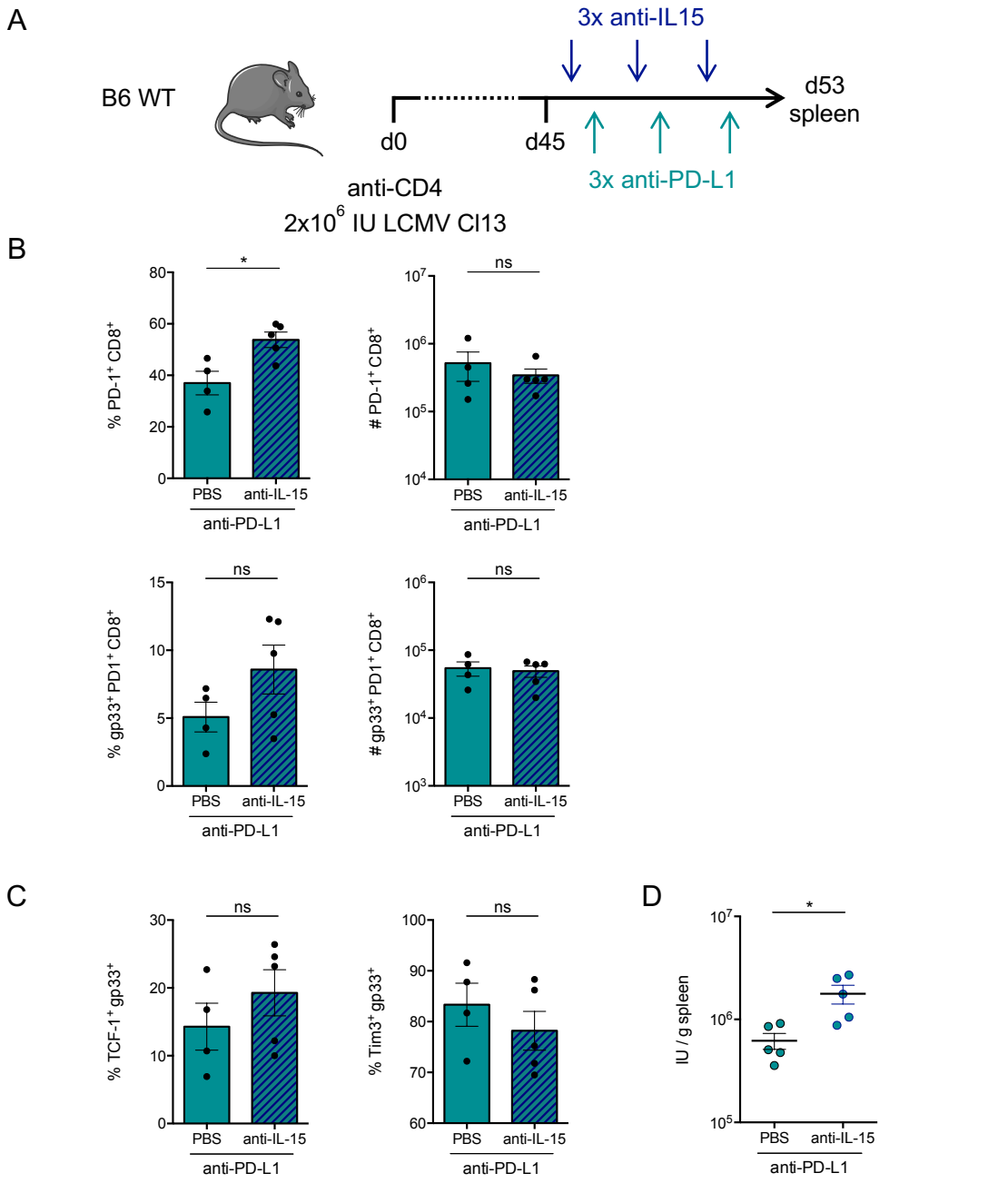


Figure 4.8: IL-15 signalling improves control of viral load during immunotherapy. (A) B6 WT mice received anti-CD4 and were i.v. infected with $2x10^6$ IU LCMV Cl13. During the chronic infection phase, mice were treated with three i.p. injections of 200 µg anti-IL-15 and three injections of 200 µg anti-PD-L1. 24 h after the last injection, spleens were isolated and analysed. **(B)** Frequency and number of LCMV-specific $CD8^+$ T cells ($PD\text{-}1^+$ and $gp33^+$). **(C)** Frequency of $TCF\text{-}1^+$ and $Tim3^+$ $gp33^+$ cells. **(D)** Splenic viral load depicted as IU / g spleen. Data present one experiment (n ≥ 4 mice/group). Error bars indicate the mean ± SEM. Comparison between groups was calculated using the unpaired Student's t-test. * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Collectively, these data reveal a supreme role of XCR1$^+$ DC for the maintenance of memory-like CD8$^+$ T cells during immunotherapy. The decrease of the TCF-1$^+$ cell pool during ongoing anti-PD-L1 treatment described in chapter 3, is pronounced in mice depleted of XCR1$^+$ DC during PD-L1 blockade, suggesting a role for these DC to support CD8$^+$ T cells in regaining their effector function. However, we did not observe this effect during the chronic infection phase when mice did not receive anti-PD-L1 treatment.

In an effort to identify the underlying molecular mechanism we investigated three likely pathways: XCR1-XCL1, CD27-CD70 and IL-15R-IL-15 interactions.

Signalling via the XCR1-XCL1 axis is not essential for the expansion of exhausted CD8$^+$ T cells during immunotherapy. Despite XCL1 being highly expressed by memory-like TCF-1$^+$ cells and a loss of this cell fraction observed when XCR1$^+$ DC were absent, no differences were detected when cDC1 did not express the XCR1 receptor. For the investigation of how XCR1$^+$ DC and exhausted TCF-1$^+$ cells could communicate we focused on co-stimulatory signalling via the CD27-CD70 axis and IL-15 signalling. Our results revealed that CD70 signalling is redundant during anti-PD-L1 treatment. However, IL-15 signalling supports control of the viral load and could therefore be part of the communication between DC and exhausted CD8$^+$ T cells. Nevertheless, blocking of IL-15 did not fully reflect the effects we observed when DC were depleted, hinting towards a more complex network of effector molecules and signalling pathways that are essential for CD8$^+$ T cell reactivation. Possibly, the localization and microenvironment are important prerequisites for the cells to be reinvigorated. Deciphering where DC and exhausted CD8$^+$ T cells interact, could help to better understand factors that are critical for successful checkpoint immunotherapy.

4.3 Discussion

In the previous chapter, we investigated the role of DC for $CD8^+$ T cell reinvigoration during chronic viral infection and found that they play an essential role during checkpoint immunotherapy. So far, we examined the T cell response in the absence of all conventional DC. However, our data reveal a differential role for DC subsets during immunotherapy. $XCR1^+$ DC maintain the memory-like cell population and thereby impact the differentiation of exhausted $CD8^+$ T cells. After depletion of $XCR1^+$ DC, the remaining $XCR1^-$ DC (mainly $CD11b^+$ DC) were sufficient to stimulate exhausted $CD8^+$ T cell proliferation. As mentioned, TCF-1^+ cells give rise to $Tim3^+$ cells but importantly also undergo self-renewal. This self-renewal is critical as this subset of exhausted $CD8^+$ T cells is the one that responds to immunotherapy (Im, Hashimoto *et al.* 2016). Taking this into account, $XCR1^+$ DC could play an essential role for prolonged PD-L1 blockade by maintaining the reactivatable TCF-1^+ cell pool. One of the first studies describing the memory-like subset of exhausted $CD8^+$ T cells by the expression of CXCR5, already hypothesized a role for $XCR1^+$ DC as RNA transcript levels of XCL1 (the ligand for XCR1) were strongly increased in the memory-like subset compared to the terminally exhausted one (Im, Hashimoto *et al.* 2016). Additionally, a study by Argilaguet *et al.* revealed a role for $XCR1^+$ DC in maintaining the memory-like cell TCF-1^+ population during an earlier phase of chronic LCMV infection without checkpoint immunotherapy (Argilaguet, Pedragosa *et al.* 2019). Importantly, $XCR1^+$ DC have also been investigated in various tumour models in the context of anti-PD-1 therapy. Tumour engrafted Batf3-deficient mice, which lack $XCR1^+$ DC, were not able to respond to anti-PD-1 or anti-PD-L1 treatment (Salmon, Idoyaga *et al.* 2016, Sanchez-Paulete, Cueto *et al.* 2016). Supporting these findings, the presence of $XCR1^+$ DC in the tumour microenvironment correlated with the efficacy of anti-PD-1 treatment (Broz, Binnewies *et al.* 2014, Barry, Hsu *et al.* 2018, Bottcher, Bonavita *et al.* 2018). Furthermore, transfer of $XCR1^+$ DC loaded with tumour antigen synergized with checkpoint immunotherapy (Wculek, Amores-Iniesta *et al.* 2019).

The question that is unresolved is how XCR1$^+$ DC maintain the TCF-1$^+$ cell population. There are different possibilities: XCR1$^+$ DC could directly interact with TCF-1$^+$ cells, for example by the XCR1-XCL1 axis or deliver important co-stimulatory molecules or cytokines. Furthermore, XCR1$^+$ DC could also indirectly support the TCF-1$^+$ cells by retaining them within an optimal environment for these cells. This environment would promote interactions with other cells, their surface receptors and ligands as well as their secreted molecules. In fact, we are likely dealing with a highly complex system in which the combination of multiple intrinsic and extrinsic pathways contributes to the overall outcome. We investigated three likely candidates based on their essential role for optimal priming of CD8$^+$ T cells during acute infections: XCR1-XCL1, CD27-CD70 and IL-15Rα-IL-15 signalling. None of these signalling pathways played an essential role and blocking of these did not recapitulate the results obtained with the depletion of XCR1$^+$ DC.

However, to examine the role of XCR1 signalling we used XCR1-deficient mice, which were chronically infected and analysed during the chronic infection phase compared to their XCR1-competent littermates. Notably, we cannot rule out that compensatory mechanisms for XCR1-XCL1 signalling developed in these mice. Therefore, a better approach would be to enable the development of the chronic infection in the presence of XCR1 signalling and abrogate this only during the chronic infection phase. Additionally, a comparison of XCL1-proficient vs. XCL1-deficient CD8$^+$ T cells in the same host during checkpoint immunotherapy would be most informative to address a possible function of this cytokine.

