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Characterization of novel determinants of

CD8 T cell differentiation in acute and chronic infections

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Characterization of novel determinants of CD8 T cell differentiation in acute and chronic infections

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1   Summary

Intracellular infections are usually cleared by cytotoxic CD8-positive T cells that specifically target and remove infected cells. During chronic infection and malignant tumors that cannot be cleared by the immune system, CD8 T cells differentiate into so-called exhausted CD8 T cells. This dysfunctional state is characterized by high expression of inhibitory receptors and reduced production of effector cytokines. To investigate novel determinants of CD8 T cell exhaustion, our laboratory compared the gene expression profile of exhausted CD8 T cells with CD8 T cells that developed an acute phenotype. We found, that the function of a large number of molecules that are differentially up- or down-regulated in exhausted CD8 T cells is not yet defined. This applies to the molecule of interest transmembrane protein 51 (*Tmem51*), which is significantly upregulated in CD8 T cells with an exhausted phenotype. TMEM51 is expressed in varying levels in the immune compartment and so far, remains a transmembrane protein of unknown function.

In my thesis, I demonstrated that *in vitro* activated CD8 T cells that were transduced with a *Tmem51* overexpression vector, showed a significant reduction in the production of the effector cytokines interferon-gamma (IFNγ) and tumor necrosis factor-alpha (TNFα). The impact of *Tmem51* overexpression on effector cytokine production was stronger in high affinity stimulated CD8 T cells when compared to low affinity stimulation. To a smaller extent, the reduction in IFNγ and TNFα-producing CD8 T cells that overexpress *Tmem51* could also be seen *in vivo* during acute infection with lymphocytic choriomeningitis virus strain Armstrong53b (LCMV-Arm53b). Nonetheless, the high levels of *Tmem51* did not impact effector and memory T cell development. Because reduced effector cytokine production is a hallmark of exhausted CD8 T cells, I addressed the question whether the deletion of *Tmem51* can reactivate exhausted CD8 T cells. *Tmem51*-deficient CD8 T cells were adoptively transferred into host mice that were infected with LCMV-clone13 (LCMV-cl13) causing a chronic infection. After the first 7 to 14 days post infection, I could observe a significant reduction of *Tmem51*-deficient CD8 T cells over wild-type CD8 T cells. A ratio of 1:2 of knockout over wild-type CD8 T cells was stable from 14 days onward. However, exhausted CD8 T cells were not reactivated in the absence of *Tmem51*. They displayed the same levels of IFNγ and TNFα production and acquired the same phenotype as wild-type exhausted CD8 T cells. In conclusion, the data presented in this thesis indicate *Tmem51* as a potential regulator of effector cytokine production in CD8 T cells.

The manipulation of factors that could reactivate exhausted CD8 T cells for T cell-based immunotherapies of malignant tumors, is a research topic of ever-growing interest. One of these factors is the phosphatase tyrosine-protein phosphatase non-receptor type 2 (*Ptpn2*). It

has recently been shown to be a powerful modulator of exhausted CD8 T cells. The deletion of *Ptpn2* in CD8 T cells during chronic LCMV-cl13 infection leads to enhanced proliferation of terminal exhausted CD8 T cells and results in superior viral control. However, the impact of *Ptpn2* on CD8 T cell differentiation in acute infection and therapeutically relevant settings remained unknown.

In my thesis, I could display that the absence of *Ptpn2* significantly increased the survival of KLRG1-positive and usually short-lived effector CD8 T cells without impacting their ability to form memory CD8 T cells. Interestingly, *Ptpn2*-deficient CD8 T cells showed a significantly higher efficacy in adoptive T cell transfer settings. I found that in the absence of *Ptpn2*, antigen-independent T cell expansion was augmented. This was reflected by a recovery of 4 to 9-fold the number of T cells after adoptively transferring briefly *in vitro* activated T cells into antigen-free host mice. Additionally, the transfer efficacy of *ex vivo* activated T cells into host mice under inflammatory conditions led to an up to 160-fold increase of *Ptpn2*-deficient over wild-type CD8 T cells. Subsequent analysis confirmed improved proliferation and survival but not an increase in initial engraftment to be the cause of higher T cell numbers upon adoptive transfer of *Ptpn2*-deficient CD8 T cells. The enhanced proliferation correlated with wide-ranging changes in the phosphoproteome of CD8 T cells that are deficient for *Ptpn2*. This included the signal transducer and activator of transcription (STAT) protein family, of which STAT3, STAT4, and STAT5 displayed higher transcriptional activity in the absence of *Ptpn2*. A *de novo* pathway enrichment approach linked the observed changes in a causal network and suggested that *Ptpn2*-deficient CD8 T cells are either less dependent on pro-survival signals or more effectively respond to limited cytokine sources. As a functional consequence, *Ptpn2*-deficient T cells that were briefly activated *in vitro* proved to be independent of extrinsic addition of the survival- and proliferation inducing cytokine Interleukin-2. Altogether, the presented data underline the substantial benefits of manipulating the expression of *Ptpn2* for T cell-based therapeutic approaches.

2 Introduction

2.1 Immune response to infection

The immune system's response to infection with pathogens is organized in different layers (**Table 1**). Innate immunity contains the first two layers of defense. The first layer is the non-specific defense versus the entry of pathogens and consists of the skin, membranes, mucus and secretions of antimicrobial substances and the normal microbiota. The second layer of defense is specific to either conserved pathogen-associated molecular patterns or danger-associated molecular patterns. Phagocytic and cytotoxic innate immune cells like macrophages and natural killer cells are able to sense extracellular and intracellular pathogens via conserved pathogenic features such as lipopolysaccharides or free amino acids. For example, the innate immune system senses an endosomal virus, recognizes that it is a virus and induces the expression of various inflammatory stimuli to recruit additional immune cells and activate the complement system (**Table 1**). If the innate immune system reencounters the same virus after some time has passed, it will induce the same exact response. These fast-reacting mechanisms of defense are sometimes not able to clear the infection and an adaptive immune response is initiated (**Table 1**, **Figure 1**).

Table 1 Layers of defense of the immune system

Innate Immunity		Adaptive Immunity
1st layer (non-specific)	**2nd layer** (specific to conserved pathogenic features)	**3rd layer** (specific to one pathogen)

1. Skin
2. Membranes
3. Mucus
4. Secretions of antimicrobial sub-stances
5. Normal microbiota
	1. Innate immune cells
	  a) Macrophages
	  b) Natural killer cells
	2. Inflammation
	3. Complement system
	4. Antimicrobial substances
		Antigen-specific lymphocytes
		1. B cells (Antibodies)
		2. T cells
		  a) CD4-positive helper T cells
		  b) CD8-positive cytotoxic T cells

The adaptive immune response is highly specific to one pathogen because lymphocytes can specifically recognize structural parts of pathogens, so-called antigens. To inform the adaptive immune system of an ongoing infection in distant sites in the body, specialized phagocytes, so-called dendritic cells (DC) are needed. They belong to the innate branch of the immune system but are a crucial link to adaptive immunity. They can phagocytose pathogens and then process and present pathogen-derived antigens on their surface to initiate the adaptive immune response (**Figure 1**). When dendritic cells are activated as a result of an infection,

they egress from the site of infection to specific lymphoid organs. Here, they encounter adaptive immune cells capable of mounting a specific and targeted response, thereby initiating the adaptive immune response (**Figure 1**). One key component in the clearance of intracellular pathogens like bacteria and viruses are cytotoxic cluster of differentiation 8 (CD8)-positive T cells (Bender et al., 1992; Klebanoff et al., 2006). CD8-positive T cells can specifically recognize small peptide fragments derived from the pathogen which are processed and presented by infected cells or by antigen-presenting cells, such as dendritic cells. Upon recognition they become activated effector CD8 T cells, specifically clear the body of all infected cells and build a long-lasting immunological memory of the same pathogen. When CD8 T cells reencounter the same virus after some time has passed, the long-lasting memory cells will induce a much stronger and faster response to quickly resolve the infection.

Adaptive Immunity
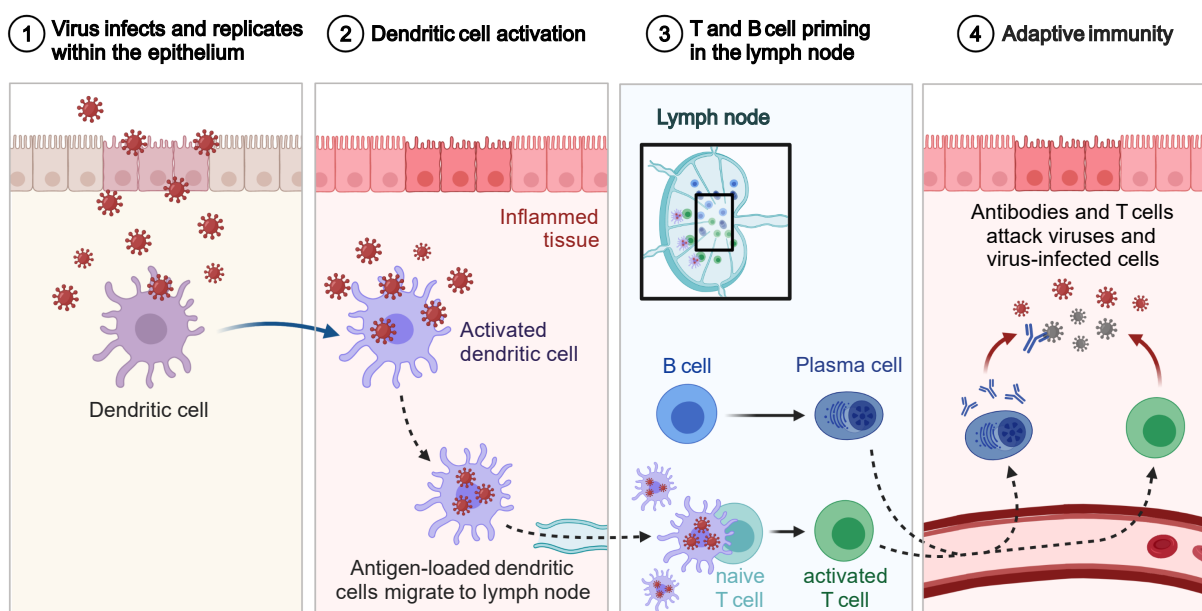
Figure 1 Adaptive immune response to viruses. (1) Infectious agents such as viruses infect and enter the body via epithelial cell layers. (2) These tissues become inflamed and recruit dendritic cells, which take up viruses in the periphery and travel via lymphatic vessels to present virus-derived and processed antigens to adaptive immune cells. (3) Adaptive immune cells such as B and T cells are primed in secondary lymphoid organs such as lymph nodes. (4) Activated plasma cells and T cells exit the lymphoid organs and travel via the blood stream to infected tissues to specifically neutralize infectious agents and kill infected cells. *Created with BioRender.com.*

2.2   Development of naïve CD8 T cells in the thymus

The term naïve describes CD8 T cells that have not yet encountered their specific antigen. First, hematopoietic stem cells differentiate into common lymphoid progenitors in the bone marrow. These egress from the bone marrow and enter the thymus (**Figure 2**). The thymus is the lymphoid organ in which T cells develop into mature but naïve CD4 or CD8-single positive

T cells (CD4 SP, CD8 SP). Thymic T cell development is a multistep process which is tightly regulated and can be monitored by the expression of specific markers of maturation (Zúñiga-Pflücker, 2004). At first, T cells in the thymus undergo maturation steps in which they do not express CD4 and CD8. These are called double negative T cells (DN). The first CD4 and CD8-double negative state (DN1) is characterized by the expression of cluster of differentiation 44 (CD44). Then, T cells in DN2 express CD44 and cluster of differentiation 25 (CD25) and T cells in DN3 only express CD25. At this stage, cells are selected for successful rearrangement of the T cell receptor (TCR)-β chain.
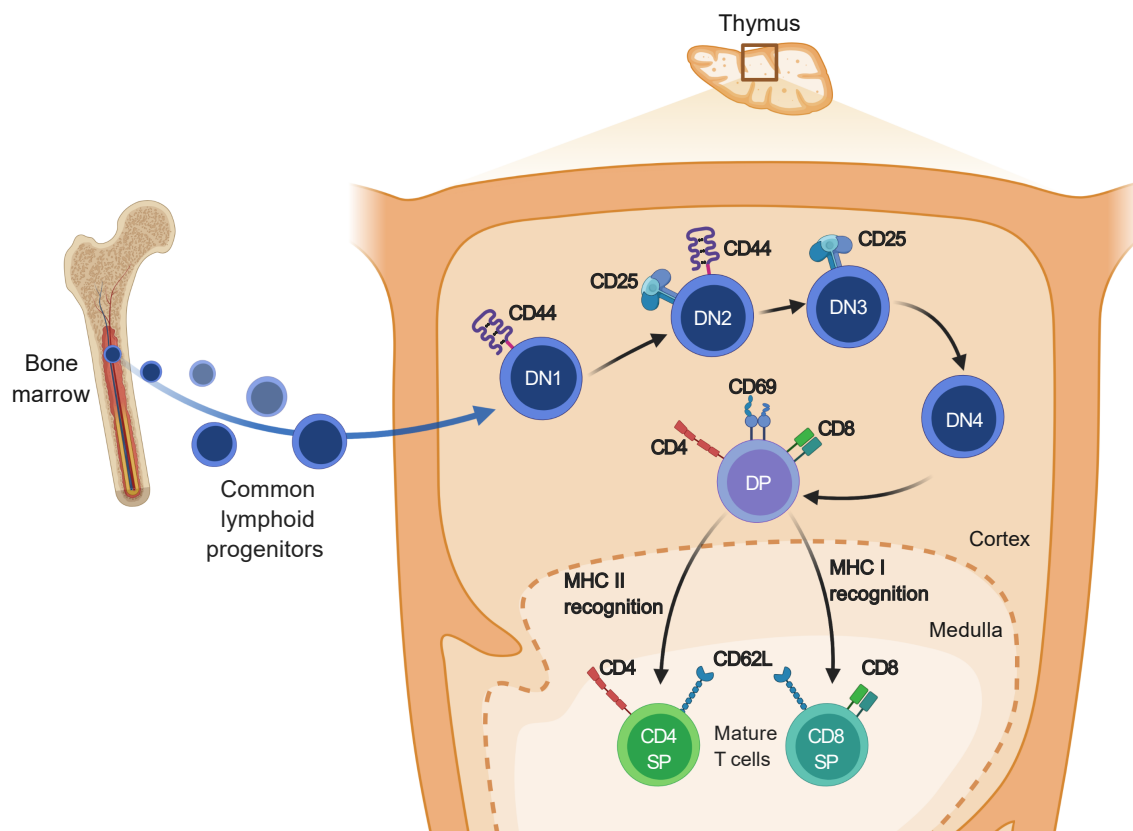
Figure 2 T cell development in the thymus. Common lymphoid progenitors derived from the bone marrow enter the thymus and undergo several maturation steps towards CD4 or CD8-single positive T cells (CD4 SP, CD8 SP). The first four steps are located in the cortex: 1) CD4 and CD8-double negative state (DN1) T cells that express cluster of differentiation 44 (CD44), 2) DN2 T cells that express CD44 and cluster of differentiation 25 (CD25), 3) DN3 T cells that only express CD25, and 4) T cells in the DN4 state do not express CD25 and CD44. These differentiate into CD4 and CD8-double positive T cells (DP). The DP T cells have assembled the T cell receptor and express cluster of differentiation 69 (CD69). The final maturation steps of DP T cells are recognition of major histocompatibility complex class I (MHCI) or MHCII and egress into the medulla where they develop into mature naïve CD4 or CD8-single positive (CD4 SP, CD8 SP) that express L-Selectin (CD62L). *Created with BioRender.com.*

After this, T cells in the DN4 state express no CD44 or CD25 and survive dependent on the TCR-β chain. They subsequently differentiate into CD4 and CD8-double positive (DP) T cells and begin rearrangement of the TCR-α chain to finally assemble the TCR-αβ heterodimer

which is in complex with cluster of differentiation 3 (CD3). Now, T cells are selected for their principal ability to bind self-peptide-MHC ligands, a process termed positive selection. During this process, the maturation marker cluster of differentiation 69 (CD69) is upregulated. While these steps take place in the cortex of the thymus, DP T cells now enter the medulla and interact with specialized DCs that either express major histocompatibility complex class I (MHCI) or MHCII and depending on their interaction T cells develop into CD4 or CD8-single positive T cells that express L-selectin (CD62L) (**Figure 2**), (Zúñiga-Pflücker, 2004). During these differentiation steps in the medulla, a process termed negative selection removes all T cells that are overly reactive to self-peptide-MHC ligands. The resulting T cells are mature naïve T cells that additionally lose the expression of cluster of differentiation 24 (CD24) (Kishimoto & Sprent, 1997; Tani-ichi et al., 2013) and migrate to the blood and secondary lymphoid organs like lymph nodes. They form a pool of polyclonal CD8 T cells in which each clone can potentially recognize a pathogen-derived antigen which is presented by infected cells or DCs.

2.3   CD8 T cell activation

CD8 T cell-based immunity to any foreign antigen is based on a limited number of naïve polyclonal CD8 T cells. They can specifically bind pathogen-derived peptide fragments which are presented on MHCI molecules of antigen presenting cells and infected cells (**Figure 3**).
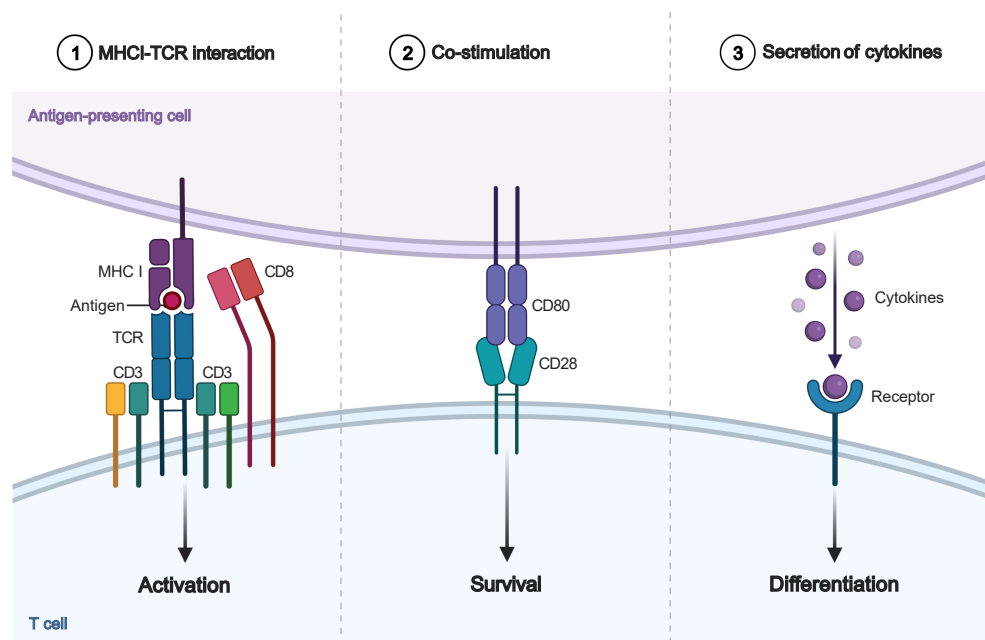
Figure 3 Three signals are required to activate CD8 T cells. The activation of CD8 T cells is dependent on three signals provided by antigen-presenting cells or infected cells. The T cell receptor (TCR) complex consists of the TCR α and β chain that are in complex with cluster of differentiation 3 (CD3) molecules. The co-receptor CD8 directly binds to major histocompatibility class I (MHCI) and the TCR itself binds to the antigen presented by MHCI.

This binding comprises signal one of CD8 T cells activation and is complemented by signal two. The second signal is co-stimulation via cluster of differentiation 80 (CD80, B7) which binds to the receptor cluster of differentiation 28 (CD28) on T cells and leads to T cell survival. The third signal is provided by inflammatory cytokines that lead to full CD8 T cell activation and differentiation. *Created with BioRender.com.*

The activation, or priming, of naïve CD8 T cells is dependent on three types of signals that are provided by antigen-presenting cells, such as dendritic cells. The first signal is generated via the aforementioned interaction of the peptide:MHCI (p:MHCI) with the T cell receptor complex. The specificity of the T cell receptor is determined by the variable T cell receptor α and β chains. By itself, these do not form a stable complex and are stabilized by the cluster of differentiation 3 (CD3) complex (Brenner et al., 1985; Call et al., 2002; Kuhns & Davis, 2012; Samelson et al., 1985). Together with the T cell receptor chains, the CD3 molecules form the stable T cell receptor complex. The intracellular signal transduction is mainly mediated by phosphorylation of cytoplasmatic regions that are called immunoreceptor tyrosine-based activation motifs (Klausner & Samelson, 1991; Kuhns & Davis, 2012; Tolar et al., 2009; Weiss & Littman, 1994). Nonetheless, the interaction of the p:MHCI with the TCR is not sufficient to fully activate CD8 T cells. The second signal required is the interaction of co-stimulatory molecules of the antigen-presenting cell with the T cell. The molecule cluster of differentiation 28 (CD28) is a well-described receptor of the co-stimulatory molecule cluster of differentiation 80 (CD80, B7). Their interaction induces T cell survival and proliferation (Bour-Jordan & Bluestone, 2002; L. Chen & Flies, 2013; Gonzalo et al., 2001; Greenwald et al., 2005; Kapsenberg, 2003; Wang et al., 2000). However, without a third signal, T cells respond poorly and are eventually depleted. For a productive response that leads to strong effector functions and survival, inflammatory cytokines are essential. These provide the third signal that is needed for full CD8 T cell activation (Mescher et al., 2006). Major cytokines that can provide signal three for CD8 T cells have been identified as Interleukin-12 (IL-12) and type I interferons (IFNα and IFNβ) (Curtsinger et al., 2005; Curtsinger & Mescher, 2010; Mescher et al., 2006; Schmidt & Mescher, 1999; Sikora et al., 2009). The source of type I interferons can be found in varying cell types, such as infected cells and DCs and IL-12 is mainly provided by antigen-presenting cells (Ali et al., 2019; Hamza et al., 2010). Fully activated CD8 T cells proliferate strongly to mount a robust and targeted response to a specific pathogen.

2.4   Magnitude of CD8 T cell responses

The extent in which CD8 T cells respond to a pathogen is called the magnitude of response and majorly depends on the range (Deng et al., 2015; van Deutekom et al., 2013) and identity (Kiepiela et al., 2007; Moutaftsi et al., 2009) of epitopes that are presented (Tscharke et al., 2015). Additionally, the amount of naïve T cells reacting to a specific epitope as well as the

strength of the interaction, namely the affinity of the TCR to the peptide:MHCI complex (pMHCI) can determine the magnitude of the immune response (Jenkins & Moon, 2012). This has been demonstrated with the use of OT-1 TCR-transgenic CD8 T cells. These T cells specifically react to the ovalbumin-derived antigen $OVA_{257-264}$ (SIINFEKL peptide) and are widely used to study the immune response of antigen-specific CD8 T cells. For example, OT-1 CD8 T cells expressing a distinct congenic marker can be adoptively transferred into host mice in low numbers. Upon infection with a pathogen that expresses the SIINFEKL peptide, the immune response of the previously transferred OT-1 CD8 T cells can be tracked with the use of the congenic marker because it is different from the host mice. To study the impact of peptide affinity on the magnitude of CD8 T cells expansion, the SIINFEKL peptide was modified to bind the TCR with varying affinity. In detail, altered peptide ligands (APL) (high to low affinity: SIINFEKL, SAINFEKL, SIYNFEKL, SIIQFEKL, SIITFEKL, SIIVFEKL) were used to determine the impact of affinity on the time in which CD8 T cells expanded during the initial expansion phase (Zehn et al., 2009). Peptides with strong affinity led to a larger magnitude of expansion, and peptides with weak interactions induced low levels of expansion. However, even very weak interactions resulted in full activation of CD8 T cells (Zehn et al., 2009). This means that the basic principle of CD8 T cell activation and differentiation into effector and memory T cells happens independently of affinity and the range of epitopes. Employing the same TCR-transgenic system, it has been demonstrated that recruitment of high affinity CD8 T cells is so efficient that almost all naïve CD8 T cells are recruited during the response phase (Van Heijst et al., 2009). The recruitment was independent of the dose of antigen and consequently, the magnitude of the response is most likely determined by strong clonal expansion of high affinity stimulated T cells.

