DISS. ETH NO. 26274

REGULATING THE ANTI-VIRAL CD8+ T CELL RESPONSE THROUGH CO-SIGNALLING MOLECULES AND CYTOKINES

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH
(Dr. sc. ETH Zurich)

presented by

PATRIZIA DIETHELM

MSc ETH Zurich

born on 28.11.1989

citizen of
Uttwil (TG)

accepted on the recommendation of

Prof. Dr. Manfred Kopf, examiner
Prof. Dr. Annette Oxenius, co-examiner
Prof. Dr. Burkhard Ludewig, co-examiner

2019

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I. SUMMARY

The immune system of multicellular organisms mediates protection against various microorganisms such as bacteria, viruses or fungi, by a diverse set of cellular and chemical components. Viral pathogens elicit a strong humoral and cell-mediated immune response, including the activation of cytotoxic T lymphocytes (CTL), which prevent virus spread through killing of virus-infected host cells. However, in order to prevent excessive tissue damage, fine tuning of the CTL response is crucial. Through soluble mediators, such as cytokines, and cell surface molecules the different stages of the CTL response, ranging from the expansion to the contraction and the long-term maintenance, are tightly regulated.

In the work presented in this thesis, we wanted to address the role of two co-signalling molecules, B and T lymphocyte attenuator (BTLA) and Herpes virus entry mediator (HVEM), and two cytokines, IL-2 and IL-21, in the CTL response in mice infected with the viral pathogen lymphocytic choriomeningitis virus (LCMV).

BTLA and HVEM are two cell-surface molecules found on several immune cells including T lymphocytes. Initially, HVEM has been identified *in vitro* as a ligand for BTLA, inhibiting T cell proliferation. Later, *in vivo* studies indicated that the BTLA-HVEM co-signalling system attenuates the immune response against a variety of different pathogens. However more recently, it has been proposed that BTLA may serve reciprocally as a ligand for HVEM, promoting T cell survival.

We aimed to study the contribution of the BTLA-HVEM system in mounting a protective CTL response in LCMV infected animals. Surprisingly, infection of BTLA or HVEM deficient mice with LCMV strains WE or Docile (DO), causing an acute respectively a persistent infection, did not affect the virus-specific CD8+ T cell response or virus clearance. Furthermore, BTLA and HVEM double deficient animals did neither show a dysregulated CD8+ T cell response nor differences in viral titers when compared to WT controls. However, we observed down-regulation of BTLA and HVEM on virus-specific effector CD8+ T cells. Through retroviral overexpression of BTLA or HVEM we wanted to assess if down regulation is crucial for the anti-viral CD8+ T cell response. Overexpression of HVEM on virus-specific CD8+ T cells resulted in reduced numbers 7 days post infection (dpi), while numbers of memory cells were increased. Virus-specific CD8+ T cells overexpressing BTLA produced

slightly more cytokines and were maintained at higher numbers during the memory phase. Thus, we revealed that down-regulation of BTLA is crucial to dampen the cytokine production but limits the memory formation whereas HVEM down-regulation is important for the accumulation of virus-specific CD8+ T lymphocytes at the peak of the response but limits the formation of memory cells.
Besides, we were wondering if BTLA and HVEM are similarly expressed on virus-specific CD8+ T cells during a mouse cytomegalovirus (MCMV) infection as during a LCMV infection. While the expression of BTLA on the classical CD8+ T cells specific for the M45 epitope of the virus followed the same pattern as on LCMV-specific CD8+ T cells, the molecule was, as predicted, low expressed on the effector-like M38-specific CD8+ T cells in MCMV infected animals. HVEM in contrast was, opposed to its low expression on LCMV-specific effector cells, rather highly expressed on effector-like inflationary M38-specific CD8+ T cells from the salivary gland. However surprisingly, infection of HVEM deficient mice with MCMV did not affect the virus-specific CD8+ T cell response or virus clearance in the salivary gland.

The second project focused on the interplay of IL-2 and IL-21 during the CD8+ T cell response during an acute LCMV infection. Previous reports indicated, that while IL-2 and IL-21 were critical for the sustained cytokine production and the maintenance of virus-specific CD8+ T cells during a chronic LCMV infection, the two cytokines were mostly dispensable for the virus-specific CD8+ T cell response during an acute LCMV infection.
Our data from mixed bone marrow chimeric mice revealed that during an acute LCMV infection, IL-2 and IL-21 can partially compensate for each other to promote the long-term persistence and the sustained cytokine response of virus-specific CD8+ T cells. Besides, combined absence of the IL-2Rα and the IL-21R directed virus-specific CD8+ T cells to a central memory phenotype, even to a higher degree than by the IL-2Rα single deficiency.

Overall, this thesis revealed a crucial role for the down-regulation of the two co-signalling molecules HVEM and BTLA on virus-specific CD8+ T cells to ensure accumulation of effector cells at the peak of the T cell response to LCMV respectively to dampen the cytokine production of virus-specific CD8+ T cells. Furthermore, we

could show that the two cytokines IL-2 and IL-21 ensure the long-term persistence and the sustained cytokine response of virus-specific CD8+ T cells during an acute LCMV infection in a partial compensatory manner.

II. ZUSAMMENFASSUNG

Das Immunsystem schützt mehrzelligen Organismen vor Mikroorganismen wie Bakterien, Viren oder Pilzen mit Hilfe von verschiedensten Zellen und chemischen Substanzen. Viren lösen eine starke humorale und zell-basierte Immunantwort aus. Unter anderem werden cytotoxische T Zellen aktiviert, welche die Verbreitung des Virus stoppen indem sie virus-infizierte Zellen töten. Es ist allerdings von grosser Bedeutung, die T Zell-Antwort genauestens zu regulieren, um eine zu starke Schädigung des Gewebes zu verhindern. Diese Regulation erfolgt durch verschieden lösliche Moleküle, wie zum Beispiel Zytokine, und Zelloberflächenproteine, welche die drei Phasen der T Zell-Antwort (Expansion, Kontraktion und langfristiger Erhalt) steuern.

Das Ziel dieser Arbeit war es herauszufinden, welche Rolle die beiden Oberflächenmoleküle B and T lymphocyte attenuator (BTLA) und Herpes virus entry mediator (HVEM) sowie die beiden Zytokine IL-2 und IL-21, in der T Zell-Antwort, ausgelöst durch eine Infektion mit dem Virus lymphocyte choriomeningitis virus (LCMV), spielen.

BTLA und HVEM sind zwei Oberflächenmoleküle welche auf mehreren verschieden Zelltypen des Immunsystems vorkommen, unter anderem auch auf T Zellen. HVEM wurde ursprünglich als Ligand für BTLA identifiziert, wobei HVEM *in vitro* die Proliferation von T Zellen inhibierte. *In vivo* drosselt das BTLA-HVEM System die Immunantwort gegen verschiedenste Pathogene. Es wurde hingegen kürzlich gezeigt, dass BTLA umgekehrt auch als Ligand für HVEM fungieren kann und dadurch das Überleben von T Zellen begünstigt.

Unser Ziel war es herauszufinden, wie das BTLA-HVEM System in der T-Zell-Antwort in LCMV infizierten Mäusen involviert ist. Mäusen welchen das BTLA oder das HVEM Molekül fehlt und mit den LCMV Stämmen WE oder Docile (DO) infiziert wurden, um eine akute oder eine chronische Infektion auszulösen, zeigten überraschenderweise keine veränderte virus-spezifische T Zell-Antwort und konnten den Virus klären. Auch Tiere welche beide Moleküle fehlen, zeigten keine veränderte virus-spezifische T Zell-Antwort und konnten den Virus klären. Wir stellten hingegen fest, dass BTLA und HVEM auf virus-spezifischen CD8+ Effektor-T-Zellen runterreguliert wurden. Durch eine retrovirale Überexpression wollten wir herausfinden, ob die Runterregulation von

BTLA oder HVEM für die anti-virale CD8+ T Zell-Antwort wichtig ist. Die retrovirale Überexpression von HVEM auf virus-spezifischen CD8+ T Zellen resultierte in einer reduzierten Anzahl Zellen 7 Tage nach der Infektion, wohingegen die Anzahl Gedächtniszellen erhöht war. Virus-spezifische CD8+ T Zellen welche BTLA überexprimieren produzierten hingegen etwas mehr Zytokine, bildeten aber ebenfalls mehr Gedächtniszellen. Wir konnten zeigen, dass die Runterregulation von BTLA wichtig ist um die Zytokineproduktion zu drosseln aber die Bildung von Gedächtniszellen limitiert. Die Runterregulation von HVEM ist hingegen wichtig für die Akkumulation von virus-spezifischen CD8+ T Zellen beim Peak der T Zell-Antwort, aber limitiert die Bildung von Gedächtniszellen.
Ausserdem wollten wir herausfinden, ob BTLA und HVEM während einer murinen cytomegalovirus (MCMV) Infektion auf virus-spezifischen CD8+ T Zellen ähnlich exprimiert sind wie auf LCMV-spezifischen CD8+ T Zellen. Während die Expression von BTLA auf den klassischen CD8+ T Zellen spezifisch für das M45 Epitop des Virus dem gleichen Muster wie auf LCMV-spezifischen CD8+ T Zellen folgte, war das Molekül, wie vermutet, sehr tief auf den Effektor-ähnlichen M38-spezifischen CD8+ T Zellen von MCMV infizierten Mäusen exprimiert. HVEM, entgegengesetzt zu seiner tiefen Expression auf LCMV-spezifischen CD8+ T Effektorzellen, war allerdings sehr hoch auf den Effektor-ähnlichen inflationären M38-spezifischen CD8+ T Zellen der Speicheldrüse exprimiert. Überraschenderweise beeinträchtigte die Abwesenheit von HVEM die virus-spezifische CD8+ T Zell-Antwort und auch die Klärung des Virus in der Speicheldrüse in MCMV infizierten Mäusen nicht.

Im zweiten Projekt fokussierten wir uns auf das Zusammenspiel von IL-2 und IL-21 während der CD8+ T Zell-Antwort gegen eine akute LCMV Infektion. Mehre Publikationen zeigten, dass IL-2 und IL-21 alleine während einer chronischen LCMV Infektion wichtig sind für die anhaltende Zytokinproduktion und den Erhalt von virus-spezifischen CD8+ T Zellen, wo hingegen die beiden Zytokine nur unwesentlich zur virus-spezifischen CD8+ T Zell-Antwort während einer akuten LCMV Infektion beitrugen.
Unsere Daten mit gemischten Knochenmarkchimären zeigten, dass während einer akuten LCMV Infektion die beiden Moleküle in einer partiellen kompensatorischen Form zum Erhalt und der Zytokinproduktion von virus-spezifischen CD8+ T Zellen beitrugen. Ausserdem wiesen virus-spezifische CD8+ T Zellen, welchen sowohl der

IL-2 Rezeptor als auch der IL-21 Rezeptor fehlt, einen erhöhten «central memory» Phänotypen auf, dies noch zu einem höheren Grad als solchen T Zellen welchen nur der IL-2 Rezeptor fehlte.

Zusammenfassend konnte in dieser Arbeit gezeigt werden, dass die Runterregulation der beiden Molekülen HVEM und BTLA auf virus-spezifischen CD8+ T Zellen wichtig ist für die Akkumulation von Effektorzellen am Peak der T Zellantwort gegen LCMV respektive wichtig ist um die Zytokinproduktion von virus-spezifischen CD8+ T Zellen zu drosseln. Ausserdem konnten wir zeigen, dass die beiden Zytokine IL-2 und IL-21 in einer partiellen kompensatorischen Form zum Erhalt und der Zytokineproduktion von virus-spezifischen CD8+ T Zellen während einer akuten LCMV Infektion beitragen.

III. GENERAL INTRODUCTION

1. The immune system

Every day we are exposed to a wide variety of different microorganisms such as bacteria, fungi or viruses, which could be potentially harmful. Our immune system eliminates these threats through a diverse set of cells and chemical mediators. It can be divided into two types: The innate and the adaptive immunity.

The innate immune system hinders, as a first line of defense, the entry of pathogens through physiological barriers like the skin or mucosal tissues. In case of penetration, innate immune cells and soluble molecules (e.g. complement) recognize common structures of pathogens, so called pathogen associated molecular patterns (PAMPs), through pattern recognition receptors (PRRs), inducing an inflammatory response, that attracts additional innate immune mediators. Besides, activated innate immune cells will migrate to adjacent lymph nodes, where they activate highly pathogen-specific adaptive immune cells.

2. Innate immune cells

Cells from the innate immune system include granulocytes, macrophages, dendritic cells (DCs) and natural killer (NK) cells. They detect pathogens by a wide variety of different soluble and membrane-bound PRRs.

Neutrophils, eosinophils and basophils are classified as granulocytes. They contain cytoplasmic vesicles (granules) filled with lytic and anti-microbial peptides or pharmacologically active substances, which can kill ingested pathogens respectively induce allergic responses upon secretion.

NK cells can kill alter-self (e.g. tumour cells) and infected host cells upon recognition of altered surface structures on the target cell membrane. They release cytotoxic molecules like perforin and granzyme for target cell lysis and secrete IFNγ that can activate macrophages. The latter, together with DCs and neutrophils, are phagocytic cells that can internalize pathogens for elimination and, in case of macrophages and DCs, activate T cells from the adaptive immune system. Besides macrophages and DCs, also B cells, belonging to the adaptive immune system, can activate T cells.

3. Adaptive immune cells

Adaptive immune cells recognize specific structures of pathogens, inducing a targeted immune response against a particular microbe. In contrast to germ-line encoded PRRs expressed on innate immune cells, receptors of adaptive immune cells are generated by genetic recombination, creating a highly diverse set of receptors. Adaptive immune cells require several days to acquire their full effector function upon activation, while innate immune cells can respond immediately upon pathogen encounter. However, the adaptive immune system is capable of forming an immunological memory, whereby antigen-experienced cells are maintained long-term and, upon pathogen re-encounter, can more rapidly and vigorously induce a secondary immune response.
The adaptive immune system is comprised of the two cell types B and T lymphocytes.

3.1. B lymphocytes

B cells are antibody-producing lymphocytes that develop in the bone marrow (BM). After rearrangement of the heavy and light chain of the Immunoglobulin (Ig) molecule and passing negative selection, which would delete autoreactive B cells, they exit the BM as immature membrane-IgM (mIgM) expressing B cells and enter the spleen where they mature to naïve mIgM and mIgD expressing cells. Mature naïve B cells will migrate through the body, entering secondary lymphoid organs to search for antigen (pathogen fragments). Upon antigen recognition by the membrane-bound Immunoglobulins, which is independent of antigen processing and presentation, the associated Ig-α/Ig-β (CD79a/b) B cell receptor (BCR) component mediates downstream signalling.
For few antigens (e.g. LPS), the sole recognition by mIg is sufficient to activate B cells (T cell-independent activation; TI). However, B cells specific to the vast majority of antigens depend on T cell help (T cell-dependent activation; TD) for their activation, where T cells will express stimulatory molecules like CD40L and secret cytokines [1]. Subsequently, B cells proliferate and differentiate into antibody-secreting plasma cells. Some activated B cells, together with T helper cells and follicular DCs, with latter able to retain antigen bound to antibodies or complement, will form follicles know as germinal centres. In these structures, class switching to other Ig isotypes, affinity

maturation through somatic hypermutation of the rearranged Ig genes and the generation of long-lasting memory B cells is taking place.

3.2. T lymphocytes

The second type of adaptive immune cells are T lymphocytes. Whereas B cells can directly recognize antigens, the T cell receptor (TCR) of T cells engage an antigen only if it is bound to a MHC molecule. The two types of T cells, CD4+ and CD8+, thereby interact with different MHCs. Whereas CD4+ T cells engage MHC class II molecules, expressed solely by antigen presenting cells (APCs), CD8+ T cells bind to MHC class I, found on nearly all nucleated cells.

T cells initially develop in the bone marrow, however they fully mature in the thymus, where the rearrangement of the two TCR segments and a selection process occurs. TCR rearrangement generates T cells either expressing a γ and δ or a α and β chain. After rearrangement, $\gamma\delta$ T cells will leave the thymus and migrate primarily to epithelial barriers to search for antigen. In contrast, $\alpha\beta$ T cells undergo positive and negative selection, obtaining cells recognizing self MHC but not reacting to self-peptides. $\alpha\beta$ T cells initially co-express the CD4 and CD8 molecule (double positive T cells; DP). Depending on the MHC type engaged during selection, T cells will subsequently down-regulate either of the two co-receptors, leaving the thymus as mature CD4 or CD8 single-positive (SP) T cells. These naïve T cells will migrate through the body, entering lymphoid tissues to search for antigen.

3.2.1. CD4+ T cell subsets

CD4+ T cells, also termed T helper (Th) cells, shape the response of other immune cells through production of various cytokines. Depending on the cytokine milieu present during their activation and the strength of TCR stimulation, different CD4+ T cells subsets form, which produce a specific set of cytokines. As depicted in Fig. 1, classification into six different Th subsets have been widely accepted: Th1, Th2, Th17, regulator T cells (Treg), T follicular helper (Tfh) and Th9 cells.

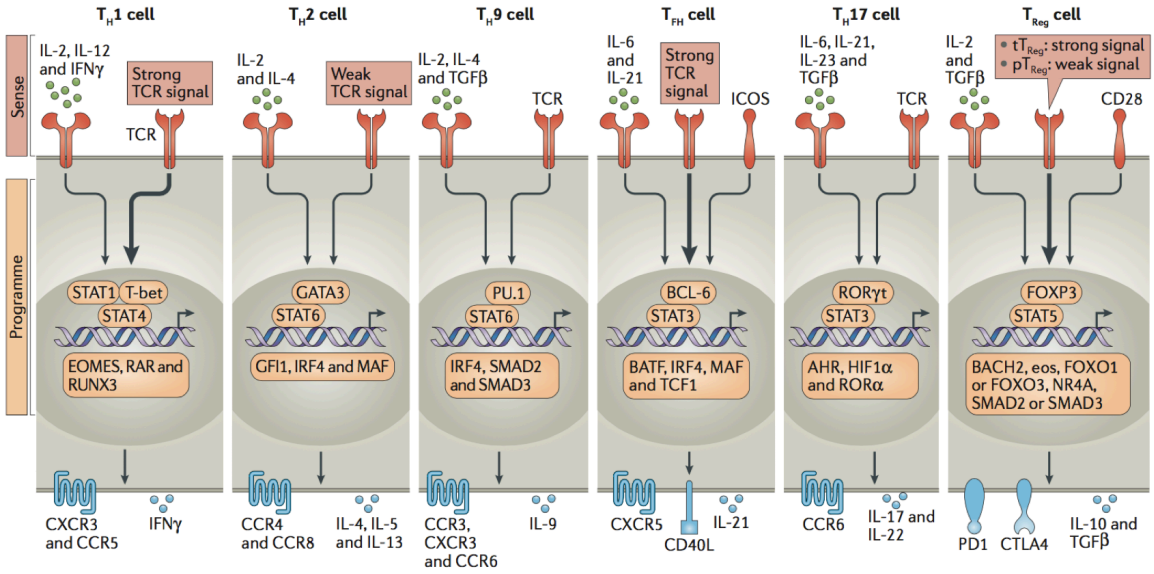

Fig. 1: CD4+ T cell subsets. Adapted from DuPage et al., 2016 [2]

Th1 cells shape the immune response against viral and intracellular bacterial infections. The hallmark cytokine IFNγ produced by Th1 cells is involved in B cell class switching from IgM to IgG2a [3] and the activation of macrophages [4]. Besides, Th1 cells also produce substantial amounts of IL-2, a cytokine critical for the CD8+ T response against a virus infection [5, 6]. Polarization into Th1 cells is mediated by IL-12 [7, 8] and IFNγ [9]. Induction of the transducer and activator of transcription (STAT) protein 4 by IL-12 [10] and STAT1 by IFNγ [4], subsequently induces T-bet [11] which mediates the expression of IFNγ by Th1 cells.