We also investigated if co-stimulatory signalling of CD27 enabled by binding of its ligand CD70 impacted the CD8$^+$ T cell response during chronic LCMV infection. This was not the case during anti-PD-L1 treatment. These negative results need to be cautiously interpreted, however, we applied an antibody clone that proved to be efficient in other experimental settings at a similar dose and application route (Penaloza-MacMaster, Ur Rasheed *et al.* 2011). Interestingly, other studies even described a rather inhibitory role of the CD27-CD70 axis during chronic LCMV infection. MacMaster *et al.* blocked this signalling during the onset of chronic infection with anti-CD70 blocking

antibodies, which led to an increase in the absolute number of antigen-specific $CD8^+$ T cells (Penaloza-MacMaster, Ur Rasheed *et al.* 2011). In line with these observations are results obtained with anti-CD27 blockade during the early phase of LCMV Cl13 infection, which reduced the viral load (Matter, Odermatt *et al.* 2006). By contrast, CD70-deficient mice were impaired in their ability to resolve an acute LCMV infection. However, ultimately the generation of memory $CD8^+$ T cells was not impacted (Munitic, Kuka *et al.* 2013). These findings support the notion that CD27-CD70 signalling is critical during the primary response of a viral infection but less so for memory responses. During chronic infections, this co-stimulatory signalling could even drive exhaustion. This is in contrast to other co-stimulatory signalling pathways such as CD28-CD80/CD86, which are essential for the reinvigoration of exhausted $CD8^+$ T cells by checkpoint immunotherapy. Notably, CD28 signalling has been put in context with cell cycle entry whereas CD27 signalling has been functionally connected with effector and memory differentiation and survival (Denoeud and Moser 2011).

In addition to possible co-stimulatory signalling pathways instructed by $XCR1^+$ DC, we investigated the role of the common gamma chain cytokine IL-15 in the context of anti-PD-L1 treatment. Blocking of IL-15 did not reflect the results obtained with $XCR1^+$ DC depletion. However, as we observed a slight increase in the frequency of antigen-specific cells when blocking IL-15, it is possible that IL-15 signalling can contribute to exhaustion. This, however, is somewhat in conflict with the increased viral load post IL-15 blockade during anti-PD-L1 treatment. Importantly, terminally exhausted $CD8^+$ T cells have been described to be less responsive to common gamma chain cytokines as some of the receptors are downregulated (Fuller, Hildeman *et al.* 2005, Lang, Recher *et al.* 2005), while IL-2Rβ is maintained (Beltra, Bourbonnais *et al.* 2016). Signalling via IL-2 and IL-15 has been described to drive terminal exhaustion rather than to maintain $CD8^+$ T cell function during chronic infection (Beltra, Bourbonnais *et al.* 2016). In vitro, IL-15 has been shown to induce the expression of PD-L1 on various immune cells (Kinter, Godbout *et al.* 2008). However, recombinant IL-15 or IL-15 superagonists and fusion complexes with IL-15Rα administered in

combination with anti-PD-1 treatment of various murine and human cancers showed synergistic effects and lowered tumour burden (Kim, Kwilas *et al.* 2016, Mathios, Park *et al.* 2016, Wrangle, Velcheti *et al.* 2018, Knudson, Hicks *et al.* 2019). Notably, the beneficial effects of IL-15 administration were not only based on $CD8^+$ T cell functions but also on increased numbers of NK cells and improved function. NK cells display a very complex role during acute and chronic LCMV infection and this might not reflect their role during cancer. For example, it has been described that NK cells shape the adaptive immune response during acute LCMV infection by targeting $CD8^+$ T cells and thereby to some extent prevent immunopathology (Pallmer, Barnstorf *et al.* 2019). Similar findings were made in the context of chronic viral infections with LCMV. Here, the depletion of NK cells improved $CD8^+$ T cell responses, prevented severe T cell exhaustion and led to an improved control of the infection (Cook and Whitmire 2013). By contrast, the depletion of NK cells in cancer models, highly increased tumour burden (Knudson, Hicks *et al.* 2019). Therefore, the interpretation of the results obtained by IL-15 blockade during anti-PD-L1 treatment is complicated as there are various cellular recipients which contribute differently to viral clearance. Additionally, it has been shown that missing IL-15 signalling can be compensated by other common gamma chain cytokines, such as IL7, to maintain the memory T cell response (DeGottardi, Okoye *et al.* 2016).

In order to identify key molecules that are essential for the communication between $XCR1^+$ DC and memory-like $TCF\text{-}1^+$ cells, an unbiased approach seems to be most straight forward. In particular, we anticipate that the analysis of scRNAseq data from $TCF\text{-}1^+$ cells after anti-PD-L1 treatment in the presence or absence of $XCR1^+$ DC may be highly informative in this regard. In the next chapter we first concentrated on deciphering the tissue context in which DC and exhausted $CD8^+$ T cells meet and communicate and how checkpoint immunotherapy influences these interactions.

5. Localization of XCR1$^+$ DC and memory-like cell populations during chronic infection and checkpoint immunotherapy

5.1 Introduction

Findings of the former two chapters revealed that DC play an essential role during immunotherapy. Importantly, the two main resident subsets of conventional DC, XCR1$^+$ DC and CD11b$^+$ DC, exhibited complementary functions during anti-PD-L1 blockade. XCR1$^+$ DC impacted on the differentiation of exhausted CD8$^+$ T cells by maintaining the memory-like cell population during immunotherapy. When depleting XCR1$^+$ DC during treatment, the remaining XCR1$^-$ DC (mainly CD11b$^+$ DC) were sufficient in supporting the proliferation of CD8$^+$ T cells.

The question that remains is which signals XCR1$^+$ DC and CD11b$^+$ DC deliver and how these differ. In chapter 3, we could show that both DC subsets present viral antigen and likely deliver costimulatory signals via CD28 as naïve P14 proliferated upon co-culture with XCR1$^+$ DC and CD11b$^+$ DC from chronically infected animals. There are different possibilities of how XCR1$^+$ DC and CD11b$^+$ DC could impact the proliferation and differentiation of exhausted CD8$^+$ T cells. First, they could directly provide distinct additional signals. Second, they could indirectly impact on CD8$^+$ T cells by interacting with them within different microenvironments. In this case external signals in the vicinity of XCR1$^+$ DC or CD11b$^+$ DC affect the CD8$^+$ T cells. Third, it could be that XCR1$^+$ DC and CD11b$^+$ DC deliver similar signals but communicate with different subsets of memory-like TCF-1$^+$ cells, hinting towards heterogeneity within this cell population. Notably, these different options are not mutually exclusive. Based on the notion that differential impact of XCR1$^+$ DC vs. CD11b$^+$ DC on CD8$^+$ T cells is caused by additional signals provided by XCR1$^+$ DC, we have directly tested two obvious pathways. In the previous chapter, we have shown that CD27-CD70 signalling is not essential during PD-L1 blockade. However, IL-15 signalling improved the control of viral load during immunotherapy. Nevertheless, blocking of the IL-15 signalling axis did not fully reflect results

obtained with the depletion of XCR1$^+$DC during anti-PD-L1 treatment. This argues that IL-15 signalling provided by XCR1$^+$ DC may contribute but is not the critical pathway underlying the maintenance of the memory-like TCF-1$^+$ compartment. Therefore, we hypothesized that additional signalling molecules are involved in the communication between DC and T cells during chronic infection and checkpoint immunotherapy. To better understand this complex interaction network including intrinsic and extrinsic (environmental) signals, we aimed to investigate the localization of XCR1$^+$ DC and CD8$^+$ T cells in the context of chronic viral infection and anti-PD-L1 treatment. During steady state, XCR1$^+$ DC are mainly located in the T cell zones of the white pulp in the spleen (Bachem, Hartung *et al.* 2012). Additionally, Im *et al.* described a predominant localization of memory-like TCF-1$^+$ in the same location (Im, Hashimoto *et al.* 2016). By contrast, terminally exhausted Tim3$^+$ are described to be located in the red pulp. Using XCR1 reporter mice, we investigated the localisation of XCR1$^+$ DC in the spleen during chronic LCMV infection and checkpoint immunotherapy. Furthermore, we aimed to elucidate the localization of viral antigen-presenting cells in the spleen and if this changes during the course of anti-PD-L1 treatment and in the absence of XCR1$^+$ DC. In addition, we investigated memory-like TCF-1$^+$ cells by single-cell RNA sequencing to address whether these cells represent a homogenous population or can be divided into further subpopulations which differ in their expression of localization-associated molecules. These insights will help to better understand the dynamics of communication between DC and memory-like CD8$^+$ T cells in the tissue context and thereby decipher essential factors which impact the success of checkpoint immunotherapy.

5.2 Results

5.2.1 Impact of chronic LCMV infection and checkpoint immunotherapy on localization of XCR1$^+$ DC and in vivo antigen presentation

The XCL1-XCR1 axis plays an important role in the context of CD8$^+$ T cell priming during acute viral infections (Brewitz, Eickhoff *et al.* 2017). Surprisingly, we were unable to find a crucial role for this signalling pathway in the context of checkpoint immunotherapy during chronic LCMV infection (Chapter 4.2.2). Nevertheless, XCR1$^+$ DC are critical cellular interaction partners that maintain the memory-like TCF-1$^+$ cell pool of exhausted CD8$^+$ T cells during anti-PD-L1 treatment (Chapter 4.2.1). To address this interaction in more detail, we first aimed to elucidate the localization of XCR1$^+$ DC during the course of chronic LCMV infection and anti PD L1 therapy. To this end, we analysed XCR1 Venus reporter mice. These mice express the fluorescent protein Venus under the Xcr1 promoter. Confocal microscopy of spleens from uninfected mice revealed that XCR1$^+$ DC are mainly located in the T cell zones of the white pulp of the spleen (Figure 5.1A). Fewer cells were located in the red pulp. Mueller *et al.* described that the splenic architecture is permanently altered during chronic LCMV infection (Mueller, Matloubian *et al.* 2007). In line with this, we observed less intact T cell zones in the spleens of chronically infected mice. Additionally, localization of XCR1$^+$ DC was strongly affected. Besides the typical localization of this DC subset in the T cell zones of the white pulp, many XCR1$^+$ DC were also detected in the red pulp of the spleen (Figure 5.1B).