2.5   Differentiation of CD8 T cells in acute infections

When naïve CD8 T cells are activated by antigen, co-stimulation and inflammation they undergo a specific program of clonal expansion and differentiate into short-lived effector and long-lasting memory CD8 T cells. This differentiation leads to distinct changes in cell cycle, metabolism, effector function, and immune cell migration that are governed by the expression of a set of molecules that defines each specific T cell state (**Figure 4**) (Doering et al., 2012; Kaech et al., 2002; Kaech & Cui, 2012; Muroyama & Wherry, 2021; Windt & Pearce, 2012). These changes are dependent on a network of transcription factors downstream of TCR-signaling. Differentiation into activated precursors and functional effector T cells is dependent on the Basic Leucine Zipper ATF-Like Transcription Factor (BATF) (Kurachi et al., 2014). Especially, early differentiation into effector CD8 T cells is dependent on BATF and it can activate important transcription factors for effector development, such as T-box expressed in

T cells (T-bet) and B-lymphocyte-induced maturation protein 1 (Blimp-1) (Nutt et al., 2007; Welsh, 2009). During expansion, CD8 T cells segregate into cluster of differentiation 127-positive and killer cell lectin-like receptor G1-negative ($CD127^+KLRG1^-$) cells, which are thought to develop into memory T cells (Kaech et al., 2003), and $CD127^-KLRG1^+$ cells, which are thought to be terminally differentiated effector cells (**Figure 4**) (Joshi et al., 2007). There are several transcription factors necessary for the development of these subsets. On the one hand, the $CD127^-KLRG1^+$ effector phenotype is mainly driven by the transcription factors Blimp-1, T-bet and inhibitor of DNA binding 2 (Id2) (Cannarile et al., 2006; Intlekofer et al., 2005; Joshi et al., 2007; Nutt et al., 2007; Sullivan et al., 2003; Welsh, 2009). After multiple cycles of proliferation, terminally differentiated CD8 T cells acquire a reduced proliferative potential and become senescent with expression of high levels of KLRG1. On the other hand, the $CD127^+KLRG1^-$ memory phenotype is dependent on expression of T cell factor 1 (TCF1), Eomesodermin (Eomes), as well as several others (**Figure 4**) (Banerjee et al., 2010; Intlekofer et al., 2005; Kaech & Cui, 2012; Pearce et al., 2003; Sullivan et al., 2003; Zhou et al., 2010). The majority of cells in the effector and memory lineages seem to be committed to their specific program. Nonetheless, there is also growing evidence for the plasticity of CD8 T cells. For example, $CD127^-KLRG1^+$ cells were previously believed to be terminal effector cells because they seemed to be unable to re-expand upon secondary challenge. But Herndler-Brandstetter et al., 2018, were able to demonstrate that $CD127^-KLRG1^+$ can differentiate into all memory cell lineages using an advanced fate mapping approach. This plasticity was evident in a small subset of the $CD127^-KLRG1^+$ but nonetheless demonstrates that the complexity of CD8 T cell development is not fully understood to date. Upon activation and clonal expansion, effector populations are able to effectively recognize the foreign antigen and eliminate infected cells. After the complete elimination of the foreign antigen, the so-called pathogenic clearance, the adaptive immune response returns to a homeostatic state (**Figure 4**). In this state, a heterogeneous central memory CD8 T cell population persists. This population is maintained by the expression of TCF1 and Eomes and marked by the expression of the markers CD127 and L-selectin (CD62L) (Banerjee et al., 2010; Z. Chen et al., 2019; Jeannet et al., 2010). The central memory CD8 T cells persist for a long time and are essential for rapid responses to the same antigen.
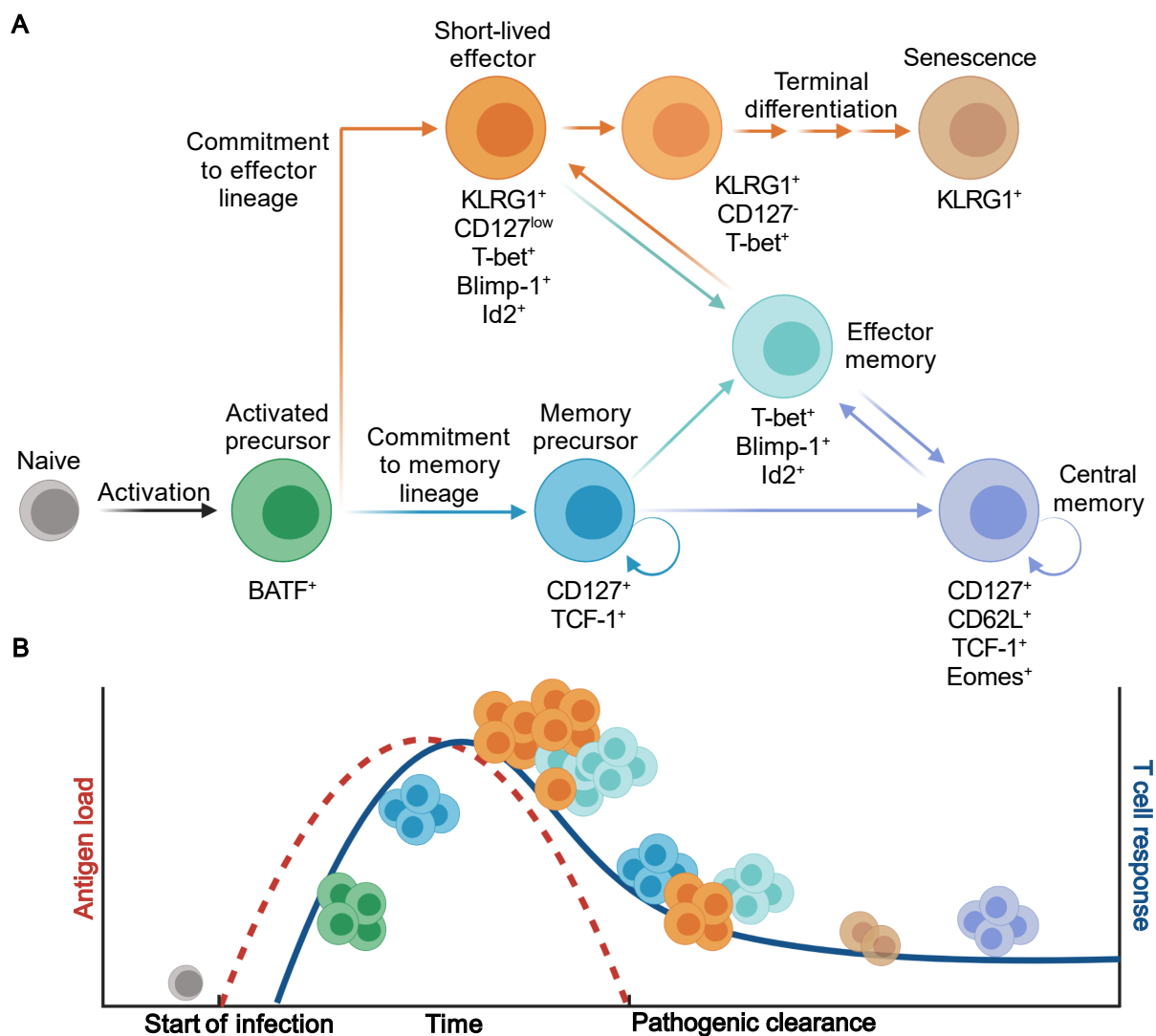
Figure 4 T cell response and differentiation in acute infections. A) Antigen, co-stimulation, and inflammation activated CD8 T cells first develop into effector precursor CD8 T cells. This is dependent upon the Basic Leucine Zipper ATF-Like Transcription Factor (BATF). In turn, BATF recruits T-box expressed in T cells (T-bet) and B-lymphocyte-induced maturation protein 1 (Blimp-1) and CD8 T cells commit to the effector lineage. At the same time, activated precursors also differentiate towards the memory lineage. Memory T cells are cluster of differentiation 127-positive (CD127+) and effector CD8 T cells are killer cell lectin-like receptor G1-positive (KLRG1+). The effector KLRG1+ phenotype is driven by the transcription factors Blimp-1, T-bet and inhibitor of DNA binding 2 (Id2). The terminally differentiated effector cells become senescent and are marked by reduced proliferation and high expression of KLRG1. The differentiation of CD127+ memory precursor CD8 T cells depends on expression of T cell factor 1 (TCF1) and Eomesodermin (Eomes). These memory precursors can directly develop into central memory CD8 T cells marked by the expression of CD127, L-selectin (CD62L), TCF1, and Eomes or into effector memory CD8 T cells. Effector memory CD8 T cells show features of effector and memory CD8 T cells and can give rise to effector as well as central memory CD8 T cells. **B)** Schematic of T cell response magnitude and occurrence of effector and memory CD8 T cell populations in relative dependence of the time after infection. The color-coding refers to the different subsets portrayed in A). *Created with Biorender.com.*

2.6   Differentiation of CD8 T cells in chronic infection

Whereas acute infections are rapidly resolved by the removal of the pathogen, the situation during chronic infections and cancer is different. Here, key factors that determine CD8 T cell differentiation are persisting antigen stimulation, a different level of inflammation, and inhibitory co-stimulation (**Figure 5**). In the setting of chronic infection, CD8 T cells form highly specialized, so-called exhausted CD8 T cells (**Figure 5**) (Blank et al., 2019; McLane et al., 2019; Muroyama & Wherry, 2021). Exhausted CD8 T cells are characterized by a reduction of effector functions, ongoing expression of inhibitory receptors (IRs), and novel epigenetic and metabolic profiles (Blank et al., 2019; McLane et al., 2019; Muroyama & Wherry, 2021). Many discoveries about exhausted CD8 T cells stem from acute and chronic lymphocytic choriomeningitis virus (LCMV) infection models in mice (Gallimore et al., 1998; Zajac et al., 1998). However, CD8 T cell exhaustion has also been shown to play a role in human chronic viral infections, such as human immunodeficiency virus (HIV) (Day et al., 2006; Goepfert et al., 2000; Pietersma et al., 2010; Shankar et al., 2000), hepatitis C virus (HCV) (Gruener et al., 2001; Lechner et al., 2000), and hepatitis B virus (HBV) (Ye et al., 2015). Exhausted CD8 T cells also arise in cancer and are thought to significantly contribute to immune dysfunction (Zarour, 2016). A major focus lies on immunotherapeutic interventions that rely on reactivating exhausted CD8 T cells for treatment of chronic infection and cancer. The first step was the discovery that blocking the inhibitory receptor programmed cell death (PD1) led to a functional recovery of exhausted CD8 T cells (Barber et al., 2006). As a consequence, research in the last years focused on advancing the knowledge about how exactly exhausted CD8 T cells develop to further advance the benefits of T cell-based therapeutic approaches. Initially, it was proposed that exhausted CD8 T cells are terminally differentiated and previously functional effector CD8 T cells. However, it became evident that exhausted CD8 T cells contain a subset of progenitor cells that constantly replenishes the pool of terminal exhausted CD8 T cells (Im et al., 2016; Utzschneider et al., 2016). These findings suggested that a revision of the earlier models was necessary and that the population of exhausted CD8 T cells is phenotypically heterogenous. The exact molecular mechanisms of the development of exhausted CD8 T cells are still being investigated but several key factors and developmental stages have been identified (**Figure 5**). The very early fate decisions of development towards the exhausted lineage can be found shortly after chronic infection and first manifests in the development of **activated precursor** CD8 T cells that express the transcriptional regulator T cell factor 1 (TCF1) (**Figure 5**) (Beltra et al., 2020; Z. Chen et al., 2019; Utzschneider et al., 2020). These commit towards development of the exhausted T cell lineage and give rise to TCF1-positive **exhaustion progenitor 1** CD8 T cells. They display a strong re-expansion potential, are

maintained long term and give rise to **exhaustion progenitor 2** CD8 T cells (Beltra et al., 2020; Romee et al., 2016; Utzschneider et al., 2013, 2016) (**Figure 5**).

A

Commitment to exhausted lineage

Exhaustion progenitor 1	Exhaustion progenitor 2	Exhaustion intermediate	Exhaustion terminal

Naive → Activation → Activated precursor (TCF-1$^+$)

Exhaustion progenitor 1:
TCF-1$^+$
PD-1^{low}
Tox$^+$
Ly108$^+$
CD69$^+$

Exhaustion progenitor 2:
TCF-1$^+$
PD-1^{low}
Tox$^+$
Ly108$^+$
CD69$^-$

Exhaustion intermediate:
TCF-1^{low}
PD-1^{int}
Tox^{low}
Ly108$^-$
CD69$^-$
TIM3$^+$
T-bet$^+$

Exhaustion terminal:
TCF-1$^-$
PD-1^{high}
Tox^{high}
Ly108$^-$
CD69$^+$
TIM3$^+$
Eomes$^+$

B

Antigen load / T cell response vs Time

Start of infection — Partly controlled infection — No pathogenic clearance

Figure 5 T cell response and differentiation in chronic infection. A) The complex dynamics of differentiation towards the exhausted lineage begins with the development of progenitor exhausted CD8 T cells that express T cell factor 1 (TCF1), SLAM family member 6 (Ly108), and cluster of differentiation 69 (CD69). The initial development and maintenance of exhausted CD8 T cells is dependent on the transcription factor thymocyte selection-associated high mobility group box protein (TOX) and TCF1. The two exhaustion progenitor subsets 1 and 2 can convert into each other. A distinction is the loss of CD69 expression and increased proliferation of progenitor exhausted subset 2. The second subset is also thought to be the direct progeny of the intermediate differentiated exhausted CD8 T cells. These express the inhibitory receptors programmed cell death 1 (PD1) and T cell immunoglobulin domain and mucin domain 3 (TIM3) and lose expression of Ly108 and TCF1. The exhaustion intermediate subset also shows reduced TOX expression and increased levels of T-box expressed in T cells (T-bet). The terminal differentiation state of exhausted CD8 T cells is accompanied by an increased expression of Eomesdermin (Eomes), TOX, PD1, TIM3, and a reduced expression of T-bet. **B)** Schematic of T cell response magnitude and occurrence of exhausted CD8 T cell subsets in relative dependence of the time after chronic infection. The color-coding refers to the different subsets portrayed in A). *Created with Biorender.com.*

The two subsets of exhaustion progenitor CD8 T cell subsets can be identified by the expression of the SLAM family member 6 (SLAMF6, Ly108), and C-X-C chemokine receptor type 5 (CXCR5) (Brummelman et al., 2018; Im et al., 2016). They have similar characteristics and can convert into each other but differ in their location and proliferative potential. On the

one hand, **exhaustion progenitor 1** CD8 T cells are mainly found in lymphoid organs and show the expression of the activation and tissue retention marker cluster of differentiation 69 (CD69) (**Figure 5**) (Beltra et al., 2020). On the other hand, **exhaustion progenitor 2** CD8 T cells lose the expression of CD69, show increased proliferation and are mainly found in circulation (Beltra et al., 2020). One major transcriptional regulator that is essential for the development and maintenance of all exhausted CD8 T cell subsets is thymocyte selection-associated high mobility group box protein (TOX) (Alfei et al., 2019; Khan et al., 2019; Scott et al., 2019; Seo et al., 2019; Yao et al., 2019). The expression of TOX is upregulated upon stimulation of the TCR and its expression can be detected at high levels in exhausted CD8 T cell subsets (**Figure 5**) (Alfei et al., 2019; Beltra et al., 2020; Blank et al., 2019). To track the LCMV-specific response of CD8 T cells, TCR-transgenic CD8 T cells that recognize the LCMV-derived glycoprotein[33-41] epitope (gp33), so-called P14 CD8 T cells were used (Mombaerts et al., 1992; Pircher et al., 1989). In adoptive transfer experiments, the deletion of TOX initially increases the number of polyfunctional P14 T cells that mediate increased immunopathology. This response is short-lived and all subsets of exhausted P14 T cells rapidly decline in their numbers (Alfei et al., 2019; Khan et al., 2019; Scott et al., 2019; Seo et al., 2019; Yao et al., 2019). These findings indicate that TOX is necessary for the development of both **exhaustion progenitor** subsets as well as a persistent antiviral CD8 T cell response. The **exhaustion progenitor 2** CD8 T cells continuously give rise to the **intermediate differentiated exhausted** CD8 T cells that express higher levels of the inhibitory receptors PD1 and are the first subset to express T cell immunoglobulin domain and mucin domain 3 (TIM3) while losing the expression of Ly108 and TCF1. The transition towards **intermediate differentiated exhausted** CD8 T cells is thought to be regulated by a reduction of TOX expression which leads to increased expression and activity of T-bet which in turn enforces the maintenance of the intermediate exhausted CD8 T cells (**Figure 5**) (Beltra et al., 2020; Buggert et al., 2014; Hudson et al., 2019). **Intermediate exhausted** CD8 T cells show a higher cytotoxic activity in the form of expression of Interferon-γ (IFN-γ), tumor necrosis factor-alpha (TNF-α) and higher levels of granzyme B (Gzmb) and are able to partly control the chronic infection by keeping viral titers at a stable level. The final differentiation step towards **terminally exhausted** CD8 T cells is accompanied by an increased expression of TOX, PD1 and other inhibitory receptors, and a reduced expression of T-bet and high expression of the transcription factor Eomesdermin (Eomes) (**Figure 5**) (Beltra et al., 2020; Buggert et al., 2014; Hudson et al., 2019). The **terminally exhausted** CD8 T cell subset has less proliferative potential and reduced cytotoxic activity compared to the intermediate subset (Beltra et al., 2020; Buggert et al., 2014; Hudson et al., 2019). In summary, the exhausted CD8 T cell lineage has complex developmental characteristics and distinct subsets with specific features. Despite these wide-ranging findings about the molecular mechanism by which exhausted CD8

T cells develop, the first developmental steps right after activation are still not clearly defined. In addition, the mechanism by which the production of cytotoxic cytokines is reduced in exhausted cells is not fully understood. In this thesis, I aimed to explore immediate effectors by which the production of cytotoxic cytokines such as IFN-γ and TNF-α is reduced.

2.7   Transmembrane protein 51 – an unknown factor in T cell development and function

To further elucidate the early development of exhausted CD8 T cells and to find molecules that immediately regulate specific functions of exhausted T cells, our laboratory performed comparative gene-array analysis of CD8 T cells derived from mice infected chronically with lymphocytic choriomeningitis virus clone 13 (LCMV-cl13) and CD8 T cells that developed during acute LCMV-Armstrong53b (LCMV-Arm53b) infection. We found that the functions in respect to the exhausted phenotype of a majority of molecules that are differentially up- or down-regulated in chronic infection remain poorly defined. One of these molecules is transmembrane protein 51 (*Tmem51*), which is significantly upregulated in P14 T cells differentiated in chronic LCMV-cl13 infection compared to acute infection with LCMV-Arm53b.

TMEM51 is a transmembrane protein of, so far, unknown function. It is almost ubiquitously expressed (Papatheodorou et al., 2018) in varying levels and in the immune compartment it shows high expression in macrophages, dendritic cells, monocytes and gets upregulated upon infection in T cells (Heng et al., 2008; Shay & Kang, 2013). Nonetheless, the molecules' function is unknown and it is only implicated in some studies to be strongly mutated in cutaneous squamous cell carcinomas which have a high mutational burden (Inman et al., 2018).

Structurally, it consists of a small N-terminal part followed by two helical transmembrane domains and a larger C-terminal part in which predictions show several protein binding sites. Based on a high-throughput human interactome network (Rolland et al., 2014), TMEM51 has several immunologically relevant interaction partners. For instance, protein tyrosine phosphatase, non-receptor type 5 (PTPN5), linking TMEM51 to MAPK1/ERF signaling as well as programmed cell death 4 (PDCD4), linking TMEM51 to Pi3K/Akt signaling (Palamarchuk et al., 2005; Zhen et al., 2016). Both pathways are integral to signal transduction upon T cell stimulation. Altogether, its expression pattern in exhausted CD8 T cells and the clear links to immunologically relevant interaction partners highlight the importance of the investigation of TMEM51 as a protein of interest in CD8 T cell responses to infection.

2.8   Protein tyrosine phosphatase non-receptor type 2 impacts autoimmune diseases and key pathways of CD8 T cell signaling

A potent regulator of the development of exhausted CD8 T cells in chronic infection has recently been found in the phosphatase protein tyrosine phosphatase non-receptor type 2 (PTPN2). LaFleur et al., demonstrated in 2019 that PTPN2 can have an impact on the response of exhausted CD8 T cells in chronic infection. They established that PTPN2 attenuates the generation, proliferation, and cytotoxicity of the TIM3-positive intermediate and terminal exhausted CD8 T cell subsets without impacting the numbers and function of the Ly108-positive progenitor exhausted CD8 T cells in chronic LCMV-cl13 infection.

PTPN2 is ubiquitously expressed but resting and activated T cells express especially high levels of PTPN2 (Lu et al., 2007; Shields et al., 2013). Initially, research on PTPN2 was increased as several single nucleotide polymorphisms (SNP) could be linked to a predisposition for the development of autoimmune diseases. In detail, genome-wide association studies (GWAS) connected a susceptibility to develop type 1 diabetes (Burton et al., 2007; Todd et al., 2007), rheumatoid arthritis, Crohn's diseases, and celiac disease (Espino-Paisan et al., 2011; Festen et al., 2011; Smyth et al., 2008) to several associated SNP in the locus of *Ptpn2*. For example, individuals that carry a single copy of the intronic SNP rs1893217(C) can display a decrease in mRNA expression levels of *Ptpn2* by up to -0.44 in CD4 T cells (Long et al., 2011). Patients that carry this risk allele express a fully functional PTPN2 that is reduced in its abundance. However, they are still more prone to develop autoimmune disorders, which highlights the impact of PTPN2 on immune cell activity.

Consequent studies provided evidence of the mechanism of action of PTPN2 via demonstrating direct dephosphorylation of Src-family kinases lymphocyte-specific protein tyrosine kinase (Lck) and proto-oncogene tyrosine-protein kinase (Fyn) by PTPN2 (Wiede et al., 2011; Zikherman & Weiss, 2011). Both, Lck and Fyn are key regulatory molecules downstream of TCR signaling in CD4 and CD8 T cells. Earlier, a non-conditional homozygous knockout of *Ptpn2* in mice was lethal by three to five weeks of age, with symptoms of runting, splenomegaly, and lymphadenopathy (You-Ten et al., 1997). More specifically, defects in bone marrow (BM), B cell lymphopoiesis, and compromised T and B cell functions were present. Nonetheless, T cell development in the thymus was not affected (You-Ten et al., 1997). A conditional, T cell-specific knockout in mice based on Cre recombinase expression under the Lck promoter led to an increased antigen sensitivity *in vitro* and *in vivo*. In homozygous as well as heterozygous mice, autoreactive T cells developed and displayed enhanced proliferative potential (Wiede et al., 2011, 2014). Additional studies demonstrate the

effect of PTPN2 on cytokine signaling. It is able to directly dephosphorylate Janus-activated kinases (JAK)-1,3 and signal transducers and activator of transcription (STAT)-1,3,5,6 (Fukushima et al., 2010; Loh et al., 2011; Shields et al., 2013; Tiganis & Bennett, 2007). Altogether, reduced activity of PTPN2 can increase TCR as well as cytokine signaling. The impact can be so profound, that mice carrying *Ptpn2*-deficient CD4 and CD8 T cells can develop progressive inflammation and spontaneous autoimmunity (Wiede et al., 2011; Zikherman and Weiss, 2011).

These extensive effects of PTPN2 on T cell signaling in the context of autoimmunity and the impact on the function of exhausted CD8 T cell subsets highlighted PTPN2 as an attractive target for T cell-based immunotherapies of malignant diseases (LaFleur et al., 2019; Manguso et al., 2017; Wiede & Tiganis, 2017). Nonetheless, it was not known how PTPN2 affects T cell differentiation in acute infections. To date, no apparent deficits of *Ptpn2*-deficient CD8 T cells have been described in terms of clonal expansion, effector function, and the formation of auto- or foreign antigen-specific memory T cells (Bousso et al., 1999; Busch et al., 1998; Hou et al., 1994; Murali-Krishna et al., 1998).

2.9   Aim of this thesis

During chronic infection and cancer, CD8 T cells can develop a state of dysfunction known as T cell exhaustion. Exhausted CD8 T cells are thought to play a major role in immune dysfunction and reactivating them is a major goal in immunotherapies. A more detailed understanding of the molecular mechanisms that govern the differentiation and specific features of exhausted CD8 T cells in these chronic diseases are needed to further advance T cell-focused therapeutic approaches.

Gene expression analysis of our laboratory marked a large number of molecules of interest with differential expression patterns in chronic versus acute infection models in mice. For many of these, functions in the development of CD8 T cells have not been clearly defined. One of these factors is the molecule transmembrane protein 51 (*Tmem51*) which is expressed in higher levels in CD8 T cells derived from chronically infected mice. In this thesis, I aimed at elucidating the specific mechanisms by which the insufficiently described factor *Tmem51* contributes to the CD8 T cell response during acute and chronic infections. In detail, I aimed to address the following questions:

1.) Does overexpression of *Tmem51* in *in vitro* activated CD8 T cells lead to the acquisition of distinct phenotypic or functional characteristics of exhausted CD8 T cells?

2.) Can *in vivo* deletion of *Tmem51* during chronic infection lead to reactivation in terms of cytotoxic and proliferative capacity of exhausted CD8 T cells?