IL-4 drives the differentiation of naïve CD4+ T cells into Th2 cells by inducing the transcription factors STAT6 [12] and Gata3 [13]. Th2 cells produce IL-4, IL-5 and IL-13 and are essential for the protection against extracellular parasites such as helminths [14], assist in B cell class switching to the IgE isotype but can also induce allergic responses [4].

Differentiation of Th17 cells is promoted through TGFβ in combination with IL-6, IL-23 and IL-21, which induce the transcription factors STAT3 and RORγt [15]. Th17 cells produce IL-17A and IL-17F as well as IL-22 and are critical for the protection against

fungal infections. However, they also play a role in autoimmune diseases (e.g. experimental autoimmune encephalomyelitis [EAE]) [15].

In contrast to the other Th subsets, Tregs rather suppress T cell responses. The cells are crucial to inhibit autoimmunity and limit the response of effector T cells, ensuring tissue homeostasis. Tregs acquired several tools to dampen the immune response including the production of suppressive cytokines like IL-10 and TGFβ, expression of inhibitory ligands (e.g. CTLA-4) [16] or consumption of IL-2 [17], a cytokine required for proliferation of activated T cells. The master regulator for induction of the Treg program is the transcription factor Foxp3 [18]. Tregs can develop in the thymus, then termed natural regulatory T (nTreg) cells, or they differentiate from naïve CD4+ T cells in the periphery, becoming induced regulator T (iTreg) cells [19]. Whereas both subtypes rely on IL-2 for their development and maintenance (see section 4.1.1), iTregs require TGFβ for differentiation [19].

Tfh cells are found in the germinal centre of secondary lymphoid organs, where they provide help to B cells, promoting class switching and affinity maturation [20]. Tfh produces cytokines like IL-4 and IL-21 and express surface receptors like CD40L [20]. Tfh differentiation is mainly regulated by the transcription factor Bcl6, which is induced by IL-21, IL-6 and ICOS, and promotes the expression of the chemokine receptor CXCR5, directing the cells to the germinal centre [20].

Th9 cells are defined by secretion of IL-9. Not only IL-4-STAT6 but also IL-2-STAT5 signalling, inducing the transcription factors IRF4 and BATF, are crucial for the differentiation of Th9 cells [21]. Furthermore, presence of TGFβ and activation of the transcription factor PU.1 are important to induce the Th9 program in CD4+ T cells [21]. However, if they truly represent a distinct T helper cell subset or are rather IL-9 producing Th2 cells is still controversial. Thus, it is difficult to assign a specific protective function *in vivo* to these cells. However, they are believed to be involved in the immune response against parasites but can also take part in inflammatory diseases of the intestinal tract and allergic responses [21].

3.2.2. CD8+ T cells

Upon activation, CD8+ T cells differentiate into cytotoxic T cells (CTLs) able to kill infected or altered self-cells (e.g. tumour cells). Elimination of the target cell, initiated upon peptide-MHC I complex engagement, is mediated either through the release of cytotoxic proteins, like perforin or granzymes, or through the interaction with cell surface molecules e.g. FasL binding to Fas. Whereas perforin can insert pores in the plasma membrane of the target cell, granzymes activate cell death pathways (e.g. the caspase pathway) [22]. Induction of apoptosis by Fas, interacting with FasL expressed on the CTL, is mediated over the Fas-associated death domain protein (FADD) that induces the Caspase cascade over Caspase 8 [23]. Additionally, the immune response of CTLs includes the production of IFNγ and TNFα, two cytokines exerting pleiotropic functions (see section 4).

3.2.3. T cell activation

Activation of T cells depends on antigen recognition by the TCR, co-stimulatory signals and inflammatory cytokines [24]. First, the TCR recognizes a specific peptide, obtained by processing of antigens, presented on a MHC class I (CD8+ T cells) or MHC class II (CD4+ T cells) molecule expressed on antigen presenting cells (APCs) like macrophages, DCs or B cells. The co-receptor CD8 respectively CD4 binds to the MHC molecule and clusters with the TCR, providing the $p56^{Lck}$ kinase required for downstream signalling via the TCR associated CD3 molecule. Through engagement of co-stimulatory molecules, T cell activation progresses and is fine-tuned by co-inhibitory molecules. Besides the activation, co-signalling molecules can also regulate the subsequent expansion, the survival and the effector response of T cells as well as the formation and long-term maintenance of memory T cells.

According to their structure, co-signalling molecules can be classified into two protein superfamilies: The immunoglobulin (Ig) superfamily, characterized one or several extracellular immunoglobulin domains, and the tumour necrosis factor/tumour necrosis factor receptor (TNF/TNFR) superfamily, which includes members that contain extracellular cysteine rich domains (CRD) [25]. TNF/TNFR superfamily members include 4-1BB, OX40, LTβR, LTα, LIGHT and HVEM, whereas the Ig superfamily include CD28, CD80, CD86, ICOS, PD-1, CD160 and BTLA [25]. Typically, only members of the same superfamily interact with each other, however

there are some exceptions e.g. the TNFR superfamily member HVEM can interact with the Ig superfamily members BTLA and CD160 [26-28].

Last, the proliferation and differentiation of activated T cells is induced through inflammatory cytokines. IL-2 is one of the first cytokines produced, inducing the expansion of activated CD4+ and CD8+ T cells (see section 4.1.1). In contrast, T cell differentiation is regulated by a specific set of cytokines. Whereas the cytokine milieu determines the subset into which an antigen-primed CD4+ T cell develops (see section 3.2.1), CD8+ T cells depend on type I IFNs and IL-12 for the differentiation into cytotoxic T cells [29-33]. Besides regulating the initial steps of the T cell response, inflammatory cytokines also control the contraction and the subsequent formation and persistence of memory T cells.

3.2.3.1. HVEM

Initially, HVEM has been identified as the receptor for the herpes simplex virus (HSV) glycoprotein D (gD), mediating virus entry into the host cell [34]. Its structure classifies HVEM as a member of the TNFR superfamily. The extracellular domain contains three complete and one partial CRD [34, 35]. The cytoplasmic tail contains a binding site for the TNF receptor-associated factor (TRAF) proteins TRAF1, TRAF2, TRAF3 and TRAF5, which mediate downstream signalling via the transcription factors NF-κB and activator protein 1 (AP-1) [35, 36], controlling genes involved in cell survival and inflammation. Alternatively, it has been proposed that HVEM can activate Akt (also known as protein kinase B, PKB), possibly over phosphoinositide 3-kinase (PI3K), inducing the expression of anti-apoptotic molecules like Bcl-2 that mediate T cell survival [37].

In humans, HVEM is expressed in the lung, liver, kidney, spleen and on peripheral blood leukocytes (PBL) [34, 36] and can be found on T cells, B cells, DCs, NK cells, monocytes and neutrophils [38-40]. In mice, HVEM has been detected on CD4+ and CD8+ T cells [27], where upon activation, the molecule is down-regulated [41-43], and at low levels on B cells [27]. Furthermore, epithelial cells [44] as well as splenic CD8α+, CD4+ and double negative DCs [45] express HVEM in mice. Besides the spleen, HVEM expression was detected in the intestine, lung, thymus and the BM [35].

As depicted in Fig. 2, HVEM interacts with the two TNF superfamily members LIGHT (lymphotoxin-like, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes) and LTα (lymphotoxin α) [46], where for latter the exact role in the HVEM pathway has not yet been discovered. Unusual for a TNFR superfamily member, HVEM can also interact with Ig superfamily members namely BTLA (B and T lymphocyte attenuator) [26, 27] and CD160 [28].

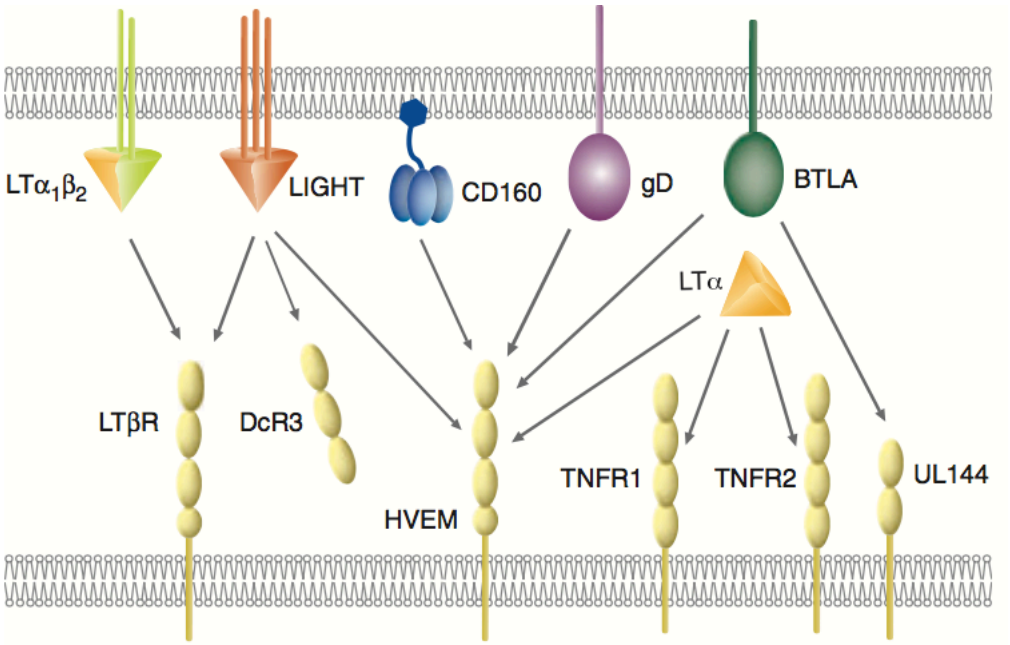

Fig. 2: The HVEM interaction network. Adapted from Steinberg et al., 2011 [47]

HVEM serves as a receptor for the two TNF superfamily members LIGHT and LTα [46], with LIGHT inducing a co-stimulatory signal through HVEM (see section 3.2.3.5). In contrast, when HVEM interacts with CD160 or BTLA, it rather serves as a ligand [26-28], inducing a co-inhibitory signal through either of the two Ig superfamily members (see sections 3.2.3.6 and 3.2.3.7). Adding an additional level of complexity to the system, a recent report suggests that *in vitro* CD160 and BTLA can reciprocally trigger HVEM [48], which was later also reported *in vivo* (see sections 3.2.3.6 and 3.2.3.7).

3.2.3.2. LIGHT

The TNF superfamily member LIGHT is a type II transmembrane protein present as a trimer at the cell surface [46], inducing the trimerization of its binding partners upon ligation. By alternative splicing, a cytosolic version with unknown function, and by proteolytic cleavage, a soluble extracellular form, that retains its receptor binding ability, can be generated [49]. Whereas both, soluble extracellular and membrane-bound and LIGHT can bind to HVEM, only the soluble form allows additional engagement of BTLA by HVEM [26, 47, 50].

LIGHT is expressed in the spleen and the brain in humans [46]. It can be found at low levels on human T cells, where it gets upregulated upon activation [41]. In contrast, maturation of human DCs causes LIGHT down-regulation [51]. The expression of the molecule in mice is less well studied.

So far, no signalling motif in its cytoplasmic domain has been identified, thus LIGHT has only been proposed to serve as a ligand for HVEM or LTβR [46, 52]. In humans, it can also engage DcR3 [53], a soluble protein most likely interfering with the interaction of LIGHT with HVEM or LTβR.

Initially, LIGHT has been shown to act co-stimulatory, promoting the proliferation, the cytokine response and the cytotoxic activity of T cells [51, 54-57], and its constitutive expression causing autoimmunity [58, 59]. LIGHT expression on tumour cells leads to infiltration of T cells and rejection of the tumour [60]. However, it was reported that the presence of LIGHT rather limits disease progression in EAE [61], possibly due to engagement of a different receptor.
LIGHT binding to LTβR positively regulates splenic microarchitecture and germinal centre formation [62] and promotes migration of DCs into the spleen and tumour tissue [63]. The co-stimulatory LIGHT-LTβR pathway can also be detrimental, causing lethal hepatitis in *Listeria monocytogenes* infected mice [64]. In contrast, triggering of LTβR by LIGHT can also induce apoptosis, namely in tumour cells [65, 66] and of high-affinity TCR expressing thymocytes during negative selection [67].

3.2.3.3. BTLA

The type I transmembrane glycoprotein BTLA contains an extracellular immunoglobulin domain, a characteristic of Ig superfamily members. The intracellular domain contains an ITIM (immunoreceptor tyrosine-based inhibitory motifs) and an ITSM-like (immunoreceptor tyrosine-based switch motif) sequence [68, 69]. Upon their phosphorylation, the two proteins SHP-1 and SHP-2 (Src homology domain 1 and 2) will bind and induce a negative downstream signal [68, 69], a feature shared with the co-inhibitory molecules CTLA-4 and PD-1. Besides, the cytoplasmic domain of BTLA contains a third motif that serves as a binding site for Grb2 and the p85 subunit of PI3K [68, 70], which potentially mediate cell survival.

BTLA mRNA was detected in the spleen and lymph node and at lower levels in the thymus, lung, small intestine and the liver of mice [68, 71]. Expression of BTLA could be detected on murine positively selected CD4+ and CD8+ T cells, but also on peripheral naïve T cells. Upon activation of CD4+ and CD8+ T cells with Con A, BTLA expression increases. Additionally, the molecule is expressed on $\gamma\delta$ T cells, B cells and MHCII+ innate immune cells [71]. In $\gamma\delta$ T cells, BTLA controls the peripheral homeostasis of the cells and dampens the inflammatory response upon activation, thus limiting pathology [72]. B cells in the bone marrow progressively upregulate BTLA from the pro- to the pre- and the immature B cell stage and express it highest on mature B cells [71], where it gets slightly down-regulated upon activation. BTLA expression on splenic DCs balance subset distribution during homeostasis [45].
In humans, BTLA can be found on B cells [73], where it is associated with the BCR to dampen the downstream signalling upon activation [74], and on T cells isolated from the blood [73]. Whereas BTLA levels are not changed upon activation of B cells, the molecule is gradually decreased on CD4+ and CD8+ T cells after activation [73].

Besides HVEM (see section 3.2.3.7), BTLA can also interact with UL144 [50]. This human cytomegalovirus (HCMV) encoded protein contains two CRDs that are homologous to CRD1 and CRD2 of HVEM [75]. UL144-Fc protein inhibited the CD4+ T cell proliferation *in vitro*, even more potently than the HVEM-Fc protein [50].

UL144 is retained intracellularly in infected cells [75], thus the interaction with BTLA possibly occurs during the lytic phase of the virus, potentially suppressing the anti-viral immune response.

Deficiency in BTLA increased the proliferation of T and B cells *in vitro* and B cells in $BTLA^{-/-}$ mice immunized with a T-cell dependent antigen showed an enhanced IgG response [68], possibly due to an increase in IL-21, a cytokine that promotes class switching (see section 4.1.2), produced by T follicular helper cells in the absence of BTLA [76]. Furthermore, absence of BTLA is associated with an increased susceptibility to MOG-induced EAE [68], development of spontaneous autoimmune-hepatitis in aged mice [77] and, due to an enhanced pathogenicity of NKT cells [78], an increased susceptibility to induced hepatitis upon ConA treatment [79]. The suppressive role of BTLA on the immune response has also been shown during an experimental asthma model, where treatment of BTLA KO mice with ovalbumin (OVA) exacerbated the allergic airway inflammation [80], due to an increased survival of pathogenic CD4+ T cells and an augmented accumulation of eosinophils in the bronchoalveolar lavage (BAL) [81]. Furthermore, therapeutic administration of an agonistic BTLA antibody attenuated the allergic response in a model of allergic dermatitis [82]. Interestingly, naïve BTLA deficient mice, as naïve $HVEM^{-/-}$ animals, contained more CD44+ memory CD8+ T cells and, during homeostatic expansion, BTLA KO CD8+ T cells outcompeted the co-transferred WT ones [83]. Furthermore, $BTLA^{-/-}$ CD8+ T cells did not only show an enhance cytotoxic activity after peptide-pulsed DC immunization, but also formed more memory CD8+ T cells and had an advantage in re-expanding upon a secondary challenge [83].

3.2.3.4. CD160

CD160 is an Ig superfamily member containing a single extracellular Ig domain. Through alternative splicing of the mRNA a glycosylphosphatidylinositol (GPI)-anchored [84] and a transmembrane isoform [85] can be generated. Additionally, the GPI-linked protein can be cleaved of, giving rise to a soluble form [86]. While the GPI-anchored molecule does not contain an intracellular domain and a possible downstream signalling pathway has not yet been identified, the cytoplasmic residue of the transmembrane isoform activates the Erk1/2 signalling pathway [85].

In humans and mice, CD160 is expressed on NK, NKT and T cells [84, 87] and can be found in the spleen, the small intestine and in humans also on PBL [84, 88].

Beside the interaction of CD160 with HVEM (see section 3.2.3.6), MHC I can weakly bind to CD160, inducing a cytotoxic response in NK cells [89] and the proliferation of CD8+ T cells [90, 91]. In contrast, the soluble form of CD160 has shown to inhibit the cytotoxic activity of NK as well as of CD8+ T cells [86], possibly by outcompeting the NK cell receptors respectively the TCR for MHC I binding.