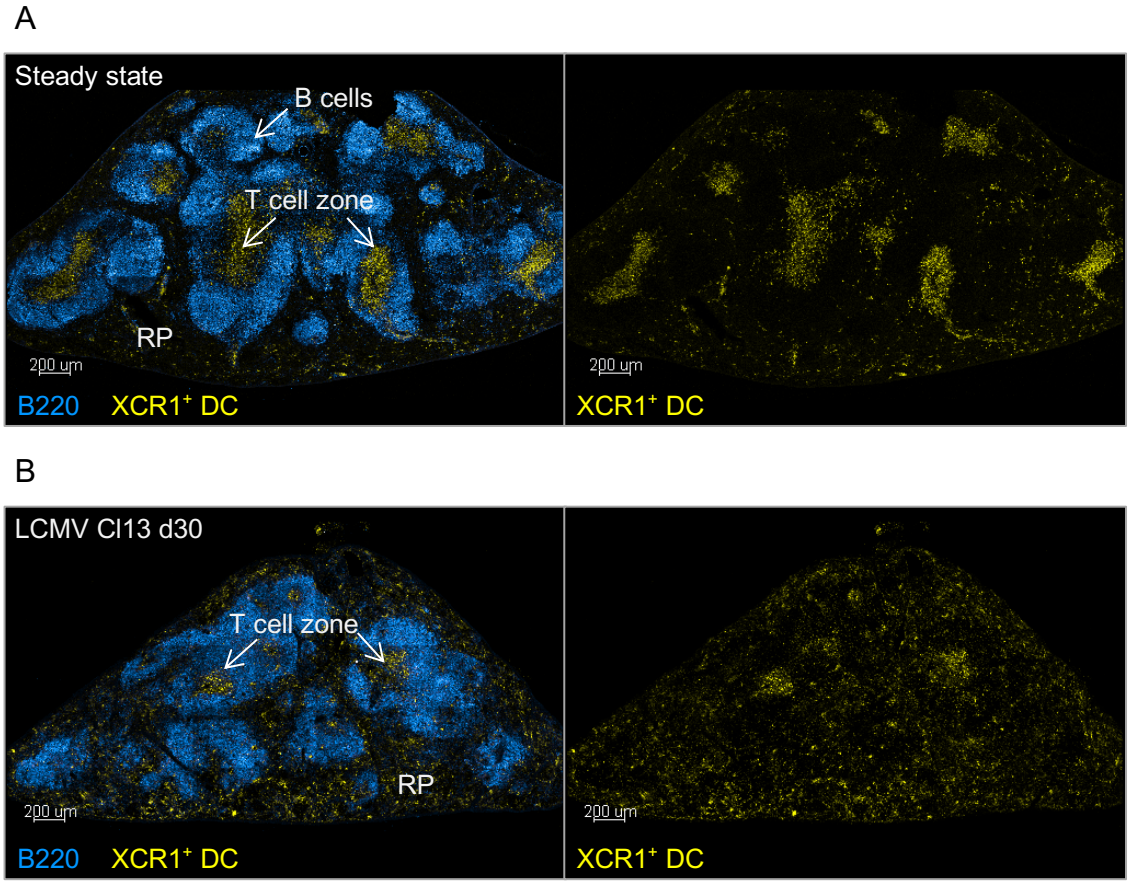
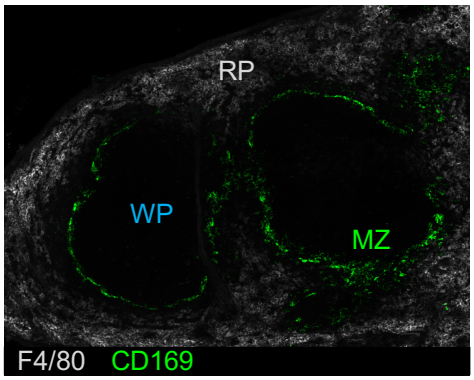


Figure 5.1: Chronic LCMV infection impacts localization of XCR1⁺ DC in the spleen. (A) IF images of the spleen of naive XCR1$^{Venus/+}$ mice. B cells were stained with anti-B220 (blue) and XCR1⁺ DC are depicted in yellow. **(B)** XCR1$^{Venus/+}$ mice received anti-CD4 and were i.v. infected with 2x10^6 IU LCMV Cl13. IF images of the spleen analysed during the chronic infection phase. Data are representative of two independent experiments (n = 3 mice/group).

Interestingly, the localization of XCR1$^+$ DC was further changed by anti-PD-L1 treatment. After three injections of 200 µg anti-PD-L1 during the chronic infection phase, XCR1$^+$ DC were still found in the T cell zone and the red pulp. However, we observed an augmented accumulation of these DC in the marginal zone (MZ) of the spleen, when compared to PBS-treated animals (Figure 5.2B and 5.3). The marginal zone is an area, which is located between the white pulp and the red pulp of the spleen indicated in Figure 5.2A. Additionally to the localization of XCR1$^+$ DC, the MZ was affected by anti-PD-L1 treatment as well. The expression of CD169, used for the identification of marginal zone macrophages, was decreased in the spleens of animals, which received immunotherapy (Figure 5.3). It remains currently unclear whether the accumulation of XCR1$^+$ DC in the MZ is based on an additional recruitment of preDC from the blood or reflects a reorganization of the existing splenic network or a combination of both.

A Steady state



B LCMV Cl13

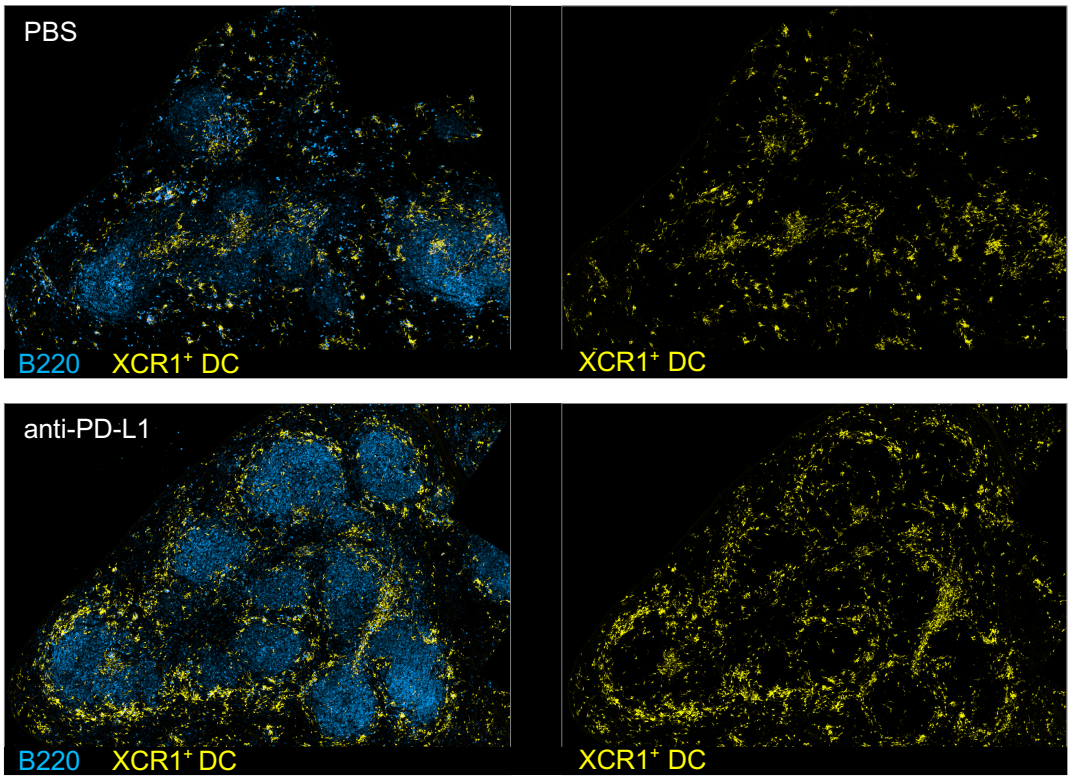


Figure 5.2: Localization of XCR1⁺ DC is influenced by anti-PD-L1 treatment during chronic LCMV infection. (A) IF image depicting the red pulp (RP) and the marginal zone (MZ) of the spleen during steady state. **(B)** Chronically infected XCR1$^{Venus/+}$ mice received three anti-PD-L1 treatments (200 µg i.p.). IF images show localization of XCR1⁺ DC in the spleen of PBS-treated animals and animals which received anti-PD-L1. B cells were stained with anti-B220 (blue) and XCR1⁺ DC are depicted in yellow. Data are representative of one experiment (n = 3 mice/group).