An additional molecule, that has recently been described to play a major role in the context of the development of exhausted CD8 T cells is protein tyrosine phosphatase non-receptor type 2 (*Ptpn2*). It has also been highlighted as a potential target for T cell-based immunotherapies. To strengthen this notion, I aimed to elucidate the impact of the loss of *Ptpn2* in CD8 T cells during acute infections and in the setting of adoptive T cell therapies by addressing the following questions:

1.) How does the loss of *Ptpn2* impact the differentiation of effector versus memory CD8 T cells during acute infection?

2.) Can the deletion of *Ptpn2* increase the efficacy of adoptive T cell therapies?

3.) Which molecular mechanisms in activated CD8 T cells are altered in the absence of *Ptpn2*?

3   Material and methods

3.1   Surface and intracellular protein antibody staining and flow cytometry analysis of murine cells

Extracellular proteins were stained with the listed antibodies (**Table 2 Antibodies**) for 30 min at 4 °C in fluorescence-activated cell sorting (FACS) buffer (2% fetal calf serum (FCS) (Sigma Aldrich) was added to phosphate buffered saline (PBS) (Invitrogen) with 0.01% sodium azide (Sigma-Aldrich). Afterwards, cells were fixed for 10 min at room temperature (RT) in PBS which was supplemented with 4% formaldehyde. When intracellular proteins were stained, the cells were permeabilized with FACS buffer supplemented with 0,02% saponin (permeabilization buffer). The antibodies used for intracellular labeling of proteins and cytokines were diluted in permeabilization buffer and the cells were stained for 40 min at 4 °C. The staining of transcription factors was performed with the FoxP3 TF staining kit (eBioscience, 00-5523-00) in accordance with the manufacturer's instructions. Then, the cells were resuspended in FACS buffer and the flow cytometry analysis was performed on an LSR-Fortessa. Data analysis were performed using FlowJo (v10.3, TreeStar).

Table 2 Antibodies used in this work

Antibody (clone)	Source	Identifier
CD44 (IM7)	Biolegend	103012
CD25 (PC61.5)	eBioscience	25-0251-82
CD8α (53-6.7)	Biolegend	100714
CD45.1 (A20)	eBioscience	48-0453-82
CD45.2 (104)	eBioscience	17-0454-82
CD127 (A7R34)	eBioscience	17-1271-82
KLRG1 (2F1)	eBioscience	48-5893-82
CD69 (H1.2F3)	Biolegend	104502
CD4 (RM4-4)	Biolegend	116013
PD-1 (J43)	eBioscience	14-9985-82
CD62L (MEL-14)	eBioscience	12-0621-82
APC-conjugated gp33 tetramers	TCMetrix	N/A
IFNγ (XMG1.2)	eBioscience	12-7311-82
TNFalpha (MP6-XT22)	eBioscience	14-7321-81
EOMES (Dan11mag)	eBioscience	12-4875-82
T-bet (eBio4B10)	eBioscience	eBio4B10

3.2   Mice and adoptive T cell transfers

OT-1;*Lck*-Cre;*Ptpn2*$^{fl/fl}$ (knockout: ko) and OT-1;*Ptpn2*$^{fl/fl}$ (wild-type: wt) mice have been described previously (Wiede et al., 2011). Briefly, Lymphocyte-specific protein tyrosine kinase (Lck) driven expression of Cre recombinase leads to LoxP-mediated excision of gene

expression of the fifth exon of *Ptpn2*. The active site of *Ptpn2* is encoded by the fifth exon and no active protein is expressed in CD8 T cells derived from these mice (Wiede et al., 2011). *Ptpn2*-deficient donor cells were adoptively transferred into CD45.1 congenic C57BL/6J host mice (Schluns et al., 2002) for proliferation tests and infection experiments. In some experiments, wt (CD45.1/2) and *Ptpn2*-deficient OT-1 T cells (CD45.2/2) were co-transferred into CD45.1 congenic host mice. Mice for the work with *Tmem51* were bred in our mouse facility at the Technical University of Munich. The TCRαβ-transgenic P14 mice that are specific for the LCMV gp33 peptide were provided by A. Oxenius (Pircher et al., 1989). The gp33 peptide is one of three immunodominant LCMV-derived epitopes and in polyclonal mice, about 50 % of all T cells that react to LCMV are specific for gp33 (Hudrisier et al., 1997; Moskophidis & Zinkernagel, 1995). The initial *Tmem51*-knockout mouse strain C57BL/6N-*Tmem51*[tm1a(EUCOMM)Hmgu] was generated by the trans-NIH Knockout Mouse Project (KOMP) in cooperation with the EUCOMM/KOMP-CSD ES cell resource program and obtained from the MRC Harwell institute with the specific identifier HEPD0725_6_C05. To acquire conditional knockout mice, the C57BL/6N-*Tmem51*[tm1a(EUCOMM)Hmgu] line was crossed with a flippase (FLP)-deleter strain to eliminate the LacZ reporter. This converted their designation into C57BL/6N-*Tmem51*[tm1c(EUCOMM)Hmgu] mice. These are mice where the first exon of *Tmem51* is flanked by LoxP sites and are further designated as *Tmem51*^{fl/fl}. The progeny of these mice was crossed with Mx1[cre], or ERT2[cre], or Cd4[cre], -Rosa26[STOP-eYFP] (Jackson Laboratories) and P14 transgenic mice. Subsequent crossing led to the generation of three strains of quadruple transgenic mice:

(1) P14;Mx1-Cre;Rosa26-Stop^{fl/fl}-eYFP;*Tmem51*^{fl/fl}
(2) P14;CD4-Cre;Rosa26-Stop^{fl/fl}-eYFP;*Tmem51*^{fl/fl}
(3) P14;Gzmb-ERT2/Cre;Rosa26-Stop^{fl/fl}-eYFP;*Tmem51*^{fl/fl}

These three mouse strains were used as genetically matched wild-type controls:

(1) P14;Mx1-Cre;Rosa26-Stop^{fl/fl}-eYFP
(2) P14;CD4-Cre;Rosa26-Stop^{fl/fl}-eYFP
(3) P14;Gzmb-ERT2/Cre;Rosa26-Stop^{fl/fl}-eYFP

The conditional knockout of *Tmem51* is mediated as follows:

Strain (1) conditional knockout and wild-type control mice that express Cre recombinase under the Mx dynamin-like GTPase 1 (Mx1)-promoter were treated with three times 200 µg polyinosinic:polycytidylic acid (P1530, Sigma Aldrich) (polyI:C) via i.p. injection to activate the promotor in an interferon-dependent manner. PolyI:C was dissolved in PBS to 20mg/ml. Donor cells expressing eYFP as the reporter for successful deletion of the target gene, were then transferred into host mice which were infected with LCMV-cl13 **(Figure 11A)**.

Strain (2) conditional knockout and wild-type control mice that express Cre recombinase under the CD4-promoter. This leads to *Tmem51* deletion in all cells that express CD4. The specific deletion in CD8 T cells takes place in the double positive stage during thymic development (**Figure 10A**).

Strain (3): Mice that express ERT2/Cre under the promoter of Granzyme B. ERT2/Cre is a Cre recombinase that is fused to a mutant of the human estrogen receptor (ERT2) which cannot bind its natural ligand. ERT2/Cre binds the synthetic ligand 4-hydroxytamoxifen (4-OHT). Treating mice with the compound Tamoxifen, which gets processed to its active form 4-OHT in the liver leads to cellular translocation to the nucleolus of the Cre-ERT2 construct where Cre recombinase acts on the LoxP-flanked sequence of interest. For conditional deletion of *Tmem51*, tamoxifen was administered to CD45.1 mice carrying *Tmem51*$^{fl/fl}$ or to wild-type control P14 mice (3). Mice received 100mg/kg Tamoxifen (T5648, Sigma Aldrich) via intraperitoneal (i.p.) injection **(Figure 12A)**. Tamoxifen was dissolved at 20mg/ml in corn oil.

Tmem51-deficient donor cells were transferred into CD45.1 congenic C57BL/6NxJ host mice. These host mice are the F1-generation of a mating of C57BL/6N and C57BL/6J mice and were needed to avoid rejection of the mixed genetic background of the *Tmem51*-deficient T cells.

All mouse breeding and experiments were performed in specific pathogen-free facilities. Host mice were six to sixteen-week-old female or male mice. The experiments were done at the Technical University of Munich in Germany and in accordance with institutional and governmental regulations of the Regierung von Oberbayern. Experimental groups were non-blinded, animals were randomly assigned to experimental groups and no specific method was used to calculate sample size.

3.3   Acute infection model with wild-type and recombinant *Listeria monocytogenes*

For studies of *Ptpn2*, I used *Listeria monocytogenes (Lm)* which are gram-positive facultative anaerobic intracellular bacteria, making them a well-suited model system to study CD8 T cell responses. *Listeria* elicit a potent immune response with classical response phases of expansion, contraction, and memory phase with potent recall potential. Recombinant, modified SIINFEKL expressing Lm-N4 bacteria are used to induce a specific immune response of previously transferred TCR-transgenic CD8 OT-1 T cells, which recognize the ovalbumin-derived and presented SIINFEKL peptide fragment. The generation of the recombinant *Listeria* has been described previously (Zehn et al., 2009). Before infection of host mice, *Listeria* were grown in brain heart infusion broth (BHI) without antibiotics at 37°C and continuously stirred in an incubator (Oxoid, Thermo Fisher). Via measurement of the

OD_{600}, bacterial growth was monitored until the mid-log phase which corresponds to an OD_{600} of 0.3 to 0.6. An OD_{600} of Lm in BHI corresponds to 1×10^8 CFU/ml (CFU: colony forming units). *Listeria* were diluted in PBS to inject 1000 CFU / 300 µl per mouse intra venously (i.v.). As a control 30 µl of the same dilution was plated on BHI agar plates containing 200 µg/ml Streptomycin for Lm-wt and 200 µg/ml Streptomycin + 7.5 µg/ml Chloramphenicol for Lm-N4 overnight at 37°C. The next day the colonies were counted to control for the exact infectious dose, which had to be in the range of 950-1050 CFU/mouse.

3.4   Lymphocytic choriomeningites virus model for acute and chronic viral infections

The infection model is based on two different lymphocytic choriomeningitis virus (LCMV) strains. LCMV-Armstrong53b (Arm53b) is the strain that causes an acute infection and is the parental viral strain of LCMV-clone13 (cl13) which causes a chronic infection in mice. LCMV-cl13 is mutated in 5 nucleotides that lead to mutations in the polymerase and glycoprotein of LCMV-cl13 (Klepper & Branch, 2015; Smelt et al., 2001).   The changed glycoprotein allows for higher affinity binding to the cell target receptor α-dystroglycan which is thought be one cause of the increased virulence of LCMV-cl13 (Klepper & Branch, 2015; Smelt et al., 2001). The mutations allow LCMV-cl13 to infecT cells more efficiently and to replicate faster leading to a chronic infection which causes CD8 T cells to acquire an exhausted phenotype. The infectious dose of LCMV-Arm53b used in this study was 2×10^5 plaque forming units (PFU)/mouse which was injected intraperitoneal (i.p.) and the dose for LCMV-cl13 was 5×10^6 PFU/mouse which was injected intravenously (i.v.).

3.5   *In vitro* T cell activation and proliferation assays of *Ptpn2*-deficient CD8 T cells

In accordance with the manufacturer's instructions, Dynabeads™ Mouse T-Activator antiCD3/antiCD28 beads (Thermo Fisher) were used to isolate and activate naïve *Ptpn2*-deficient and wt CD8 T cells with varying concentrations of recombinant IL-2 in RPMI1640 (Gibco, 11875093) medium supplemented with 10% heat inactivated FCS, 5 mM Hepes (Invitrogen), 50 µM β-Mercaptoethanol (Invitrogen), and 100 U/ml of Penicillin and Streptomycin (Amimed) (RPMI1640-10). Cell numbers were determined in Neubauer counting chambers as well as by flow cytometry. The ilastik object classification and segmentation software (Sommer et al., 2011) was used for determination of proliferative clusters. These clusters were counted automatically using the ilastik segmentation classification with the

criteria for proliferative clusters (size over 200 μm) in ImageJ (v1.5). *Ptpn2*-deficient and wt OT-1 T cells were isolated and activated for testing T cell programing and autonomous expansion. This was performed in accordance with an *in vitro* stimulation system described by Van Stipdonk et al., 2001: OT-1 T cells were co-cultured with activated MEC.B7.SigOVA fibroblasts that are manipulated to express SIINFEKL, H-2K^b and CD80 (Van Stipdonk et al., 2001). As indicated above, cells were cultured in IL-2 supplemented medium. Afterwards, 10^5 activated T cells were transferred into antigen-free CD45-congenic C57BL/6J host mice. 5-7 days later, the organs of the host mice were harvested.

3.6   Sample preparation for mass spectrometry

Ptpn2-deficient and wt OT-1 CD8 T cells were isolated from three separate donors each and activated with Dynabeads™ Mouse T-Activator antiCD3/antiCD28 beads (Thermo Fisher) for 30 hours in 50 U/ml of recombinant IL-2 in RPMI media supplemented with 10% heat inactivated FCS in accordance with the manufacturer's manual. Activated cells were harvested and resuspended in urea lysis buffer (8 M urea, 40 mM Tris-HCl at pH 7.6, protease and phosphatase inhibitor cocktail) to extracT cellular proteins. Subsequently, the lysates were centrifuged for 15 min, at 20.000 × g at 4°C. Further protein extraction, enzymatic digestion, tandem mass tag (TMT) labeling, phosphopeptide enrichment, high pH reversed phase (RP) tip fractionation of the phosphoproteome, and Trinity P1 fractionation of the full proteome were performed by our cooperation partner Jana Zecha in the laboratory of Professor Bernhard Küster as described previously (Ruprecht et al., 2017; Yu et al., 2017; Zecha et al., 2019). The identification and quantification of peptides and proteins were performed by Jana Zecha using MaxQuant (v1.6.0.16) and the perseus software suite (v.1.6.6.1) to perform two-sided, unpaired t-tests for phosphosite and protein intensities identified in at least two out of the three replicates (Flosbach et al., 2020).

3.7   *De novo* pathway enrichment of phosphoproteome data

To build a connected network from the proteomic data, I employed the *de novo* network enrichment tool KeyPathwayMiner (List et al., 2016). As a basic interactome, I used interaction data supplied by the STRING network (v. 11), filtered for regulatory interactions (post-translational modification, activation, or inhibition) of high confidence (combined score >600). This network was enhanced with known interactions of differentially phosphorylated tyrosine phosphoproteins curated in the Ingenuity pathway analysis tool (IPA, v. 46901286, between January and March 2019, QIAGEN Inc., (Krämer et al., 2014)) database (filtered for interaction that are: experimentally observed, downstream, murine targets). This combined STRING and

IPA interactome was loaded into the KeyPathwayMiner tool together with the following datasets: (1) differentially phosphorylated tyrosine phosphoproteins (log_2 ko/wt fold change of > 0.5 and p < 0.05) and (2) differential protein expression (log_2 fold change of >0.5 and p < 0.05). KeyPathwayMiner extracted several networks out of this data. I overlaid the largest subnetwork extracted by KeyPathwayMiner with protein expression data and manually added annotations for known effects of site-specific tyrosine phosphorylations using Cytoscape (Shannon et al., 2003), v. 3.7.1).

3.8   Cell culture

The Phoenix E cell line used in this work was cultivated in Dulbecco's Modified Eagle Medium (DMEM) medium (Thermofisher, 41965039) supplemented with 10 % FCS, 2 mM L-glutamine (Thermofisher, A2916801), 1x non-essential amino acids (NEAA) (Thermofisher, 12084947), and 1 % penicillin/streptomycin (Thermofisher, 15140122) (DMEM-10) at 37°C and 7% CO_2. The cells were passaged at a confluence of about 70-80 %, for which cells were washed with sterile phosphate buffered saline (PBS) and detached with 0.05% Trypsin/ Ethylenediaminetetraacetic acid (EDTA) (Gibco, 25300054) at 37°C. The cell number of cells in suspension was determined in a Neubauer improved haemocytometer. The counting chamber was filled with an aliquot of 10 µl cell suspension and cells in 16 small squares were counted. To obtain the number of cells per milliliter (ml), the count was multiplied with 1×10^4.

3.9   Cryoconservation of cells

For a permanent cryoconservation of cells, they were detached as previously described, pelleted at 300 × g for 5 min and resuspended in FCS supplemented with 10 % dimethyl sulfoxide (DMSO, Sigma-Aldrich, CAS 67-68-5). Afterwards, the aliquoted cells were transferred to a freezing container and slowly frozen at -80°C for 24 h and then transferred to liquid nitrogen at -150°C for permanent storage. To recultivate frozen cells, they were quickly thawed at 37°C and resuspended in DMEM-10. Then the cells were pelleted at 300 × g for 5 min, resuspend in DMEM-10 and transferred to tissue culture flasks.

3.10 *Tmem51* overexpression vector generation

The coding sequence for *Tmem51* was obtained from ensembl.org with the transcript identifier ENSMUST00000036572.4. Using the Geneious software, the forward primer: CAGTCA CTCGAG TGACACAGGAGAGTCTCTTTGC   (XhoI) and the reverse primer: AGTCA CAATTG GGAGTGTAGTCAGAGAGTCCTTC (EcoRI) with an overhang for the indicated

restriction enzyme sites were designed to bind at the start and stop codon of the *Tmem51* transcript. As a template, cDNA was isolated from activated CD8 T cells. The primers were used at an annealing temperature of 65°C. The PCR was performed with high fidelity Phusion enzyme (NEB) with 0,5 µM primer and in accordance with the manufacturer's instructions. The
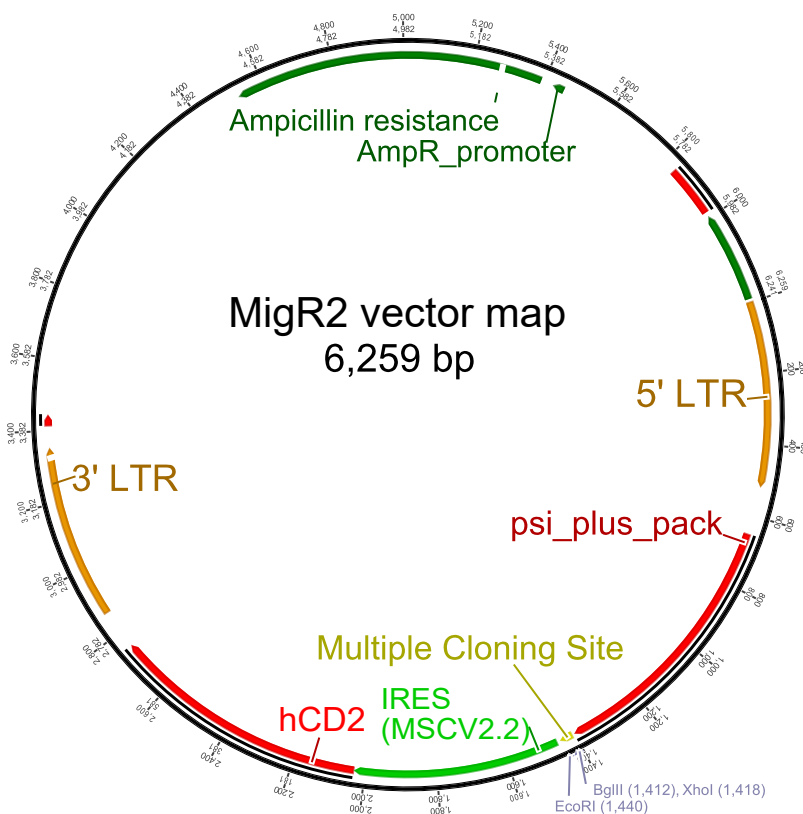
Figure 6 MigR2 vector map. The vector carries an ampicillin resistance for bacterial selection. The 5'- and 3'- long terminal repeat (LTR) contain viral structures that facilitate the integration into the host genome. The internal ribosome entry site (IRES) is based on the murine stem cell virus (MSCV) and drives continuous expression of the human CD2 (hCD2) reporter and *Tmem51* which has been integrated at the multiple cloning site for the MigR2-*Tmem51* vector. The psi_plus_pack confers packaging into retroviral particles when the vector is expressed in Phoenix E cells.

PCR program was set up as follows: 98°C 30sec, 34 cycles of: (98°C 10sec, 65°C 20sec, 72°C 1min), 72°C 5min, 4°C unlimited. The product was purified with the QIAquick PCR Purification Kit (Qiagen) in accordance with the manufacturer's instructions and digested with XhoI and EcoRI at 37 °C for 2 h. At the same time the vector MigR2-empty was digested with XhoI and EcoRI at 37 °C for 2 h and also dephosphorylated using shrimp alkaline phosphatase (SAP, NEB) in accordance with the manufacturer's instructions. The digested samples were separated on a 1.5 % agarose gel and afterwards ligated using the T4 ligase (NEB) in accordance with the manufacturer's instructions. The resulting MigR2-*Tmem51* vector was used to transform Top10 competent bacteria (Thermofisher) in accordance with the manufacturer's instructions. The transformed bacteria have an ampicillin resistance and were selected on agar plates and then single colonies were used to grow bacteria overnight. The plasmid was purified the next day with the Qiagen plasmid mini kit (Qiagen) in accordance with the manufacturer's instructions. The insertion of the *Tmem51* coding sequence was verified via sequencing of the product of the PCR with the primers GCCTCGATCCTCCCTTTATCC MigR2(1365-1379):forward and GACAAACGCACACCGGC

CTTA MigR2(1516-1496):reverse which bind directly outside of the multiple cloning site of the vector. The pMSCV-based retroviral vector encodes for a bicistronic transcript that encodes for *Tmem51* and human CD2 (hCD2) separated by an internal ribosome entry site (IRES). This allows for identification of transduced T cells via the hCD2 reporter (**Figure 6**). Primer design and analysis of sequencing data of cloning samples was performed with Geneious v.9.

3.11 Generation of retroviral particles

To produce retroviral particles, Phoenix E cells were seeded in DMEM media (Thermofisher, 41965039) supplemented with 10 % FCS, 2 mM L-glutamine (Thermofisher, A2916801), 1x non-essential amino acids (NEAA) (Thermofisher, 12084947), 1 % penicillin/streptomycin (Thermofisher, 15140122) (DMEM-10) at a cell count of 3.5×10^5 cells per well of a 6-well plate. For the production of retroviral particles, 1 µg plasmid-DNA encoding for *Tmem51* or empty vector were transfected 24 hours later using Fugene 6 (Promega) according to manufacturer's instructions. The Phoenix E packaging cell line encodes for retroviral group antigens (gag), the reverse transcriptase (pol), and the ecotropic envelope protein (env). The retroviral particles generated by this cell line were used to transduce activated and proliferating OT-1 CD8 T cells with retroviral expression vectors either containing the coding sequence for *Tmem51* (MigR2-*Tmem51*) or an empty vector control (MigR2-empty).

3.12 Transduction of activated CD8 T cells with retroviral particles

Donor T cells were isolated from spleens taken from 6 to 16-week-old mice. The spleens were minced and mashed through a 100 µm filter, afterwards red blood cell lysis was performed with ammonium-chloride-potassium lysing buffer (ACK; 0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4) for 3 minutes at room temperature. After washing with DMEM-10, negative isolation of CD8 T cells was performed with mouse CD8 T cell enrichment MACS kit (Miltenyi) according to manufacturer's instructions. Then, the CD8 T cells were resuspended in RPMI1640 medium supplemented with 10% heat inactivated FCS, 5 mM Hepes (Invitrogen), 50 µM β-mercaptoethanol (Invitrogen), and 100 U/ml of penicillin and streptomycin (Amimed) (RPMI1640-10) and 1.0×10^6 CD8 T cells were plated in 24 well plates with addition of 50 Units/ml IL-2 and CD3/CD28-coupled activation beads (Dynabeads mouse T-activator, 114.56D Thermo Fisher) in accordance with the manufacturer's instructions. 30 hours later, the activated T cells were transduced with retroviral particles in the presence of 10 µg/ml polybrene at 700 × g für 90 minutes at 32 °C. Polybrene is a cationic polymer that allows for stronger binding of the retroviral particles to the targeT cells by neutralizing the repulsion of positively charged retroviral particles and the cell surface. Afterwards cells were resuspended

in PBS and injected into host mice or resuspended in fresh RPMI1640-10 and cultivated further *in vitro.*

3.13 Peptide ligand stimulation of TCR transgenic T cells

OT-1 CD8 T cells have a fixed TCRVα2 and TCRVβ5 chain and specifically recognize the ovalbumin residues 257-264 (SIINFEKL) peptide. 48 hours after retroviral transduction, the OT-1 T cells were stimulated with RMA cells that were loaded with the altered peptide ligands (APL) SIINFEKL, SIIQFEKL, SIITFEKL, and SIIVFEKL. These APL stimulate the TCR of OT-1 TCR-transgenic CD8 T cells with different affinity, high to low: SIINFEKL>SIIQFEKL>SIITFEKL>SIIVFEKL (Zehn et al., 2009). The APL were used in the concentration of 10^{-6} M to 10^{-13} M and the RMA cells present the peptides via MHCI to the TCR of OT-1 T cells. P14 TCR-transgenic CD8 T cells have a fixed TCRVα2 and TCRVβ8 chain and specifically recognize the LCMV derived gp33-41 peptide (KAVYNFATC). P14 T cells that were derived from LCMV-infected mice were stimulated with 10^{-6} M gp33 peptide. In both cases, Brefeldin A (BFA) was added to the cells 30 minutes afterwards. Another 5 hours later, the T cells were stained and analyzed via flow cytometry.