3.2.3.5. HVEM-LIGHT interaction

LIGHT binds, as LTα, to the CRD2 and CRD3 domain of HVEM [92]. Since LIGHT exists as a trimer [46] it engages three HVEM molecules.
Whereas soluble LIGHT and BTLA can simultaneously bind to HVEM [26, 50], suggesting different binding sites (see sections 3.2.3.7), membrane-bound LIGHT ligated to HVEM blocks the formation of the BTLA-HVEM complex, presumably due to steric hindrance [47].

Binding of LIGHT to HVEM induces a co-stimulatory signal, pivotal for the protection against a *Leishmania* infection by Th1 cells [93, 94], killing of LIGHT-expressing tumours by CD8+ T cells activated by HVEM expressing NK cells [38] and the anti-bacterial response of HVEM expressing monocytes, neutrophils [39] and ILC3 [95]. Furthermore, long-term maintenance of memory CD8+ T cells after respiratory virus infection [96] and of memory CD4+ T cells [37] is highly dependent on the HVEM-LIGHT axis. In contrast, absence of LIGHT increased the longevity of transplanted allografts [97, 98] and reduced the anti-host CTL response and the survival of donor T cell in a model of graft-versus-host disease (GVHD) [99]. In turn, transfer of LIGHT overexpressing transgenic T cells into $Rag^{-/-}$ mice caused intestinal inflammation due to an increased Th1 cytokine response, induced by the interaction of LIGHT with HVEM and LTβR [100]. In allergic mouse models, HVEM promoted keratinocyte-induced dermatitis [101] and inflammation and remodelling of the airway by mast cells [102, 103] through engaging LIGHT. Thus, LIGHT binding to HVEM induces a co-

stimulatory signalling mediating protection against infections but promoting graft rejection and anti-host as well as allergic responses.

3.2.3.6. HVEM-CD160 interaction

Besides TNF superfamily members, HVEM can also interact with the Ig superfamily member CD160 [28]. Interaction studies identified the CRD1 domain of HVEM being crucial for the engagement of CD160 [28]. CD160 is present as a monomer at the surface of the cells, thus interacting with one HVEM molecule, forming a 1:1 complex [104].

Binding of HVEM to CD160+ CD4+ T cells inhibited their proliferation *in vitro*, whereas blocking of the CD160-HVEM interaction, augmented their cytokine response [28]. It has been suggested that the co-inhibitory action of the HVEM-CD160 system attenuates the pathology in the brain of *Plasmodium berghei* ANKA infected animals, through limiting the IFNγ and Granzyme B production of CD8+ T cells [105]. Furthermore, CD160 has been detected on CD8+ T cells from chronic virus infected mice and humans, attenuating T cell responses [106-109], possibly through the engagement of HVEM. Indeed, upon blocking of the HVEM-CD160 interaction, T cell proliferation and cytokine production was enhanced [107, 108].
However, CD160 can also transmit a positive downstream signal, promoting T cell activation *in vitro* [110]. In NK cells, CD160 can promote the cytokine production [111, 112] leading to tumour control [111].
The observed discrepancy in the transmission of a positive or negative signal by CD160 might be ascribed to the different isoforms of the molecule, the GPI-anchored and the transmembrane one.

Reciprocally, CD160 can serve as a ligand for HVEM, promoting NF-κB downstream signalling [48]. This co-stimulatory pathway has shown to be important during bacterial mucosal infections, where binding of CD160, found on innate-like intraepithelial lymphocytes, to HVEM expressed on epithelial cells mediates protection [44].

3.2.3.7. HVEM-BTLA interaction

BTLA, as CD160 [28] and the gD protein of HSV [26, 50], binds to the CRD1 domain of HVEM [27]. The molecule is expressed as a monomer, thus forming a 1:1 complex with HVEM [113]. gD and CD160 compete with BTLA for the interaction with HVEM due to an overlapping binding sequence [26, 28, 50]. Since the BTLA/gD binding site is on the opposite side, the DARC (gD and BTLA binding site on the TNFR HVEM in CRD1) side, from the one of LIGHT [50], a trimeric complex with soluble, but not membrane bound, LIGHT can form [26, 50] (see section 3.2.3.5). Besides binding in *trans*, BTLA and HVEM expressed on the same cell might interact in *cis*, as shown for T cells, inhibiting HVEM-mediated NF-κB downstream activation and blocking the interaction in *trans* [114]. Soluble LIGHT, but not CD160, can bind to HVEM-BTLA *cis* complex, though NF-κB activation is not induced.

Binding of HVEM to BTLA in *trans* has been shown to dampen the immune response, initially reported *in vitro*, reducing the T cell proliferation [26, 27]. *In vivo*, absence of BTLA or administration of a blocking HVEM-BTLA antibody augmented clearance of the parasitic nematode *Strongyloides ratti* [115] and of *Listeria monocytogenes* [116], latter due to an enhanced cytokine response of innate immune cells. Furthermore, BTLA deficient mice were better protected from a blood-stage malaria infection through an increased innate and adaptive immune response [117]. Besides, the role of the BTLA-HVEM pathway has also been assessed in transplantation models, where $BTLA^{-/-}$ recipients showed an increased rejection of a partially MHC-mismatched allograft due to an augmented proliferation and cytokine response of host anti-graft CD4+ T cells [118]. Surprisingly, absence of BTLA in a fully MHC-mismatched cardiac allograft model rather revealed a protective role, possibly due to the increased PD-1 expression detected on alloreactive CD4+ and CD8+ T cells, that would dampen the anti-graft response [118].

More recently, various studies have addressed the role of the BTLA-HVEM pathway in the anti-tumour immunity. Expression of BTLA has been reported on various tumour-reactive cell types, including CD8+ T cells [119, 120] and NKT cells [121]. The induction of BTLA down-regulation or the blockage of the HVEM-BTLA interaction enhanced the infiltration of cells and their anti-tumour response, which could induce tumour regression in mouse models [119-123]. In contrast, development of a GC

lymphoma is promoted in the absence of HVEM on B cells [124, 125] through loss of the B cell intrinsic inhibitory BTLA signal [124], elicited by B cell inter-cellular interactions, and through an increased help of Tfh to B cells [125]. Haematological malignancies are often treated by allogeneic stem cell transplantations to induce donor anti-tumour T cell responses. By blocking the interaction between BTLA expressing allogeneic T cells and HVEM+ tumour cells or by mutating the HVEM gene, donor anti-tumour T cell proliferation and cytokine response can be augmented [126, 127].
Furthermore, the inhibitory action of the BTLA-HVEM system has also been shown in a colitis model, where transfer of WT CD4+ T cells into HVEM$^{-/-}$ Rag$^{-/-}$ mice exacerbated the intestinal inflammation due to an increased proliferation and cytokine response of the transferred cells, which was abolished by the administration of an agonistic BTLA antibody [128].

However unexpectedly, transfer of BTLA$^{-/-}$ CD4+ T cells into Rag$^{-/-}$ mice did not induce exacerbated colitis when compared to WT CD4+ T cells recipients [128]. Furthermore, whereas in BDF1 mice (F1 generation of B6 [H-2^{b}] x DBA/2 [H-2^{d}] mice) receiving B6 T cells and a treatment with an agonistic BTLA antibody, the GVHD pathogenicity was attenuated, transfer of BTLA KO B6 T cells into BDF1 mice surprisingly rather diminished than augmented donor anti-host CTL responses [129]. Similarly, in a parent-into-irradiated F1 model of allogeneic hematopoietic stem cell transplantation [130], GVHD and the expansion of alloreactive T cells was not augmented in the absence of BTLA. The observation that numbers of transferred BTLA$^{-/-}$ donor C57BL/6 or BALB/c T cells in CB6F$_1$ recipient mice, inducing acute respectively chronic GVHD in recipients, started to decrease around 9 days post transfer due to a decreased proliferation and a slightly increased death of KO T cells [131], revealed a stimulatory function of BTLA. Whereas co-transfer of BTLA sufficient together with BTLA deficient CD4+ T cells into Rag$^{-/-}$ mice [128] suggests an intrinsic pro-survival role of the molecule, the restoration of the BTLA ligand but not receptor ability in T cells cultured *in vitro*, suggested rather a cell-extrinsic role [129]. Intrinsically, triggering of BTLA may promote the survival through recruitment of Grb2 to the cytoplasmic tail of the molecule and subsequent activation of the PI3K pathway [68, 70]. Cell-extrinsically, HVEM has been identified *in vitro* as a receptor for BTLA, activating the NF-κB pathway through HVEM [48] to promote the survival [48] and the proliferation [129] of

cells. Indeed, binding of BTLA on CD8α+ DCs to HVEM expressed on Vaccinia virus (VV)- and *Listeria monocytogenes*-specific CD8+ T cells is crucial for their survival during the effector and memory phase [42, 43]. A recent study suggests, that a decreased *Listeria* burden observed in BTLA deficient compared to WT CD8α+ DCs [132], caused by and increased expression of FasL on KO CD8α+ DCs [132], might impair T cell priming and thus to the reduction in T cell numbers.

Thus, mutual signalling can occur in the BTLA-HVEM system, where HVEM binding to BTLA induces in most cases a negative signal, dampening the immune response, and binding of BTLA to HVEM acts co-stimulatory, regulating the survival or the proliferation of cells.
Interestingly, whereas the induction of peripheral Tregs is mediated through the co-stimulatory action of BTLA on DCs binding to HVEM on Tregs [133], Tregs themselves rather use the co-inhibitory ability of HVEM binding to BTLA on effector T cells to suppress the proliferation of latter [134].

3.2.4. CD8+ T cell differentiation

After activation, CD8+ T cells undergo repeated rounds of cell divisions giving rise to a heterogenous population of effector cells. Using killer cell lectin-like receptor G 1 (KLRG-1) and IL-7Rα (CD127) these effector subsets can be distinguished [135-137]. The first effector cells that form are KLRG-1- and CD127-. These early effector cells (EEC) give rise to the KLRG-1+ CD127- short lived effector cells (SLEC) and KLRG-1- CD127+ memory precursor effector cells (MPEC) [137]. Initially, these two subsets show comparable effector functions and proliferation ability [136]. However, towards the end of the T cell response, the two subsets start to diverge, where the SLEC continue to divide and produce effector molecules like Granzyme B, whereas the MPEC show an enhanced IL-2 production [135, 136]. After antigen clearance, most of the SLEC die, whereas the majority of MPEC survive and give rise to long-lasting memory cells [135-138].
The two T-box family transcription factors T-bet and EOMES are critical for accumulation of effector cells and commitment to the different effector subsets. Both, T-bet and EOMES, are required for effector cell formation [139, 140]. However, whereas SLEC sustain T-bet expression, possibly due to prolonged exposure to IL-12

[135, 141], T-bet is reduced in MPECs [142] and EOMES expression is sustained, with latter being critical for the long-term maintenance of CD8+ T cells after antigen clearance [143].

Memory cells arising from MPECs can be divided into two subsets according to the expression of CD62L and CCR7: The CD62L+ CCR7+ CD127+ central memory (T_{CM}) and the CD62L- CCR7- CD127+ effector memory (T_{EM}) cells [144-146]. T_{CM} are located mainly in lymphoid tissues, are able to produce IL-2 and can proliferate vigorously upon antigen recognition [144-147]. In contrast, T_{EM} can enter peripheral tissues and mediate protection against local infection by rapid production of cytokines. Some evidences suggest that through homeostatic proliferation, T_{EM} gradually convert to T_{CM} which then may persist lifelong [145, 147].

Recently, a third population of memory cells has been identified, residing exclusively in the tissue, thus termed tissue resident memory T cell (T_{RM}) [148]. As T_{EM}, T_{RM} are negative for CD62L and CCR7 and positive for CD127 but in contrast express CD69, a specific set of transcription factors like Hobit and Blimp-1 and, depending on the tissue, CD103 [148]. There localization and ability to rapidly exert an effector response provides protective immunity at the site of pathogen re-entry.

4. Cytokines

Cytokines are a heterogenous group of proteins involved in the development and homeostasis of cells and the regulation of the immune response against invading pathogens. They can act directly on the secreting cell (autocrine), on a cell in close proximity (paracrine) or, in a few cases, on a cell in a different part of the body (endocrine). A target cell is exposed to a set of cytokines, which may have overlapping functions (being redundant) or act together in an additive manner, each contributing equally. Besides, two cytokines can also act synergistically, exceeding the additive effect of two. In contrast, cytokines that inhibit each other's action are termed antagonists.

The respective cytokine receptors can be assigned to one of the five superfamilies: the immunoglobulin, the class I cytokine, the class II cytokine, the TNF receptor or the chemokine receptor superfamily.

Chemokine receptors are polypeptides containing seven transmembrane domains and associating intracellularly with G proteins that mediate downstream signalling [149]. Chemokine receptors can be divided into four subfamily according to their interaction partner: The CC receptors (CCRs) engaging CC chemokines, the XC receptors (XCR) interacting with XC chemokines, the CXC receptors (CXCR) engaging CXC chemokines and the CX_3C receptors (CX_3CR) interacting with the CX_3C ligands. They are involved in leukocyte recruitment to site of inflammation or injury and are expressed on activated T cells and on a wide variety of innate immune cells.

Few cytokine receptors belong to the immunoglobulin superfamily, but amongst them are the receptors for IL-18, IL-33, IL-36 and IL-1.This family also includes, amongst others, some co-signalling molecules.

The majority of the cytokine receptors belong to the class I cytokine receptor superfamily. It includes the receptors for the cytokines IL-4, IL-5, IL-6, IL-12 and for the γ-chain cytokines IL-2, IL-9, IL-7, IL-15 and IL-21 (discussed in more detail in section 4.1). The receptors have a conserved extracellular domain containing four cysteine residues (CCCC) and a tryptophan-serine-(variable amino acid)-tryptophan-serine (WSXWS) sequence.
The receptor for IL-12 consist of two subunits, the IL-12Rβ1 and the IL-12Rβ2, and is expressed on a variety of cells including T and NK cells [150]. Whereas the cytokine is critical for the activation of CD8+ T cells [29-31] and NK cells [151], IL-12 is crucial for the polarization of naïve CD4+ T cells into Th1 cells [7, 8] (see section 3.2.1).
As the IL-12R, most of the class I cytokine receptors are composed of multiple subunits. Their cytoplasmic tail is constitutively associated with Janus (JAKs) and/or TYK kinases [152], which, upon binding of the respective cytokine, phosphorylate each other and activate STAT proteins, that will subsequently induce target gene transcription.

The receptors for the type I IFNs IFNα and IFNβ, which are involved in CD8+ T cell activation [31-33], and the receptors for IL-10 and IFNγ belong to the class II cytokine receptors. In contrast to the class I cytokine receptors, the class II members only share

the CCCC sequence in their extracellular domain but lack the WSXWS motif. IFNγ is produced by Th1, CD8+ and NK cells and exerts a pleiotropic function [153]. It not only stimulates the anti-microbial action of macrophages and increases the expression of MHCII on DCs and macrophages, but also enhances the cytotoxic activity of NK cells and the antibody production of B cells.
As for the class I cytokine receptors, also for the class II receptors downstream signalling is induced through auto-phosphorylation of constitutively associated JAKs, which then in turn activated STATs that induce transcription of target genes [154].

The TNF receptor superfamily comprise the receptor for TNFα but also co-signalling receptors like HVEM (see section 3.2.3.1) or CD40 and death receptors like FAS and TRAIL [155]. The extracellular domain of TNF receptors consists of one to four CRDs. Upon binding to the respective TNF ligand trimer, receptor trimerization is induced. Downstream signalling for the activating receptors is mediated via NF-κB or mitogen-activated protein kinase (MAPK) whereas death receptors recruit FADD adaptor proteins that induce apoptosis through activation of the caspase cascade [23].
Binding of TNFα to its receptors can induce a pro-inflammatory signal, activating neutrophils and macrophages but also induce chemokine expression on endothelial cells and macrophages for the recruitment of neutrophils, or induce apoptosis. Binding of TNFα to the TNFR1 or TNFR2 recruits TRAF2 which can activate the NF-κB or MAPK pathway, inducing a pro-inflammatory signal [156]. However in contrast to the TNFR2 which lacks a death domain [156], binding of TNFα to TNFR1 can recruit the adaptor protein FADD and induce, together with several other proteins, apoptosis over a caspase cascade [157].

4.1. The γ-chain (γ_c) cytokine receptors

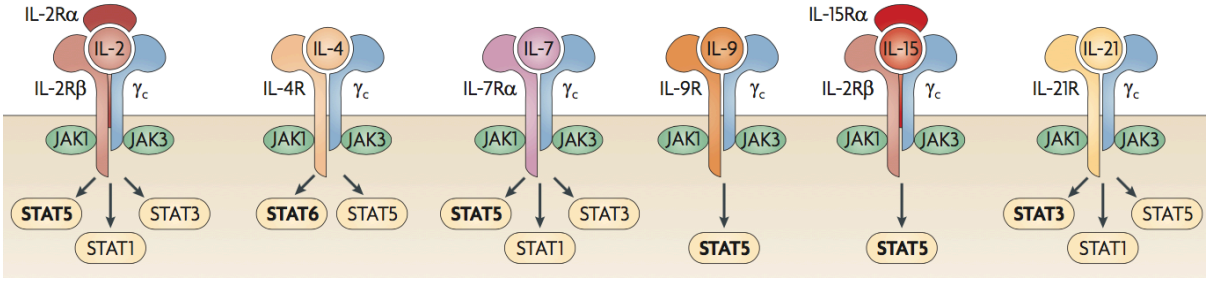

Fig. 3: The class I cytokine receptor subfamily γc: Adapted from Rochman et al., 2009 [158]

A subfamily of class I cytokine receptors is the γ-chain (γ_c) family (Fig. 3), characterized by a common γ_c subunit as part of their receptor complex. Whereas the receptors for IL-4, IL-7, IL-9 and IL-21 are composed of the γ_c and a cytokine-specific subunit, IL-2 and IL-15 engage heterotrimeric receptors, consisting of the γ_c, a cytokine-specific and the shared IL-2Rβ chains [152]. While for the heterodimeric family members, the cytokine-specific and the γ_c subunit are involved in downstream signalling, for the IL-2 and the IL-15 receptor rather the two shared subunits transduce the signal [159]. The IL-2Rα and IL-15Rα chains increase the affinity for the respective cytokine.

All family members use the JAK-STAT pathway for downstream signalling, but also the PI3K pathway can be activated [159]. Upon formation of the cytokine-receptor complex, trans-phosphorylation of JAK1, associated with the IL-2Rβ, IL-4R, IL-7Rα, IL-9R or the IL-21R subunit, and JAK3, bound to the γ_c chain, occurs [159]. Subsequently, the activated kinases phosphorylate tyrosine residues in the cytoplasmic tail of their bound receptor chains, allowing the association with and the activation of STAT proteins, which eventually regulate the transcription of target genes [159].