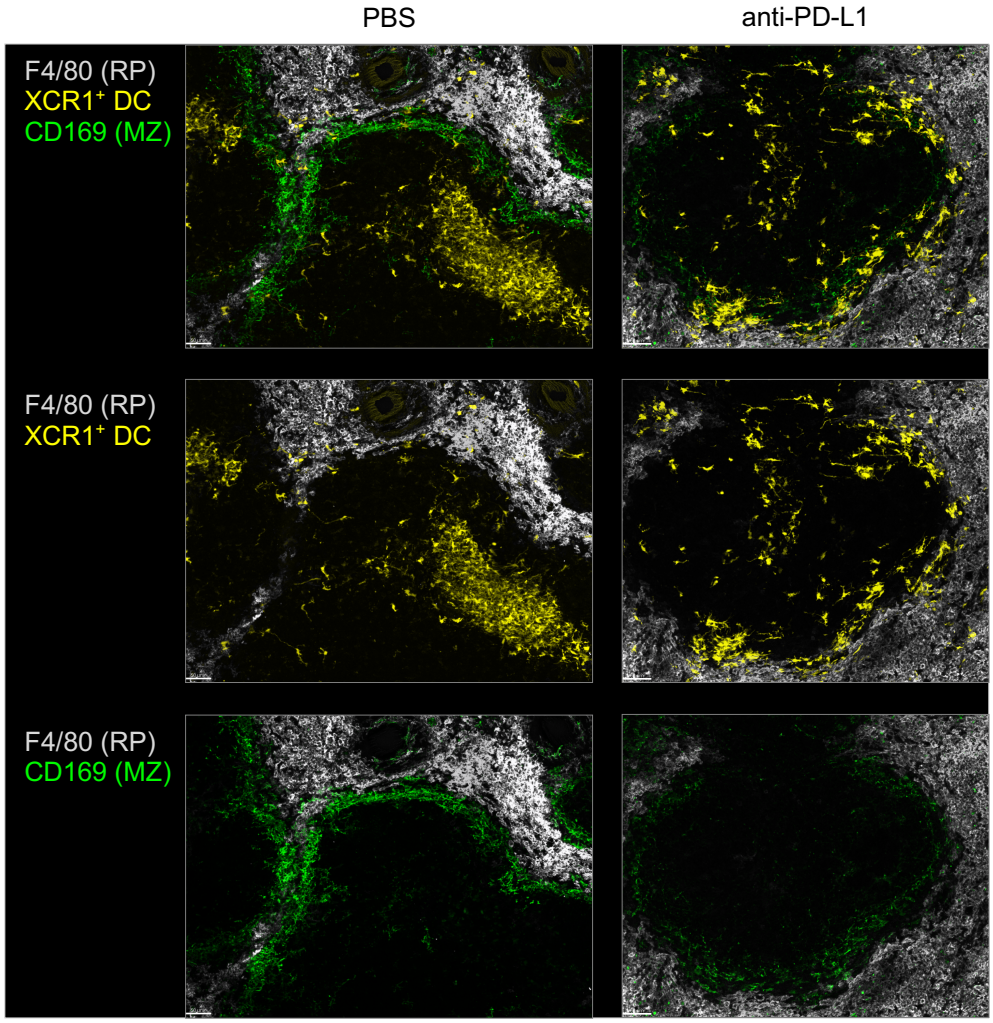


Figure 5.3: Anti-PD-L1 treatment affects marginal zone and localization of XCR1⁺ DC in the spleen. Chronically infected XCR1$^{Venus/+}$ mice received PBS (left) or three anti-PD-L1 treatments (200 µg i.p.) (right). IF images show localization of XCR1⁺ DC (yellow) in the white pulp and marginal zone. Marginal zone is depicted in green and red pulp in grey. Data are representative of one experiment (n = 3 mice/group).

As the localization of XCR1$^+$ DC is affected by checkpoint immunotherapy, we aimed to examine where antigen is presented during anti-PD-L1 treatment in the presence and absence of XCR1$^+$ DC. In order to visualize the localisation of cells that present viral antigen in the context of MHC I, we transferred naive LCMV-specific P14 CD8$^+$ T cells. Naive T cells actively migrate and search the tissue for the presence of antigen-presenting cells. Once they have engaged peptide-MHC I complexes they arrest and upregulate the early activation marker CD69, which hinders cells from egress from the lymphoid tissue. We further combined this approach with the XCR1 Venus DTR mouse line, which allows depletion of XCR1$^+$ DC. Specifically, mice were chronically infected with LCMV Cl13 in combination with CD4 depletion. During the chronic infection phase, mice received three injections of anti-PD-L1 in combination with DTX to deplete XCR1$^+$ DC. As a readout of antigen presentation, 2.5x10^6 naïve P14 cells, which express the fluorescent protein tdTomato, were transferred post treatment and spleens isolated for analysis by confocal microscopy 12 h post transfer. In anti-PD-L1-treated animals, which still harboured XCR1$^+$ DC, P14 cells were detected in the central areas of the T cell zone as well as the MZ and fewer in the red pulp of the spleen (Figure 5.4A). By contrast, in the absence of XCR1$^+$ DC and in the context of anti-PD-L1 treatment, few P14 cells localized to the T cell zone and most of them were detected in the MZ and red pulp (Figure 5.4B). This argues that XCR1$^+$ DC could be the predominant DC population that presents antigen within the T zone, while other DC and possibly MZM (marginal zone macrophages) preferentially do so in the MZ and the red pulp. However, as we observed a strong decrease of antigen presentation by XCR1$^+$ DC during anti-PD-L1 treatment *ex vivo* (Chapter 3), we speculate that XCR1$^+$ DC provide certain retention signals that lead to the accumulation of P14 cells in the T cell zones. Having identified three areas of the spleen (white pulp, marginal zone and red pulp), in which viral antigen presentation takes place as well as possible retention signals are delivered, we next wanted to address whether TCF1$^+$ CD8$^+$ T cells comprise a heterogeneous population and if so whether subsets thereof could be distinguished based on chemokine or adhesion molecules that indicate a preferential localization in the spleen.

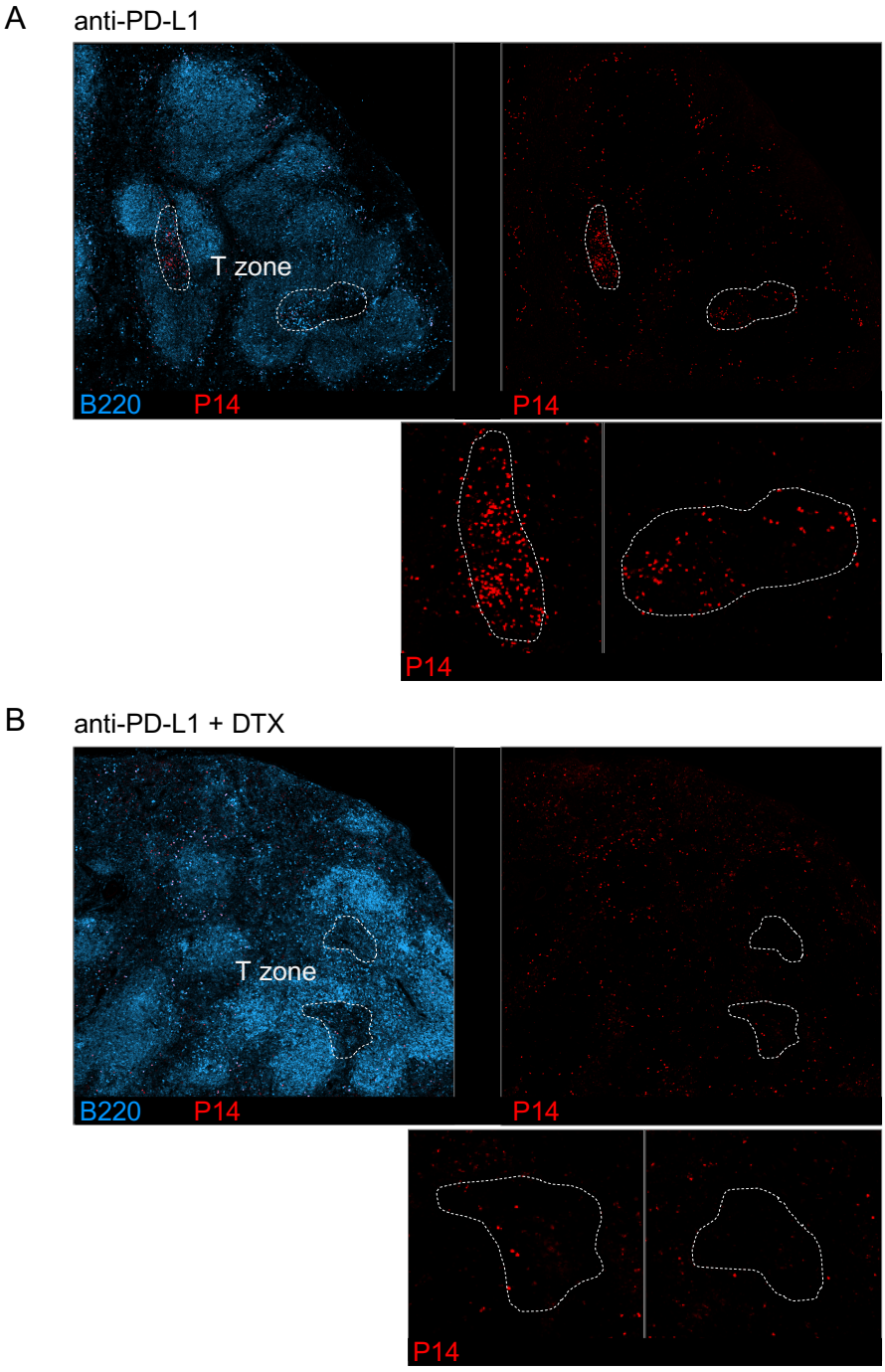


Figure 5.4: Splenic sublocalization of naïve P14 cells is dependent on the presence of XCR1$^+$ DC during immunotherapy. 2.5x10^6 naïve P14 cells (tdTomato) were transferred into chronically infected XCR1 Venus DTR after treatment. IF images show localization of P14 cells (red) 12 h post transfer in the spleens of mice which received **(A)** anti-PD-L1 treatment alone or **(B)** in combination with DTX. B cells are depicted in blue and T cell zones are encircled. Data are representative of one experiment (n = 3 mice/group).

5.2.2 Single-cell RNA sequencing deciphers subpopulations of memory-like exhausted CD8$^+$ T cells

Memory-like TCF-1$^+$ CD8$^+$ T cells have been described to be primarily localized in the white pulp. By contrast, terminally exhausted Tim3$^+$ CD8$^+$ T cells mostly reside in the red pulp of the spleen (Im, Hashimoto *et al.* 2016). Studies identifying these two subsets investigated the expression of various intracellular and surface markers. While these studies focused on differences between these two subsets, they also provided first hints at a potential heterogeneity within the memory-like TCF-1$^+$ compartment. Some of the expressed receptors are known to regulate the localization of lymphocytes within tissues. For example, the expression of the chemokine receptors CXCR5, CCR7, CXCR3 and the adhesion molecule CD62L have been described for a fraction of TCF-1$^+$ cells (Im, Hashimoto *et al.* 2016). To investigate the heterogeneity of the memory-like TCF-1$^+$ cell pool in more detail and in order to put this into context of their localization in secondary lymphoid organs, we performed single-cell RNA sequencing (scRNAseq) of splenic Tim3$^-$ PD-1$^+$ CD8$^+$ T cells from chronically infected mice. Analysis of these data revealed nine distinct clusters within the Tim3$^-$ cells displayed in a UMAP projection shown in Figure 5.5A. These clusters could be distinguished by a variety of differentially expressed genes. Importantly, clusters 1 and 3 were more distinct from the other clusters and could be identified by the expression of the *2B4* (CD244) and the absence of *Tcf7* (TCF-1) (Figure 5.5B). As we aimed to focus on the heterogeneity of the TCF-1$^+$ subset, we further on excluded clusters 1 and 3 from the analysis.