3.14 General data analysis

Dot plots and statistical analyses were, unless specified differently, prepared and calculated with Prism v8.0 and v9.0 (Graphpad Software). Unpaired t-tests were used to compare two groups and $p \leq 0.05$ was considered significant and $p > 0.05$ was considered not significant. The analysis of flow cytometry data was performed with FlowJo v.10 (BD).

4   Statement of contributions

The data presented in this thesis are based on experiments performed and analyzed by myself. This is expressed in the text in the singular first person. The data of the investigation of *Tmem51* is unpublished. The results presented on *Ptpn2* are published in (Flosbach et al., 2020). Additional related results that are published in this paper and which were performed by cooperation partners (data not shown in this thesis) are referred to in the related results section with the plural first person. Sample preparation and peptide quantification of the phosphoproteome presented in Figure 18 were performed by Jana Zecha in the laboratory of Professor Dr. Bernhard Küster. The pathway presented in Figure 19 was generated with KeyPathwayMiner and Dr. Markus List and Dr. Josch Pauling in the laboratory of Professor Dr. Jan Baumbach introduced me to the tool. Final data analysis, writing of this thesis and the design of figures presented in this thesis were always performed by myself.

5   Results

5.1   Transmembrane protein 51 is a potential regulator of cytokine production in CD8 T cells

5.1.1 Overexpression of *Tmem51* in CD8 T cells *in vitro* leads to significant reduction of effector cytokine production

There is a number of molecules in CD8 T cells that are differentially up- or down-regulated when comparing chronic versus acute infection. Many of these have no defined function in the development of exhausted CD8 T cells. One such molecule of interest is transmembrane protein 51 (*Tmem51*). The expression of *Tmem51* is significantly higher in P14 T cells derived from chronic LCMV-cl13 versus P14 T cells derived from acute LCMV-Arm53b infection. Consequently, I first investigated whether the overexpression of *Tmem51* in CD8 T cells can lead to the development of features of exhausted CD8 T cells. *In vitro* activated CD8 T cells are typically able to produce high levels of inflammatory cytokines. I addressed the question, if the overexpression of *Tmem51* can lead to reduced levels of cytokine expression in an *in vitro* setting. To induce the expression of *Tmem51* in CD8 T cells, the retroviral expression vector MigR2 was used. The cDNA of *Tmem51* was cloned into the MigR2 vector. The retroviral vector encodes for a bicistronic transcript that encodes for *Tmem51* and human CD2 (hCD2) separated by an internal ribosome entry site (IRES). This allows for indirect identification of transduced T cells via the hCD2 reporter. For the transduction of CD8 T cells, this retroviral vector was transfected into the Phoenix E packaging cell line, which encodes for retroviral group antigens (gag), the reverse transcriptase (pol), and the ecotropic envelope protein (env). The retroviral particles generated by this cell line were used to transduce OT-1 CD8 T cells that were activated 30 hours before with CD3/CD28-coupled beads. The transduction efficiency of the empty vector control (MigR2-empty) was about 32 % and the transduction efficiency of the retroviral expression vector that carried the coding sequence for *Tmem51* (MigR2-*Tmem51*) was about 42 % **(Figure 7A)**. This is displayed by the percentage of CD8 T cells that express the marker hCD2 **(Figure 7A)**. To stimulate the OT-1 T cells with their cognate antigen, RMA cells were loaded with the altered peptide ligands (APL) SIINFEKL, SIIQFEKL, SIITFEKL, and SIIVFEKL. The APL were then presented via MHCI to the previously activated and transduced OT-1 CD8 T cells. These APL stimulate the TCR of OT-1 TCR-transgenic CD8 T cells with different functional affinity, high to low: SIINFEKL>SIIQFEKL>SIITFEKL>SIIVFEKL. For full activation of T cells, small concentrations of the high affinity SIINFEKL peptide are sufficient. To induce the same response with the low

affinity SIIVFEKL peptide, higher concentrations are needed. The clearly defined APL system allows the characterization of the antigen sensitivity of T cells. In this case, I used it to test whether *Tmem51*-overexpressing OT-1 T cells reacted differently to varying amounts of the altered peptide ligands SIINFEKL, SIIQFEKL, SIITFEKL, and SIIVFEKL. The peptide titration curves show a significantly reduced maximum response of the effector cytokines IFNγ and TNFα in CD8 T cells that overexpress *Tmem51* when comparing the expression to the empty vector control **(Figure 7B).** The maximum response of IFNγ and TNFα expression at 10^{-6} M peptide is up to 30 % lower in T cells that overexpress *Tmem51*. This is similar among all affinities of APL that were used. To directly compare the impact of *Tmem51* overexpression on the antigen sensitivity of OT-1 T cells, I normalized the expression values and used a nonlinear regression curve fit model to calculate the half maximum effective concentration (EC50) **(Figure 7C).** The difference between empty vector control and *Tmem51*-overexpressing OT-1 T cells is the largest with the natural high affinity ligand SIINFEKL, indicating that *Tmem51* has the strongest impact on the high affinity stimulated T cells **(Figure 7C).** A reduced production of effector cytokines is a hallmark of exhausted CD8 T cells. The reduced production of IFNγ and TNFα in CD8 T cells overexpressing *Tmem51* is in line with the observed upregulation of *Tmem51* during chronic infection.
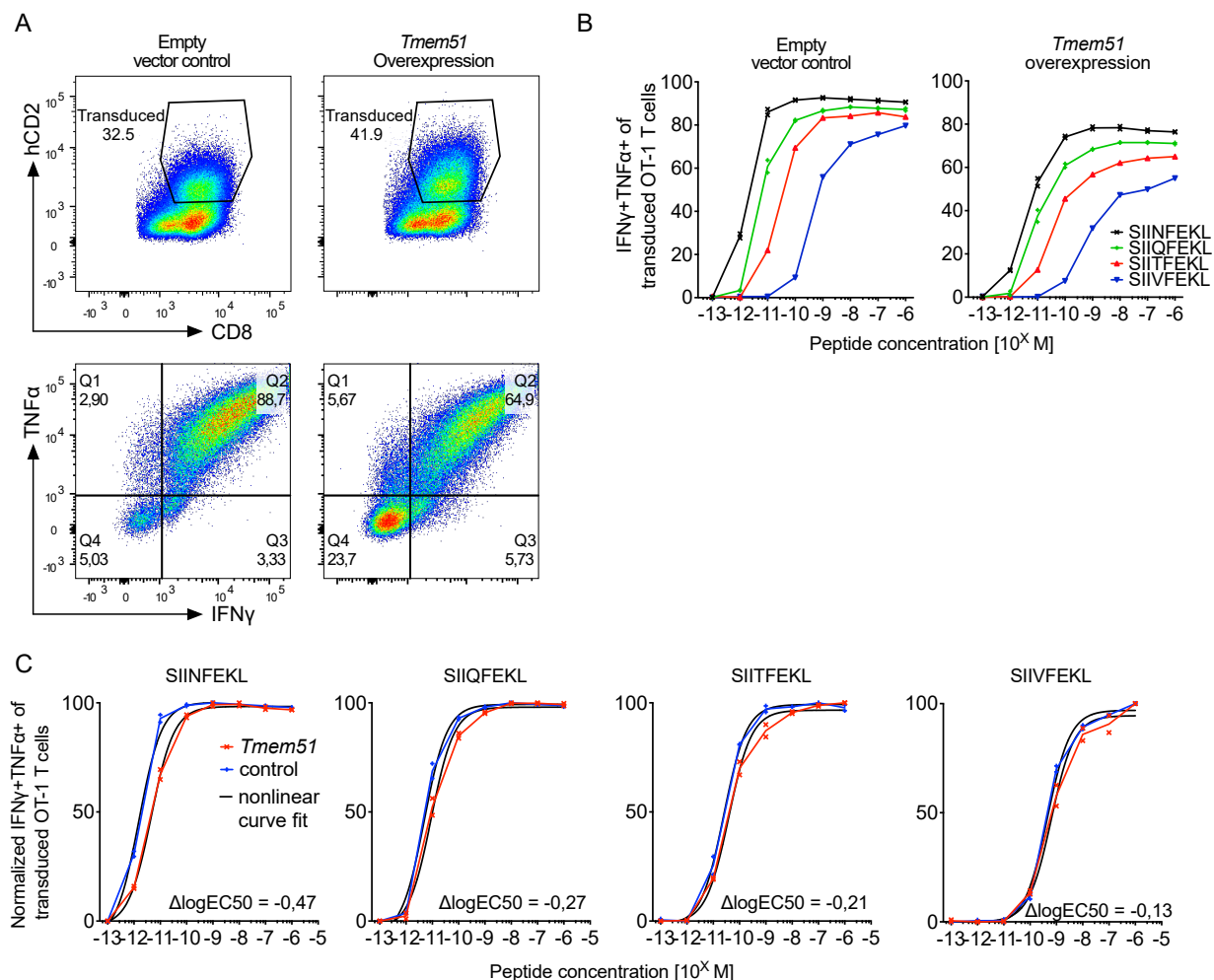
Figure 7 *Tmem51* overexpression leads to reduced production of effector cytokines of peptide-stimulated CD8 T cells. A-B) Spleens of donor mice were taken at the age of 6-18 weeks. Naive OT-1 CD8 T cells (T cells) were isolated via negative selection and separated by the use of magnetic beads. Then, the T cells were activated for 30 hours with 50 U/ml IL-2 and CD3/CD28-coupled beads. After activation, T cells were transduced with retroviral vectors to overexpress *Tmem51* or an empty vector control. 48 hours after retroviral transduction, the T cells were stimulated with RMA cells that were pulsed with altered peptide ligands (APL) with 10^{-6} M to 10^{-13} M concentrations. 30 minutes later, Brefeldin A (BFA) was added to the cells and 5 hours later, the T cells were stained and analyzed via flow cytometry. **A)** Representative fluorescence activated cell sorting (FACS) plots of *Tmem51* overexpressing or empty vector control T cells. The top panel shows the transduction efficiency as determined by human CD2 (hCD2) expression and the lower panel shows the percentage of interferon-gamma (IFNγ) and tumor necrosis factor – alpha (TNFα) producing hCD2-positive T cells that were stimulated with 10^{-9} M SIIQFEKL peptide. **B)** The plots show titrated IFNγ+TNFα+ production of *Tmem51*-overexpressing or empty vector control transduced T cells that were stimulated with the indicated concentrations of SIINFEKL (black), SIIQFEKL (green), SIITFEKL (red), or SIIVFEKL (blue) APLs. **C)** The plots show the normalized percent expression of IFNγ and TNFα of empty vector control (blue) and *Tmem51*-overexpressing T cells (red). The black line represents a nonlinear regression analysis of the normalized samples, this was used to determine the EC50 and the delta(log)EC50 between empty vector control and *Tmem51*-overexpresing T cells. The data are representative of two independent experiments with two donor mice each.

5.1.2 Overexpression of *Tmem51* in CD8 T cells leads to significantly reduced effector cytokine response in acute infection

T cells overexpressing *Tmem51* show a deficit in their ability to produce effector cytokines. As this is one feature of exhausted CD8 T cells in chronic infection, I wanted to test whether the overexpression of *Tmem51* is sufficient to lead T cells in acute infection towards the development of features of exhaustion. To investigate this, I used the lymphocytic choriomeningitis virus (LCMV) infection model. It is based on two different LCMV strains that cause acute (LCMV-Arm53b) or chronic (LCMV-cl13) infection in mice. LCMV-Arm53b is the parental viral strain of LCMV-cl13 which differs in 5 nucleotides that lead to mutations in the polymerase and glycoprotein of LCMV-cl13. These small changes allow LCMV-cl13 to infect T cells more efficiently, replicate faster and consequently allows LCMV-cl13 to establish a chronic infection causing CD8 T cells to acquire an exhausted phenotype. In contrast, the acute infection initiated by LCMV-Arm53b is cleared by the immune system and leads to the development of effector and memory T cells. To follow the immune response to LCMV infection, I used P14 TCR transgenic CD8 T cells, which are specific to the gp33 peptide derived from LCMV. The P14 CD8 T cells were activated *in vitro* as described before, but directly after transduction with MigR2-*Tmem51* or MigR2-empty, the CD45.1 congenic P14 T cells were adoptively transferred into CD45.2 congenic C57BL/6 hosts mice. This allowed for the convenient tracking of the T cell response using the congenic marker system. The host mice were infected with LCMV-Arm53b causing an acute infection. After the *in vivo* transfer, the transduction efficiency was lower than *in vitro* and about 4 % hCD2-positive, i.e. transduced cells could be recovered **(Figure 8A)**. At 26 days post infection, the production of IFNγ and TNFα in CD8 T cells that overexpress *Tmem51* was significantly lower when comparing the expression to the empty vector control **(Figure 8A)**. At the same time, the development of CD127$^+$ memory and KLRG1$^+$ effector T cells was not significantly impacted by the overexpression of *Tmem51* **(Figure 8B-C)**. This was evident in the blood at 8 and 14 days post infection and in the spleen, lymph node, and liver at 26 days post infection **(Figure 8B-C)**. Of note and in line with the reduced effector cytokine production, there is a slightly lower percentage of KLRG1$^+$ effector cells among T cells that overexpress *Tmem51* at 14 days post infection in the blood and 26 days post infection in the liver **(Figure 8B-C)**. The data indicate *Tmem51* as a regulator of effector cytokine production in CD8 T cells during acute infection.
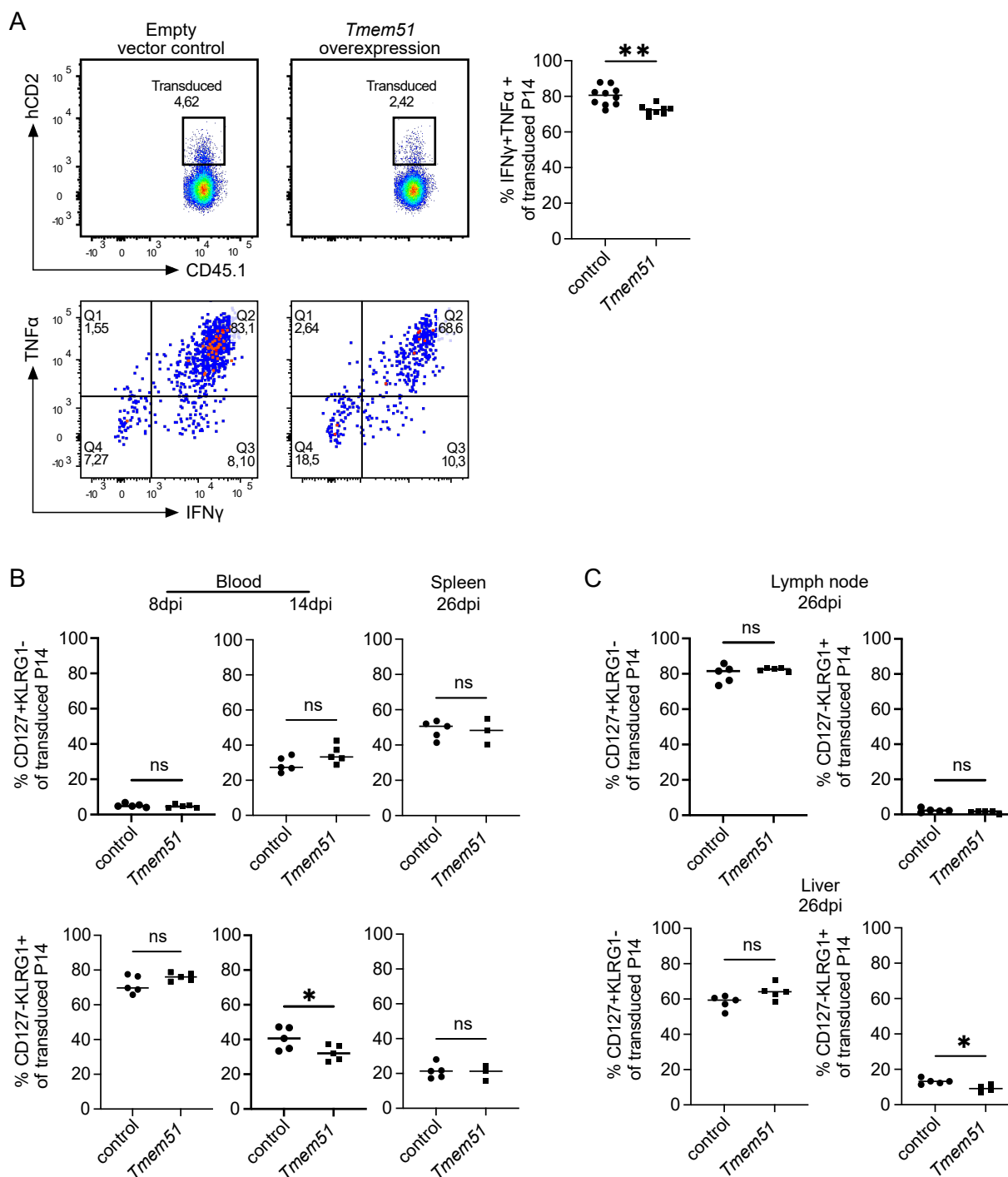
Figure 8 Overexpression of *Tmem51* in CD8 T cells reduces IFNγ and TNFα production without impacting effector and memory T cell development. A-C) LCMV-specific CD45.1 congenic P14 T cells were isolated, activated and transduced as described in **Figure 7**, but 2 × 10⁴ T cells were transferred into CD45.2 congenic C57BL/6J host mice three hours after transduction. The next day, the host mice were infected with 2 × 10⁵ PFU LCMV-Arm53b. The development of the T cells was monitored at 8 and 14 days post infection (dpi) in the blood and spleen, lymph node and liver were analyzed at 26 dpi. **A)** The four plots on the left show representative fluorescence activated cell sorting (FACS) plots of CD45.1-positive CD8 T cells derived at 26 dpi from spleens. The top panel demonstrates the transduction efficiency of *Tmem51* overexpressing and empty vector control T cells as determined by human CD2 (hCD2) expression and the lower panel shows the percentage of interferon-gamma (IFNγ) and tumor necrosis factor – alpha (TNFα) producing hCD2-positive T cells that were stimulated with 10⁻⁶ M

gp33 peptide. The dot plot to the right depicts a summary of the percentage of IFNγ+TNFα+ T cells, each dot represents one of two technical replicates per mouse. **B)** The dot plots in the upper panel display the percentage of cluster of differentiation 127 (CD127)-positive of transduced P14 T cells and the lower panel shows the percentage of killer cell lektin like receptor G1 (KLRG1)-positive of transduced P14 T cells at 8, 14 and 26 dpi. **C)** The dot plots depict the percentages of CD127+ or KLRG1+ transduced P14 T cells at 26 dpi in the lymph node and the liver. Each dot represents one individual mouse, the horizontal line the mean. The data are representative of at least two independent experiments with five mice each. Statistical analysis: unpaired t-test, **p ≤ 0.001, *p ≤ 0.01, ns (not significant) p ≥ 0.05.

5.1.3 *Tmem51*-deficient CD8 T cells still acquire an exhausted phenotype during chronic infection

The reduced capability of T cells overexpressing *Tmem51* to produce effector cytokines prompted me to test whether the deletion of *Tmem51* could reactivate exhausted CD8 T cells via restoring their ability to produce effector cytokines. To this end, I generated TCR transgenic P14 *Tmem51* conditional knockout mice. These mice carry a *Tmem51* mutation in which the first exon is LoxP-flanked. Three different Cre recombinase expression systems allow for the timed deletion of *Tmem51*. The strains are quadruple transgenic and additionally carry a LoxP-flanked stop;enhanced yellow fluorescent protein (eYFP) under the ubiquitous Rosa26 locus (Rosa26-Stop$^{fl/fl}$-eYFP). This allows for the identification of cells that express the Cre recombinase via eYFP. Three knockout strains are designated as: (1) P14;Mx1-Cre;Rosa26-Stop$^{fl/fl}$-eYFP;*Tmem51*$^{fl/fl}$, (2) P14;CD4-Cre;Rosa26-Stop$^{fl/fl}$-eYFP;*Tmem51*$^{fl/fl}$, and (3) P14;Gzmb-ERT2/Cre;Rosa26-Stop$^{fl/fl}$-eYFP;*Tmem51*$^{fl/fl}$. And the following three mouse strains were used as genetically matched wild-type controls: P14;Mx1-Cre;Rosa26-Stop$^{fl/fl}$-eYFP, P14;CD4-Cre;Rosa26-Stop$^{fl/fl}$-eYFP, and P14;Gzmb-ERT2/Cre;Rosa26-Stop$^{fl/fl}$-eYFP. Strain (2) has a CD4-mediated Cre recombinase expression. This allows for testing whether a *Tmem51* deletion at the CD4CD8 double positive stage has an impact on thymic development of CD8 T cells. To this end, I analyzed the T cell development of thymocytes derived from 6 to 16-week old donor mice **(Figure 9)**. I could demonstrate that the cellularity of CD4$^+$CD8$^+$ double positive (DP) and single positive CD8 T cells (CD8SP) is not impacted in *Tmem51*-deficient T cells **(Figure 9)**. As determined by CD44 and CD25 expression, there is also no difference in the percentages of cells that are in the double negative (DN) stages of T cell development **(Figure 9)**. Overall, the data show that thymocyte development is not impacted in the absence of *Tmem51*.
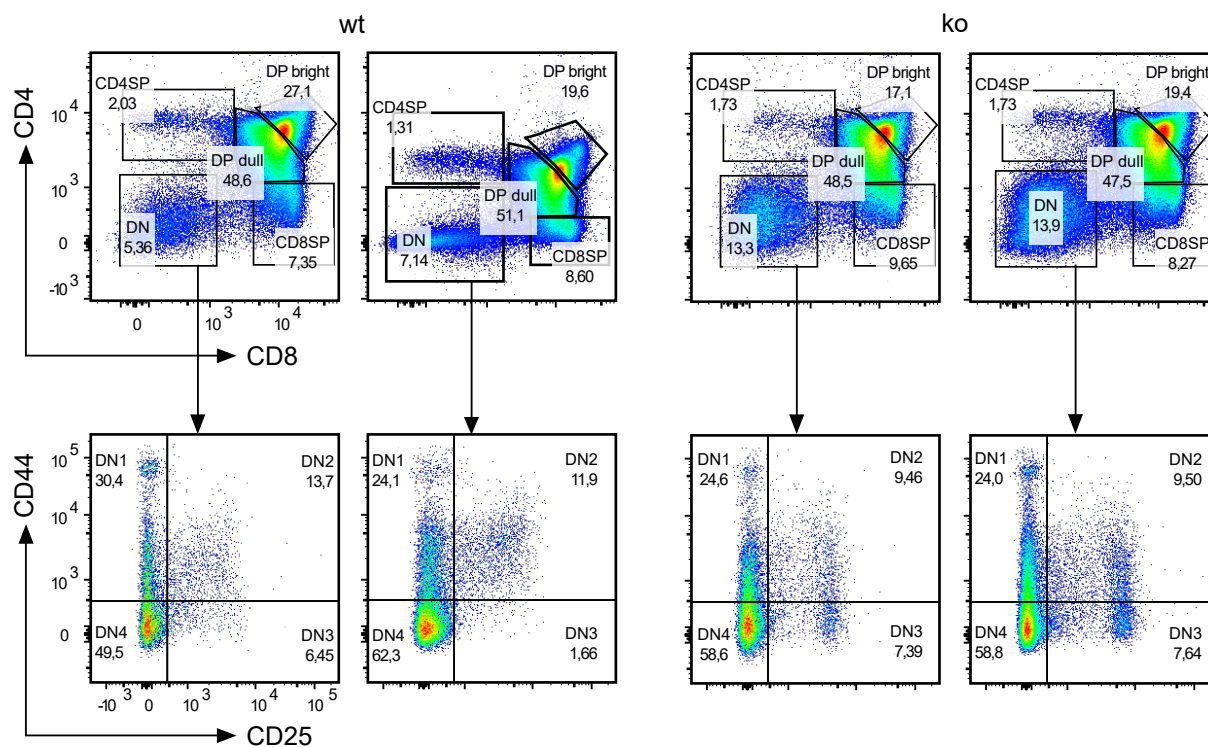
Figure 9 CD4-Cre mediated deletion of *Tmem51* does not alter thymocyte development. The thymi of P14;CD4-Cre;Rosa26-Stop^{fl/fl}-eYFP;*Tmem51*^{fl/fl} (ko) and P14;CD4-Cre;Rosa26-Stop^{fl/fl}-eYFP (wt) were taken from donor mice at the age of 6-18 weeks. A) Frequencies of developing T cells among total thymocytes were determined by flow cytometry. FACS plots of the major developmental stages of two wild-type (wt) (left) or knockout (ko) (right) mice are shown in the top panel. The four double negative (DN) T cell developmental stages are depicted in the FACS plots in the lower panel as determined by CD44 and CD25 expression. SP: single positive, DP: double positive.