Recently, an alternative spliced γ_c protein lacking the cytoplasmic and the transmembrane domain, has been reported [159]. This secreted protein can bind to the other membrane-bound subunit involved in signalling and prevent signal transduction, thus representing a mechanism to regulate the activity of γ_c cytokine receptors.

The γ_c family members display pleiotropic function by regulating the development, proliferation and effector response, but also mediating long-term maintenance of a variety of innate and adaptive immune cells. Briefly, whereas IL-4 is crucial for the polarization of naïve CD4+ T cells into Th2 cells (see section 3.2.1) and plays a role in the induction of allergic responses and immunoglobulin class switching, IL-7 is involved in the development of T and B cells but is also pivotal for the maintenance of naïve T cells, and together with IL-15, the long-term persistence of memory T cells by regulating their homeostatic proliferation [158]. IL-15 is furthermore important for the development of NK cells and the survival and function of DCs. IL-9 is a growth factor for T and mast cells but also acts on epithelial cells [158]. The action of receptors for IL-2 and IL-21 are described in the two subsequent chapters.

Giving the wide variety of functions of γ_c receptors, it is not surprising that absence of the γ_c chain (also termed IL-2Rγ) in humans causes severe defects in the immune system, e.g. non-functional B cells and complete absence of T and NK cells in the most common disease form, the X-linked severe combined immunodeficiency disease (XSCID) [160, 161].

4.1.1. IL-2R

The IL-2 receptor was the first member of the γ_c cytokine receptor family discovered, initially shown to promote the expansion of T cells [6, 162-164] but later also reported to induce apoptosis of T cells through activation induced cell death [165-167].

IL-2 can bind to the heterodimeric or the high-affinity heterotrimeric form of the receptor. Whereas the latter consists of the IL-2Rα (CD25), the IL-2Rβ (CD122) and the γ_c (CD132) subunits, the dimeric receptor is composed of the IL-2Rβ and the γ_c chains. While IL-2 binds to the IL-2Rβ with an intermediate affinity and triggers the dimerization with the γ_c subunit [159], the IL-2Rα binds the cytokine with high-affinity and subsequently interacts with the IL-2Rβ and the γ_c chain [152, 168].

Target gene transcription upon IL-2R complex ligation is mainly mediated by the JAK-STAT pathway, where activated JAK1 and JAK3 recruit primarily STAT5 to the cytoplasmic tails of the IL-2Rβ and γ_c subunits, but also signalling via the MAPK and the PI3K pathways has been reported [168].

The IL-2Rβ-γ_c dimer is present on NK cells, promoting their cytolytic activity [169], on NKT and at low levels on naïve but at high levels on memory CD8+ T cells [168, 170]. While expression of the IL-2Rβ and the γ_c subunits on memory CD8+ T cells did not differ between the two major subsets T_{CM} and T_{EM} [145], the production of the IL-2 cytokine by T_{CM} is higher than by T_{EM} [145, 146].

The trimeric receptor can be found on activated T cell, where the expression of IL-2Rα is induced by IL-2 itself [171], and is constitutively expressed on regulatory T cells [168]. The latter rely on IL-2 for their development in the thymus [172, 173], where the cytokine induces the Treg program through upregulating the expression of Foxp3 [174]. Absence of the cytokine or the receptor causes severe autoimmunity in mice [175-177]. Furthermore, the homeostasis of Tregs in the periphery is dependent on IL-2 [178]. Since Tregs produce only very low amounts of the cytokine, the main source for IL-2 in the steady-state are autoreactive T cells, where the paracrine consumption of the cytokine by Tregs additionally represents a regulatory mechanism, to suppress potentially harmful T cell responses [17].

Conventional T cells upregulate the IL-2Rα (CD25) upon activation and start producing IL-2, that acts in an autocrine and paracrine manner [168, 179] promoting T cell expansion [6, 162-164]. In case of CD8+ T cells, CD25 furthermore influences the differentiation of activated cells into the different effector subsets. A few days after activation, CD8+ T cell transiently divide into $CD25^{high}$ and $CD25^{low}$ cells, which differ in their transcriptional profile [180]. Whereas the mRNA levels of CD127, Bcl-6 and CD62L are higher in $CD25^{low}$ subset then in $CD25^{high}$, the latter rather have elevated levels of T-bet, Blimp-1 and Granzyme B [180]. Blimp-1 promotes a SLEC effector state [180, 181] associated with Granzyme B expression, while supressing memory cell formation [181] and intrinsically dampens IL-2 production through a negative feedback loop [182] to prevent excessive, potentially harmful, IL-2 mediated T cell expansion. After antigen clearance, the $CD25^{hi}$ CD8+ T cells are prone to apoptosis, whereas the $CD25^{low}$ cells are preferentially maintained long term [180].

Interestingly, the long-term persistence of memory cells seems to be mostly independent of the IL-2 receptor during an acute virus infection [5, 6], however absence of the IL-2 signal skews memory CD8+ T cells towards a central memory phenotype [6]. In contrast, the secondary response of memory CD8+ T cells [5, 6] and

the maintenance of CD8+ T cells during a chronic infection [6] were shown to be highly dependent on the IL-2 signal.

Conventional CD4+ T cells rely on IL-2 for the polarization of naïve cells into Th1 cells [183], through the induction of T-bet and IL-12Rβ2 expression, and into Th2 cells [184], by inducing the expression of the IL-4Rα and ensuring accessibility of the Il4 locus. In contrast, IL-2 inhibits the generation of Th17 cells by dampening the gene expression of the IL-6Rα and the IL-6 signal transducer gp130, both important for the polarization into IL-17 producing CD4+ T cells [184]. Recent reports also suggest a role of IL-2 in the generation of Tfh and Th9 cells, where low expression of the IL-2Ra is associated with the induction of a Tfh program [185] but in contrast IL-2 is required for the differentiation of Th9 cells [186-188].
Furthermore, IL-2 promotes the immunoglobulin production by B cells [189].

4.1.2. IL-21R

Most recently, the IL-21R has been classified as a γ_c receptor family member. The receptor is composed of the specific IL-21Rα chain and the shared γ_c subunit [158]. Like all family members, IL-21R signals via the JAK-STAT pathway through JAK1, bound to the IL-21Rα, and JAK3, associated with the γ_c subunit, that activate the STAT proteins 1,3,5 [190]. Target gene transcription is then mainly induced by STAT3. Besides, also the PI3K and the MAP kinase pathway can contributed to the signal transduction [190].

Whereas IL-21 is mainly produced by CD4+ T cells and NKT cells [190], the receptor is expressed on a wide variety of cells including B cells, T cells, NK and NKT cells, DCs, macrophages and on keratinocytes and fibroblasts [190, 191].
IL-21 modulates the fate of B cells after activation, depending on the stimuli engaged. Whereas the cytokine acts pro-apoptotic in B cells exposed solely to TLR ligands, BCR triggering and T-cell co-stimulation induces proliferation of B cells in the presence of IL-21 [192]. Furthermore, the cytokine promotes the differentiation of mouse and human B cells into plasma cells [193, 194] and the immunoglobulin class switching to IgG1 upon protein immunization [195], latter through the autocrine action of the cytokine on follicular T helper cells [196] that regulate germinal centre formation.

Besides, IL-21, induced by IL-6 in CD4+ T cells [197, 198] and maintained over a positive autocrine feedback loop [198], can contribute *in vitro* to the differentiation of CD4+ T cells into Th17 cells. IL-21 induces the expression of the IL-23R, and subsequently together with IL-23, induce RORγt in CD4+ T cells [198]. However, the relevance of IL-21 in inducing Th17 cells *in vivo* is controversial. Whereas, one study reported a reduction of IL-17 producing CD4+ T cells in IL-21 KO mice accompanied by a reduced EAE progression in mice [197], two reports showed no difference in the generation of Th17 cells and rather a slightly increased susceptibility to EAE in cytokine and receptor KO animals [199, 200].
In models of allergic airway inflammation and helminth infection, IL-21 is involved in the Th2-driven immune response by promoting the cytokine production of Th2 cells and the infiltration of eosinophils into the airways [201, 202]. Whereas it was initially suggested that IL-21 acts Th2 cell intrinsically [202], a recently published study rather proposes that IL-21 induces apoptosis in Tregs and thus, through lack of the suppressive function of latter cells, indirectly promotes the Th2-driven allergic airway inflammation [203]. Indeed, the negative function of IL-21 on the Treg-mediated suppression of conventional T cell responses has been suggest before, however by rather increasing the resistance of CD4+ CD25- T cells to suppression [204] or by reducing the production of IL-2 by conventional T cells [205], a cytokine crucial for Treg homeostasis. In mouse models, the limited Treg cell function in the presence of IL-21 is crucial for sustaining the protective CD8+ T cell response during a chronic virus infection [206], but in contrast, mediates GVHD by favouring graft anti-host T cell responses [207].

Furthermore, during a chronic LCMV infection, IL-21, mainly produced by CD4+ T cells, cell-intrinsically promotes the continuous proliferation and cytokine production of virus-specific CD8+ T cells to ensure virus clearance [208-210]. In contrast, IL-21 is dispensable for the primary and secondary CD8+ T cells response during an acute virus infection with low virulent LCMV strains [208, 209]. However during a VV infection, IL-21 has shown to be crucial for the survival and cytokine production of virus-specific CD8+ T cells [211]. Furthermore, IL-21 promotes the CD8+ T cell

response against a chronic *Toxoplasma gondii* and a chronic *Mycobacterium tuberculosis* infection [212, 213].

Besides, IL-21 promotes the cytokine response and the cytotoxic action of NK and NKT cells [214-216]. However, IL-21 inhibits the expansion of NK cells [214, 215], but promotes the survival and proliferation of NKT cells [216].

Whereas IL-21 impairs the cytokine production and the stimulatory action of bone marrow-derived dendritic cells (BMDCs) [217], the cytokine induces the expression and processing of IL-1β in conventional DCs (cDCs) [218]. Furthermore, IL-21 can promote apoptosis of cDCs [219].

5. Lymphocytic choriomeningitis virus (LCMV)

LCMV is a member of the *Arenaviridae* family, containing a large (L) and as small (S) negative stranded RNA segment [220]. Whereas the glycoprotein precursor, later cleave into the glycoprotein 1 (GP1) and 2 (GP2), and the nucleoprotein are encoded on the S RNA, the L segment encodes a RNA polymerase and a RING finger-containing Z protein. While GP2 anchors GP1 in the virion lipid envelope, latter can bind to alpha-dystroglycan (α-DG) expressed on the target cell inducing virus entry. The Z protein aligns the intracellular surface of the virion envelope and interacts with GP2. The nucleoproteins and the polymerase are bound to the RNA fragments.
LCMV is a non-cytopathic virus with the mouse as its primary host [220].
The first LCMV strains isolated were Armstrong (Arm) and Traub, both named after their discoverer, and WE. Later on, the LCMV Arm derivate Clone 13 (Cl13) [221] and the LCMV WE derivative Docile (DO) [222] were found. LCMV Cl13 differs in only two amino acids from the Arm strain [223], one located in the polymerase and the other in the glycoprotein sequence. While immunocompetent mice infected with the variants Arm and WE control the virus within 8 to 15 days (acute infection), the LCMV strains Cl13 and DO, of latter a high dose, can persist up to several month in mice, establishing a chronic infection. Persistence is shown to be promoted by increased viral replication and improved infection of the target cell by the virus, difference in target cell tropism [224] and the reduction in recognition of virus-infected cells by T cells [225].

5.1. Immune response to LCMV

Cytotoxic CD8+ T cells are indispensable for the control of a LCMV infection [226]. Through lysis of infected cells and production of pro-inflammatory cytokines (see section 3.2.2) the virus infection is resolved. During a chronic LCMV infection, induced by the strains Cl13 and DO, the CD8+ T cell response is attenuated, a process termed T cell exhaustion, a strategy of the virus to prevent killing and/or a mechanism induced by the host to prevent immunopathology of infected tissue [227]. T cell exhaustion is characterized by a continuous loss of effector functions, like the ability to produce pro-inflammatory cytokines and target-cell killing, and can eventually result in the physical deletion of the cell [228]. Several mechanisms contribute to T cell exhaustion, including the upregulation of co-inhibitory receptors like PD-1, LAG3, CD160, CTLA-4 or BTLA on virus-specific CD8+ T cells to dampen their immune response, exposure to immunosuppressive cytokines like IL-10 and TGFβ and the presence of regulatory T cells which suppress CTL functions [229]. Amongst the co-inhibitory receptors, PD-1 is the most intensively studied. Blockage of the PD-1 pathway efficiently restores CTL function [230] and reduces viral loads [230-232]. Despite its prominent role in T cell exhaustion, the underlaying mechanism is still unclear. Furthermore, a chronic infection is accompanied by a substantial induction of the immunosuppressive cytokine IL-10 [233], which has been shown to intrinsically decrease the antigen sensitivity of the TCR by enhancing the glycosylation of surface proteins on CD8+ T cells [234]. TGFβ rather dampens the cytokine production and cytotoxicity of virus-specific CD8+ T cells during a chronic infection and is able to induce Bim-mediated apoptosis in CTLs [235]. Regulatory T cells are highly induced upon a chronic LCMV infection and their depletion restores the CTL function [206]. Tregs can produce IL-10 and TGFβ. If production of immunosuppressive cytokine is the primary mechanism, promoting the T cell exhaustion by Tregs, is however still unclear.

Whereas CD4-help is dispensable for the expansion and differentiation of CD8+ T cells during an acute LCMV infection [226, 236], absence of CD4+ T cells impairs the generation and long-term maintenance of memory CD8+ T cells [236, 237]. Furthermore, absence of CD4-help leads to a defective secondary response upon re-challenge [237, 238]. In contrast, during a chronic infection absence of CD4 T cells impairs the CTL response, leading to persistence of virus [226].

B cells produce neutralizing and non-neutralizing antibodies during a LCMV infection. Neutralizing antibodies appear relatively late (after 60 dpi) whereas non-neutralizing antibodies can already be detected between 4 to 7 days after infection [239-241]. Whereas the antibody response is dispensable during an acute LCMV infection [242, 243], virus control during a persistent infection is highly dependent on the antibodies [242]. Interestingly, transfer of non-neutralizing antibodies into chronically infected mice could significantly reduce viral loads already early during the infection [244, 245].

IV. AIM OF THIS THESIS

Upon activation, CD8+ T cell differentiate into cytotoxic lymphocytes, which are able to kill virus-infected and altered self-cells (e.g. tumours cells) and produce a set of pro-inflammatory cytokines. Following pathogen clearance, most CD8+ T cells die, however some survive and form a pool of long-lasting memory cells, which upon re-infection, will rapidly mount a secondary immune response.

The major aim of this thesis was to elucidate the contribution of the two co-signalling molecules B and T lymphocyte attenuator (BTLA) and Herpes virus entry mediator (HVEM) in the CD8+ T cell response induced upon a lymphocytic choriomeningitis virus (LCMV) infection in mice. Recently, BTLA and HVEM have been suggested to promote the survival of pathogen-specific effector and memory CD8+ T cells during a Vaccinia virus and *Listeria monocytogenes* infection [42, 43]. Since those two pathogens are controlled rather rapidly, we aimed to elucidate the role of BTLA and HVEM in the CD8+ T cell response not only during a rapidly resolved acute infection but also during a chronic infection, where antigen can persist up to several months. Thus, we made us of the viral pathogen LCMV, which, depending on the viral isolate and the dose, can cause an acute or a chronic infection in mice.

Besides, we aimed to assess the interplay of the two gamma chain (γ_c) cytokine family members IL-2 and IL-21 in promoting the CD8+ T cell response during an acute LCMV infection. Whereas the primary CD8+ T cell response induced upon an acute LCMV infection was shown to be mostly independent of either of the two cytokines [[5, 6] and [208, 209]], IL-2 and IL-21 were crucial for the maintenance and the effector response of virus-specific CD8+ T cells during a chronic LCMV infection [[6] and [208-210]].

V. RESULTS

1. Down-regulation of BTLA and HVEM on effector CD8+ T cells interferes with efficient maintenance of memory against LCMV

Patrizia Diethelm[1], Iwana Schmitz[1], Jan Kisielow[1], and Manfred Kopf[1]

[1] Institute of Molecular Health Sciences, Department of Biology, ETH Zurich, 8093, Zurich, Switzerland

Key words: BTLA, HVEM, LCMV, CTL

1.1. Abstract

Regulating the expression of co-signalling molecules is pivotal for shaping the T cell response against pathogens. While BTLA (B and T lymphocyte attenuator) intrinsically inhibits the T cell proliferation when interacting with HVEM (Herpes virus entry mediator), binding of BTLA to HVEM on antigen-specific CD8+ T cells ensures survival of effector and memory cells. Surprisingly, the absence of BTLA and HVEM did not affect anti-viral T cell responses and viral clearance upon acute and chronic LCMV infection. However, virus-specific T cells effectively down-regulated BTLA and HVEM at the peak of the acute response. Forced expression of HVEM on virus-specific CD8+ T cells resulted in a reduction of effector cells, whereas numbers of memory cells were increased. In contrast, BTLA overexpression on virus-specific CD8+ T cells only led to an increase in memory cells. Overall, this study indicates that down-regulation of HVEM ensures an efficient acute response at the price of impaired maintenance of T cell memory. Moreover, down-regulation of BTLA during the effector response also limits memory formation.

1.2. Introduction

The protective immune response against virus infections includes the activation of CD8+ T cells which, upon antigen encounter, expand and become cytotoxic. These effectors are able to kill infected cells and produce large amounts of pro-inflammatory cytokines like IFNγ and TNFα. After pathogen clearance, most CD8+ T cells die, however some survive and give rise to self-renewing memory T cells, which persist long-term and mount a rapid secondary immune response upon re-infection (reviewed in [246]).The different stages of a CD8+ T cell response are regulated by co-signalling molecules from the TNF/TNFR and the CD28 Ig superfamilies that can either promote (co-stimulatory) or limit (co-inhibitory) T cell responses. Whereas the co-stimulatory molecules CD28, 4-1BB and OX-40 promote the survival, expansion or long term maintenance, respectively, of antigen-specific T cells [247-250], the co-inhibitory molecules CTLA-4 and PD-1 rather limit the proliferation of T cells [251-253] and the latter also the effecter response upon antigen encounter [252-254].