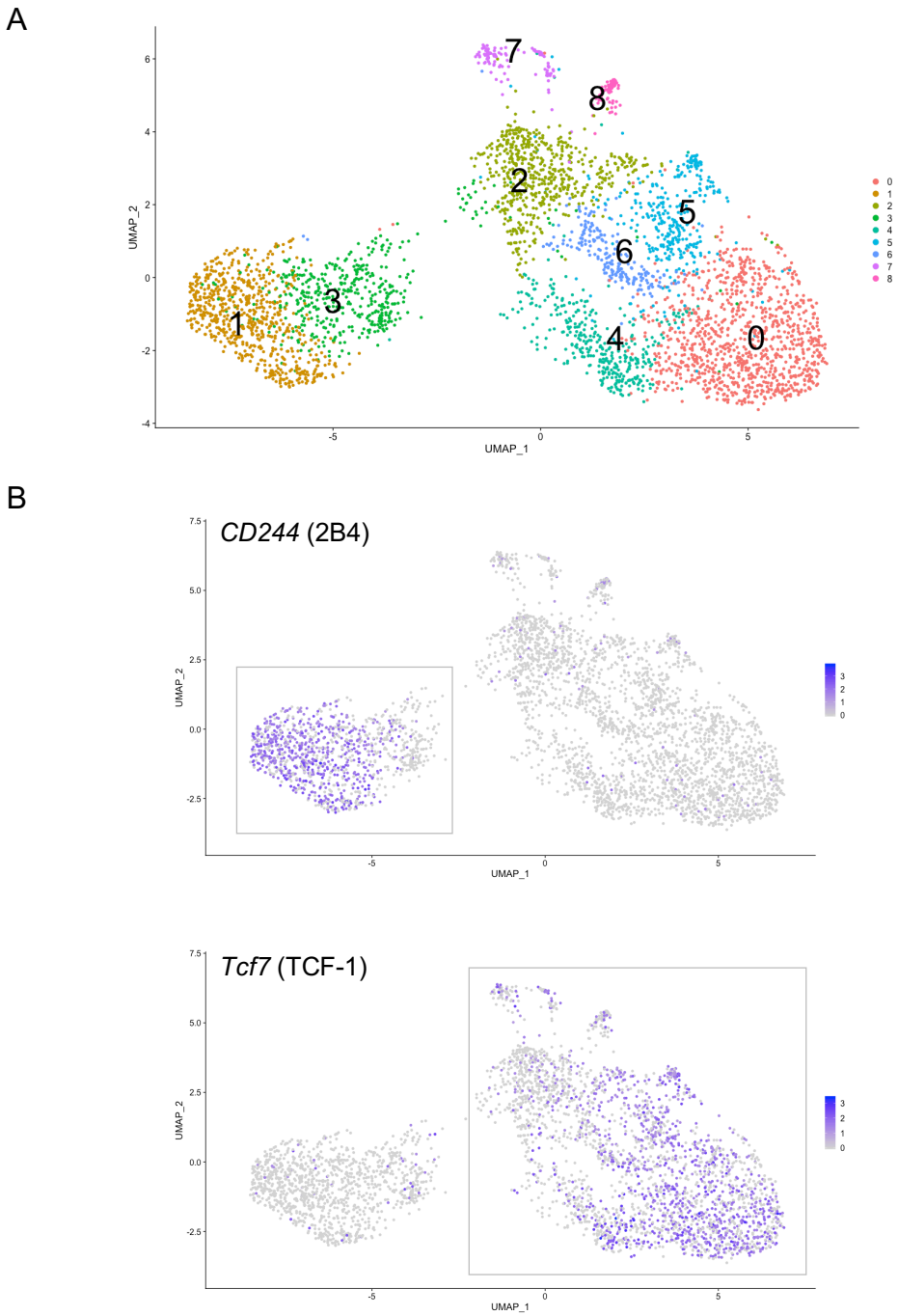


Figure 5.5: Single-cell RNA sequencing analysis of splenic Tim3$^-$ PD-1$^+$ CD8$^+$ T cells from chronically infected mice. Mice were infected with 2x10^6 IU LCMV Cl13 in combination with CD4 depletion. On day 30 post infection, spleen was isolated and Tim3$^-$ PD-1$^+$ CD8$^+$ T cells sorted for scRNAseq analysis. **(A)** UMAP projection of nine distinct colour-coded clusters of Tim3$^-$ cells determined by Seurat v.3. **(B)** UMAP projection of *CD244* and *Tcf7* transcript levels in distinct clusters. Colour scale: 0 (grey) to 3 (blue). Each dot corresponds to one individual cell.

Analysing the TCF-1$^+$ cells, eight distinct clusters could be revealed. These are displayed in a UMAP projection in Figure 5.6A. The clusters could be distinguished by a variety of differentially expressed genes. A heatmap of the top ten differentially expressed genes for each cluster is shown in Figure 5.6B. The top ten expressed genes are additionally listed in Table 5.1. This gene expression profile provides insights on the function and possible tissue localization of the different memory-like TCF-1$^+$ subpopulations as well as possible signals the cells receive and cellular contacts they could have. For example, cluster 0 displays cells which express *Xcl1*. XCL1 is the ligand for XCR1, which could indicate communication of this cell cluster with XCR1$^+$ DC. In addition, *Cxcr5* is expressed, which hints towards a possible localization close to B cell follicles (Leong, Chen *et al.* 2016). Also, *Slamf6*, which on protein level regulates T cell activation (Dragovich, Adam *et al.* 2019). Other clusters display a role in effector functions by the expression of *Gzma* and *Gzmb* (cluster 1 and 7). Cells within cluster 2 express *Sell*, which codes for the adhesion molecule CD62L that is required for circulation within secondary lymphoid organs as well as *Il7r* (CD127), an important survival cytokine. Both are described as important markers for memory cells. Cluster 4 harbors cells which upregulated genes for different interferon-induced proteins. This could indicate increased responsiveness or exposure of this cluster to IFNα/β and IFNγ signals. Additional in-depth analysis of the different subsets of memory-like TCF-1$^+$ cells will enhance our understanding of its various functions. Further on, we concentrated on the expression of certain chemokine receptors and adhesion molecules which enable a better understanding of the possible localization of the different TCF-1$^+$ subpopulations.

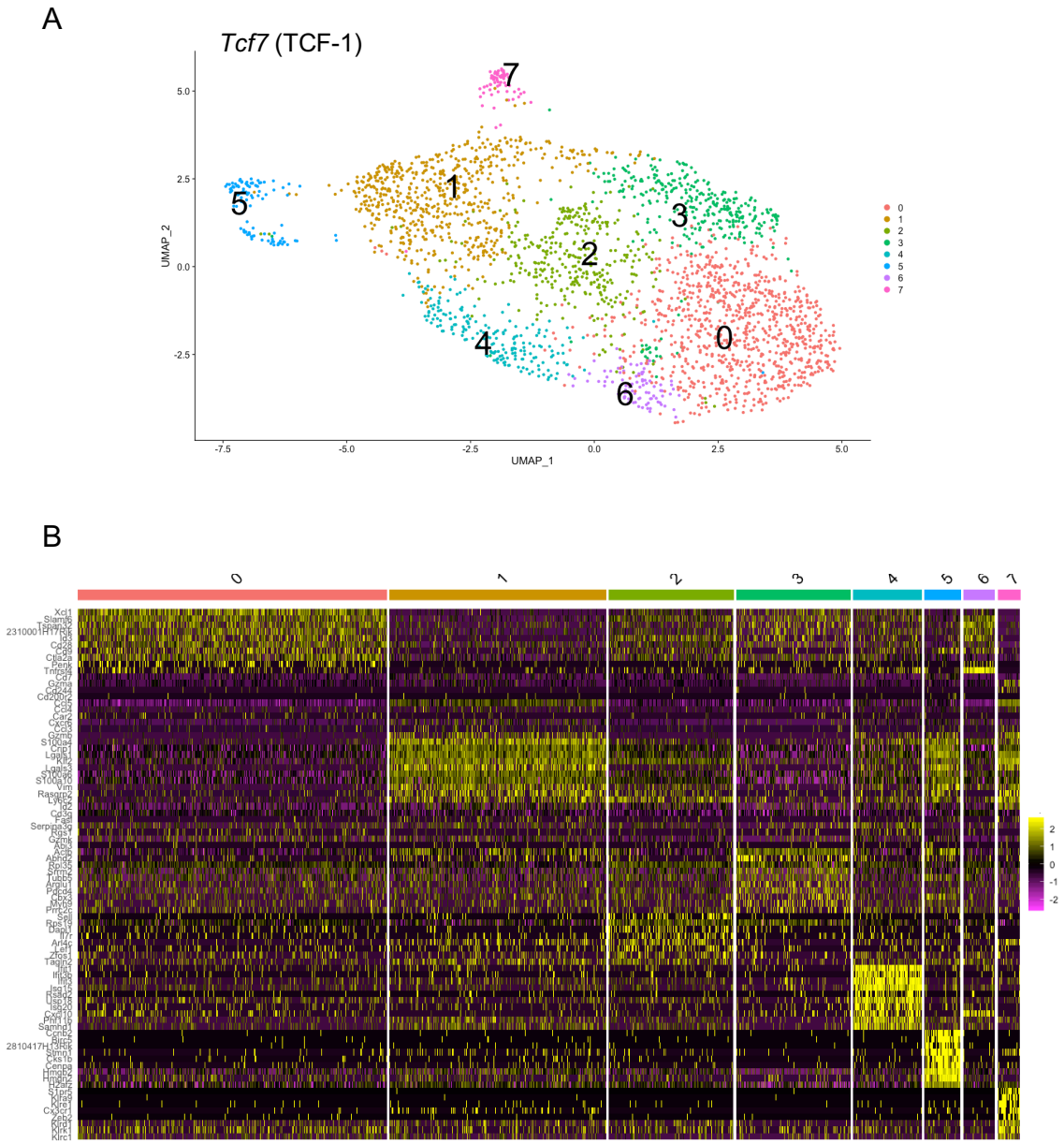


Figure 5.6: Single-cell RNA sequencing analysis of *Tcf7*-expressing cells **(A)** UMAP projection of eight distinct colour-coded clusters of *Tcf7*-expressing cells determined by Seurat v.3. Each dot corresponds to one individual cell. **(B)** Heatmap displaying the top 10 genes expressed in each cluster. The columns correspond to the cells in each cluster, the rows correspond to the genes. The colour scale is based on a z-score distribution from −2 (purple) to 2 (yellow).

cluster	gene
0	Xcl1 Ctla2a Slamf6 2310001H17Rik Cxcr5 Tspan32 Lag3 Penk Eomes Tnfrsf9
1	Crip1 Ccl5 S100a6 Lgals1 Lgals3 S100a4 Klf2 Gzmb Ifngr1 Vim
2	Sell Rps28 Rps19 Ly6c2 Dapl1 Il7r Rpl12 Zfos1 Arl4c Lef1
3	Actb Malat1 Abhd2 Srrm2 Tubb5 Arglu1 Pdcd4 Myh9 Cbx3 Prrc2c
4	Ifit1 Ifit3b Ifit3 Isg15 Rsad2 Usp18 Isg20 Stat1 Cxcl10 Samhd1
5	Ccnb2 Birc5 2810417H13Rik Cks1b Stmn1 Cenpa Hmgb2 Hmgn2 Ptma Cks2
6	Bace2 Aicda Il1r2 Tnfrsf4 Cxcl10 Podnl1 Cd81 Cd83 Nfkbia Tnfsf8
7	S1pr5 Klra9 Klre1 Zeb2 Cx3cr1 Ccl5 Gzmb Klrd1 Klrc1 Gzma

Table 5.1: Top ten differentially expressed genes for each cluster of memory-like TCF-1$^+$ cells from chronically infected mice. Mice were infected with 2x10^6 IU LCMV Cl13 in combination with CD4 depletion. On day 30 post infection, spleen was isolated and Tim3$^-$ PD-1$^+$ CD8$^+$ T cells sorted for scRNAseq analysis. *Tcf7*- expressing clusters were analysed and listed are the top ten differentially expressed genes for each cluster sorted by the log-fold change (avg logFC) in gene expression.