I could demonstrate that the thymic development of mature and naïve T cells is not impacted when *Tmem51* is deleted in the double positive stage. This verifies that the mouse strain P14;CD4-Cre;Rosa26-Stop^{fl/fl}-eYFP;*Tmem51*^{fl/fl} can serve as an experimental system to investigate the impact of *Tmem51* on T cell differentiation in chronic infection. To monitor the T cell response of wild-type P14;CD4-Cre;Rosa26-Stop^{fl/fl}-eYFP and knockout P14;CD4-Cre;Rosa26-Stop^{fl/fl}-eYFP;*Tmem51*^{fl/fl} in the same host, low numbers of congenic *Tmem51*-deficient P14 and wild-type P14 were transferred into congenic C57BL/6 hosts. Then, the host mice were infected with lymphocytic choriomeningitis virus clone 13 (LCMV-cl13) causing a chronic infection **(Figure 10A)**. The co-transfer of wild-type and knockout T cells allowed for the convenient tracking of population dynamics **(Figure 10A)**. Initially, the same numbers of wild-type and knockout T cells were transferred, but already at 7 days post infection more wild-type than knockout T cells were detectable **(Figure 10A)**. From 14 days post infection, this discrepancy stabilized until 30 days post infection at about 70 % wild-type to 30 % knockout T cells of all eYFP-positive T cells **(Figure 10A)**. To investigate whether the *Tmem51*-deficient T cells were able to produce more cytokine than their wild-type counterpart, T cells from liver

and spleen were stimulated with gp33 peptide at 30 days post infection **(Figure 10B)**. The deletion of *Tmem51* led to significant increase of about 35 % of IFNγ and TNFα-producing T cells at 30 days post infection. In comparison, this is similar to splenic wild-type T cells, of which about 30 % produced IFNγ and TNFα at 30 days post infection. There was no significant change in cytokine production of T cells isolated from the liver **(Figure 10B)**. The absence of *Tmem51* had no impact on the level of PD1 in T cells derived from spleen, lymph node, and liver at 30 days post infection **(Figure 10C)**. The percentage of natural killer receptor 2B4 (2B4)-positive *Tmem51*-knockout T cells from the liver was about 70 % and significantly lower when compared to 80 % 2B4-positive wild-type T cells **(Figure 10D)**. Additionally, the level of T-bet expression was significantly lower in *Tmem51*-deficient T cells derived from the liver while the expression of Eomes was unchanged from wild-type to knockout T cells **(Figure 10E)**. The difference in T-bet expressing T cells could not be observed in the spleen. Overall, these changes indicate that *Tmem51* has an impact on population dynamics and a minor impact on effector cytokine production in exhausted CD8 T cells. The deletion of *Tmem51* did not induce changes in the expression of inhibitory receptors in the spleen and the changes in the liver were small but significant.
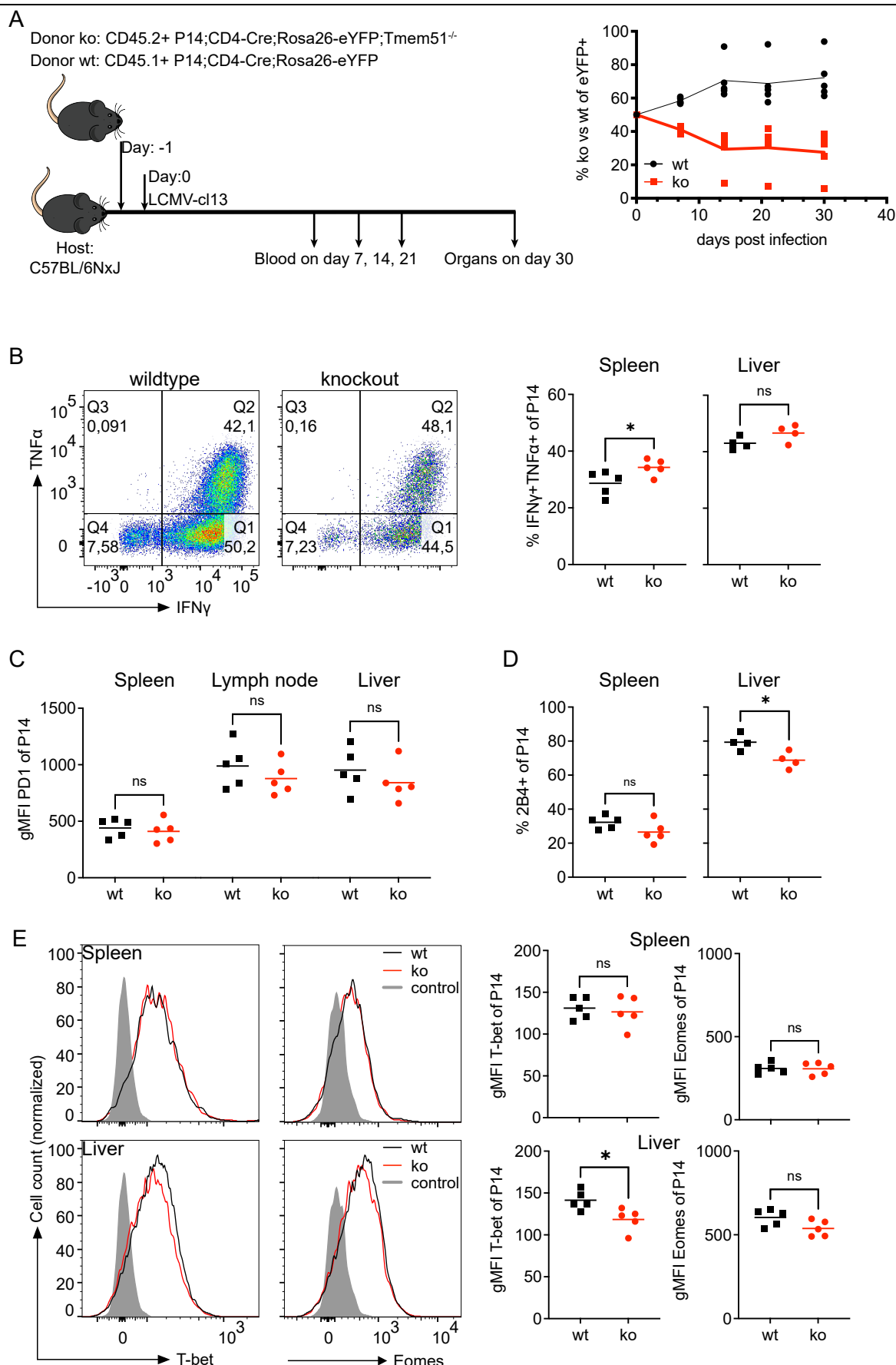
Figure 10 Previously not activated *Tmem51*-deficient T cells differentiate into exhausted T cells during chronic viral infection. CD45 congenic C57BL/6NxJ host mice were grafted with 2 × 10³ P14;CD4-Cre;Rosa26-eYFP;*Tmem51*^fl/fl (ko) and P14;CD4-Cre;Rosa26-eYFP (wt) P14 T cells. 24 hours later the host mice were infected
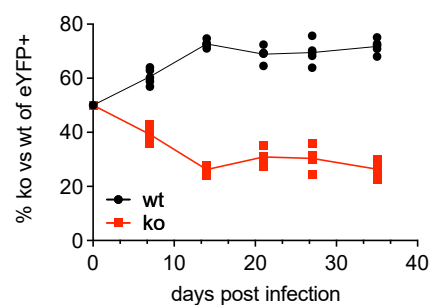
with 5×10^6 PFU LCMV-cl13. Peripheral blood T cells were analyzed by flow cytometry at 7, 14, and 21 days post infection and splenic, lymph node, and liver T cells at 28 days post infection. **A)** Schematic representation of the experimental setup. **B)** The FACS plots to the left are representative of liver samples analyzed at 30 days post infection. P14 T cells were stimulated with 10^{-7} M gp33 peptide and the percentage of IFNγ+TNFα+ T cells are shown. The dot plots to the right show the percentage of IFNγ+TNFα+ T cells stimulated with 10^{-7} M gp33 peptide of five mice. **C)** The dot plots display geometric mean fluorescent intensity (gMFI) of wt and ko P14 T cells derived from spleen, lymph node, and liver at 30 days post infection. **D)** The dot plots demonstrate the percentage of wt and ko P14 T cells expressing natural killer cell receptor 2B4 (2B4). **E)** The histograms to the left depict the representative overlay of wt, ko and unstained control cells analyzed for the expression of T-box expressed in T cells (T-bet) and Eomesdermin (Eomes) in liver and spleen derived T cells. The dot plots to the right show the gMFI of T-bet and Eomes expression of 5 individual mice in the spleen and liver. Each dot in the dot plots represents one mouse, horizontal lines the mean. Statistical analysis: unpaired t-test, *p ≤ 0.01, ns (not significant) p ≥ 0.05.

5.1.4 Interferon-dependent activation of CD8 T cells and deletion of *Tmem51* prior to chronic infection has no impact on exhaustion

Next, I investigated whether the timing of *Tmem51*-deletion has an impact on T cell differentiation in chronic infection. Therefore, P14;Mx1-Cre;Rosa26-Stop$^{fl/fl}$-eYFP;*Tmem51*$^{fl/fl}$ and P14;Mx1-Cre;Rosa26-Stop$^{fl/fl}$-eYFP donor mice were treated with polyI:C. This leads to an interferon-dependent activation of the Mx1 locus, inducing Cre recombinase expression and deletion of *Tmem51*. This way, T cells become pre-activated in a TCR-independent manner, ablate gene expression of *Tmem51* and can be adoptively transferred into host mice, which were then infected with LCMV-cl13. As before, wild-type T cells were more abundant from 7 to 35 days post infection **(Figure 11A)**. This was evident in the blood, spleen, lymph node and liver. The differences in the liver were most pronounced with a ratio of 90 % wild-type over 10 % *Tmem51*-deficient T cells **(Figure 11B)**. To further analyze the effector cytokine profile of *Tmem51*-deficient T cells, I performed a gp33 peptide titration with T cells derived from the spleen at 35 days post infection **(Figure 11C)**. There was no significant difference in the percentage of IFNγ and TNFα producing T cells when comparing *Tmem51*-deficient to wild-type P14 T cells **(Figure 11C)**. The exhausted phenotype was also not impacted by deletion of *Tmem51*, as evident by same levels of PD1 and TIM3 expression in T cells from the spleen at 35 days post infection **(Figure 11D)**. The data indicates, that the timing and mechanism of *Tmem51* deletion has no impact on its function in T cell differentiation during chronic infection.

A

Donor ko: CD45.2+ P14;Mx1-Cre;Rosa26-Stop$^{fl/fl}$-eYFP;Tmem51$^{fl/fl}$
Donor wt: CD45.1+ P14;Mx1-Cre;Rosa26-Stop$^{fl/fl}$-eYFP

3 x polyI:C

ko: P14;Mx1-Cre;Rosa26-eYFP;Tmem51$^{-/-}$
wt: P14;Mx1-Cre;Rosa26-eYFP

Day: -1

Day:0
LCMV-cl13

Host:
C57BL/6NxJ   Blood on day 7, 14, 21, 28   Organs on day 35

B

CD8 (Spleen 35 dpi)

eYFP+ 20,0

wt 73,0
ko 26,9

35 days post infection

**** **** ****

Spleen   Lymph node   Liver

C

Spleen

ns

Peptide Concentration [10^X M]

D

Spleen

ns   ns
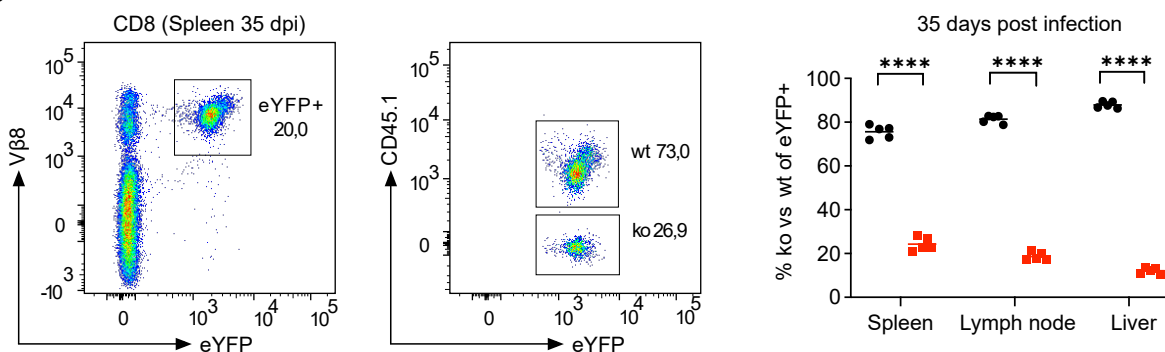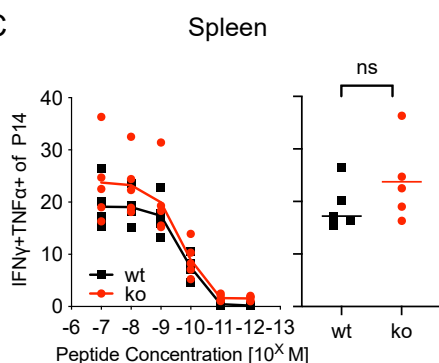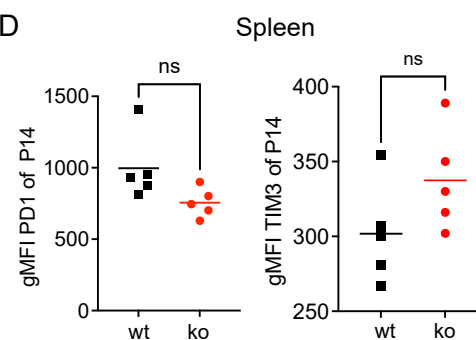
Figure 11 Absence of *Tmem51* reduces T cell numbers but does not alter differentiation of previously activated T cells during chronic viral infection. A-D) CD45 congenic C57BL/6NxJ host mice were grafted with 2×10^3 wild-type (wt) and knockout (ko) P14 T cells derived from donor mice that were treated with polyinosinic-polycytidylic acid (polyI:C) to induce Mx1-Cre mediated deletion of *Tmem51*. 24 hours 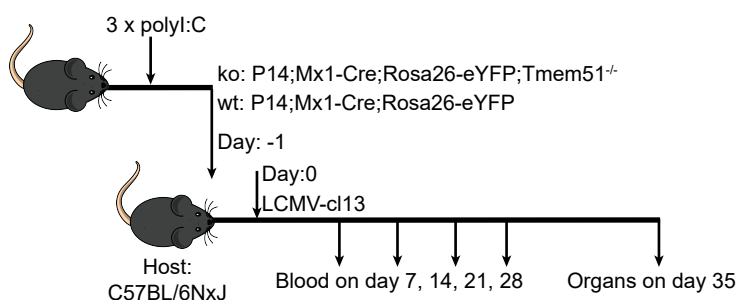later, the host mice were infected with 5×10^6 PFU LCMV-cl13. Peripheral blood T cells were analyzed by flow cytometry at 7, 14, 21 and 28 days post infection (dpi) and splenic, lymph node, and liver T cells at 28 dpi. **A)** The left depicts a schematic description of the experimental procedure. The diagram to the right demonstrates the population dynamics of ko versus wt T cells over 35 days of infection. **B)** The depicted FACS plots to the left are representative of spleen samples analyzed at 35 days post infection and show how wt and ko cells were identified by expression of enhanced yellow fluorescent protein (eYFP), T cell receptor variable beta chain 8 (Vβ8), and CD45.1. The dot plot to the right displays the frequencies of wt and ko T cells among 5 mice. **C)** Splenic T cells were stimulated with 10^{-7} M to 10^{-12} M gp33 peptide. The plot to the left shows the percentage of interferon-gamma-positive (IFNγ+) tumor necrosis factor-alpha-positive (TNFα+) T cells in relation to the gp33 concentration. The dot plot to the right demonstrates the percentage of IFNγ+TNFα+ T cells stimulated with 10^{-7} M gp33 peptide. Each dot represents one mouse, horizontal lines the mean. **D)** Splenic T cells were analyzed for their expression of programmed cell death

1 (PD1) and T cell immunoglobulin and mucin-domain containing-3 (TIM3). Each dot represents one mouse, horizontal lines the mean. The data are representative of at least two independent experiments with five mice each. Statistical analysis: unpaired t-test, ****p ≤ 0.00001, *p ≤ 0.01, ns (not significant) p ≥ 0.05.

To verify whether the engraftment of *Tmem51*-deficient T cells or subsequent events lead to their reduced cell numbers in chronic infection, I used the Gzmb-ERT2/Cre system. It has the benefit, that on the hand ERT2/Cre gets expressed upon T cell activation and that deletion of *Tmem51* happens after the transfer into host mice. Specifically, tamoxifen was used to delete *Tmem51* at 0-5 days post infection in P14;Gzmb-ERT2/Cre;Rosa26-Stop$^{fl/fl}$-eYFP;*Tmem51*$^{fl/fl}$ T cells that were previously transferred into host mice that were infected with LCMV-cl13 **(Figure 12A)**. As before, wild-type T cells were evident in significantly higher numbers than *Tmem51*-deficient T cells at 7 and 14 days post infection in the blood and at 28 days post infection in the spleen, lymph node, and liver **(Figure 12B)**.

A

B
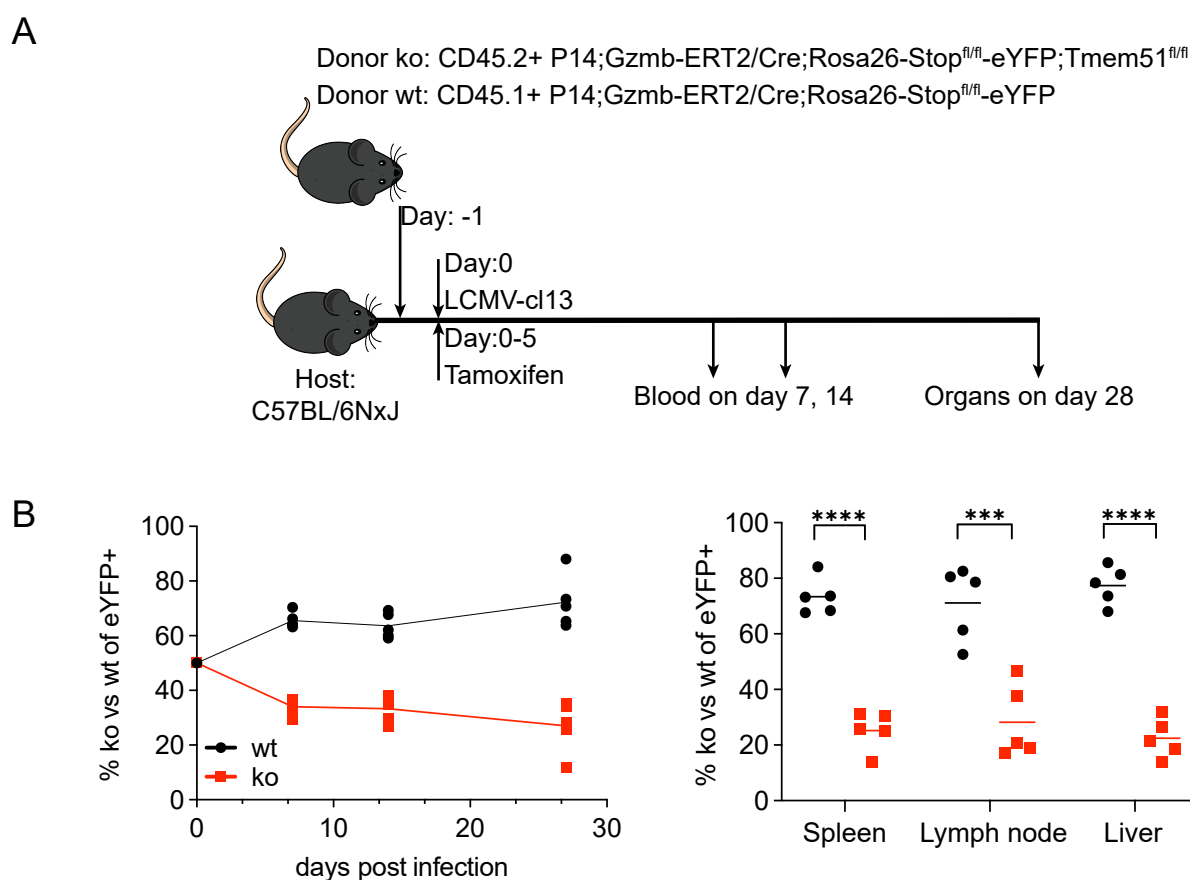
Figure 12 Deletion of *Tmem51* leads to reduced T cell numbers in chronic infection. A-B) CD45 congenic C57BL/6NxJ host mice were grafted with 2 × 10^3 wild-type (wt) and knockout (ko) P14 T cells. 24 hours later the host mice were infected with 5 × 10^6 PFU LCMV-cl13 and treated with Tamoxifen to induce ERT2/Cre mediated deletion of *Tmem51*. Peripheral blood T cells were analyzed by flow cytometry at 7 and 14 days post infection (dpi) and splenic, lymph node, and liver T cells at 28 dpi. **A)** Schematic of the experimental procedure. **B)** The plots to the left demonstrate the population dynamics of wt and ko P14 T cells at 7, 14 and 28 days post infection. The dot plot to the right shows the frequencies of wt and ko T cells in the spleen, lymph node, and liver among 5 mice. Each dot represents one mouse and the horizontal line the mean. The data are representative of at least two

independent experiments with five mice each. Statistical analysis: unpaired t-test, ****p ≤ 0.00001, ***p ≤ 0.0001, ns (not significant) p ≥ 0.05.

These data show that the reduced cell numbers of *Tmem51*-deficient T cells compared to wild-type T cells do not develop because of differences in engraftment of the populations. Moreover, the data indicate that the impact of *Tmem51* on CD8 T cell differentiation in chronic infection is not dependent on the timing of its deletion.

5.1.5 The absence of *Tmem51* in all CD4-expressing immune cells does not impact the CD8 T cell response to chronic LCMV-cl13 infection

The limited impact of *Tmem51*-deficiency on CD8 T cells response led me to investigate the impact of the deletion of *Tmem51* in other immune cell subsets. To this end, I employed the CD4-Cre expression system to delete *Tmem51*. Immune cells that express CD4 include subsets of dendritic cells and macrophages. For this experimental setup, mice that do not carry CD8 T cells that are TCR transgenic were selected. As knockout host mice, C57BL/6N;CD4-Cre;Rosa26-eYFP;Tmem51$^{fl/fl}$ and as wild-type host mice, C57BL/6N;Rosa26-Stop$^{fl/fl}$-eYFP mice were used. In these mice, it is additionally possible to monitor the polyclonal CD8 T cell response to infection. Although, CD8 T cells that respond to stimulation with the gp33 peptide already make up about 50 % of all T cells that respond to LCMV (Hudrisier et al., 1997). Altogether, this experimental design allows for the investigation of a broader impact of the deletion of *Tmem51* in the immune compartment. In detail, I infected P14-negative host mice that deleted *Tmem51* under the CD4 promotor with LCMV-cl13 and compared their polyclonal T cell response to that of CD4-Cre-negative host mice as a wild-type control **(Figure 13A)**. In peripheral blood T cells at 7 days post infection, there is no significant difference in the production of the effector cytokines IFNγ and TNFα by CD8 T cells responding to stimulation with the gp33 peptide **(Figure 13B)**. At 14- and 30-days post infection, wild-type and knockout host mice show similar levels of TIM-3 and PD1 expression in T cells specific for LCMV as identified by MHC multimer staining for gp33 (Tetramer+) **(Figure 13C, E)**. CD8 T cells derived from spleens at 30 days post infection and stimulated with gp33 showed no significant difference in their ability to produce effector cytokines **(Figure 13D)**. These finding indicate that the deletion of *Tmem51* in other immune compartments as well as CD8 T cells has no significant impact on the development of the exhausted phenotype of CD8 T cells during chronic infection with LCMV-cl13.