Herpes virus entry mediator (HVEM) has initially been identified as the main target for entry of Herpes simplex virus into host cells [34]. Later, the TNF family members LIGHT (lymphotoxin-like, exhibits inducible expression, and competes with herpes

simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes) and lymphotoxin alpha (LTα) have been described as a co-stimulatory ligands for HVEM [46], where former inducing T cell proliferation and cytokine secretion [51, 57].
Besides, HVEM can also engage Ig superfamily members like CD160 and BTLA. Binding of CD160 to HVEM induces a positive signal [48], promoting protection against mucosal bacterial infections [44]. However, HVEM can serve reciprocally as a ligand for CD160, impairing the proliferation and cytokine response of T cells [28, 108].
Initially, HVEM binding to BTLA has been shown to inhibit the T cell proliferation *in vitro* [26, 27]. Absence of the BTLA signal induces intestinal inflammation [128], autoimmunity [68, 77, 79] and cardiac allograft rejection [118], though augments pathogen clearance [115, 116] and anti-tumor T cell responses [126]. However surprisingly, transfer of BTLA deficient T cells did not increase the severity of colitis [128] and transfer of BTLA deficient splenocytes rather diminished the anti-host effector T cell response in a model of graft-versus-host-disease (GVHD) [129], latter due to an impaired survival/proliferation of the transferred cells [129, 131]. Indeed, BTLA can serve reciprocally as a ligand for HVEM promoting survival of T cells [48]. Recently, it was shown that during infection with Vaccinia virus or *Listeria monocytogenes*, the survival of effector and memory pathogen-specific CD8+ T cells was dependent on the binding of BTLA to HVEM expressed on T cells [42, 43].
Since the HVEM-BTLA pathway was crucial for the CD8+ T cell response during vaccinia virus infection [42], we aimed to elucidate the importance of the two molecules during acute and chronic LCMV infection. Our data indicate that down-regulation of HVEM on virus-specific CD8+ T cells assures effector cell accumulation whereas down-regulation of BTLA dampens the cytokine response during LCMV infection. However, memory formation is impaired by the low expression of either of the molecules.

1.3. Material and Methods

Mice

All mouse strains including BTLA$^{-/-}$ [68] and HVEM$^{-/-}$ mice [134] (kindly provided by Kenneth M. Murphy (Washington University School of Medicine, St. Louis, USA)), Smarta1 (CD45.1+) [255], P14 (CD45.1+) [256] and OT-I (CD45.2+) [257] transgenics and C57BL/6 mice were housed in individually ventilated cages under specific

pathogen-free conditions at the ETH Phenomics Center (EPIC, Zurich, Switzerland). BTLA$^{-/-}$/HVEM$^{-/-}$ mice were generated by crossing in-house bred BTLA$^{-/-}$ and HVEM$^{-/-}$ animals. For experiments, age-matched and sex-matched mice were used at an age of 6 to 9 weeks. Mice were euthanized with CO_2. All experiments were performed according to institutional guidelines and the Swiss animal protection law (Swiss Animal Protection Ordinance [TschV and TschG] Switzerland) and had been approved by local animal ethics committee of Kantonales Veterinäramt in Zurich, Switzerland (permissions 113/20112 and ZH135/15).

Virus and infection

Mice were infected *i.v.* with indicated doses of the LCMV strains WE, Clone-13 or Docile. Viruses were originally provided by Rolf Zinkernagel (University of Zurich, Zurich, Switzerland) and propagated on L-929, BHK-21 or MDCK cells, respectively. For LCMV production, the respective cell line was grown until confluency in MEM medium (Gibco, Massachusetts, USA) containing 5% heat-inactivated FBS (Gibco), 100 U/ml Penicillin/100 µg/ml Streptomycin (Gibco) and 2mM L-Glutamine (Sigma-Aldrich, Missouri, USA) for BHK-21 cells, in RPMI medium (Gibco) containing 5% heat-inactivated FBS (Gibco), 100 U/ml Penicillin/100 µg/ml Streptomycin (Gibco) and 2mM L-Glutamine (Sigma-Aldrich) for MDCK or L-929 cells. Cells were counted in the morning (cells per one tissue culture flask represents 100% confluency) and appropriate numbers were seeded to get 70-80% confluency. In the afternoon, virus in 5 ml medium per flask at a MOI (multiplicity of infection) of 0.01 was added to the adherent cells and incubated for 1h at room temperature. Tissue culture flasks were tapped every 15 min. Subsequently, 15 ml of fresh medium was added to the cells and incubated for 48h at 37°C, 5% CO_2. Medium was harvested and centrifuged. The supernatant was aliquoted and frozen at -80°C. 20 ml of fresh medium was added to the cells and incubated for an additional day to receive a second batch of virus. The virus titer of the supernatants was determined by plaque assay.

Retroviral expression of BTLA and HVEM

Btla and *Hvem* genes were PCR amplified from mouse tissue cDNA, followed by cloning into the moloney murine leukemia based retroviral expression vector pMYs-IRES-GFP upstream of IRES. Retroviruses were produced in the Phoenix-eco packaging cell line, kindly provided by Garry Nolan (Standford University, California,

USA), by transfection of cells with the pMYs-BTLA-IRES-GFP (rvBTLA-GFP), pMYs-HVEM-IRES-GPF (rvHVEM-GFP) or, as a control, the pMYs-IRES-GFP vector (rvGFP) and retroviral supernatants were harvested on day two and three. Until further use, supernatants were frozen at -80°C. Retrovirus was concentrated by centrifugation at 10'000g for 1h with a Sorvall RC 6+ (Fisher scientific, New Hampshire, USA).

Generation of BTLA and HVEM overexpressing CD8+ T cells

Overexpressing CD8+ T cells were obtained as described by Kurachi M. et al. [258]. Briefly, MACS sorted splenic CD8+ T cells of tg P14 mice were activated for 18h with 1 ug/ml anti-CD3 (home-made), 0.5 ug/ml anti-CD28 (BioLegend, California USA) and 20 ng/ml IL-2 (home-made) at 37°C, 5% CO_2. The next day, activated CD8+ T cells were enriched by Percoll (GE Healthcare, Illinois, USA) density centrifugation, followed by retrovirus transduction of enriched cells and incubation for 4h at 37°C, 5% CO_2. Subsequently, retroviral supernatant was removed, transduced cells were transferred to a tissue-cultured treated 12-well plate and incubated overnight at 37°C, 5% CO_2 in medium used for activation. The day after, overexpressing GFP+ cells were FACS sorted with a BD FACS Aria III (BD Bioscience, California, USA) and adoptively transferred into WT animals.

Adoptive T cell transfer

BTLA and HVEM expression on virus-specific CD8+ respectively CD4+ T cells during a LCMV infection was assessed by transfer of $1x10^4$ MACS sorted splenic CD8+ T cells from tg P14 mice or $5x10^5$ MACS sorted splenic CD4+ T cells from tg Smarta1 mice into LCMV WE (2000 pfu) infected CD45.2+ WT mice (day 1 post infection).
Kinetics of overexpressing virus-specific CD8+ T cells during an acute infection were examined by adoptive transfer of $1x10^4$ retrovirally transduced CD8+ T cells into naïve CD45.2+ WT mice, which were infected with 2000 pfu LCMV WE the day after. To study the effector response of overexpressing CD8+ T cells during a chronic infection, $1x10^4$ retrovirally transduced CD8+ T cells were transferred into CD45.2+ OT-I mice, which were infected with $1x10^6$ pfu LCMV Cl13 the next day.

Generation of BMDCs

BMDCs were obtained by differentiating bone marrow cells isolated from the hind legs of WT, $BTLA^{-/-}$, $HVEM^{-/-}$ or $BTLA^{-/-}/HVEM^{-/-}$ mice *in vitro* for 7 days with 2 ng/ml GM-

CSF (home-made) in RPMI (Gibco) containing 2mM L-Glutamine (Sigma-Aldrich), 10% heat-inactivated FBS (Gibco), 100 U/ml Penicillin/100 µg/ml Streptomycin (Gibco), 50 µM 2-Mercaptoethanol (Gibco), and 10 mM HEPES (Lonza, Basel, CH). Cells were frozen in liquid nitrogen until use.

Co-culture

One day prior to the set-up of the co-culture, BMDCs were thawed. The next day, cells were counted and seeded in IMDM (1X) + GlutaMAX-I (Gibco) containing 10% heat-inactivated FBS (Gibco), 100 U/ml Penicillin/100 µg/ml Streptomycin (Gibco) and 50 µM 2-Mercaptoethanol (Gibco) at a concentration of $4x10^4$ cells/well in a round bottom 96-well plate. Gp33 or its altered peptide ligands A4Y or V4Y (both from Mimotopes, Victoria, Australia) were added at the indicated concentration. 20min later, $5x10^4$ MACS sorted splenic CD8+ T cells from P14 mice were added and co-cultured for 1, 2 or 3 days at 37°C, 5% CO_2.

VLP immunization

WT or $BTLA^{-/-}/HVEM^{-/-}$ mice were injected with 10 µg Qß-virus-like particles (VLPs) *i.p.*, which were kindly provided by Martin Bachmann (University of Zürich, Zürich, CH). Blood samples were collected at indicated days and frozen at -80°C until further use.

LCMV ELISA

LCMV Cl13 containing supernatant of BHK-21 cell culture was produced as described earlier with the difference, that after 24h of incubation, the cell medium was replaced with FBS-free medium. For coating, FBS-free supernatant collected 24h later was added to wells of NUNC ELISA plates (Thermo Fisher Scientific, Massachusetts, USA), which were subsequently irradiated with 900'000 µJ/cm^2 with a Spectrolinker XL-1500 UV cross-linker (Spectronics Corporation, New York, USA) and incubated overnight at 4°C. The next day, plates were washed and blocked with PBS, 1% BSA (Sigma-Aldrich) at RT for 1h. In the meantime, blood samples from LCMV Cl13 infected mice were titrated over 7 1:3 dilution steps in PBS, 0.1% BSA. After incubation, ELISA plates were washed with PBS, samples were added and plates were once more irradiated with a Spectrolinker. After 1h of incubation at RT, plates were washed and IgG1- (1:1000; SouthernBiotech, Birmingham, USA), IgG2a- (1:1000; BD Biosciences), or IgG2b-AP (1:1000; SouthernBiotech) antibodies were

added in PBS, 0.1% BSA and incubated for 1h at RT. Plates were washed and the pNPP substrate (Sigma-Aldrich) dissolved in ELISA buffer (96 ml 1M Diethanolamine [Sigma-Aldrich],101.6 mg $MgCl_2$ [Merck], filled up to 1000 ml with ddH_2O, pH 9.8) was added to wells. Absorbance was afterwards measured at 405nm with a SpectraMax 190 (Bucher Biotec, Basel, CH).

VLP ELISA

A sandwich ELISA to detected IgG1 and IgG2c levels was performed as described previously, except plates were coated with 1 µg/ml VLPs dissolved in PBS and titrated samples added to the ELISA plates were not irradiated. IgG2c-AP antibody was purchased from SouthernBiotech and used 1:1000.

Determination of virus titer

Viral titers were determined on MC57 cells as previously described [259]. Briefly, organ samples were frozen at -80°C in 1 ml MEM medium (Gibco) supplemented with 5% heat-inactivated FBS (Gibco), 100 U/ml Penicillin/100 µg/ml Streptomycin (Gibco) together with a metal bead, whereas blood was diluted 1/5 with MEM medium, 5% FBS, Penicillin/Streptomycin (Gibco) containing Heparin (Liquemin, 25 000 U.I./5 ml, Drossa Pharm, Basel, Switzerland). To determine virus titer, samples were thawed and organs were lysed using a tissue lyser (Qiagen AG, Hilden, Germany) for 4 min at 25 Hz. Samples were diluted over 5 (organs) or 3 (blood) 1:10 steps and mixed 1:1 with MC57 cells in a 24-well plate. After 2-4h of incubation at 37°C, 5% CO_2, overlay (26.5 g/l DMEM [Gibco], 10% heat-inactivated FBS (Gibco), 100 U/ml Penicillin/100 µg/ml Streptomycin (Gibco), and 2% Methylcellulose [Sigma-Aldrich]) was added and plates were incubated for two days at 37°C, 5% CO_2. To detect viral plaques, wells were fixed with PBS (Life Technologies, California, USA) containing 4% Formalin (Sigma-Aldrich) for 30 min at RT, followed by fixation with PBS containing 1% Triton-X (BioRad, California, USA) for 20 min at RT. Unspecific binding was prevented by blocking with PBS, 10% FBS (Gibco) for 1h at RT. Plaques were stained with supernatant from VL-4 cells for 1h at RT, followed by the incubation with a peroxidase-conjugated AffiniPure Goat anti-Rat IgG antibody (Jackson ImmunoResearch Laboratories, INC., Pennsylvania, USA) for 1h at RT. Last, a solution of 0.2M Na_2HPO_4 $2H_2O$ (Merck, Darmstadt, Germany), 0.1M Citric Acid (Sigma-Aldrich) together with a tablet of OPD (Sigma-Aldrich) was used for visualization.

The detection limit (DtL) correlates to 50 plaques per organ respectively per ml blood. For samples with a PFU below the DtL, the value was set to 10 pfu.

Cell preparation

Blood samples were collected in Heparin (Liquemin, 25 000 U.I./5 ml, Drossa Pharm, Basel, Switzerland) and subsequently treated twice with ACK buffer for 5 min at 4°C to remove erythrocytes. Single cell suspension of the spleen was obtained by smashing organs through a 70 μm cell strainer followed by lysis of erythrocytes with ACK buffer for 5 min at 4°C. The liver was minced and smashed through a 70 μm cell strainer. After centrifugation, the cell pellet was resuspended in 5 ml of pure PBS (Life Technologies, California, USA) and centrifuged at 20g for 5 min at RT to exclude hepatocytes. The upper layer was transferred into a new tube, underlaid with 5 ml of Lympholyte-M solution (Cedarlane, Ontario, Canada) and centrifuged at 836g for 20 min without break. The intermediate phase was washed with PBS containing 2% heat-inactivated FBS (Gibco). Before FACS staining, spleen and liver cells were filtrated through a 35 μm cell strainer.

The lung was removed after perfusion of the mouse with PBS. The organ was minced with a gentleMax (Miltenyi Biotec, Bergisch Gladbach, Germany) and digested in IMDM + GlutaMAX-I (Gibco), 100 U/ml Penicillin/100 µg/ml Streptomycin (Gibco) containing 600 U/ml Collagenase IV (Worthington, New Jersey, USA) and 200 U/ml DNase I (Sigma-Aldrich) for 30 min at 37°C and 130 rpm. The tissue was homogenized with the gentleMax and passed through a 70 μm cell strainer. Red blood cells were removed by ACK and the single cell suspension was filtered through 35 μm cell strainer.

Reagents

The gp33-41 peptide (KAVYNFATM) and the gp61-80 (GLNGPDIYKGVYQFKSVEFD) peptide were bought from Mimotopes (Victoria, Australia). The PE-conjugated gp33-MHC class I tetramer (H-2Db/gp33-41) was kindly provided by the National Institute of Health tetramer core facility (Bethesda, Maryland, USA). For exclusion of dead cells, efluor780 (eBioscience, Massachusetts, USA) or ZombieAqua (BioLegend) was used. Antibodies used were as follows: PD-1-FITC (J43; Thermo Fisher Scientific, Massachusetts, USA), BTLA-APC (8F5;

BioLegend), HVEM-APC (LH1; eBioscience), CD19-APC-Cy7 (6D5; BioLegend), CD4-PE (RM4-5; BD Bioscience), CD4-PerCP-Cy5.5 (RM4-5; BioLegend), CD8a-FITC (53-6.7, eBioscience), CD8a-PE-Cy7 (53-6.7; BioLegend), CD8a-APC-Cy7 (53-6.7; BioLegend), CD8a-PE (53-6.7; BD Bioscience), IFNγ-APC (XMG1.2; BioLegend), CD127-APC (SB/199; BioLegend), CD25-PE (PC61; eBioscience), Ki-67-PE (16A8; BioLegend), KLRG-1-biotin (2F1/KLRG1; BioLegend), Streptavidin-BV711 (BD Bioscience), CD62L-BV421 (MEL-14; BioLegend), CD45.1-PerCP-Cy5.5 (A20; BioLegend), TNFα-PE (MP6-XT22; BD Bioscience), TNFα-FITC (MP6-XT22; BioLegend)

Flow cytometry

All stainings were performed in FACS buffer (PBS + 0.5% heat-inactivated FBS) unless otherwise stated. First, cells were incubated for 5 min at 4°C with anti-CD16/CD32 mAb (2.4G2, homemade) to block FcγIII/II receptors. Cells were stained with gp33-tetramer for 15 min at room temperature in the dark followed by viability dye staining together with surface markers for 20 min at 4°C. Before acquisition, samples were fixed with 4% Formalin at room temperature for 5 min.

For staining Ki-67, co-cultured T cells were incubated with anti-CD16/CD32 mAb (2.4G2, homemade) diluted in FACS buffer (PBS + 0.5% heat-inactivated FBS) to block FcγIII/II receptors for 5min at 4°C, followed by viability dye efluor780 staining for 20min at 4°C in FACS buffer. Cells were washed twice with pure PBS and fixed with PBS diluted 1:10 in 70% Ethanol (Merck) at -20°C until FACS analysis. Before acquisition, cells were washed twice with FACS buffer followed by staining of Ki-67 diluted in FACS buffer for 30min at 4°C.

For analysis of the cytokine production, spleen and liver cells were stimulated with 2 μg/ml Monensin (Sigma-Aldrich), 1 μM gp33 and 1μM gp61 peptide in IMDM (1X) + GlutaMAX-I (Gibco) containing 10% heat-inactivated FBS (Gibco), 100 U/ml Penicillin/100 µg/ml Streptomycin (Gibco) and 50 µM 2-Mercaptoethanol (Gibco) for 3-4h at 37°C. Before viability dye staining together with surface marker staining for 20 min at 4°C, FcγR was blocked as described above. Subsequently, cells were fixed with 4% Formalin for 5 min at room temperature followed by permeabilization with permeabilization buffer (PBS + 0.5% heat-inactivated FBS + 0.5% saponin [Sigma-Aldrich]) for 5 min at room temperature. Intracellular staining was performed for 20 min

at 4°C with antibodies diluted in permeabilization buffer. Cells were washed twice with permeabilization buffer and once with FACS buffer.
Data were acquired on a BD FACSCanto II (BD Bioscience) or LSRFortessa (BD Bioscience) and analysed with FlowJo software version 9.9 (TreeStar; Oregon, USA).