Interestingly, the expression of the chemokine receptors CXCR6, CXCR5, CX3CR1 and the adhesion molecule CD62L significantly distinguished the clusters of TCF-1$^+$ cells within the data set (Figure 5.7). In addition, analysis of the scRNAseq data also revealed *Mki67* (Ki67) expression specifically in cluster 5 (Figure 5.7). *Cxcr6* expression was found in nearly all of the TCF-1$^+$ cell cluster but was most dominantly expressed in cluster 1 and 5, in which *Cx3cr1* and *Mki67* expression were enriched as well. In contrast, *Cxcr5*-expressing cells were found in cluster 0, but the expression of this chemokine receptor was highly decreased in the clusters expressing *Cxcr6*, *Cx3cr1* and *Mki67*. Cluster 2 harbors cells, which show an increased expression of *Sell* (CD62L). To validate if the high transcript levels of these molecules are reflected by protein expression on the surface of cells, we analysed for their presence on Tim3$^-$ PD-1$^+$ CD8$^+$ T cells from chronically infected mice by flow cytometry. Indeed, surface expression of CXCR6, CXCR5, CX3CR1 and CD62L reflected the results obtained with scRNAseq and confirmed the existence of different subpopulations of memory-like TCF 1$^+$ CD8$^+$ T cells (Figure 5.8). We observed distinct clusters of TCF-1$^+$ cells with these markers with an overlap between CXCR6$^+$ and CX3CR1$^+$ cells. Notably, in comparison to the small cluster of Mki67-expressing cells revealed with scRNAseq, a higher proportion of Ki67$^+$ cells were detected with flow cytometry with some of these being CXCR6$^+$ and CX3CR1$^+$ (Figure 5.8). Based on the critical role of chemokine receptors and adhesion molecules that define these subsets, we hypothesize that these TCF-1$^+$ CD8$^+$ T cells subsets are found in distinct areas of the spleen.

In summary, confocal microscopy and scRNAseq data of this chapter show that chronic LMCV infection and immunotherapy with anti-PD-L1 impact on the localization of XCR1$^+$ DC and that memory-like TCF-1$^+$ cells display a heterogenous group with subpopulations expressing distinct localization-associated molecules. These new findings provide an important step forward in better resolving where exactly these subsets are localized and which DC subsets reside in the same area to impact on proliferation and differentiation during chronic infection and checkpoint immunotherapy.

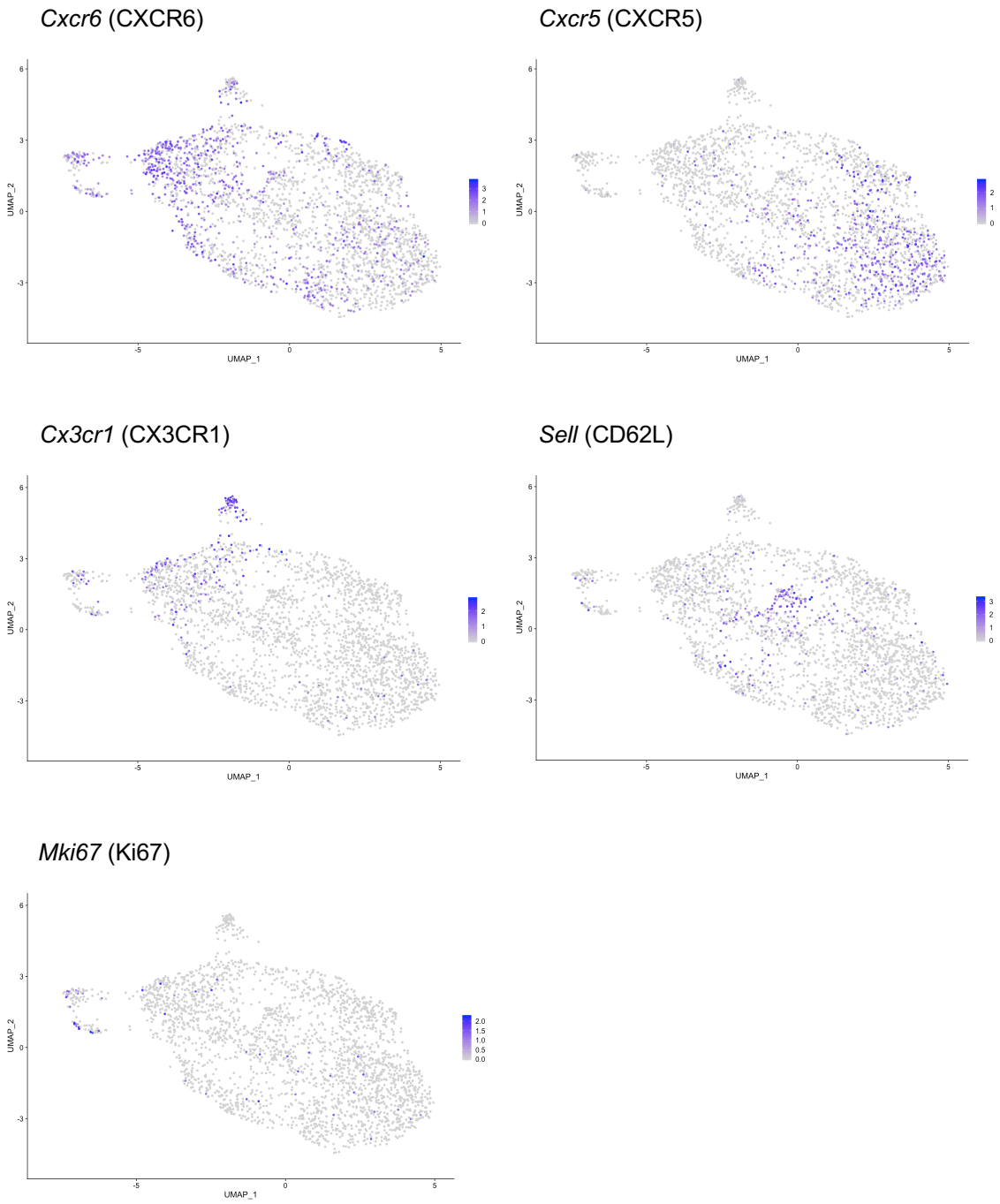


Figure 5.7: Subpopulations of TCF-1⁺ cells express distinct transcription levels of chemokine receptors and adhesion molecules. UMAP projections of *Cxcr6, Cxcr5, Cx3cr1*, *Sell* and *Miki67* transcript levels in distinct clusters of the *Tcf7*-expressing clusters. Each dot corresponds to one individual cell. Colour scale: 0 (grey) to 3 (blue).