A

Host ko: C57BL/6N;CD4-Cre;Rosa26-eYFP;Tmem51^{fl/fl}
Host wt: C57BL/6N;Rosa26-Stop^{fl/fl}-eYFP
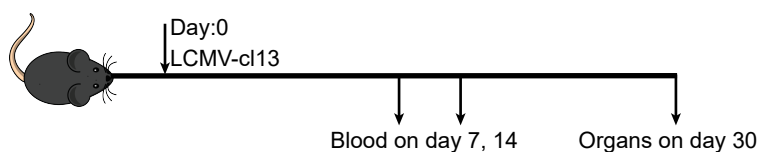
B
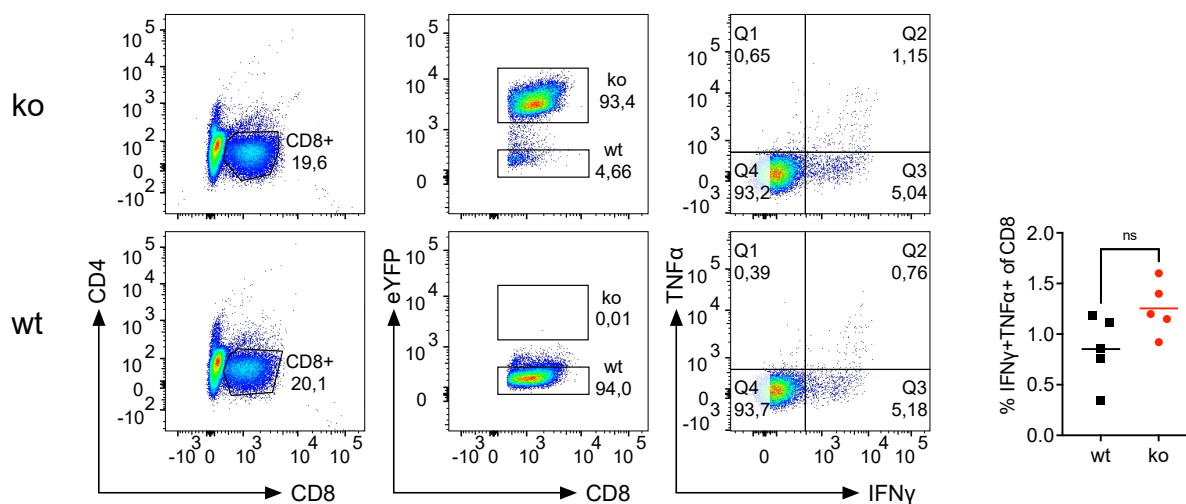
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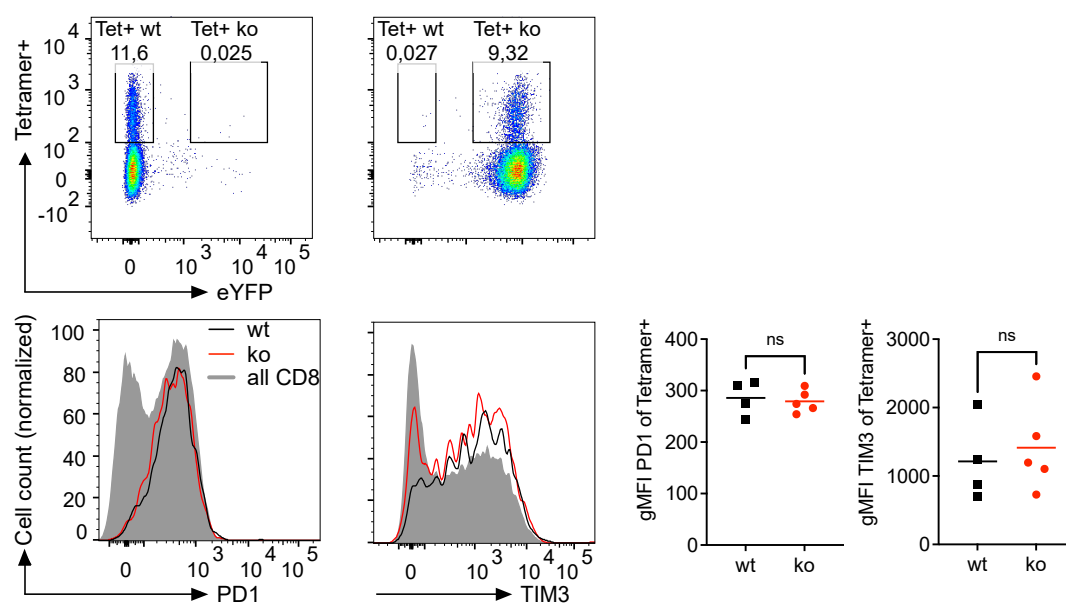
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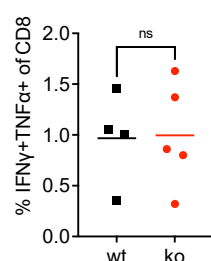
E
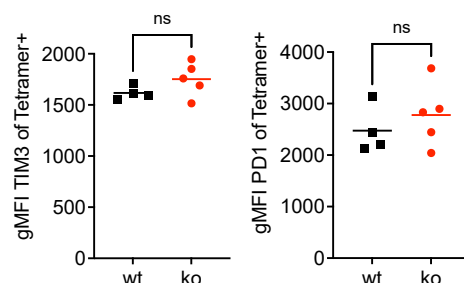
Figure 13 The CD8 T cell response to chronic infection is not altered in the absence of *Tmem51* in CD4-expressing immune cells. A-D) C57BL/6N;CD4-Cre;Rosa26-eYFP;*Tmem51*^{-/-} (ko) and C57BL/6N;Rosa26-Stop^{fl/fl}-eYFP;*Tmem51*^{fl/fl} (wt) mice were infected with 5 × 10⁶ PFU LCMV-cl13. Peripheral blood T cells were

analyzed by flow cytometry at 7 and 14 days post infection (dpi) and splenic T cells at 30 days post infection. **A)** Schematic representation of the experimental setup. **B)** Peripheral blood T cells at 7 days post infection were stimulated with with 10^{-7} M gp33 peptide and analyzed via flow cytometry. The identification of knockout versus wild-type mice via eYFP expression and the percentage of IFNγ+TNFα+ T cells are demonstrated on representative FACS plots. The dot plot to the right shows the percentage of IFNγ+TNFα+ T cells stimulated with 10^{-7} M gp33 peptide of ten mice. **C)** Peripheral blood T cells at 14 days post infection were analyzed via flow cytometry and LCMV-specific T cells were identified with a staining for gp33-specific MHC multimers (tetramer) as depicted on the representative FACS plots. The T cells were also analyzed for their expression of programmed cell death 1 (PD1) and T cell immunoglobulin and mucin domain-containing protein 3 (TIM3) as shown representative histogram overlays on the left as well as summarizing dot plots to the right. **D)** Splenic T cells at 30 days post infection were stimulated with 10^{-7} M gp33 peptide and analyzed via flow cytometry. The dot plot depicts the percentage of IFNγ+TNFα+ T cells stimulated with 10^{-7} M gp33 peptide of nine mice. **E)** Splenic T cells at 30 days post infection were analyzed via flow cytometry and the geometric mean fluorescent intensity (gMFI) of TIM3 and PD1 of nine mice is shown on the dot plots. Each dot represents one mouse and horizontal lines the mean. Statistical analysis: unpaired t-test, ns (not significant) $p \geq 0.05$.

In summary, the data show that the overexpression of *Tmem51* leads to significant downregulation of effector cytokine production *in vitro* and *in vivo*. These high levels of *Tmem51* had no impact on effector and memory T cell development in acute infection. The findings suggest that *Tmem51* has the potential to regulate the production of effector cytokines when expressed at high enough levels. When the expression of *Tmem51* is ablated during chronic infection, CD8 T cells accumulate in significantly lower numbers. Additionally, T*mem51*-deficient CD8 T cells display a small but overall insignificant increase in the production of effector cytokines. Overall, the exhausted phenotype of CD8 T cells in chronic infection cannot be reverted by the deletion of *Tmem51*.

5.2   *Ptpn2*-deficient CD8 T cells are less dependent on proliferative stimuli and show increased survival effector cells

5.2.1   *Ptpn2* deficiency does not alter thymocyte and peripheral T cell development

The reactivation of exhausted CD8 T cells for T cell-based immunotherapies of malignant tumors is based on the manipulation of factors that drive the exhausted phenotype. The molecule of interest, phosphatase tyrosine-protein phosphatase non-receptor type 2 (*Ptpn2*), has recently been identified to control the proliferation and survival of terminal exhausted CD8 T cells during chronic LCMV-cl13 infection (LaFleur et al., 2019). However, the impact of *Ptpn2* on CD8 T cell differentiation in acute infection and therapeutically relevant settings remained unknown. To address this question, I used an established mouse line that leads to T cell-specific deletion of *Ptpn2* through Lymphocyte-specific protein tyrosine kinase (Lck) driven expression of Cre recombinase (Wiede et al., 2011). In these transgenic mice, the fifth exon of *Ptpn2* is flanked by LoxP sites (Loh et al., 2011). The fifth exon encodes for the active site of *Ptpn2* and it has been demonstrated that no active protein is expressed (Wiede et al., 2011). Employing this specific system to express the Cre recombinase, *Ptpn2* is deleted in the thymus at double negative stage 3/4 (DN3/4) (Wiede et al., 2017). Consequently, I investigated if thymic development of *Ptpn2*-deficient OT-1 T cells is impaired. To this end, I analyzed thymocytes from 6-12 weeks old donor mice and could demonstrate that the cellularity of $CD4^+CD8^+$ double positive (DP) and single positive CD8 T cells (CD8SP) is not impacted by the loss of *Ptpn2* (**Figure 14A-D**). In addition, looking at the percent of mature $CD24^-$ cells among CD8 single positive OT-1 T cells and the CD69 expression level among bright and dull double positive OT-1 T cells, no differences were evident when comparing wild-type to *Ptpn2*-deficient T cells (**Figure 14A-D**). T cells in the so-called DP bright stage co-express high levels of CD8 and CD4 and are the thymocytes that enter positive selection. They then express less CD4 and CD8 after encountering antigen and move to the DP dull stage. As OT-1 donor cells for all consequent experiments were obtained from spleens from 6 to 10-week old mice, I also characterized the amount of CD8 T cells showing a naïve phenotype by demonstration of the ratio of $CD44^{low}$ $CD62L^{high}$ to $CD44^{high}$ $CD62L^{low}$ CD8 OT-1 T cells (**Figure 14E**). Donor cells that were used for the following experiments, showed a comparable phenotype when comparing wild-type to knockout CD8 OT-1 T cells. In conclusion, there is no evidence for a disturbance of CD8 T cell development in thymic as well as peripheral OT-1 T cells.
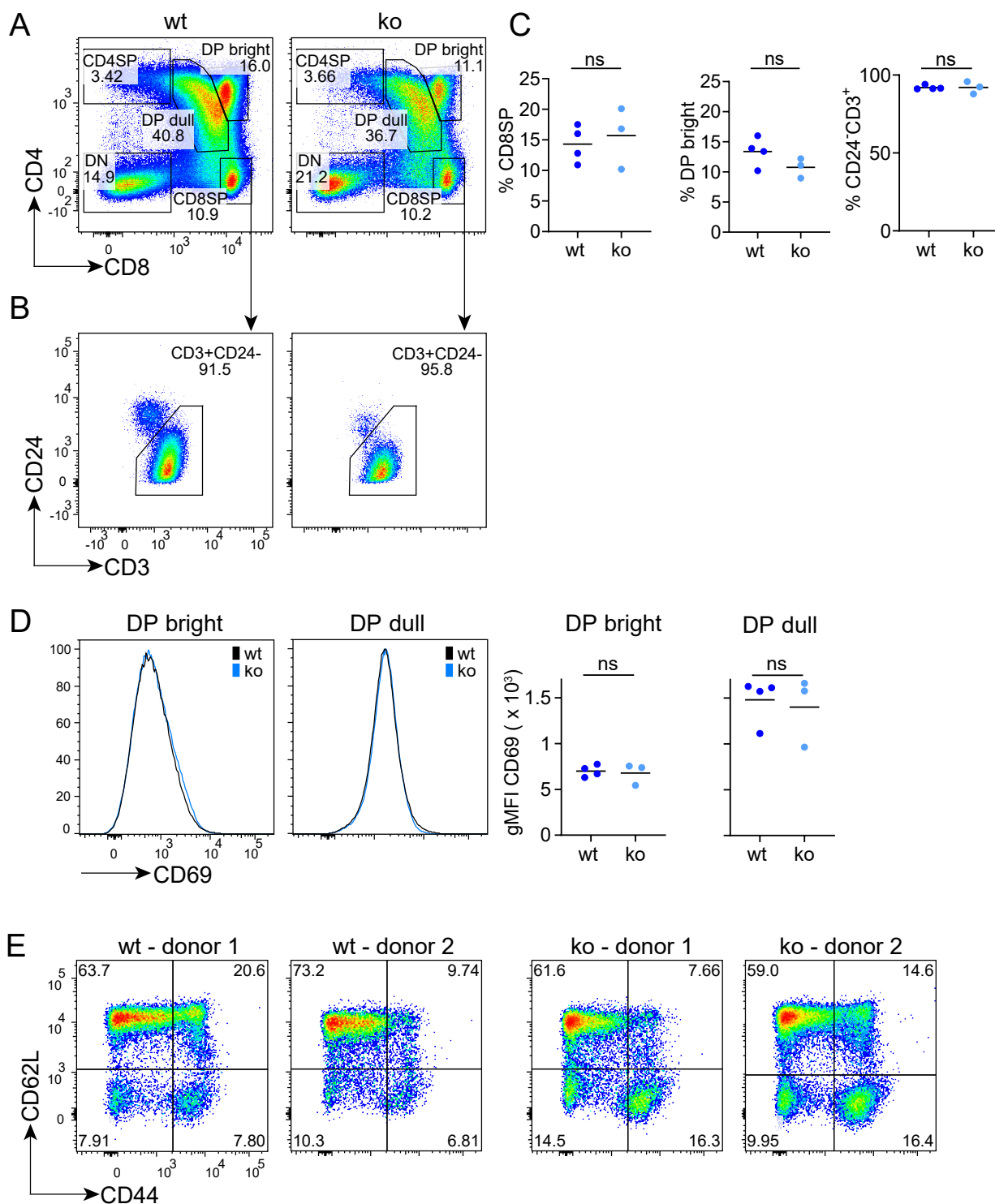
Figure 14 Absence of *Ptpn2* does not impair thymic development. The thymi of OT-1;Lck-Cre;*Ptpn2*fl/fl (ko) and OT-1;*Ptpn2*fl/fl (wt) OT-1 were taken from donor mice at the age of 6-12 weeks. **A)** Frequencies of developing T cells among total thymocytes were determined by flow cytometry and FACS plots of the major developmental stages of two mice are shown. Data are representative of seven donor mice. SP: single positive, DP: double positive, DN: double negative **B)** The FACS plots show the percentage of CD24-CD3+ and the histogram shows the level of CD69 expression of CD8-single positive cells (CD8SP). **C)** The dot plots show the summary of all 7 animals and each dots represents one individual mouse, horizontal lines the mean. **D)** The histograms show a representative overlay of the CD69 expression levels of the CD8+CD4+ bright and dull populations and the dot plots show the geometric mean fluorescent intensity (gMFI) of all seven animals. **E)** The FACS plots show the pre-

activation status of isolated OT-1 CD8 T cells by means of CD44 and CD62L expression of 4 individual donor mice that were used in the following experiments. Statistical analysis: unpaired t test. ns (not significant) p ≥ 0.05. From „*Ptpn2* Deficiency Enhances Programmed T cell Expansion and Survival Capacity of Activated T cells" by Flosbach et al., 2020, Cell Reports 32, 107957 July 28, 2020 © 2020, The Author(s). https://doi.org/10.1016/j.celrep.2020.107957.

5.2.2 CD8 T cells lacking *Ptpn2* preferentially develop into terminal effector CD8 T cells

The previously presented data provided evidence that naive *Ptpn2*-deficient T cells show no apparent deficits in their development. As a consequence, they can serve as a valuable experimental system to study the impact of *Ptpn2* on T cell differentiation in response to pathogenic infection. To monitor the pathogen-specific response of one clone of T cells, I transferred low numbers of CD45.2 congenic *Ptpn2*-deficient *Lck*-Cre;*Ptpn2*$^{fl/fl}$ OT-1 (ko) and control OT-1;*Ptpn2*$^{fl/fl}$ CD8 T cells (wt) into CD45.1 congenic C57BL/6 hosts. These host mice were then infected with recombinant *Listeria monocytogenes* (Lm) which was modified to express the ovalbumin derived SIINFEKL ligand (N4). This is the natural high-affinity ligand of the TCR transgenic OT-1 T cells. Using the congenic marker system, I was able to conveniently track the pathogen-specific response of the transferred T cells. At 7 days post infection, I could clearly show that *Ptpn2*-deficient CD8 T cells develop primarily into CD127$^-$KLRG1$^+$ terminal effector CD8 T cells after infection with Lm-N4 **(Figure 15A, B)**. Similarly, the generation of CD127$^+$KLRG1$^-$ memory precursor CD8 T cells was reduced **(Figure 15A, B)**. This clear shift between the effector and memory precursors was evident until day 28 and was apparent in the spleen and blood of infected animals (**Figure 15B**). The principal ability to develop memory cells was not compromised as a clear CD127$^+$ CD8 T cell population developed in the *Ptpn2*-deficient CD8 T cells (**Figure 15A**). Despite these major phenotypic differences between wild-type and *Ptpn2*-deficient OT-1 T cells, their magnitude of expansion was comparable (Flosbach et al., 2020). Additionally, we were able to show that upon low affinity stimulation, *Ptpn2*-deficient temporarily showed enhanced expansion when compared to their wild-type counterpart (Flosbach et al., 2020). Interestingly, we could also demonstrate that *Ptpn2*-deficient KLRG1$^+$ CD8 T cells were stable for several weeks and even able to robustly re-expand upon secondary infection (Flosbach et al., 2020). Overall, the data show that deletion of *Ptpn2* increases long-term survival as well as expansion capacity of normally short-lived effector CD8 T cells. At the same time, memory formation was not impacted and *Ptpn2*-deficient CD8 T cells showed no functional deficits.
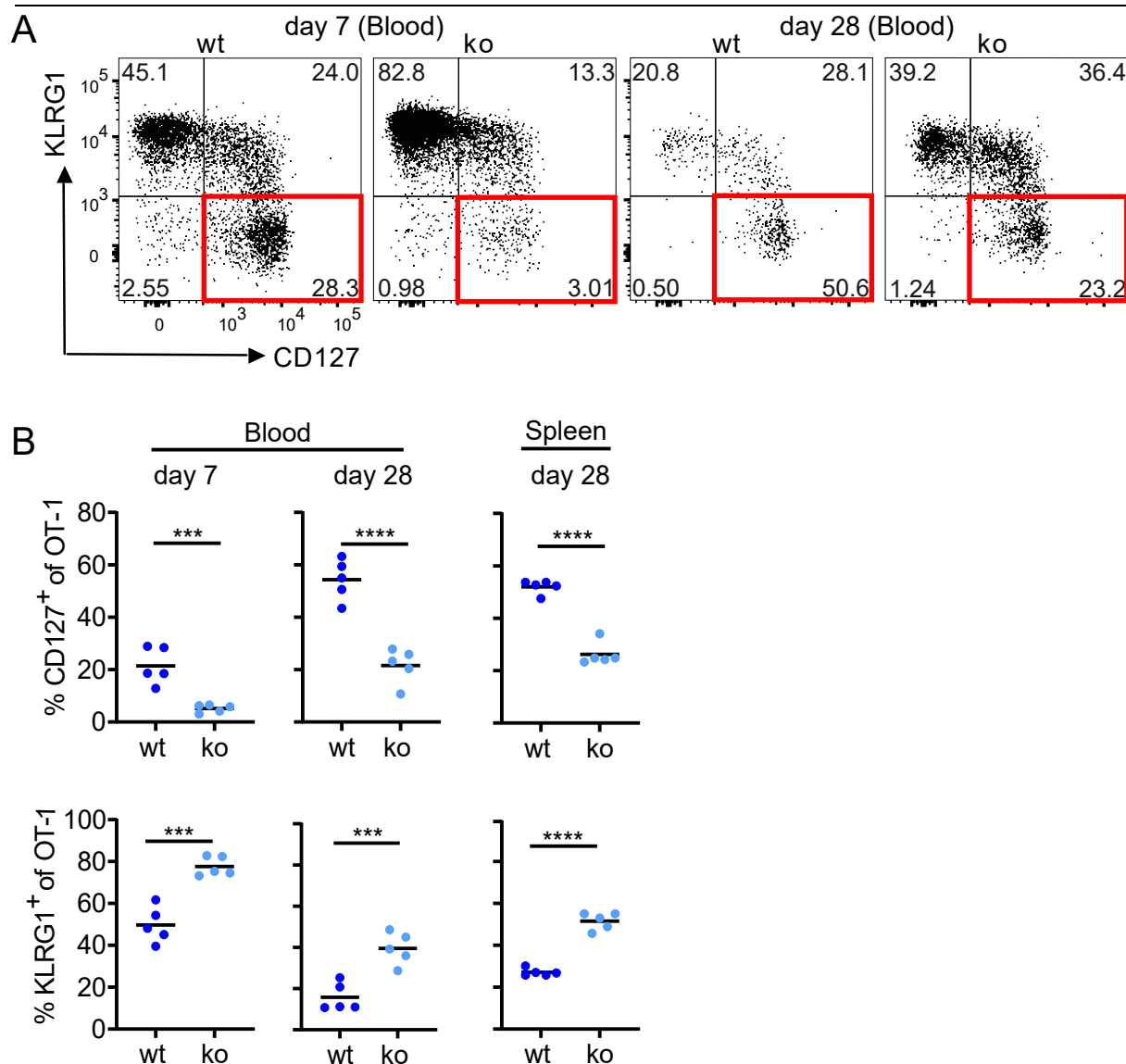
Figure 15 *Ptpn2* alters the ratio of terminal effector versus memory precursor T cells. CD45 congenic C57BL/6J host mice were grafted with 10^4 wild-type (wt) or knockout (ko) OT-1 T cells and infected with 1000 CFU Lm-N4 24 hours later. **A, B)** Peripheral blood T cells were analyzed by flow cytometry at 7 and 28 days post infection and splenic T cells at 28 days post infection. The depicted FACS plots are representative of blood samples analyzed at 7 and 28 days post infection and the dot plots show the frequencies of KLRG1+ (upper row) and CD127+ (lower row) OT-1 T cells of five mice. The data are representative of at least two independent experiments with four to five mice in each group and the horizontal line represents the mean. Statistical analysis: unpaired t-test, ****p ≤ 0.00001, ***p ≤ 0.0001, ns (not significant) p ≥ 0.05.   Adapted from „*Ptpn2* Deficiency Enhances Programmed T cell Expansion and Survival Capacity of Activated T cells" by Flosbach et al., 2020, Cell Reports 32, 107957 July 28, 2020 © 2020, The Author(s). https://doi.org/10.1016/j.celrep.2020.107957.