Statistics

Data are shown as average ±SD. For statistical analysis ordinary one-way ANOVA (multiple comparisons) or two-tailed Student *t* test was performed using Prism 7 software (GraphPad software, California, USA). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Author Contributions

P.D. and I.S. performed the experiments. P.D., I.S., J.K. and M.K. designed the experiments. P.D. and M.K. wrote the manuscript.

1.4. Results

In vitro expansion and cytokine response of CD8+ T cells is independent of BTLA and HVEM

Initially, the BTLA-HVEM system has been proposed to inhibit the T cell expansion [26, 27]. *In vitro* culturing of CD4+ T cells expressing BTLA with antigen presenting cells (APCs) expressing HVEM impaired the proliferation of T cells [27].
To study if priming of antigen-specific CD8+ T cells is dependent on BTLA or HVEM expression on antigen presenting cells, we co-cultured bone marrow-derived dendritic cells (BMDCs), lacking either or both molecules, loaded with different amounts of gp33 peptide together with gp33-specific CD8+ T cells. T cell activation, effector function, and proliferation/expansion, as measured by CD25 up-regulation (after 24h) (Fig. 1, A), IFNγ production (Fig. 1, B) and Ki-67 expression/number of cells (after 48h or 72h) (Fig. 1, C, D), respectively was largely unaffected by antigen presentation of BMDCs lacking HVEM and/or BTLA. It was reported that T cell proliferation in the presence of low affinity TCR peptides depends strongly on the co-stimulatory molecule CD28 [260]. Thus, we compared the T cell response when wildtype and BTLA- or HVEM-deficient DCs presented the gp33 peptide or the low affinity altered peptide ligands (APLs) [261], A4Y and V4Y. However, again, no differences were found in IFNγ

production (Fig. 1, E) and T cell expansion (Fig. 1, F). Thus, our data indicate that BTLA and HVEM expression on DCs is dispensable for T cell priming and effector responses.

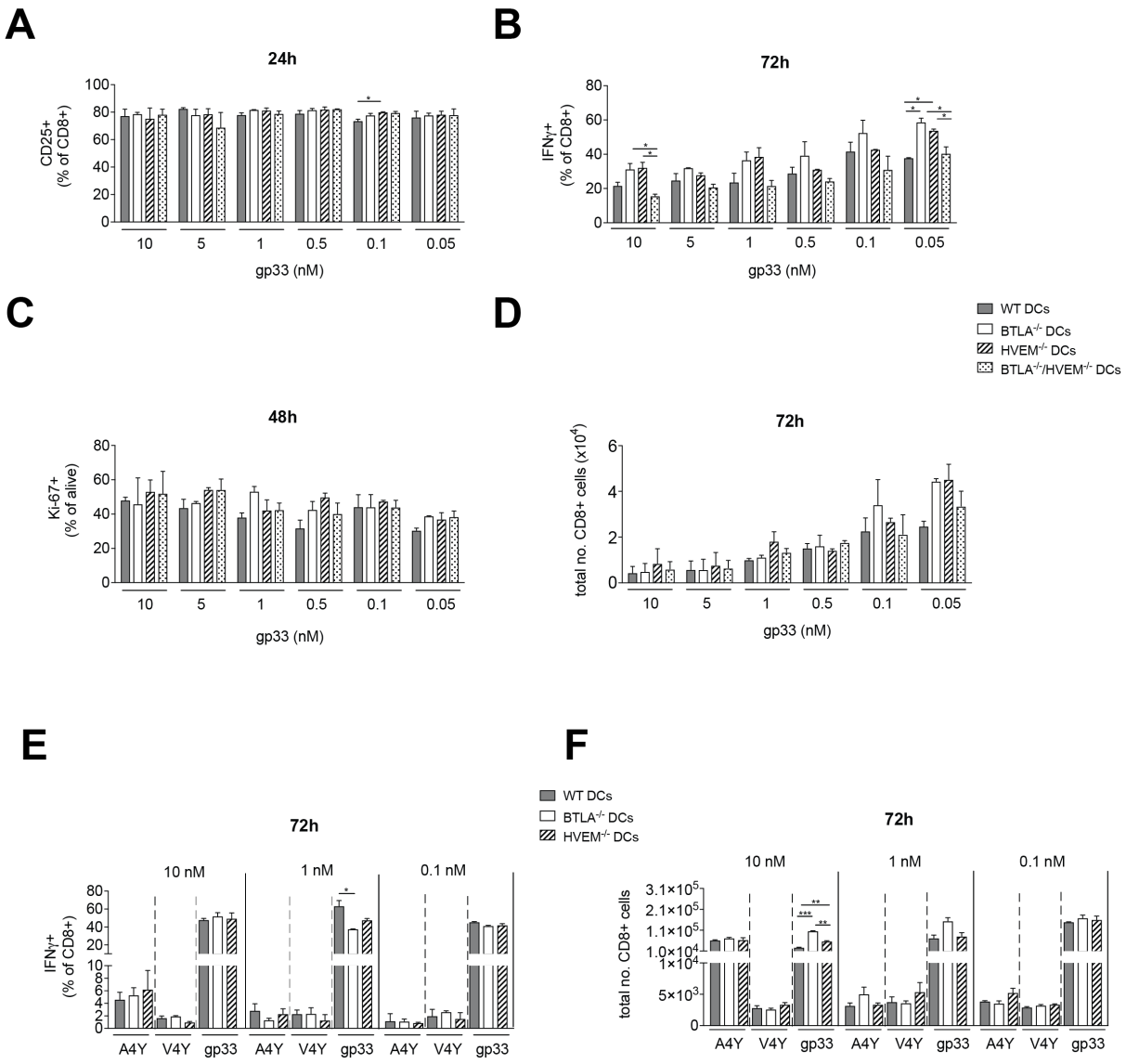

Fig. 1: BTLA and HVEM are dispensable for the *in vitro* activation, expansion and effector response of gp33-specific CD8+ T cells. *In vitro* differentiated BMDCs deficient in BTLA, HVEM or both molecules loaded with (A-D) indicated amounts of gp33 peptide or (E, F) indicated amounts of gp33, A4Y or V4Y peptide were co-cultured with gp33-specific CD8+ T cells, isolated from the spleen of naïve tg P14 mice. (A) Frequency of CD25+ CD8+ T cells after 24h of culturing. (B) Frequency of IFNγ producing CD8+ T cells after 72h of culturing were determined by in vitro restimulation with gp33 peptide, followed by intracellular cytokine staining. (C) Frequency of Ki-67+ alive cells after 48h of culturing. (D) Total numbers of co-cultured CD8+ T cells after 72h. (E) Frequency of IFNγ producing CD8+ T cells after 72h of

culturing with WT, BTLA$^{-/-}$ or HVEM$^{-/-}$ BMDCs loaded with gp33, A4Y or V4Y peptide were determined by in vitro restimulation with gp33 peptide, followed by intracellular cytokine staining. (F) Total numbers of CD8+ T cells after 72h of culturing. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (one-way ANOVA, multiple-comparison). All culture conditions were performed in duplicates. Data are shown as mean values ± SD. (A-D) Data are representative of two independent experiments. (E, F) Data are representative of one experiment.

BTLA-HVEM interactions are dispensable for the CD8+ T cell response during an acute and a chronic LCMV infection

HVEM and BTLA have been suggested to regulate survival of effector and memory CD8+ T cells during VV infection [42]. Therefore, we aimed to assess the quantity and quality of the virus-specific CD8+ T cell response during the course of LCMV infection in BTLA$^{-/-}$ and HVEM$^{-/-}$ mice. Surprisingly, the absence of BTLA or HVEM did not affect the expansion, contraction and long-term maintenance of virus-specific gp33+ and total CD8+ T cells (Fig. 2, A, B). Killer cell lectin-like receptor G 1(KLRG-1) together with IL-7Rα (CD127) can serve as markers to predict the fate of a T cell after antigen clearance [135, 136]. Whereas most of the KLRG-1+ CD127- short-lived effector cell (SLEC) die after pathogen control, KLRG-1- CD127+ memory precursor effector cells (MPEC) survive and give rise to long-lasting memory cells. Frequency of virus-specific CD8+ MPECs and SLECs were comparable in WT, BTLA$^{-/-}$ and HVEM$^{-/-}$ mice 8 dpi in the blood (Fig. 2, C). CD127+ T cells can be divided into different memory subsets by the expression of L-selectin (CD62L) [144-147]. CD62L- CD127+ effector memory T cells (T_{EM}) patrol peripheral tissues and mediate protection against local infection by rapid production of cytokines, whereas the CD62L+ CD127+ central memory T cells (T_{CM}) reside mostly in lymphoid tissues and proliferate extensively upon antigen re-encounter. While the frequency of those memory subsets in the blood was not affected by the absence of HVEM over the course of infection (Fig. 2, D), T_{CM} cells were reduced, however not significantly, in BTLA$^{-/-}$ mice in the blood and spleen > 4 weeks after infection (Fig. 2, D, lower). However, two additional experiments revealed no difference in frequency of T_{CM} in the blood and the spleen, thus the observed reduction is experiment dependent. Last, absence of BTLA or HVEM did not interfere with the cytokine production of splenic or lung-resident CD8+ T cells (Fig. 2, E).

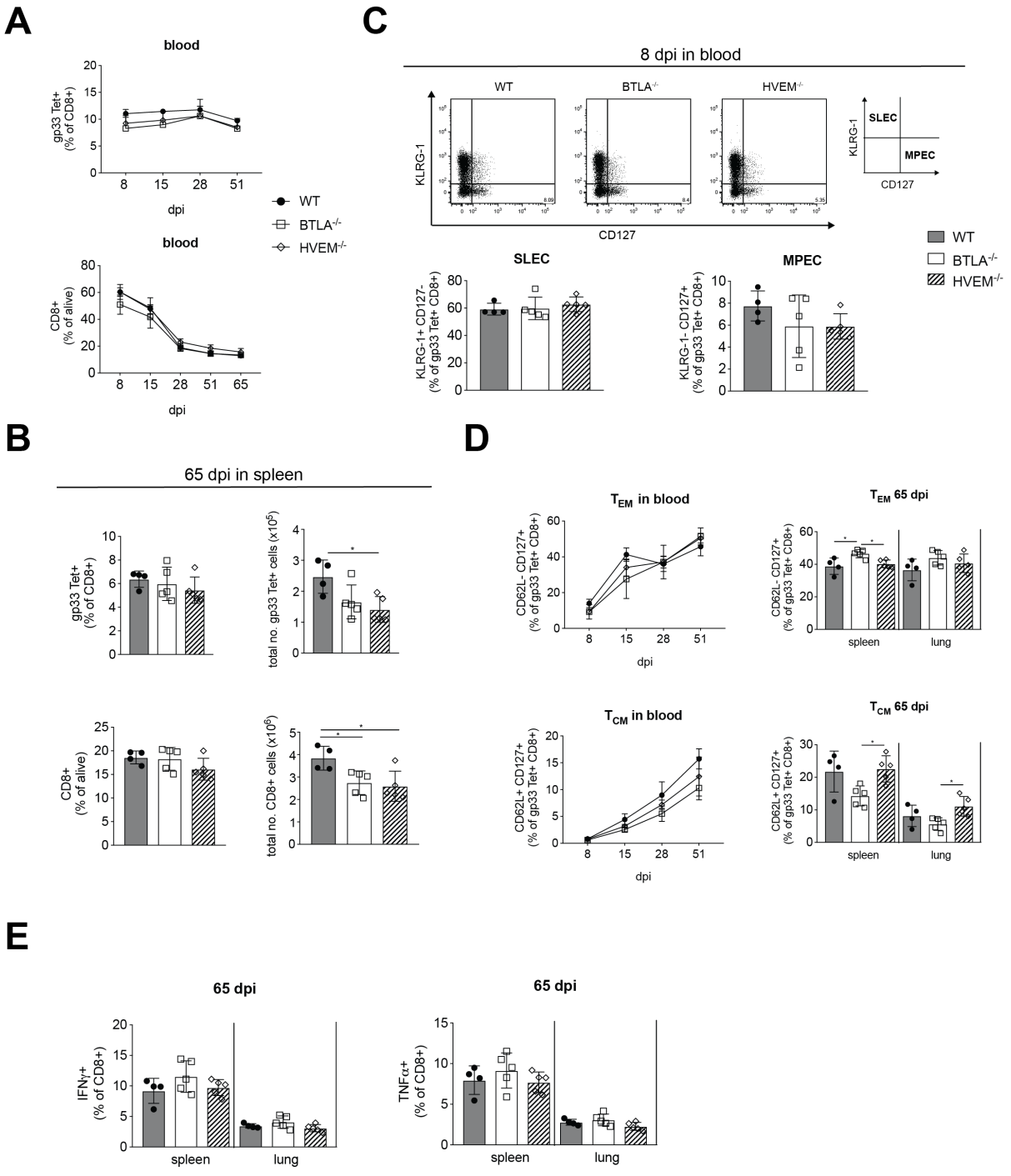

Fig. 2: Absence of BTLA or HVEM does not impair the anti-viral T cell response during acute LCMV infection. WT, BTLA$^{-/-}$ and HVEM$^{-/-}$ mice were infected with 2000 pfu LCMV WE *i.v.*. Frequency of gp33-specific and total CD8+ T cells over time in the blood (A) and frequency (B, left) and total numbers (B, right) in the spleen 65 dpi. (C) Representative FACS plots of KLRG-1 and CD127 expression on gp33-specific CD8+ T cells (upper) and frequency of KRLG-1+ CD127- SLEC (left) and KLRG-1- CD127+ MPEC (right) in the blood 8 dpi. (D, left) Frequency of gp33-specific T_{EM} and T_{CM} in the blood over time and (D, right) in indicated organs 65 dpi. (E) Frequency of IFNγ and TNFα producing CD8+ T cells from the spleen and

the lung of infected animals 65 dpi after *in vitro* restimulation with gp33 peptide, followed by intracellular cytokine staining. * $p \leq 0.05$ (one-way ANOVA, multiple-comparison). (A and D, left) Symbols represent means ± SD. (B-E) Symbols represent individual mice, bars represent means ± SD. Minimum of four mice per group were used. Data are representative of two independent experiments.

Although absence of HVEM and BTLA seems not to affect the anti-viral T cell response during acute infection, we cannot rule out a potential role upon chronic infection. Thus, we infected mice with LCMV DO and monitored T cell responses over time in the blood (Fig. 3, A) and in the spleen and the liver 16 weeks post infection (Fig. 3, B, C). Similar to acute infection, expansion, contraction, and exhaustion as well as the cytokine response (Fig. 3, D) of CD8+ T cells were comparable in $HVEM^{-/-}$ or $BTLA^{-/-}$ and wildtype controls. Accordingly, virus titers in the blood (Fig. 3, E, upper) and organs (Fig. 3, E, lower) remained unaffected in the absence of BTLA or HVEM.
Overall, our data indicate that the two co-signalling molecules BTLA and HVEM are dispensable for the CD8+ T cell response against an acute and a chronic LCMV infection.

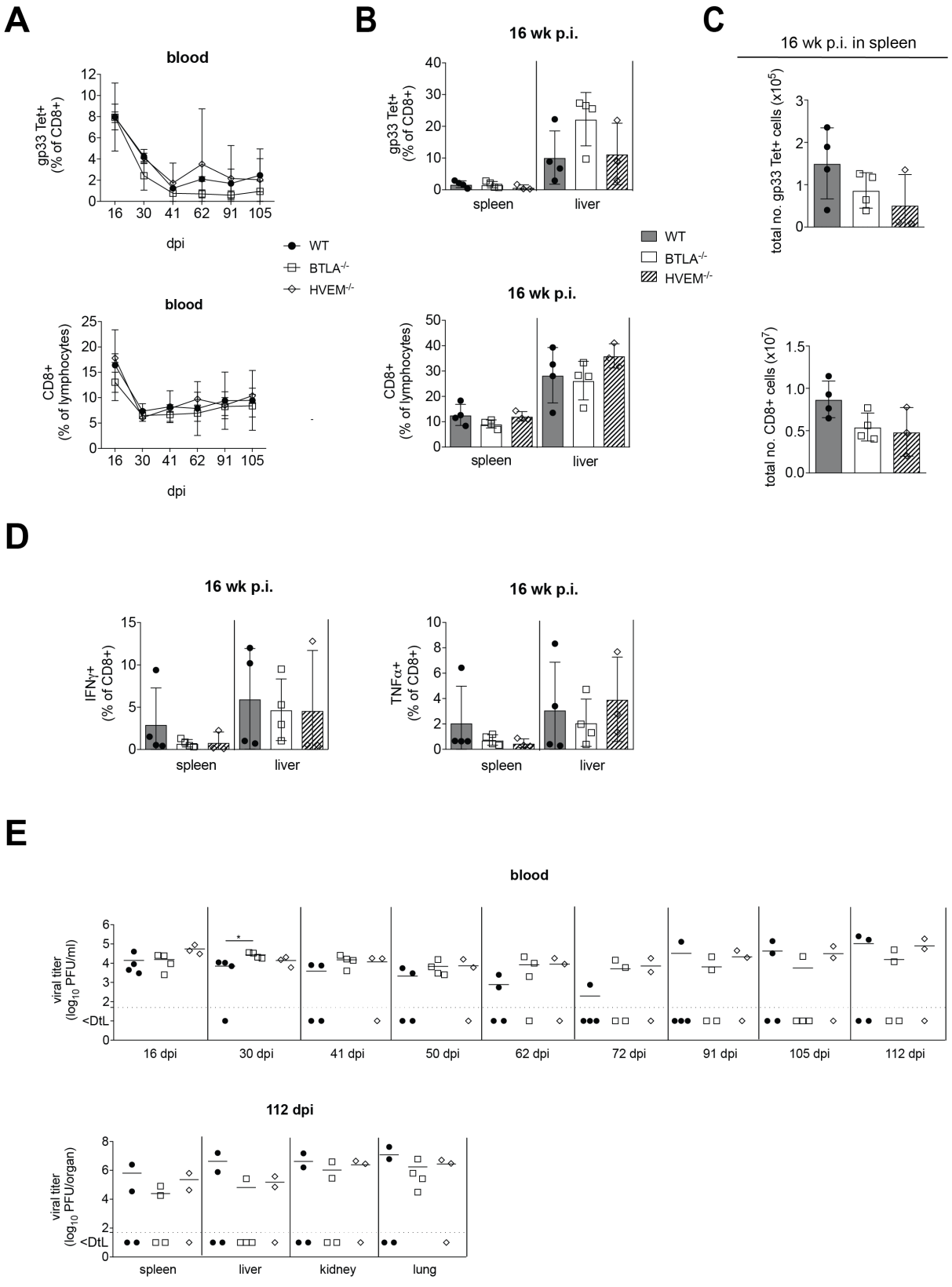

Fig. 3: Protection against a chronic LCMV infection is independent of BTLA and HVEM. WT, BTLA$^{-/-}$ and HVEM$^{-/-}$ were infected with 2x10^6 pfu LCMV DO *i.v.*. Frequency of over time in the blood (A) and frequency in indicated organs (B) and numbers in the spleen (C) 16 wk p.i. of gp33-specific and total CD8+ T cells. (D) Frequency of IFNγ and TNFα producing CD8+

T cells from the spleen and the liver of infected animals 16 wk p.i. after *in vitro* restimulation with gp33 peptide, followed by intracellular cytokine staining. (E) Viral titers in the blood over time (upper) and in indicated organs 16 wk p.i. (lower). * $p \leq 0.05$ (one-way ANOVA, multiple-comparison). (A) Symbols represent means ± SD. (B-E) Symbols represent individual mice, bars represent means ± SD. Dotted lines represent detection limit (DtL). Minimum of four mice per group were used. Data are representative of one experiment.