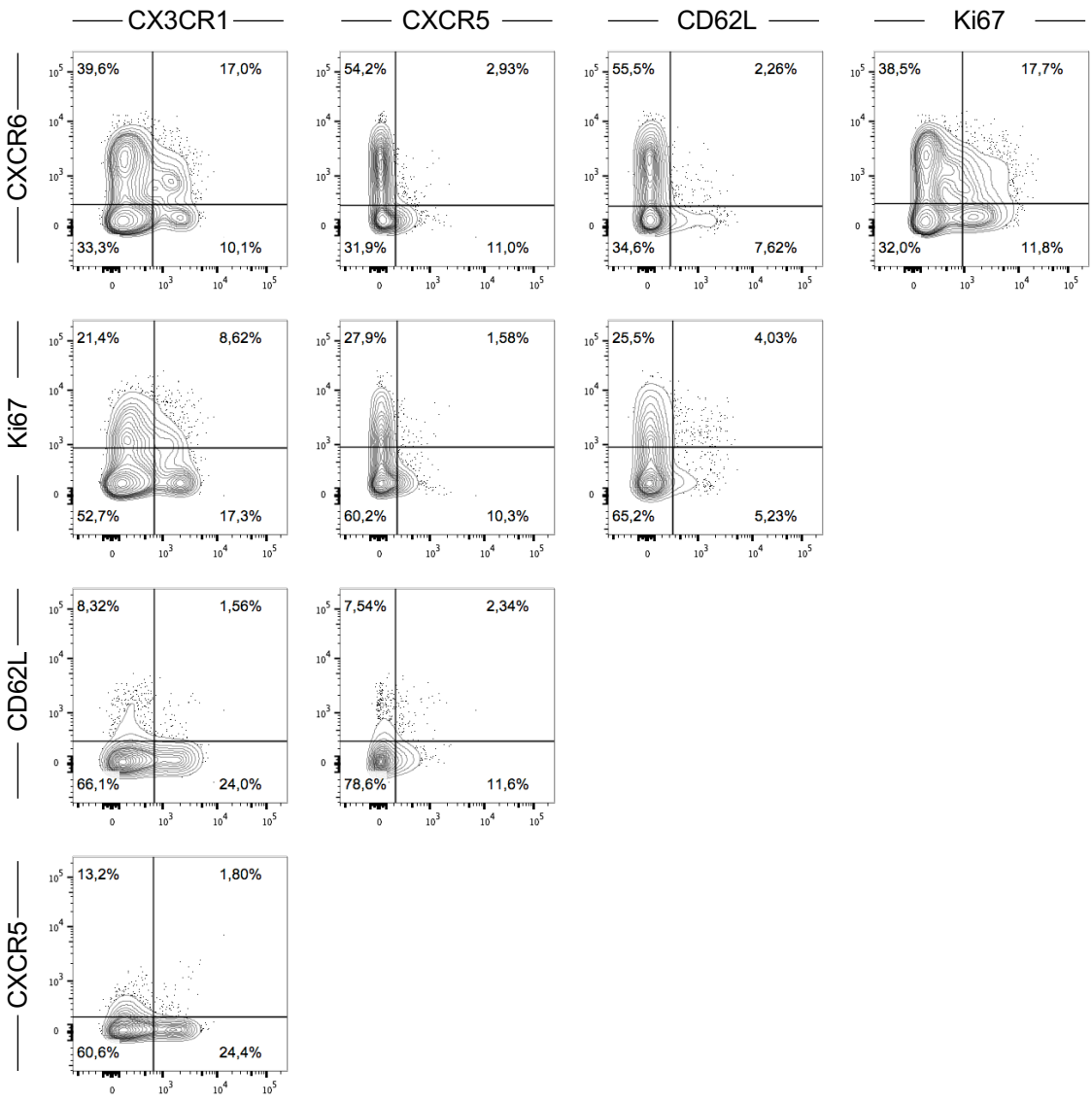


Figure 5.8: TCF-1$^+$ subpopulations can be distinguished by the expression of distinct chemokine receptors and adhesion molecules. Mice were infected with 2x10^6 IU LCMV Cl13 in combination with CD4 depletion. On day 30 post infection, spleen was isolated and Tim3$^-$ PD-1$^+$ CD8$^+$ T cells stained for flow cytometry analysis. Representative flow cytometry plots showing the expression of the chemokine receptors CXCR6, CX3CR1 and CXCR5, the adhesion molecule CD62L and the proliferation marker Ki67 Data are representative of two independent experiments (n = 1-3 mice/group).

5.3 Discussion

Examining the localization of certain cells can greatly improve our understanding of cellular communication pathways. This not only provides important clues about whether cells need to co-exist in similar locations as a prerequisite of cell-cell interaction, but also deciphers the impact of the local microenvironment the cells reside in. In this chapter, we aimed to elucidate the impact of chronic LCMV infection and checkpoint immunotherapy on the localization of XCR1$^+$ DC and memory-like TCF-1$^+$ cells. Different DC subsets could influence the memory-like population owing to either distinct signals delivered directly or by altering the local microenvironment and thereby the conditions in which the T cells respond to infection and therapy. Moreover, different subgroups within the memory-like TCF-1$^+$ cell pool could reside in different areas and therefore differ in their cellular interaction with other immune cells. In addition, the distinct microenvironments these different subsets reside in could affect their activation and differentiation.

Chronic LCMV infection disrupts the splenic architecture. In contrast to acute LCMV infection, this is likely caused by increased infection and resulting functional impairments of stromal cells, namely fibroblastic reticular cells (FRC) (Mueller, Matloubian *et al.* 2007). The white pulp of the spleen, which includes the T cell zone and the B cell follicles, is smaller and disorganized, which is partly mediated by NK and CD8$^+$ T cells (Mbanwi, Wang *et al.* 2017). XCR1$^+$ DC predominantly reside in the T cell zone of the white pulp and fewer of them are found in the red pulp (Steinman, Pack *et al.* 1997). However, we found here that more of these DC located to the red pulp of the spleen during chronic LCMV infection. The alterations of splenic architecture described above likely underlie this change in localization. It is also possible that XCR1$^+$ DC directly migrate to the red pulp, where many infected cells, such as red pulp macrophages reside. Interestingly, Argilaguet *et al.* described that the number of XCR1$^+$ DC decreases during the first five days after infection with a chronic LCMV strain (Argilaguet, Pedragosa *et al.* 2019) This this is rapidly followed by an influx as indicated by increased numbers of XCR1$^+$ DC with a peak around

d9 post infection. Thereafter, a decrease of this DC subsets was described to a level comparable to prior to infection (Argilaguet, Pedragosa *et al.* 2019). These newly incoming XCR1$^+$ DC likely migrate to different areas than under steady state conditions as a consequence of the disrupted architecture and the inflammatory environment during chronic viral infection. Notably, checkpoint immunotherapy additionally impacts their localization. We have observed that XCR1$^+$ DC accumulate in the marginal zone of the spleen. This area represents the interface between white pulp and red pulp. At the same time, we detected a loss of CD169 staining within the MZ. Whether this reflects a downregulation of this protein, a loss of MZM or even a more profound alteration of the MZ needs to be further investigated. To date, little is known about the effect of anti-PD-L1 treatment on the splenic architecture of the spleen. Marginal zone macrophages are infected with LCMV Cl13 (Mueller, Matloubian *et al.* 2007). Therefore, these cells could be direct targets of the reinvigorated cytotoxic T cell response by anti-PD-L1 treatment. This scenario would hint at an induction of immunopathology by checkpoint immunotherapy. One could speculate that reactivated CD8$^+$ T cells produce the chemokine XCL1, which could in turn recruit intrasplenic XCR1$^+$ DC to the marginal zone. However, the direct cause of the change in localization of XCR1$^+$ DC upon anti-PD-L1 treatment remains to be investigated.

Nevertheless, we still observed XCR1$^+$ DC in the T cell zones of the white pulp with and without checkpoint immunotherapy. Here, they appeared to be essential for the localization of transferred naïve P14 cells to the T cell zone of the spleen, as only few them could be detected in this area in the absence of XCR1$^+$ DC. XCR1$^+$ DC could be the main cell type responsible for LCMV-antigen presentation in the T cell zone. Conversely, *ex vivo* DC-P14 co-culture experiments presented in chapter 3 revealed that antigen presentation over the course of anti-PD-L1 treatment is strongly decreased. We cannot exclude a bias in the isolation process of DC from PBS- and anti-PD-L1-treated mice. However, XCR1$^+$ DC could also lead to the retention of naïve P14 cells by antigen-independent signals. As the memory-like TCF-1$^+$ cells have also been described to predominantly localize to the T cell zones, direct XCR1$^+$ DC and T

cell interactions could be of great importance for the reinvigoration and self-renewal of these cells. In chapter 2, we described that this memory-like T cell subset is reduced upon the depletion of XCR1$^+$ DC during anti-PD-L1 treatment. Therefore, XCR1$^+$ DC could deliver retention signals to these T cells and thereby keep them in the white pulp. It is possible that T cells receive less inflammatory, differentiation-driving signals in this area of the spleen compared to the red pulp. A possible pathway of how XCR1$^+$ DC could lead to the retention of TCF-1$^+$ cells in the white pulp is via the adhesion molecule class I-restricted T cell-associated molecule (CRTAM). During infection, activated CD8$^+$ T cells have been shown to be retained by binding of CRTAM to its ligand nectin-like molecule-2 (Necl2), which is expressed by XCR1$^+$ DC (Takeuchi, Itoh *et al.* 2009). Interestingly, this notion is supported by our scRNAseq data, which reveal the expression of *Crtam* especially in cells that are part of the clusters of *Cxcr5*- and *Sell*-expressing (CD62L) cells (data not shown). It will be of great interest to elucidate the signals which drive self-renewal of the TCF-1$^+$ cell compartment and which signals drive the differentiation into the terminally exhausted Tim3$^+$ subset of exhausted CD8$^+$ T cells.

In this chapter we further revealed that the TCF-1$^+$ compartment comprises of a heterogenous group of cells, which likely reside in different areas of the spleen. The latter conclusion is based on differences in their expression of certain chemokine receptors and adhesion molecules. The gene expression profile of the various clusters of TCF-1$^+$ cells may reflect that they all reside in different microenvironments and therefore are exposed to different signals. For example, CXCR6$^+$ TCF-1$^+$ cells are more likely to reside in the marginal zone or red pulp as CXCR6 signalling plays a role for the egress of CD8$^+$ T cells from the T cell zones and guides their migration to the red pulp (Matloubian, David *et al.* 2000). CXCR6 is also expressed by the terminally exhausted Tim3$^+$ subset (Wu, Ji *et al.* 2016). It can be speculated that the CXCR6$^+$ TCF-1$^+$ cells will ultimately differentiate into Tim3$^+$ cells that reside in the red pulp (Im, Hashimoto *et al.* 2016). In contrast to this subset of TCF-1$^+$ cells, the CD62L$^+$ and CXCR5$^+$ cells appear more likely to reside in the T cell zones of the spleen. As mentioned above, the expression of *Crtam* could guide retention. Additionally, *Sell*

expression (CD62L) overlaps with the expression of *Ccr7* (CCR7) (data not shown). This chemokine receptor enables migration of $CD8^+$ T cells and DC to the T cell zones upon secretion of its ligands CCL19 and CCL21 (Link, Vogt *et al.* 2007). Notably, the generation of memory cells has been described to be absent during chronic viral infection (Wherry, Barber *et al.* 2004, Shin and Wherry 2007). However, by the expression of *Sell* (CD62L), *Ccr7* (CCR7) and *Il7r* (CD127), these cells display key features of central memory T cells (Sallusto, Geginat *et al.* 2004). Also residing in the T cell zones but maybe more towards the B cell follicles could be the $CXCR5^+$ $TCF\text{-}1^+$ cells. These cells have also been described as T follicular cytotoxic cells (T_{FC}) and have been shown to contain chronic viral infection (He, Hou *et al.* 2016, Leong, Chen *et al.* 2016). Another cluster of cells expressed the fractalkine receptor CX3CR1. Upon acute infection, the expression levels of CX3CR1 allow to distinguish three different memory subsets (Gerlach, Moseman *et al.* 2016). Classical central memory T cells (TCM) express low levels of CX3CR1 while effector memory T cells (TEM) express high levels. A third subset of memory cells expresses intermediate levels of CX3CR1 and has high capacity to undergo self-renewal, give rise to TCM cells and additionally is found to migrate through peripheral tissues (Gerlach, Moseman *et al.* 2016). Interestingly, tumour-infiltrating $CD8^+$ T cells can also be distinguished by the expression of CX3CR1. Here, $CX3CR1^{hi}$ $CD8^+$ T cells display terminally differentiated cells and only the $CX3CR1^{int}$ and $CX3CR1^{low}$ cells proliferated upon checkpoint immunotherapy (Yamauchi 2018). Notably, results displayed in this chapter showed that some of the $CX3CR1^+$ cells expressed the proliferation marker Ki67. This argues for a low but constant proliferation of the exhausted $CD8^+$ T cells. It can be speculated that these cells are also the first responders that proliferate in the context of checkpoint immunotherapy.