5.2.3 *Ex vivo*-activated and adoptively transferred *Ptpn2*-deficient T cells show enhanced expansion capacity

The enhanced survival and maintenance of *Ptpn2*-deficient effector T cells led me to test whether the manipulation of *Ptpn2* could enhance the adoptive transfer capacity of *ex vivo* activated T cells. This is of special interest, as the efficacy of adoptive T cell therapies is in large parts determined by the number of engrafted T cells and their *in vivo* expansion capacity. Typically, *ex vivo* activated and manipulated T cells are used for adoptive T cell therapies. To address this, I used a previously established *in vitro* stimulation system in which T cells are exposed to CD3/CD28 antibody-coupled beads (Dynabeads Mouse T-Activator CD3/CD28, Gibco) that lead to a TCR-dependent T cell activation which is similar to activation by antigen presenting cells for a defined period (Ito et al., 2003). Afterwards, the activated CD8 T cells can be easily separated via magnetic separation MACS columns and then transferred into host mice. As a first step, I investigated how *ex vivo* activated OT-1 CD8 T cells would respond upon transfer to 1) antigen-free mice, 2) mice with an ongoing infection, and 3) mice carrying the cognate antigen for OT-1 T cells. Specifically, I transferred OT-1 T cells into 1) non-infected host mice or 2) mice infected with wild-type *Listeria monocytogenes* (Lm-wt) or 3) mice infected with recombinant *Listeria monocytogenes* that produce the SIINFEKL epitope (Lm-N4) recognized by OT-1 T cells (**Figure 16A**). In antigen-free host mice, *Ptpn2*-deficient OT-1 T cells underwent a significantly stronger expansion than their wild-type counterpart, reflected by 9-times the amount of T cells recovered after 6 days of *in vivo* expansion (**Figure 16B, C**). Interestingly, the ratio shifted to about 160:1 (ko:wt) following transfer into wild-type *Listeria* (Lm-wt) infected mice (**Figure 16B, C).** When the cognate antigen of the TCR transgenic OT-1 T cells was present in mice infected with Lm-N4, the expansion of *Ptpn2*-deficient and wild-type OT-1 CD8 T cells was comparable (**Figure 16B, C**). These findings demonstrate, that the *ex vivo* stimulated *Ptpn2*-deficient T cells are not in a hyperactivated state in which additional stimulation would lead to activation-induced cell death. In contrast, they are able to respond stronger to inflammatory stimuli. In conclusion, the absence of *Ptpn2* enhances the capacity of briefly activated T cells to expand after they have been separated from the *ex vivo* stimulation system.
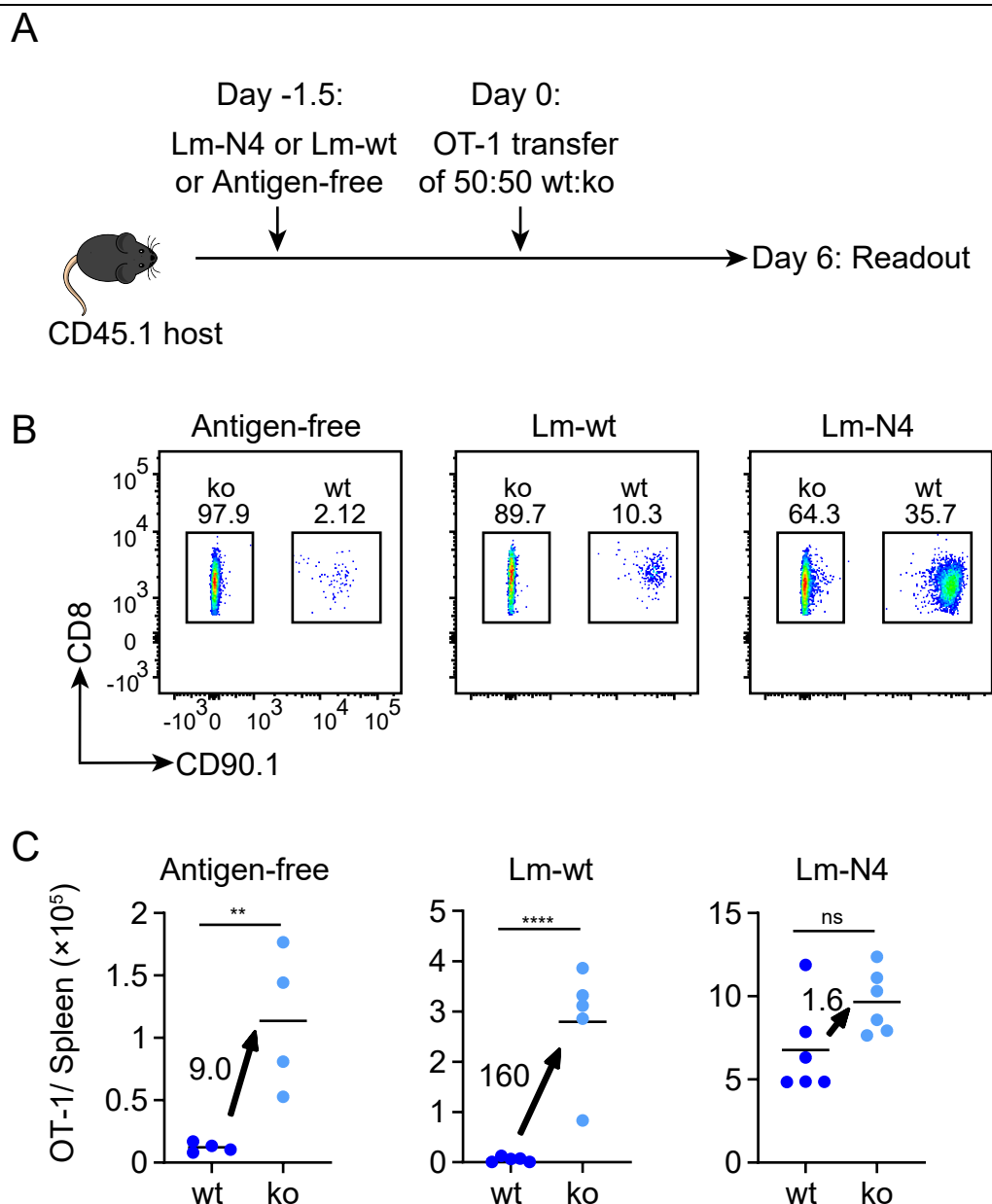
Figure 16 Pre-activated *Ptpn2*-deficient T cells expand significantly in response to antigen. **A)** CD45 congenic C57BL/6J host mice were infected with 1000 CFU Lm-N4 or Lm-wt or kept antigen-free for 36 hours before receiving 5×10^4 OT-1;Lck-Cre;*Ptpn2*$^{fl/fl}$ (ko) mixed with 5×10^4 OT-1;*Ptpn2*$^{fl/fl}$ (wt) OT-1 that had been activated *ex vivo* for 36 hours. **B)** Numbers of OT-1 T cells in the spleen were determined by flow cytometry 7.5 days after infection and representative FACS plots of one mouse of each condition are displayed. **C)** The dot plots show the absolute numbers of OT-1 T cells per spleen and each dot represents one individual mouse, horizontal lines the mean. Data are representative of two independent experiments for the transfer in antigen-free hosts and one experiment for the transfer in infected hosts, with at least four mice per group. Statistical analysis: unpaired t test. ****p ≤ 0.00001, **p ≤ 0.001, *p ≤ 0.01, ns (not significant) p ≥ 0.05. From „*Ptpn2* Deficiency Enhances Programmed T cell Expansion and Survival Capacity of Activated T cells" by Flosbach et al., 2020, Cell Reports 32, 107957 July 28, 2020 © 2020, The Author(s). https://doi.org/10.1016/j.celrep.2020.107957.

5.2.4 *Ptpn2*-deficient CD8 T cells have an enhances capacity for programmed expansion

Next, I wanted to further verify the observed enhanced expansion capacities of the shortly *ex vivo* activated T cells. To this end, I slightly changed the T cell activation protocol to activation of the TCR via a peptide loaded MHC-I molecule and also monitored T cell proliferation in blood and lymph node. In detail, I took advantage of the previously established *in vitro* stimulation system that uses SIINFEKL, H-2Kb and CD80 expressing artificial antigen presenting cells (MEC.B7.SigOVA) to activate OT-1 CD8 T cells (van Stipdonk et al., 2001). This system originally led to the discovery of the so-called programmed expansion of T cells. It describes the phenomenon that even a short burst of antigenic TCR stimulation leads to clonal expansion and differentiation of CD8 T cells (Bevan & Fink, 2001; Van Stipdonk et al., 2001). Here, OT-1 CD8 T cells were activated for 1 day and then transferred into antigen-free host mice for 7 days. I could demonstrate an increase of 5-times more *Ptpn2*-deficient T cells in the spleen compared with wild-type T cells, about 7 times more in the blood, and no significant changes in lymph nodes (**Figure 17A**). The phenotype of the recovered OT-1 CD8 T cells was comparable to the observations made during infection, as reflected by an increased frequency of KLRG1$^+$ T cells in the *Ptpn2*-deficient CD8 OT-1 T cells (**Figure 17B**). To investigate whether enhanced engraftment of the *Ptpn2*-deficient CD8 T cells leads to their increased numbers, I also showed the engraftment of activated as well as naïve CD8 T cells upon transfer into antigen-free host mice. The numbers of *Ptpn2*-deficient and wild-type OT-1 CD8 T cells were comparable at 6 hours after the transfer of naïve CD8 T cells and up to 20 hours after the transfer of activated T cells (**Figure 17C**). This excludes that differences in the transfer efficacy account for the increased numbers of *Ptpn2*-deficient T cells. Altogether, these data indicate that the enhanced numbers of *Ptpn2*-deficient T cells arise due to enhanced proliferation and survival.
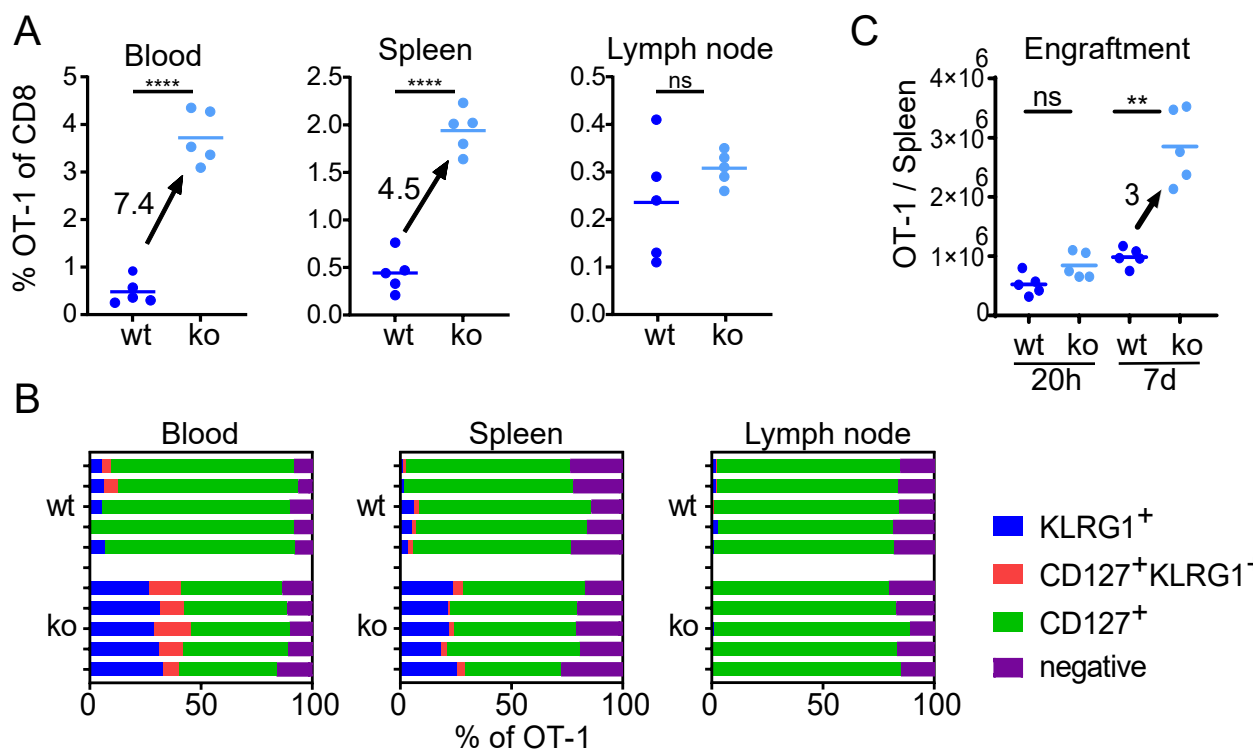
Figure 17 *Ptpn2* deficiency enhances programmed expansion of briefly stimulated effector T cells. *Ptpn2*-deficient (ko) or wild-type (wt) OT-1 T cells were activated *in vitro* with SIINFEKL, H-2Kb and CD80 expressing artificial antigen presenting cells (MEC.B7.SigOVA) for 1 day. Activated T cells (10^5) were then transferred into antigen-free CD45 congenic C57BL/6J host mice. **A)** Frequency of OT-1 among total CD8 T cells was determined 7 days post transfer. Bar graphs in **B)** show the representative phenotype of 5 individual mice of the recovered OT-1 T cells. **C)** The plot shows the OT-1 T cell numbers recovered per spleen at 20 hours post transfer and at 7 days post transfer of 10^5 activated wt versus ko OT-1 T cells. The data are representative of at least two independent experiments with 5 mice each. Dots in all panels represent data from a mouse, horizontal lines the mean. Statistical analysis: unpaired t-test, ****p≤0.00001, ***p≤0.0001, **p≤0.001, *p≤0.01, ns (not significant) p≥0.05. Adapted from „*Ptpn2* Deficiency Enhances Programmed T cell Expansion and Survival Capacity of Activated T cells" by Flosbach et al., 2020, Cell Reports 32, 107957 July 28, 2020 © 2020, The Author(s). https://doi.org/10.1016/j.celrep.2020.107957.

To verify whether the increased numbers of *Ptpn2*-deficient T cells led to functional differences in the host mice, we tested, if the *ex vivo*-activated and transferred *Ptpn2*-deficient T cells displayed higher cytotoxicity when compared to the wild-type population. The *in vivo* cytotoxic activity in host mice that received *Ptpn2*-deficient T cells was significantly higher when compared to wild-type T cells (Flosbach et al., 2020). In conclusion, the data demonstrate that *Ptpn2*-deficient CD8 OT-1 T cells have an increased capacity for programmed expansion upon transfer in antigen-free host mice. This capacity increases greatly in the favor of *Ptpn2*-deficient CD8 OT-1 T cells when the cells are transferred in mice with a non-antigen-specific ongoing infection that compromises an *in vivo* environment of higher inflammatory cytokines.

5.2.5 *Ptpn2* deficiency in recently activated CD8 T cells leads to wide-ranging changes in their phosphoproteome

The deletion of *Ptpn2* seems to lead to a greater capacity of T cells to respond to inflammatory stimuli which allows for extended survival and proliferation of effector T cells. To elucidate the involved mechanisms of action, I analyzed 30-hour *ex vivo*-activated wild-type or *Ptpn2*-deficient OT-1 T cells via proteomics. Overall, 1565 differentially expressed proteins and 2030 phosphorylated sites with differential abundance were detected (**Figure 18A**). There were 24 differentially phosphorylated phospho-tyrosine sites that include known targets of *Ptpn2* but also additional targets that were not known so far (**Figure 18B**). Compared to serine and threonine phosphorylations, the relatively low number of phospho-tyrosine target sites can be expected. Tyrosine-phosphorylations generally represent a smaller margin of overall phosphorylations (Huttlin et al., 2010; Lundby et al., 2012).
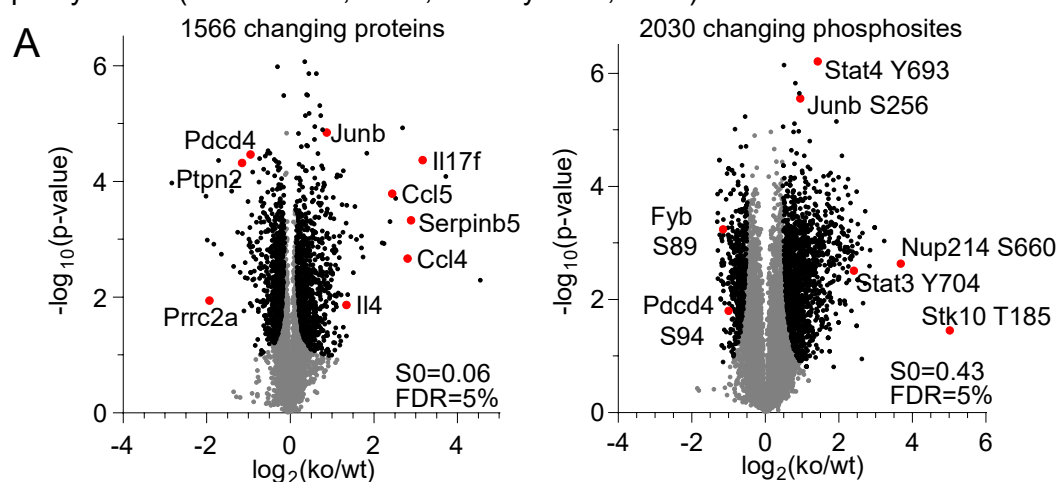
B

Proteins	Isoforms	Positions within isoforms	Amino acid	log2 ko/wt	Localization probability	Score
Stat3	P42227-3	704	Y	2.4	1.00	115
Stat3	P42227;B7ZC18;P42227-2	705;679;705	Y	2.3	1.00	138
Stat5b	P42232	699	Y	2.2	1.00	122
Stat5a	P42230	694	Y	1.9	1.00	108
Stat4	Q3V157;P42228	693;694	Y	1.4	0.99	116
Vav1	P27870;E9PXI0;Q8VDU4	844;820;805	Y	1.2	0.90	51
Ptpn18	Q3V441;Q61152	32;381	Y	1.2	0.77	119
Fyb	O35601;O35601-2	559;559	Y	1.1	1.00	122
Ptpn6	P29351;P29351-2;P29351-3;G3UYY5	564;566;525;64	Y	1.0	1.00	108
Mapk1	P63085	185	Y	1.0	1.00	190
Pdap1	Q3UHX2	70	Y	1.0	0.93	120
Srsf1	H7BX95;Q6PDM2	202;202	Y	0.9	0.98	98
Hist1h4a	P62806	52	Y	0.9	1.00	118
Pabpc1	P29341	364	Y	0.8	1.00	90
Itsn2	E9QNG1;B2RR82;Q9Z0R6;Q9Z0R6-2;A0A1W2P775	922;949;922;922;481	Y	0.8	1.00	138
Cd28	P31041	189	Y	0.7	1.00	165
Cblb	Q3TTA7;B9EKI5;Q3TTA7-2	889;845;737	Y	0.6	1.00	70
Cfl1	P18760;F8WGL3	68;68	Y	-0.7	0.87	179

Figure 18 Differentially phosphorylated and expressed proteins in briefly activated *Ptpn2*-deficient T cells. Quantitative changes of protein and phosphosite levels of *Ptpn2*-deficient (ko) versus wild-type (wt) OT-1 T cells that were activated for 30 hours *in vitro* with antiCD3/antiCD28 coupled beads. **A)** Volcano plots showing significant

hits (black) compared to non-significant hits (gray) were determined via a constant S0, which was calculated in R (version 3.4.1, function "samr") and further corrected for multiple testing by applying a permutation-based 5% FDR calculation. B) Table showing the differentially phosphorylated tyrosine-sites with notation of their specific isoform, the tyrosine position within the isoform, the $\log_2$ fold change, the localization probability, and the MS-Score, an experimental quality control parameter. Adapted from „*Ptpn2* Deficiency Enhances Programmed T cell Expansion and Survival Capacity of Activated T cells" by Flosbach et al., 2020, Cell Reports 32, 107957 July 28, 2020 © 2020, The Author(s). https://doi.org/10.1016/j.celrep.2020.107957.

5.2.6   Recently activated *Ptpn2*-deficient CD8 T cells show an altered cytokine signaling profile

The wide-ranging alterations in protein and phosphorylation levels caused by the deletion of *Ptpn2* prompted me to explore the main underlying mechanisms. To this end, I used the *de novo* pathway enrichment tool KeyPathwayMiner (List et al., 2016; Pauling et al., 2014). Firstly, known protein-protein interactions were obtained from the murine STRING network (v. 11 (Szklarczyk et al., 2019)) and combined with interactions of the curated database embedded in the Ingenuity pathway analysis tool (IPA, v. 46901286, QIAGEN Inc., (Krämer et al., 2014)). Secondly, significantly differing phosphorylated tyrosine phosphoproteins and differential protein expression values of the proteome analysis were used to define possible subnetworks with KeyPathwayMiner. As a result, several subnetworks were obtained and the largest subnetwork extracted by KeyPathwayMiner was used for additional analysis and visualization (**Figure 19A**). In detail, I used the tool Cytoscape (Shannon et al., 2003) to automatically overlay protein expression data and manually added annotated functions of differentially phosphorylated tyrosine residues (**Figure 19A**). In the absence of *Ptpn2*, central regulatory nodes in this network such as STAT3, STAT4, and STAT5 are hyperphosphorylated and in a transcriptionally active state. These key transcription factors are connected to a larger number of differentially expressed proteins. Of note, proliferation-associated proteins such as IL-2, IL-2rα, IFN-γ, I -4, CD44 as well as several more display enhanced levels of expression in recently activated *Ptpn2*-deficient CD8 T cells when compared to wild-type CD8 T cells (**Figure 19A**). To investigate how well the extracted subnetwork represents the overall impacted changes, I analyzed the data directly via IPA which determined the probability index (z-score) that links *Ptpn2* deficiency-related proteome alterations to specific functions and disease-related signatures (**Figure 19B**). These annotated functions are curated in the IPA database and the "Quantity of T lymphocytes" is highlighted with a green border as one example (**Figure 19A-B**). The *Ptpn2* deficiency-related differences in the expression of 91 out of all 1566 differentially expressed proteins leads to a positive correlation with the function „Quantity of T lymphocytes", which in the end means, that the quantity of T lymphocytes is increased in the *Ptpn2*-deficient T cells when compared to wild-type T cells. Interestingly,

about 30 % of these proteins are represented in the subnetwork extracted by KeyPathwayMiner (**Figure 19B**).
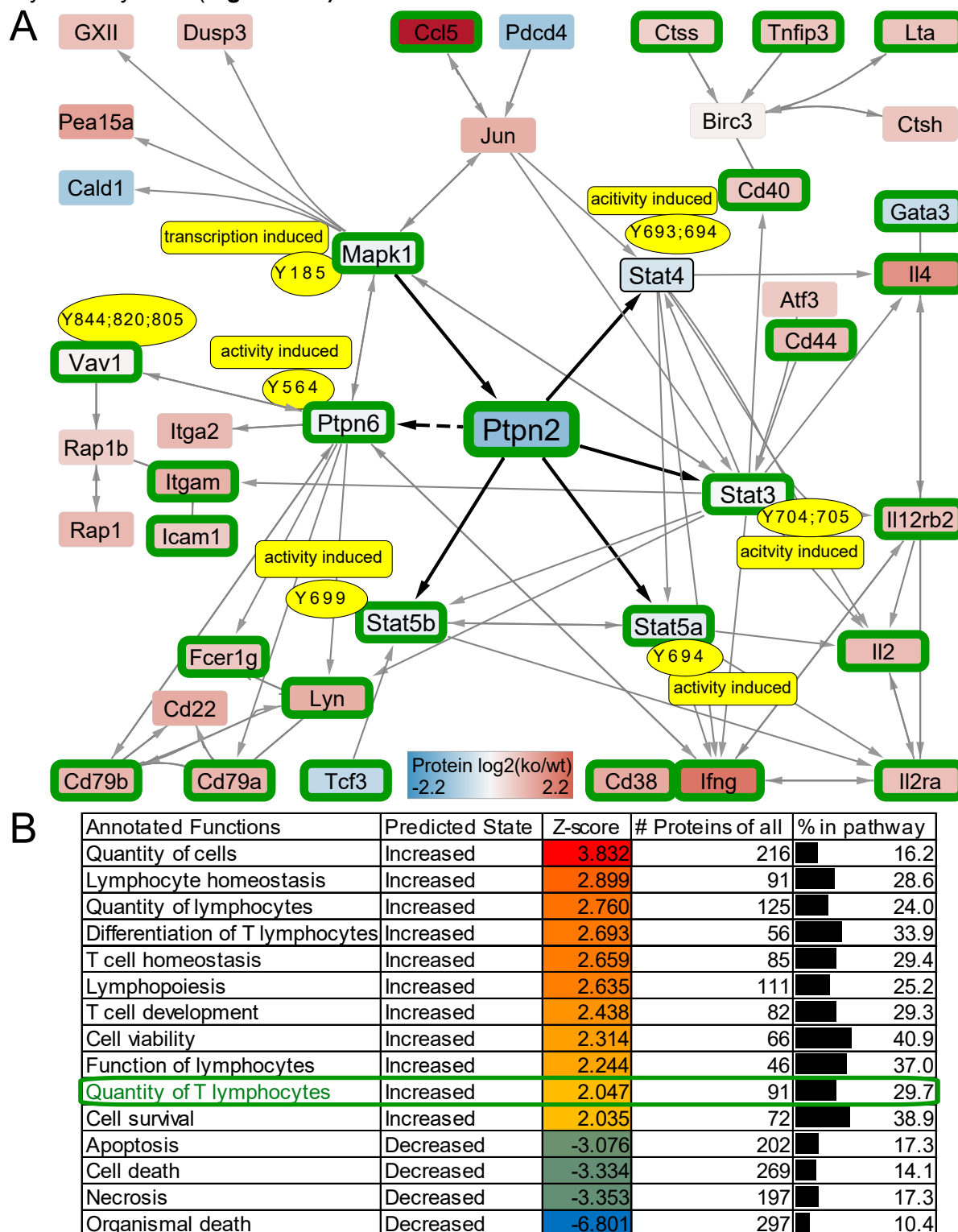
Figure 19 Proteome analysis of briefly stimulated wild-type and *Ptpn2*-deficient T cells. A) To extract signaling cascades that are markedly deregulated in the absence of *Ptpn2*, I used the *de novo* network enrichment tool KeyPathwayMiner. This used the interactome of the murine STRING network (v. 11), and known interactions of differentially phosphorylated tyrosine phosphosites curated in the Ingenuity pathway analysis (IPA) database to extract the key regulatory network of these data sets: (i) differentially phosphorylated tyrosine phosphoproteins

(absolute log2 ko/wt fold change of > 0.5 and p < 0.05) and (ii) differential protein expression (absolute log2 fold change of >0.5 and p < 0.05). Depicted is the largest subnetwork that has been extracted by KeyPathwayMiner. This has been overlaid with protein expression data and function of differentially phosphorylated tyrosine residues. Highlighted with a green border are the proteins that are associated with an increased quantity of T lymphocytes as designated by the IPA analysis. **B)** Analysis of changes in protein expression and phosphorylation status via the IPA software. Probability index (z-score) that links *Ptpn2* ko versus wt data sets to specific signatures. Adapted from „*Ptpn2* Deficiency Enhances Programmed T cell Expansion and Survival Capacity of Activated T cells" by Flosbach et al., 2020, Cell Reports 32, 107957 July 28, 2020 © 2020, The Author(s). https://doi.org/10.1016/j.celrep.2020.107957.