Combined absence of BTLA and HVEM does not impair the kinetics or the effector response of virus-specific CD8+ T cell during acute and chronic LCMV infection

Giving the complexity of the HVEM signalling network [47, 70], we wanted to assess whether the absence of BTLA can be compensated by the interaction of HVEM with a different ligand. Thus, we generated mice deficient in both BTLA and HVEM and studied the CD8+ T cell response during an acute and a chronic LCMV infection.

Absence of both molecules did not influence virus-specific and total CD8+ T cell responses monitored over the course of infection in the blood (Fig. 4, A) and spleen and liver 50 dpi (Fig. 4, B and data not shown) during an acute LCMV infection. Furthermore, frequency of cytokine producing splenic CD8+ T cells in BTLA/HVEM double-deficient animals was comparable to controls (Fig. 4, C). Besides, the absence of BTLA together with HVEM did not impair formation of virus-specific CD8+ T_{EM} or T_{CM} (Fig. 4, D).

Similarly, over the course of chronic infection with LCMV clone 13, frequencies and numbers of anti-viral and total CD8+ T cells were comparable in the presence and absence of both BTLA and HVEM as shown in Fig. 4 E and F. Notably, the frequency of IFNγ and TNFα producing splenic CD8+ T cells was slightly impaired in $BTLA^{-/-}$ /$HVEM^{-/-}$ mice (Fig. 4, G), although this did not affect virus persistence and eventual clearance (Fig. 4, H).

Thus, absence of BTLA is not compensated by HVEM interacting with another ligand during acute or chronic LCMV infection.

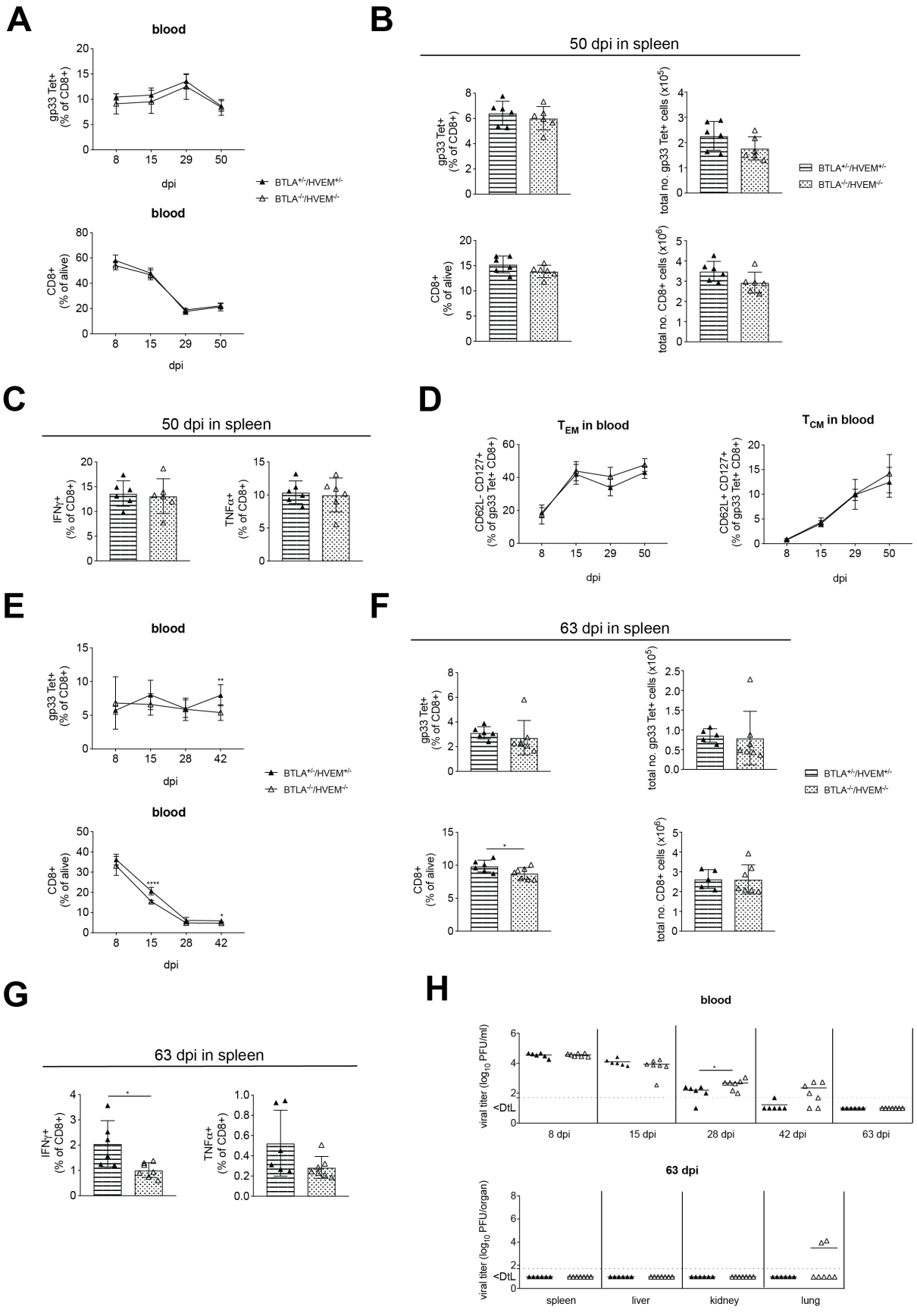

Fig. 4: Combined absence of BTLA and HVEM does not impair the CD8+ T cell response against acute or chronic LCMV infection. (A-D) BTLA$^{+/-}$/HVEM$^{+/-}$ controls and BTLA$^{-/-}$

/HVEM$^{-/-}$ mice were infected with 2000 pfu LCMV WE *i.v.*. Frequency over time in the blood (A) and frequency (left) and total numbers (right) in the spleen 50 dpi (B) of gp33-specific and total CD8+ T cells. (C) Frequency of IFNγ and TNFα producing splenic CD8+ T cells in infected animals 50 dpi after *in vitro* restimulation with gp33 peptide, followed by intracellular cytokine staining. (D) Frequency of gp33-specific T_{EM} and T_{CM} in the blood over time in infected animals. (E-H) BTLA$^{+/-}$/HVEM$^{+/-}$ and BTLA$^{-/-}$/HVEM$^{-/-}$ mice were infected with 1x10^6 pfu LCMV Cl13 *i.v.*. Frequency over time in the blood (E) and frequency (left) and total numbers (right) in the spleen 63 dpi (F) of gp33-specific and total CD8+ T cells. (G) Frequency of IFNγ and TNFα producing splenic CD8+ T cells in infected animals 63 dpi after *in vitro* restimulation with gp33 peptide, followed by intracellular cytokine staining. (H) Viral titers in the blood over time (upper) and in indicated organs 63 dpi (lower). * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$ (two-tailed Student *t* test). (A, D, E) Symbols represent means $\pm$ SD. (B, C, F-H) Symbols represent individual mice, bars represent means $\pm$ SD. Dotted lines represent detection limit (DtL). Minimum of six mice per group were used. Data are representative of one experiment.

To understand the kinetics of BTLA and HVEM surface expression on virus-specific CD4+ and CD8+ T cells over the course of an acute LCMV infection, we monitored their surface levels by flow cytometry on adoptively transferred congenically marked CD4+ and CD8+ T cells expressing a TCR specific for LCMVgp61 and LCMVgp33 from Smarta and P14 transgenic mice, respectively, in C57BL/6 mice. Both molecules were expressed on naïve P14 CD8+ T cells (Fig. 5, A) and became potently down-regulated on effector CD8+ T cells and stayed low expressed on memory CD8+ T cells (Fig. 5, B). Similarly, both molecules were expressed on naïve Smarta CD4+ T cells (Fig. 5, A). Interestingly, while HVEM expression on CD4+ T cells was suppressed in the effector and memory phase of the response, similar to CD8+ T cells, BTLA was up-regulated during the CD4+ T cell effector phase (Fig. 5, B). Moreover, while BTLA expression is much stronger on naïve B cells than on naïve T cells (Fig. 5, A) and remains unchanged over the course of infection (Fig. 5, B), HVEM expression is brighter on naïve T cells compared to naïve B cells (Fig. 5, A). However, while HVEM is suppressed on T cells it becomes up-regulated on B cells 8 dpi (Fig. 5, B).

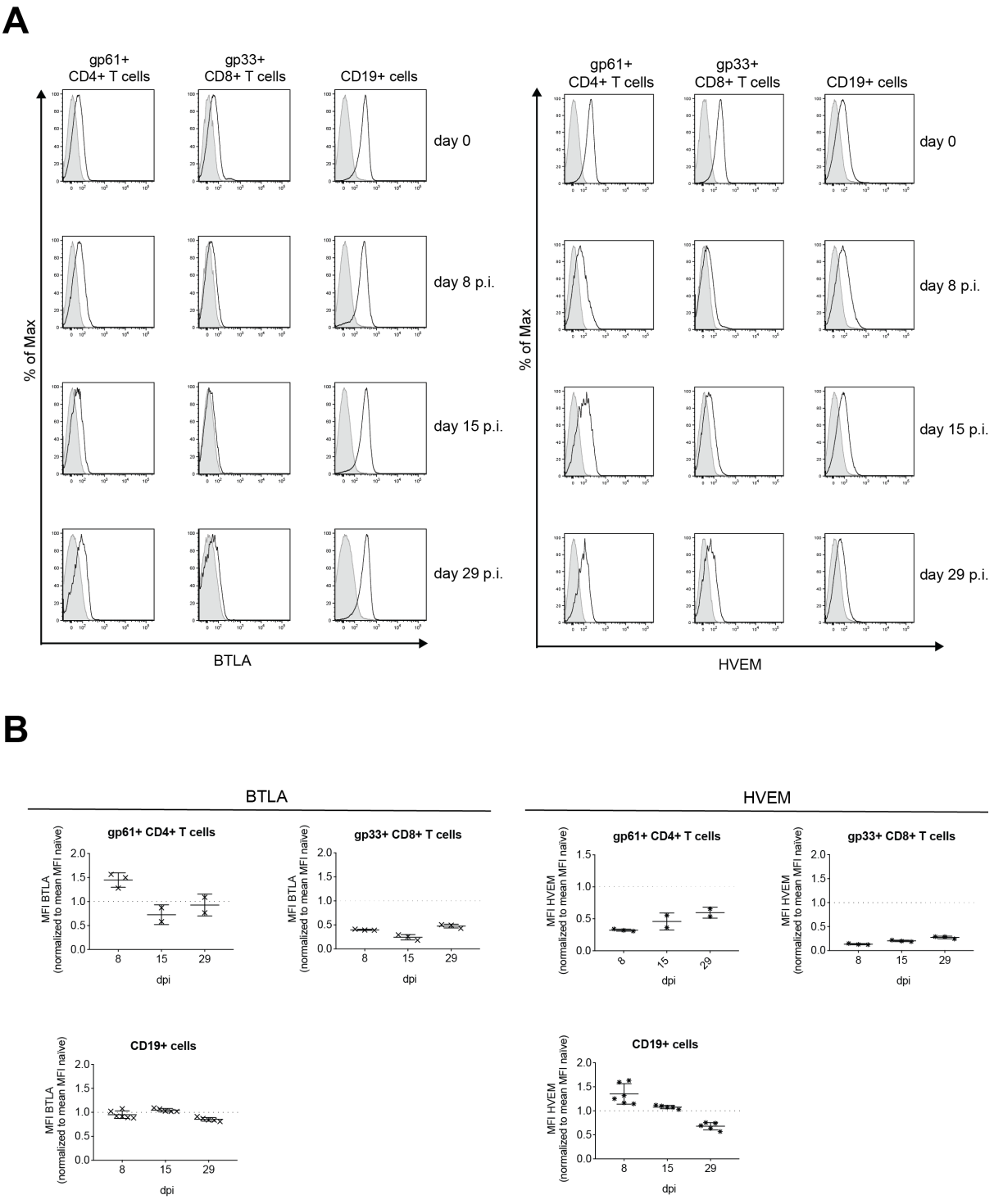

Fig. 5: Expression of BTLA and HVEM on adoptively transferred virus-specific tg CD4+ and tg CD8+ T cells and on endogenous B cells. (A) Expression of BTLA (left, black line) and HVEM (right, black line) and (B) mean fluorescent intensity (MFI) of BTLA (left) and HVEM (right) on adoptively transferred gp61-specific tg CD4+ T cells, gp33-specific tg CD8+ T cells and on endogenous CD19+ cells at indicated days post infection in WT mice infected with 2000 pfu LCMV WE *i.v.*. (A) As a control (grey), expression of BTLA or HVEM on total CD4+, CD8+ T cells or CD19+ cells from naïve BTLA$^{-/-}$/HVEM$^{-/-}$ mice was used. (B) Data are

normalized to the mean MFI of BTLA or HVEM from the respective naïve cell population (dotted line). From all values, the MFI of the respective population in DKO animals was subtracted before normalization. Symbols represent individual mice. Data are representative of three independent experiments.

High and maintained expression of HVEM and, in particular, BTLA prompted us to study B cell responses in the respective knockouts. To our surprise, antibody responses to LCMV remained unaffected in knockouts between 5 and 15 weeks post infection (Fig. 6, A-C). Similarly, HVEM and BTLA signalling in B cells is dispensable for antibody responses to non-replicating virus-like particles (Qß-VLPs) [262] (Fig. 6, D-E).

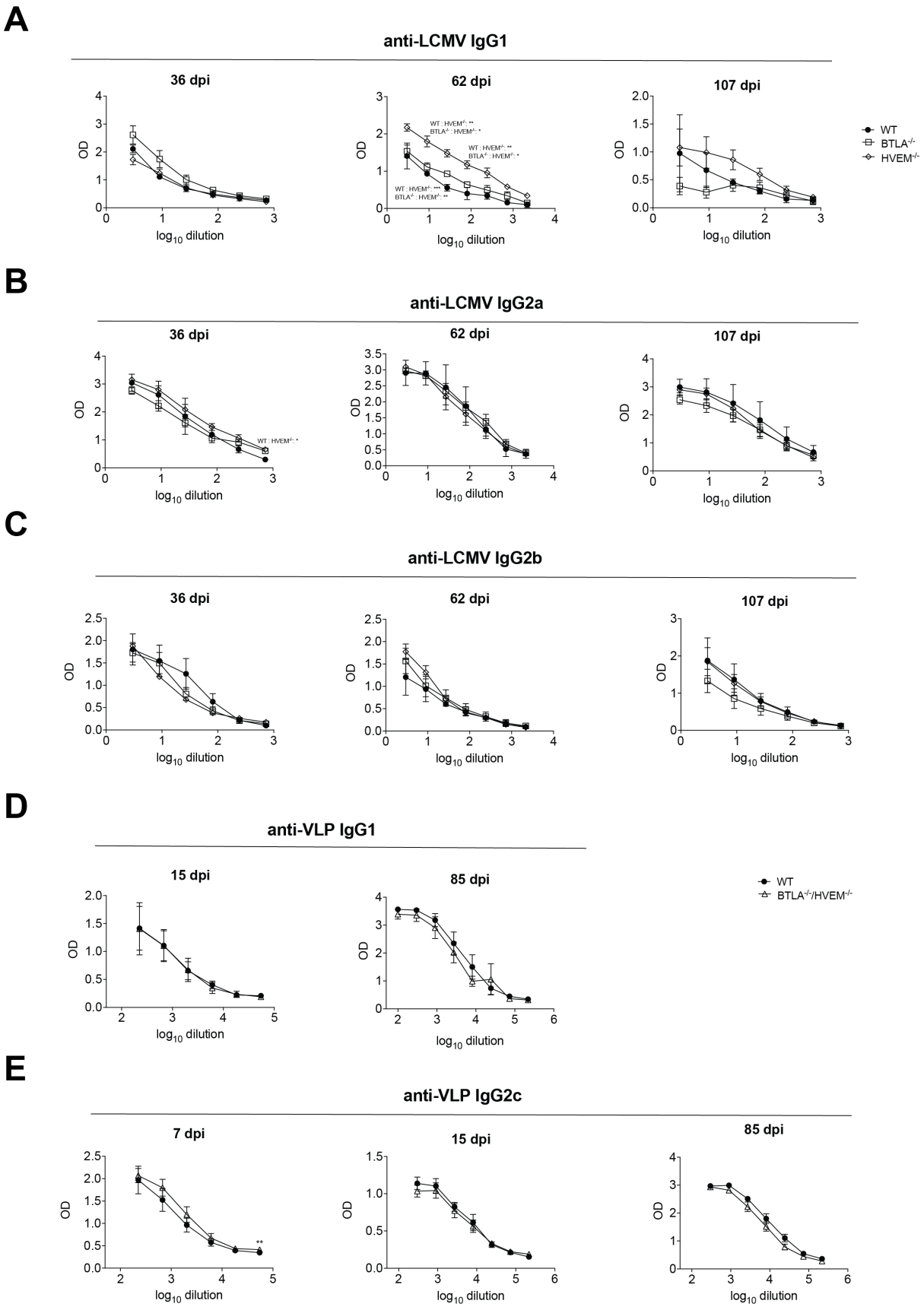

Fig. 6: The antibody response is independent of BTLA and HVEM. (A-C) WT, BTLA$^{-/-}$ and HVEM$^{-/-}$ mice were infected with 1x10^6 pfu LCMV Cl13 and IgG1 (A), IgG2a (B) and IgG2b (C)

production in the blood at indicated time points was measured by a LCMV ELISA. IgG1 (D) and IgG2c (E) production at indicated time points in the blood of WT and BTLA$^{-/-}$/HVEM$^{-/-}$ mice treated with 10 µg Qß-VLPs *i.p.* was measured by a VLP ELISA. (A-C) * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (one-way ANOVA, multiple-comparison). (D, E) ** $p \leq 0.01$ (two-tailed Student *t* test). Symbols represent means ± SD of (A-C) minimum two mice per group or (D, E) minimum four mice per group. Data are representative of one experiment.