The identification of distinct memory-like $TCF\text{-}1^+$ subsets enables better in-depth analysis of the mechanisms driving self-renewal and differentiation into terminally exhausted $CD8^+$ T cells. In addition, this can be investigated in the context of DC-T cell communication and checkpoint immunotherapy. Therefore, we aim to compare the transcription profile of memory-like $TCF\text{-}1^+$ cells from

chronically infected untreated mice with those from anti-PD-L1-treated animals. This will reveal if a distinct subset of TCF-1$^+$ cells responds to checkpoint immunotherapy. Additionally, this can be examined in the presence or absence of XCR1$^+$ DC to understand the impact of these cells for the reinvigoration of exhausted CD8$^+$ T cells.

6. General discussion

Intact $CD8^+$ T cell responses are critical for the control of viral infections. However, during chronic viral infections such as with HIV, HCV and HBV as well as during cancer, $CD8^+$ T cells progressively lose their effector functions. During this state of T cell exhaustion, a complex network of intrinsic and extrinsic factors inhibits T cell activation and proliferation to prevent immunopathology. This regulatory mechanism prevents from self-harm but at the same supports persistence as the infection cannot be cleared. The inhibitory receptor PD-1 and its ligand PD-L1 play a crucial role in this context. Infection of PD-1-deficient or PD-L1-deficient mice with a chronic LCMV strain causes lethal immunopathology (Barber, Wherry *et al.* 2006, Frebel, Nindl *et al.* 2012). In contrast, blocking this signalling pathway during an established chronic infection, can partly reverse T cell exhaustion without causing fatal immunopathology (Barber, Wherry *et al.* 2006). The $CD8^+$ T cells can be reinvigorated but still display an exhausted phenotype due to additional inhibitory receptors as well as transcriptional and epigenetic changes (Wherry and Kurachi 2015). Checkpoint immunotherapy is an enormous success for the treatment of various cancer types and the administration of checkpoint inhibitors has rapidly become a routine clinical application. Research of the past years has revealed a subset of exhausted $CD8^+$ T cells which can respond to checkpoint immunotherapy and many studies investigate how treatment can be improved. Nevertheless, the underlying mechanisms of the development of T cell exhaustion, the balance between terminal exhaustion and control of the infection as well as of the process of reactivation with anti-PD-1/anti-PD-L1 treatment remain incompletely understood.

In this study, we aimed at elucidating cellular interaction partners of exhausted $CD8^+$ T cells and found that DC are essential for the reinvigoration of such by anti-PD-L1 treatment. However, our data also hint towards a self-limiting scenario in which DC are targeted by the regained cytotoxic functions of the exhausted $CD8^+$ T cells. Moreover, we found that this was the case for the two

subsets of XCR1$^+$ DC and CD11b$^+$ DC. Additionally, this study reveals that these DC subsets display complementary roles. In the absence of XCR1$^+$ DC during checkpoint immunotherapy, exhausted CD8$^+$ T cells are still able to proliferate. These findings reveal that the remaining predominantly CD11b$^+$ DC, are sufficient to deliver critical expansion signals. Nevertheless, we found a supreme role of XCR1$^+$ DC for the maintenance of the memory-like TCF-1$^+$ cell pool during anti-PD-L1 treatment. As we showed that these memory-like TCF-1$^+$ cells display a heterogenous group of cells which could reside in distinct areas within the secondary lymphoid tissues, these subsets likely have different cellular interaction partners including DC subsets and receive diverse signals.

Checkpoint immunotherapy with combinatorial approaches targeting DC to improve therapy outcome are of great interest in the cancer field. There are various DC-targeting approaches such as the administration of adjuvants which boost the endogenous DC compartment. For example, treatments with granulocyte-macrophage colony-stimulating factor (GM-CSF) and FMS-like tyrosine kinase 3 ligand (Flt3L) are used to enhance and support DC development and maturation (Saito, Takayama *et al.* 2008, Yan, Shen *et al.* 2017). Additionally, TLR agonists are administered, which have been shown to activate dysfunctional DC during cancer treatment (Wculek, Cueto *et al.* 2019). In addition to these mediators of DC activation, delivery of tumour-associated antigens (TAAs), which can be cross-presented by DC, leads to improved control of tumour burden (Finn 2017). Both, adjuvants and antigens, can also be directly delivered by coupling to DC-specific antibodies (Bonifaz, Bonnyay *et al.* 2004, Sancho, Mourao-Sa *et al.* 2008). In addition, different DC vaccination approaches have been investigated and are used for cancer treatment. Here, *in vitro* generated or autologous DC are *ex vivo* treated and adoptively transferred into the patient. So far, for human cancer therapy mostly blood monocyte-derived DC have been used (Garg, Coulie *et al.* 2017, Saxena and Bhardwaj 2017). However, in a recent study by Wculek *et al.* XCR1$^+$ DC loaded with tumour antigen were used for this approach (Wculek, Amores-Iniesta *et al.*

2019). This treatment synergized with anti-PD-1 treatment in a murine tumour model.

There are several aspects of this study which can be used for further investigation of processes underlying checkpoint immunotherapy as well as the design of novel DC-targeting treatment approaches:

6.1 Cytotoxic targeting of DC by reactivated CD8$^+$ T cells during immunotherapy

During acute infections and tumour development, DC which activate CD8$^+$ T cells have been described to also be targets of the cytotoxic response by these cells (Hermans, Ritchie *et al.* 2000, Ritchie, Hermans *et al.* 2000, Ludewig, Bonilla *et al.* 2001). Our results point towards a similar scenario during checkpoint immunotherapy. We hypothesize that CD8$^+$ T cell reactivation limits itself by killing of DC. So far, it remains unclear how DC are targeted and unraveling the exact underlying mechanism will help to develop strategies to circumvent it. The cytotoxic CD8$^+$ T cell response during LCMV infection has been shown to be dependent on perforin (Kagi, Ledermann *et al.* 1994, Walsh, Matloubian *et al.* 1994, Nansen, Jensen *et al.* 1999). Interestingly, early studies reported that perforin regulates the expansion of antigen-specific CD4$^+$ and CD8$^+$ T cells (Matloubian, Suresh *et al.* 1999). In the context of checkpoint immunotherapy, killing of DC by reactivated CD8$^+$ T cells could be perforin-mediated as well. However, constant blocking of the perforin pathway will also prevent killing of other infected cells and hence preclude control of the viral load. Therefore, transient inhibition of perforin-mediated killing during anti-PD-L1 treatment could improve therapy outcome. In the context of DC vaccination approaches for the treatment of cancer, improving the survival of transferred DC could also promote improved CD8$^+$ T cell expansion and activation. Some immune cells, such as CD8$^+$ T cells and NK cells have evolved mechanisms to be intrinsically protected from the perforin-mediated cytotoxicity. Structural changes of the membrane including phospholipid modifications can protect

these cells from perforin-mediated killing (Lopez, Brennan *et al.* 2012). Transfer of *in vitro* generated DC, which are protected from this killing mechanism, in combination with checkpoint immunotherapy could be an approach to improve cancer therapy.

6.2 XCR1$^+$ DC preserve memory-like TCF-1$^+$ cells during immunotherapy

Elucidating how XCR1$^+$ DC maintain the pool of exhausted CD8$^+$ T cells which respond to checkpoint immunotherapy, could be used to improve treatment strategies as well. In murine tumour models, transfer of XCR1$^+$ DC synergized with anti-PD-1 treatment (Wculek, Amores-Iniesta *et al.* 2019). Utilizing this DC subset to improve therapy in the context of chronic viral infections could show similar results. Memory-like TCF-1$^+$ cells represent the subset of exhausted CD8$^+$ T cells that can be reactivated by anti-PD-1/PD-L1 treatment (Im, Hashimoto *et al.* 2016, Utzschneider, Charmoy *et al.* 2016). We hypothesize that the expansion of this cell compartment would lead to an improved therapy outcome as more cells can be reactivated by checkpoint inhibitors. This could be achieved with enhancing critical signals transmitted by XCR1$^+$ DC simultaneous to immunotherapy. In this regard, also staggered or alternating treatment regimens could be investigated. Increasing the pool of memory-like cells prior to checkpoint immunotherapy might help to further expand the cytotoxic CD8$^+$ T cell pool in order to fight infected or tumour cells.

6.3 Heterogeneity within the memory-like TCF-1$^+$ cell compartment

We found that the pool of memory-like TCF-1$^+$ cells includes distinct subsets which likely reside in different areas of lymphoid organs and therefore exist in different microenvironments in which they receive various signals by distinct cellular interaction partners. It will be of great interest to explore which of these subsets respond to checkpoint immunotherapy and how. Are these subsets functionally or developmentally connected? If so, this would point to a scenario

where exhausted CD8$^+$ T cells undergo various differentiation steps from a small progenitor memory-like cell pool to the terminally exhausted cells. In melanoma patients, the frequency of TCF-1$^+$ cells serves as a valuable biomarker for the success of checkpoint immunotherapy (Sade-Feldman, Yizhak *et al.* 2018). In case only a subset of the TCF-1$^+$ cell pool responds to anti-PD-1/anti-PD-L1 treatment, the presence and frequency of this subset could be used as an even more precise biomarker. In addition, combination therapies using anti-PD-1/anti-PD-L1 together with blocking antibodies targeting other inhibitory receptors show synergistic effects with improved therapy outcome (Kaufmann, Kavanagh *et al.* 2007, Blackburn, Shin *et al.* 2009). Which cells are directly targeted by these combinatorial treatment approaches remains unclear. However, designing new or improving combination therapies by targeting one or more subgroups of memory-like TCF-1$^+$ cells could further increase treatment efficacy.

Taken together, this study revealed an essential but complex role for dendritic cells during checkpoint immunotherapy. The presented findings provide new insights into the multifaceted parameters which impact the reinvigoration of exhausted CD8$^+$ T cells and underline the importance of different DC subsets in this context. In particular, this involves CD8$^+$ T cell heterogeneity, functional specialization of DC subsets and spatiotemporal aspects of their interaction.
The mouse model of chronic LCMV infection greatly helped to understand various immunological mechanisms in the past decades and it will be of great interest to translate the findings of this study to other chronic infections as well as cancer in humans. Delineating the exact molecular mechanisms will provide the framework for the design of novel and more effective immunotherapeutic treatments in the future.

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