Altogether, the data suggests that recently activated *Ptpn2*-deficient CD8 T cells show an increased responsiveness to cytokine stimuli leading to enhanced capacities for co-stimulation-independent expansion and survival.

5.2.7 IL-2 sensitivity and survival capacity are enhanced in *Ptpn2*-deficient CD8 T cells

The proteomics data and analysis pointed at increased responses of recently activated *Ptpn2*-deficient T cells to cytokine-related stimuli. I investigated whether this had functional consequences on T cell proliferation via exposure of *in vitro* activated CD8 T cells to titrated doses of IL-2. Because increased cell numbers can have a positive impact on T cell survival, the cells were split every 24 hours and seeded with the same cell count in fresh tissue culture wells. In addition, the cells were re-supplemented with 50% conditioned medium and 50% fresh medium and the respective amounts of IL-2. I observed that in high IL-2 concentrations of 50 Units/ml, *Ptpn2*-deficient T cells had no expansion or survival benefit when compared to wild-type T cells (**Figure 20A**). This was apparent in similar cell numbers and comparable cluster formation (**Figure 20A-B**). The formation of proliferative clusters is a hallmark of functionally intact and proliferating CD8 T cells *in vitro.* Cluster formation was determined via microscopy and automated cluster size determination and counting. After 48 hours in culture, the wild-type CD8 T cells failed to proliferate in lower IL-2 concentrations, this is reflected in stagnating cell numbers and almost no visible cluster formation (**Figure 20A-B**). In contrast, the *Ptpn2*-deficient CD8 T cells were proliferating robustly even when provided with no IL-2 after 48 hours of cultivation. They formed significantly bigger proliferative clusters in all conditions and were able to survive and expand up to 96 hours of cell culture without provision of IL-2 (**Figure 20A-B**).
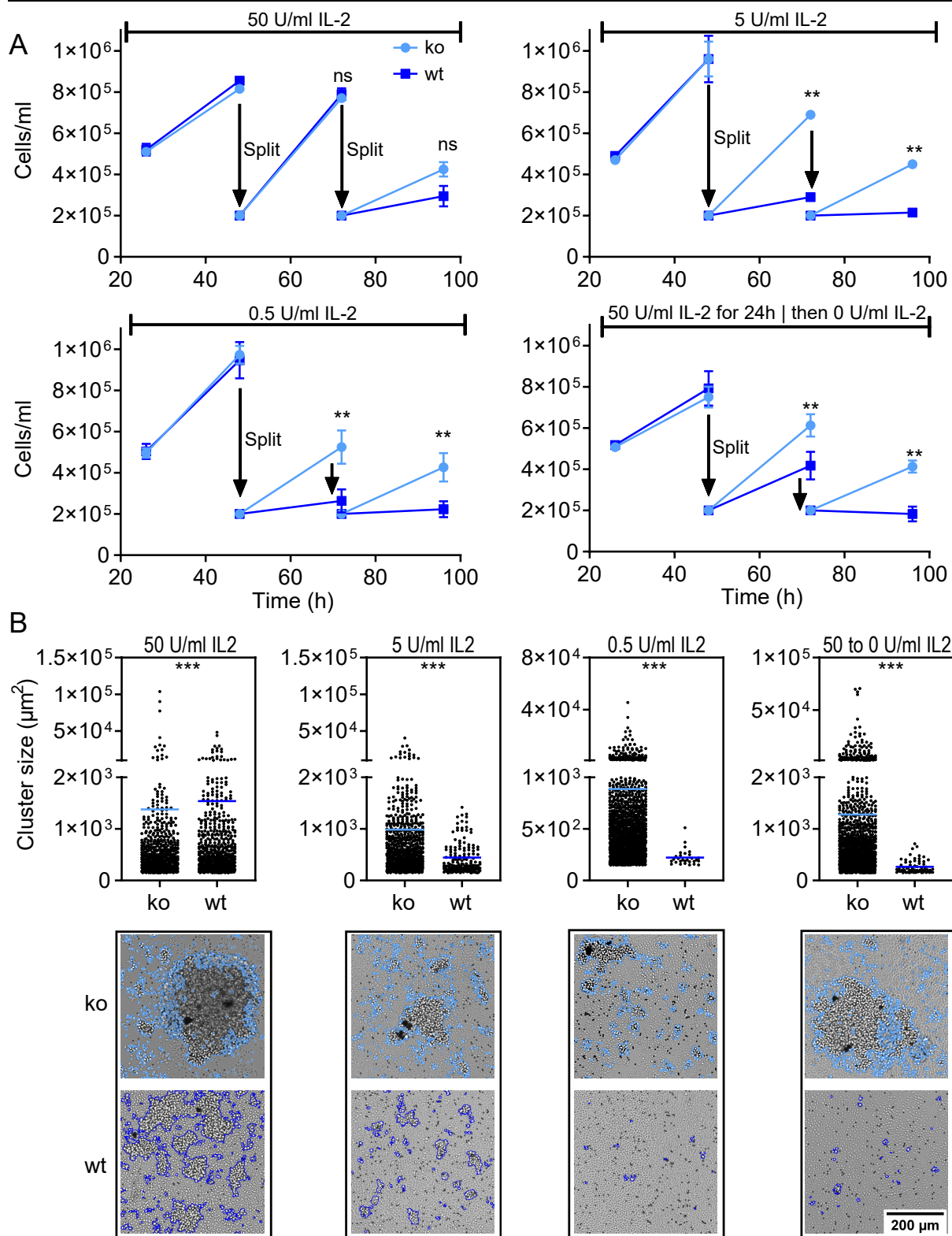
Figure 20 *Ptpn2* deficiency increases IL-2 sensitivity of recently activated T cells. A) A total of 5×10^5 *Ptpn2*-deficient (ko) or wild-type (wt) OT-1 T cells were activated with antiCD3/antiCD28 coupled beads and stimulated with the indicated concentrations of IL-2. Activated cells were split every 24 hours and 2×10^5 cells were transferred into new wells. **B**) The dot plots show the number and size of clusters at 72 hours after activation and each dot represents one cluster of a size > 200 µm. The images show a representative example of cluster determination via ilastik (v. 1.3.2) as indicated by the blue outline. The number of clusters was counted via ImageJ (v. 1.5). Shown data are three replicates that are representative of three independent experiments. Statistical analysis: **A**) unpaired t-test, ***p≤0.0001, **p ≤ 0.001, ns (not significant) p ≥ 0.05. **B**) nonparametric Mann-

Whitney-test with indicated p-values. From „*Ptpn2* Deficiency Enhances Programmed T cell Expansion and Survival Capacity of Activated T cells" by Flosbach et al., 2020, Cell Reports 32, 107957 July 28, 2020 © 2020, The Author(s). https://doi.org/10.1016/j.celrep.2020.107957.

In summary, the data demonstrate that *Ptpn2* deficiency renders recently *in vitro* activated CD8 T cells to be more sensitive to very low levels of IL-2. This allows them to maintain a highly proliferative state. Considering all observations, the data strongly suggest that a heightened sensitivity to cytokine stimuli is the key mechanism by which normally short-lived *Ptpn2*-deficient CD8 T cells are able to expand much more rigorously when compared to wild-type CD8 T cells.

6   Discussion

6.1   *Tmem51* regulates the production of effector cytokines in CD8 T cells

6.1.1 High levels of *Tmem51* significantly reduce effector cytokine production of CD8 T cells

The development of exhausted CD8 T cells is constantly being investigated and several key factors that contribute to the development and specific features of exhausted CD8 T cells have been identified. Nonetheless, there are still many molecules in CD8 T cells that are differentially up- or down-regulated when comparing chronic versus acute infection for which no defined function has been found to date. The group of molecules that are upregulated in CD8 T cells during chronic infection includes the previously undescribed transmembrane protein 51 (*Tmem51*). The data presented in this study revealed that the overexpression of *Tmem51* leads to significant downregulation of effector cytokine production *in vitro* and during acute infection *in vivo* without impacting effector and memory T cell development. In chronic infection, *Tmem51*-deficient CD8 T cells showed reduced numbers but developed an exhausted phenotype with no significant changes in their capability to produce effector cytokines. In general, the overexpression of any gene leads to unusually high expression and can be used to initially determine whether a molecule has a specific function in a defined setting. The overexpression of *Tmem51* in CD8 T cells *in vitro* led to an up to 30 % reduction of CD8 T cells that produce the effector cyto ines IFNγ and TNFα. This observation is in line with increased levels of *Tmem51* in exhausted CD8 T cells and suggests that *Tmem51* might have an impact on the reduced levels of effector cytokines found in exhausted CD8 T cells. The next step was to investigate whether *Tmem51* overexpression could lead CD8 T cells to acquire features of an exhausted phenotype in acute infection. In principal, the reduction of the effector cyto ines IFNγ and TNFα could also be recapitulated *in vivo* during acute infection with LCMV-Arm53b. However, the percent of IFNγ and TNFα producing CD8 T cells was only decreased by about 10 %. This might suggest *in vivo* compensation mechanisms that are not present in the *in vitro* stimulation setting. At the same time, effector and memory development was not impacted when *Tmem51* was overexpressed in CD8 T cells. Overall, *Tmem51* seems to have the distinct function to reduce effector cytokine production without impacting other differentiation processes of CD8 T cells. This might be of therapeutic interest for example for autoimmune disorders in which enhanced inflammation is often the cause of severe

symptoms. Here, manipulating the expression of *Tmem51* could potentially lead to reduced inflammation via downregulation of effector cytokine production.

6.1.2 CD8 T cells that lack *Tmem51* develop an exhausted phenotype during chronic infection

Inhibitory receptors, like PD1, that are upregulated on exhausted CD8 T cells, can also lead to a reduction of effector cytokine production. Blocking the interaction of PD1 with its ligand PD-L1 leads to a significant increase of IFNγ producing CD8 T cells (Barber et al., 2006). At the same time, they start to proliferate much stronger and show higher cytotoxicity (Sharpe et al., 2007). In contrast, when *Tmem51* is deleted from CD8 T cells in chronic infection with LCMV-cl13, effector cytokine production is not significantly impacted. In addition, the numbers of *Tmem51*-deficient CD8 T cells decline to a ratio of about 1:2 when compared to wild-type CD8 T cells. The decline in T cell numbers stabilizes from day 14 post infection and other phenotypic markers of exhausted CD8 T cells are not changed in the absence of *Tmem51*. These findings might indicate, that *Tmem51* has a redundant function in CD8 T cells that is rapidly compensated upon its deletion. The expression levels of *Tmem51* in different immune compartments were originally determined via microarray analysis in the gene skyline browser of the immunological genome project (immgen.org, microarray data from February 2016 (Heng et al., 2008)). The expression of *Tmem51* in activated T cell populations was comparable to that in macrophages and dendritic cells. More recent RNA sequencing approaches now revealed that the expression of *Tmem51* in T cells is relatively low when compared to dendritic cells and macrophages (immgen.org, RNA sequencing data from 2020 (Heng et al., 2008)).   Altogether, the deletion of *Tmem51*, which is already expressed at relatively low levels is probably compensated by upregulation of other inhibitory mechanism so that the exhausted phenotype of CD8 T cells is not impacted.

6.1.3 The deletion of *Tmem51* in CD4-positive immune cells has no impact on CD8 T cell function in chronic infection

The deletion of *Tmem51* via Cre recombinase expression under the CD4 locus allows for the investigation whether the absence of *Tmem51* on all cells that express CD4 has functional consequences for CD8 T cells responding to chronic LCMV-cl13 infection. CD4-positive immune cells that potentially interact with CD8 T cells include CD4 T helper cells, subsets of macrophages, and dendritic cells (Peranzoni et al., 2018; Vremec et al., 2000). The infection of mice that are not TCR-transgenic also permits for the investigation of the polyclonal CD8 T cell response to LCMV-cl13. About 50 % of all T cells that react to LCMV are specific for the immunodominant epitope gp33 (Hudrisier et al., 1997; Moskophidis & Zinkernagel, 1995). The

data obtained in this study demonstrate, that gp33-specific CD8 T cells responding to LCMV-cl13 infection in *Tmem51*-deficient host mice show a comparable phenotype to wild-type host mice. There are about 0.4 % more IFNγ and TNFα producing CD8 T cells in the absence of *Tmem51*, but the difference between wild-type and knockout T cells was not significant. These results underline that *Tmem51* is probably a regulator of effector cytokine production but at same time demonstrate that the redundancy mechanism can compensate for the deletion of *Tmem51*. The further investigation of *Tmem51*-deficient CD8 T cells might lead to better insights into its mechanisms of action. Nonetheless, there does not seem to be a significant functional impact of deleting *Tmem51* during chronic infection. This is not too surprising as there seem to be various redundancy and feedback loop mechanisms that lead to the very stable acquirement of the exhausted phenotype in CD8 T cells (Beltra et al., 2020; Bolouri et al., 2020; Kurtulus et al., 2019; Lei et al., 2020; Muroyama & Wherry, 2021).

6.2 *Ptpn2* is a major regulator of effector T cells and modulates the dependency of recently activated T cells on survival-promoting cytokines

6.2.1 The absence of *Ptpn2* favors the development of effector T cells and enhances their plasticity

Up to now, the investigation of *Ptpn2* was focused on the development of autoimmune diseases because lower levels of *Ptpn2* correlate with a higher risk of attaining an autoimmune disorder (Espino-Paisan et al., 2011; Smyth et al., 2008; Todd et al., 2007; Zikherman & Weiss, 2011). It has been demonstrated that *Ptpn2* is a negative regulator of TCR signaling that acts via dephosphorylation of proto-oncogene tyrosine-protein kinase (SRC) family kinases. Additionally, it directly dephosphorylates key molecules during JAK-STAT-mediated cytokine signaling (Fukushima et al., 2010; Loh et al., 2011; Shields et al., 2013; Tiganis & Bennett, 2007). More recently, it has been shown for the first time, that *Ptpn2* can have an impact on the response of CD8 T cells during chronic infection (LaFleur et al., 2019). They demonstrated that the deletion of *Ptpn2* favors the generation, proliferation, and cytotoxicity of the TIM3-positive intermediate and terminal exhausted CD8 T cell subsets. These characteristics highlighted *Ptpn2* as potent target for T cell-based immunotherapies (LaFleur et al., 2019; Wiede et al., 2020). However, the absence of *Ptpn2* in CD8 T cells during acute infection has not been investigated yet. It was unclear, how effector and memory differentiation as well as antigen-sensitivity of *Ptpn2*-deficient CD8 T cells are impacted. The data presented in this study revealed that *Ptpn2* i) regulates the survival and expansion capacity of short-lived

effector T cell populations, ii) attenuates the antigen-independent expansion of briefly activated CD8 T cells, and iii) controls the reliance of CD8 T cells on survival-promoting cytokines. Short-lived effector T cells that are positive for the marker KLRG1 develop in a variety of acute infections (Joshi et al., 2007; Kaech et al., 2003; Wherry et al., 2003). Generally, these effector T cells show a limited capacity of secondary expansion. When KLRG1-positive T cells are transferred out of an acute infection into secondary host mice and are re-challenged, they cannot re-expand as potently as CD127-positive memory T cells (Sallusto et al., 2000; Sarkar et al., 2008; Schluns et al., 2000; Weinreich et al., 2009). An advanced fate mapping approach allowed for the clarification, that a small subset of CD127$^-$ KLRG1$^+$ CD8 T cells can indeed differentiate into all memory cell lineages (Herndler-Brandstetter et al., 2018). The data in my thesis demonstrate that the absence of *Ptpn2* enhances the proliferation and survival of KLRG1-positive CD8 T cells during acute infection. These findings complement the recent discovery that in chronic infection, the biologically comparable TIM3$^+$ terminal effector exhausted CD8 T cells arise in enhanced numbers (LaFleur et al., 2019). Overall, this supports the conclusion, that *Ptpn2*-deficient CD8 T cells are favored to develop into effector cells which have a greater capacity for survival. Both are characteristics that can potentially be used to advance T cell-based immunotherapies.

6.2.2 The efficacy of adoptive T cell transfers is greatly enhanced in the absence of *Ptpn2*

Furthermore, the data presented here strengthen the notion that *Ptpn2* is a potent target for T cell-based immunotherapies. I demonstrate, that deleting *Ptpn2* enables T cells to expand significantly better following a limited period of stimulation. Notably, not the initial engraftment but the subsequent survival and proliferation of *Ptpn2*-deficient CD8 T cells was significantly higher. A brief, TCR-dependent stimulation period of up to 36 hours and transfer into host mice under inflammatory conditions, showed a benefit of expansion of up to 160-fold in T cells deficient for *Ptpn2*. When adoptively transferring the T cells into antigen-free host mice, the expansion was still enhanced by up to 9-fold, indicating that *Ptpn2*-deficient CD8 T cells are augmented in their capacity to undergo programmed T cell expansion. The effect was reduced upon adoptive transfer into host mice that carry the cognate antigen of the transferred T cells. However, the *Ptpn2*-deficient CD8 T cells still expanded 1.6 times stronger than the wild-type T cells. These enhancements constitute a major advantage, as it could be clinically beneficial to engraft larger numbers of functional T cells. Especially, T cell-based therapies that rely on chimeric antigen receptors (CAR) to target tumor-specific antigens, have been hindered by inadequate T cell activation and the immunosuppressive environment often found in tumors (Ribas & Wolchok, 2018; Yong et al., 2017). Very recently, it could be demonstrated that the

deletion of *Ptpn2* enhances the activation of CAR T cells targeting solid tumors to such an extent, that they can be eradicated (Wiede et al., 2020). This is in line with my observations that activated T cells that are deficient for *Ptpn2* show enhanced survival.

6.2.3 Briefly activated *Ptpn2*-deficient CD8 T cells have a highly stimulated cytokine signaling profile

The enhanced proliferation and survival of recently activated *Ptpn2*-deficient CD8 T cells prompted me to investigate the underlying mechanisms using a proteomics approach. As a result, an increased phosphorylation status of known and potentially newly identified interaction partners of *Ptpn2* could be observed. The alterations in total protein levels correlate strongly with the enhanced activity of these interaction partners. As an example, the heightened tyrosine-phosphorylation status in STAT3, STAT4, and STAT5 induces their transcriptional activity which in turn enhances the expression of proliferation-associated proteins. These include IL-2, Interleukin 2 receptor-alpha (IL-2rα, CD25), IFN-γ, Interleu in-4 (IL-4), and CD44. Using the *de novo* network enrichment tool KeyPathwayMiner, I was able to build a causal network of changes in the phosphoproteome of recently activated *Ptpn2*-deficient CD8 T cells. The resulting network suggests potential novel interactors or downstream affected molecules of *Ptpn2*. This includes the tyrosine-protein kinase Lyn (Lyn), which is a member of the Src family of protein kinases and highly expressed in the absence of *Ptpn2*. Lyn has been shown to directly phosphorylate and increase the activity of Src homology region 2 (SH-2) domain-containing phosphatase 1 (SHP-1, PTPN6) at Tyrosine-564 (Ingley, 2012; Li et al., 1995; Neel, 1993), which shows increased phosphorylation levels in the phosphoproteome. SHP-1 has been extensively studied as a negative regulator of TCR signaling and cell activation and proliferation (Carter et al., 1999; Johnson et al., 2013). The upregulated activity of SHP-1 might constitute a regulatory mechanism that is induced to dampen the hyperactive state of *Ptpn2*-deficient CD8 T cells. An additional downstream affected molecule is programmed cell death protein 4 (PDCD4), which is a tumor suppressor and apoptosis-inducing factor (Shibahara et al., 1995; Yang et al., 2003). In the absence of *Ptpn2*, its expression is significantly decreased and it has been demonstrated that cytotoxic T cells lacking PDCD4 display an increased expression of effector molecules and superior tumor control (Lingel et al., 2017). Taken together, PDCD4 might play a role in the increased survival of CD8 T cells deficient for *Ptpn2*. Overall, the observed changes indicate that the absence of *Ptpn2* renders T cells less dependent on receiving pro-survival signals. Additionally, *Ptpn2* deficiency might allow T cells to more effectively response to limiting cytokine sources. This is in line with the 160-fold enhanced expansion of recently activated

Ptpn2-deficient CD8 T cells in hosts under inflammatory stimuli. Both mechanisms could explain the improved transfer efficacy of *Ptpn2*-deficient over wild-type CD8 T cells.

6.2.4 *Ptpn2*-deficient CD8 T cells are independent of extrinsic sources of Interleukin-2 *in vitro*

CD8 T cells that are activated *in vitro* die of apoptosis without the addition of growth factors. IL-2 is the major cytokine that on its own is sufficient to allow for long-time survival and proliferation of CD8 T cells *in vitro* (D'Souza & efrançois, 2003 Gillis & Smith, 1977). On a functional level, I demonstrated that the wide-ranging changes observed in the phosphoproteome of *Ptpn2*-deficient CD8 T cells, renders them independent of extrinsic sources of IL-2 *in vitro.* Additionally, the absence of *Ptpn2* increases the capacity of CD8 T cells to form proliferative clusters in *in vitro* cultures. The occurrence of these clusters is a hallmark of robust T cell proliferation and might be explained by the enhanced expression of the cell adhesion molecules Integrin alpha-2 (ITGA2), Integrin alpha-M (ITGAM), and Intercellular adhesion molecule 1 (ICAM1) in *Ptpn2*-deficient T cells (Zumwalde et al., 2013). Taking these effects together, the manipulation of *Ptpn2* might be especially beneficial in combinatorial therapies in which IL-2 is pharmacologically applied to bolster T cell-based immunotherapies (Jiang et al., 2016).

7 Conclusion

The data presented in my thesis demonstrate, that *Tmem51* has the potential to modulate the production of effector cytokines in CD8 T cells. At the same time, the impact of *Tmem51* on CD8 T cell differentiation in acute and chronic infection is limited. It is likely, that redundancy mechanisms *in vivo* can compensate for the deletion and overexpression of *Tmem51*. Of note, the deletion of *Tmem51* is not sufficient to reactivate CD8 T cells that acquired an exhausted phenotype. Nonetheless, the further investigation of *Tmem51* in other immune cells like CD4 T cells, macrophages, and dendritic cells might point towards additional functions of *Tmem51*.

Additionally, I could reveal key functions of the phosphatase *Ptpn2* in acute infection and in therapeutically relevant settings. *Ptpn2* has recently been identified as a modulator of terminal exhausted CD8 T cells. In line with this observation, the absence of *Ptpn2* specifically increases the numbers, survival and plasticity of biologically comparable effector CD8 T cells. Notably, the deletion of *Ptpn2* significantly improves the survival and expansion capacity of briefly activated and adoptively transferred CD8 T cells. These results support the further investigation and development of *Ptpn2* inhibitors for T cell-based therapies.

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Abbreviations

°C	Degree Celsius
µg	Microgram
µl	Microliter
2B4	natural killer cell receptor 2B4
BATF	Basic Leucine Zipper ATF-Like Transcription Factor
BCL6	B-cell lymphoma 6 protein
Blimp-1	B-lymphocyte-induced maturation protein 1
BM	bone marrow
BSA	Bovine Serum Albumin
CD127	Cluster of differentiation 127
CD24	cluster of differentiation 24
CD62L	L-Selectin
CD69	Cluster of differentiation 69
CD8	Cluster of differentiation 8
Da	Dalton
DN	double negative
DP	CD4, CD8-double positive
Eomes	Eomesdermin
eYFP	enhanced yellow fluorescent protein
FACS	Fluorescence activated cell sorting
FCS	fetal calf serum
Fyn	proto-oncogene tyrosine-protein kinase
g	relative centrifugal force
g	gram
gp33	LCMV-derived glycoprotein[33-41] epitope
GWAS	Genome wide association studies
GZMB	Granzyme B
h	Hour/s
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
Id2	inhibitor of DNA binding 2
IFN-γ	Interferon-γ
IL-7	Interleukin 7
ISP	immature single positive
kDa	Kilodalton
KLRG1	Killer cell lectin-like receptor G1
L	Liter
Lck	lymphocyte-specific protein tyrosine kinase
LCMV	Lymphocytic choriomeningitis virus
M	Molar
mg	Milligramm
MHCI	major histocompatibility complex class I

min	Minute/s
ml	Milliliter
mM	Millimolar
NEAA	non-essential amino acids (NEAA)
nmol	Nanomol
P14 T cells	TCR-transgenic CD8 T cells specific for gp33
PCR	Polymerase chain reaction
PD1	programmed cell death protein 1
pMHCI	peptide:MHCI complex
Ptpn2	Protein tyrosine phosphatase non-receptor type 2
RNA	Ribonucleicacid
RT	room temperature
s	Second/s
SLAMF6	Ly108, SLAM family member 6
SNP	single nucleotide polymorphisms
STAT	signal transducer and activator of transcription
T-bet	T-box expressed in T cells
TCF1	T cell factor 1
TCR	T cell receptor
TIM3	T cell immunoglobulin domain and mucin domain 3
Tmem51	Transmembrane protein 51
TNF-α	tumor necrosis factor-alpha
TOX	thymocyte selection-associated high mobility group box protein
OT-1 T cells	TCR-transgenic CD8 T cells specific for SIINFEKL

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