Overexpression of BTLA augments memory formation and moderately increases the cytokine production of virus-specific CD8+ T cells

Our data show that surface expression of BTLA and HVEM on naïve CD8+ T cells is down-regulated on anti-viral effector CD8+ T cells and stays low on memory cells, probably explaining why complete absence of both molecules does not significantly alter anti-viral T cell responses. Thus, we were wondering whether down-regulation of both molecules on anti-viral CD8+ T cells during infection of wildtype mice has a consequence for effector and memory responses. To study this possibility, we retrovirally overexpressed BTLA or HVEM on gp33-specific CD8+ T cells harvested from P14 transgenic (CD45.1) mice and adoptively transferred them into C57BL/6 recipients (CD45.2) prior to infection and measured T cell responses during acute LCMV infection. To sort transduced P14 cells, BTLA and HVEM expression was linked to GFP, separated by IRES in the viral vectors (P14$^{rvBTLA\text{-}GFP}$ and P14$^{rvHVEM\text{-}GFP}$) and controls expressing GFP alone (P14^{rvGFP}).

While 7 dpi transferred P14$^{rvBTLA\text{-}GFP}$ highly expressed GFP and BTLA or P14^{rvGFP} GFP respectively (Fig. 7, A, left), expression of both molecules decreased over time (Fig. 7, A, middle and right). Possibly, the retroviral integration sites in transduced P14 cells get silenced over time, thus expression of GFP and BTLA respectively GFP alone decreases.

Interestingly, while BTLA overexpression did not alter expansion of the transduced P14$^{rvBTLA\text{-}GFP}$ cells in the acute phase of infection with LMCV (Fig. 7, B, upper and C), it improved maintenance of the memory cells measured in the spleen (Fig. 7, B, middle and lower) and the liver (Fig. 7, C) at days 14 and 28 post infection, as compared to control mice harboring P14^{rvGFP} cells. No dysregulation of SLEC or MPEC development in the spleen (Fig. 7, D) and in the liver (data not shown) was observed. Interestingly, the frequency of TNFα producing P14$^{rvBTLA\text{-}GFP}$ cells was strongly

increased in the spleen (Fig. 7, F) and the liver (data not shown) until 14 dpi and elevated 28 dpi, although IFN-γ producers were only slightly increased when compared to $P14^{rvGFP}$ cells (Fig. 7, E).

We next studied the effects of BTLA overexpression during a chronic LCMV infection when antigen persists. In this case, however, we transferred $P14^{rvBTLA\text{-}GFP}$ cells to OT-I mice to avoid that endogenous CD8+ T cells participate in the response against LCMV. Notably, the frequencies of BTLA overexpressing $P14^{rvBTLA\text{-}GFP}$ cells monitored over the course of infection was comparable to controls (Fig. 7, G). Similarly, frequencies (Fig. 7, H) and numbers (Fig. 7, I) of BTLA-GFP transgenic P14 cells in the spleen and the liver were comparable to OT-1 mice harbouring $P14^{rvGFP}$ cells at day 62 post infection.

Thus, overexpression of BTLA on virus-specific CD8+ T cells results in an increased memory formation and a moderate increase in the cytokine response during an acute LCMV infection.

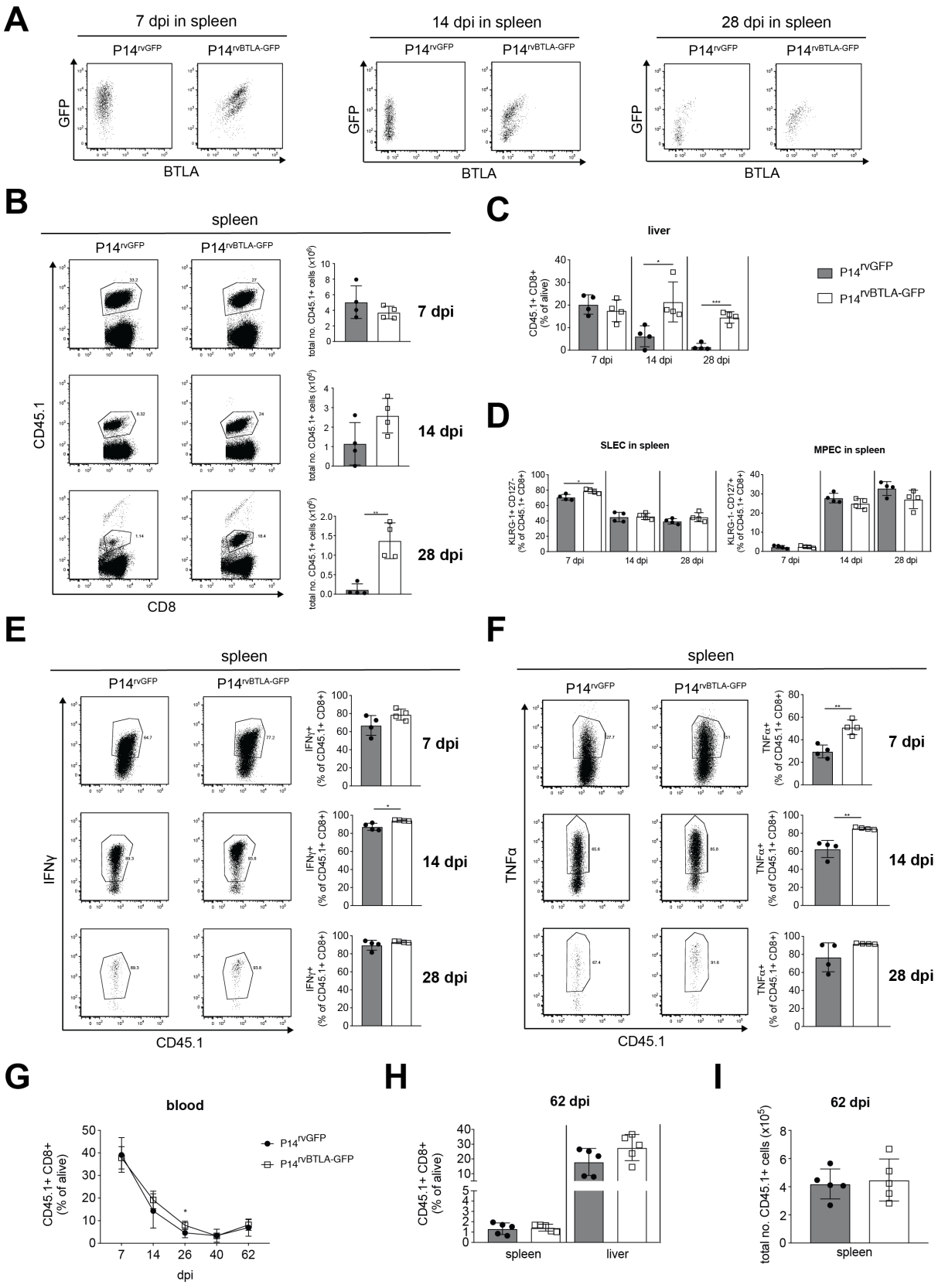

Fig. 7: Numbers of memory BTLA overexpressing virus-specific CD8+ T cells is increased during an acute LCMV infection. (A-F) 1x10^4 CD45.1+ gp33-specific tg CD8+ T cells (P14) transfected with a BTLA-GFP or control GFP retrovirus were transferred *i.v.* into

WT mice, which were infected with 2000 pfu LCMV WE *i.v.* the following day. (A) Representative FACS plots of the expression of BTLA and GFP on transferred P14 cells isolated from the spleen of WT hosts 7 (left), 14 (middle) or 28 dpi (right). Pre-gated on alive, CD45.1+ CD8+. (B) Representative FACS plots (left), pre-gated on alive and CD8+, and total numbers (right) of CD45.1+ $P14^{rvGFP}$ and $P14^{rvBTLA\text{-}GFP}$ cells in the spleen of WT hosts 7 (upper), 14 (middle) and 28 dpi (lower). (C) Frequency of transferred cells in the liver of WT hosts 7, 14 or 28 dpi. (D) Frequency of CD45.1+ $P14^{rvGFP}$ and $P14^{rvBTLA\text{-}GFP}$ SLEC (left) and MPEC (right) in the spleen of WT hosts at indicated time points. Representative FACS plots (left), pre-gated on alive CD45.1+ CD8+, and frequency (right) of IFNγ (E) and TNFα (F) producing CD45.1+ $P14^{rvGFP}$ and $P14^{rvBTLA\text{-}GFP}$ cells in the spleen of WT hosts 7 (upper), 14 (middle) and 28 dpi (lower) after in vitro restimulation with gp33 peptide, followed by intracellular cytokine staining. (G-I) $1x10^4$ CD45.1+ gp33-specific tg CD8+ T cells (P14) transfected with a BTLA-GFP or control GFP retrovirus were transferred *i.v.* into OT-I mice, which were infected with $1x10^6$ pfu LCMV Cl13 *i.v.* the day after. (G) Frequency of $P14^{rvGFP}$ and $P14^{rvBTLA\text{-}GFP}$ cells in the blood of OT-I hosts over time. Frequency of retrovirally transfected P14 cells in the spleen and liver (H) and total numbers in the spleen (I) of host mice 62 dpi. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (two-tailed Student *t* test). (G) Symbols represent means $\pm$ SD. (B-F and H-I) Symbols represent individual mice, bars represent means $\pm$ SD. Five mice per group were used. Data are representative of two independent experiments.

Virus-specific CD8+ T cells overexpressing HVEM accumulate at lower numbers at the peak of the T cell response but are increased during the memory phase

Next we studied the consequences of HVEM overexpression. Hence, we adoptively transferred $P14^{rvHVEM\text{-}GFP}$ cells or control $P14^{rvGFP}$ to C57BL/6 mice prior to infection with LCMV WE.

Analysis of the expression of GFP and HVEM or GFP only on $P14^{rvHVEM\text{-}GFP}$ cells or control $P14^{rvGFP}$ respectively revealed a marked decrease in levels from 7 to 14 and 28 dpi (Fig. 8, A). As hypothesized before, silencing of the integration sites of the retrovirus in transduced P14 cells might be the cause of the observed decrease in expression of GFP and HVEM respectively GFP alone.

Interestingly, overexpression of HVEM reduced the expansion of $P14^{rvHVEM\text{-}GFP}$ cells in the spleen (Fig. 8, B, upper) and the liver (Fig. 8, B) in the acute phase of an LCMV infection. However, maintenance of $P14^{rvHVEM\text{-}GFP}$ cells 28 dpi was increased when compared to $P14^{rvGFP}$ controls (Fig. 8, B, lower and C). No dysregulation in the cytokine response of $P14^{rvHVEM\text{-}GFP}$ cells in the spleen was observed (Fig. 8, D).

However, overexpression of HVEM on P14 cells led to an increase in MPECs and a decrease in SLEC in the spleen (Fig. 8, E) and the liver (data not shown) of C57BL/6 recipients at all timepoints analysed.

Next, we studied the effects of HVEM overexpression during a chronic LCMV infection. In order to avoid the participation of endogenous CD8+ T cells in the immune response, we transferred $P14^{rvHVEM\text{-}GFP}$ or control $P14^{rvGFP}$ cells into OT-I mice. Notably, the frequency of HVEM overexpressing $P14^{rvHVEM\text{-}GFP}$ cells over the course of infection was decreased when compared to controls (Fig. 8, F). Similarly, frequencies (Fig. 8, G) and numbers (Fig. 8, H) of HVEM-GFP transgenic P14 cells in the spleen and the liver of OT-I mice 62 dpi were reduced.

Thus, overexpression of HVEM on virus-specific CD8+ T cells results in reduced numbers of effector cells but increased formation of memory cells during an acute LCMV infection.

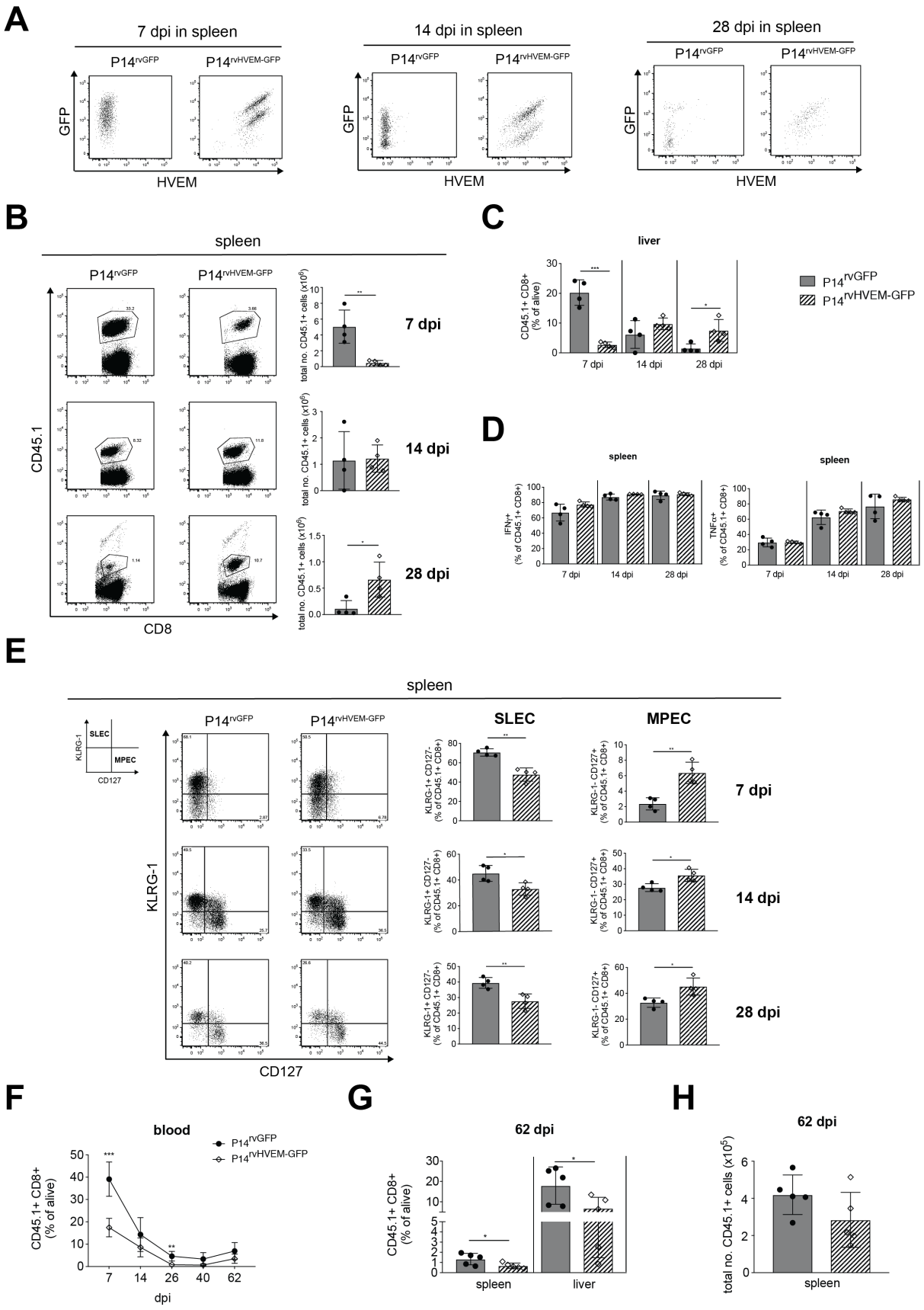

Fig. 8: Reduced expansion but increased memory formation of HVEM overexpressing virus-specific CD8+ T cells. (A-E) 1x10^4 CD45.1+ gp33-specific tg CD8+ T cells (P14)

transfected with a HVEM-GFP or control GFP retrovirus were transferred *i.v.* into WT mice, which were infected with 2000 pfu LCMV WE *i.v.* the following day. (A) Representative FACS plots of the expression of HVEM and GFP on transferred P14 cells isolated from the spleen of WT hosts 7 (left), 14 (middle) or 28 dpi (right). Pre-gated on alive, CD45.1+ CD8+. (B) Representative FACS plots (left), pre-gated on alive CD8+, and total numbers (right) of CD45.1+ $P14^{rvGFP}$ and $P14^{rvHVEM\text{-}GFP}$ cells in the spleen of WT hosts 7 (upper), 14 (middle) and 28 dpi (lower). (C) Frequency of transferred cells in the liver of WT hosts 7, 14 or 28 dpi. (D) Frequency of IFNγ (left) and TNFα (right) producing CD45.1+ $P14^{rvGFP}$ and $P14^{rvHVEM\text{-}GFP}$ cells in the spleen of WT hosts at indicated time points after in vitro restimulation with gp33 peptide, followed by intracellular cytokine staining. (E) Representative FACS plots (left) of the KLRG-1 and CD127 expression on transferred CD45.1+ P14 T cells, pre-gated on alive CD45.1+ CD8+, and frequency (right) of $P14^{rvGFP}$ and $P14^{rvHVEM\text{-}GFP}$ SLECs and MPECs in the spleen of WT hosts 7 (upper), 14 (middle) and 28 dpi (lower). (F-H) $1x10^4$ CD45.1+ gp33-specific tg CD8+ T cells (P14) transfected with a HVEM-GFP or control GFP retrovirus were transferred *i.v.* into OT-I mice, which were infected with $1x10^6$ pfu LCMV Cl13 *i.v.* the day after. (F) Frequency of CD45.1+ $P14^{rvGFP}$ and $P14^{rvHVEM\text{-}GFP}$ cells in the blood of OT-I host over time. Frequency of retrovirally transfected P14 cells in the spleen and liver (G) and total numbers in the spleen (H) of host mice 62 dpi. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (two-tailed Student *t* test). (F) Symbols represent means $\pm$ SD. (B-E and G-H) Symbols represent individual mice, bars represent means $\pm$ SD. Five mice per group were used. Data are representative of two independent experiments.

1.5. Discussion

Initially, HVEM has been identified as a ligand for BTLA, inhibiting T cell proliferation [26, 27], preventing autoimmunity [68, 77, 79] and transplant rejection [118] but dampening anti-tumour responses [126] and bacterial and parasite clearance [115, 116]. However, contradictory results were observed when transferred $BTLA^{-/-}$ cells did not exacerbate colitis in $Rag^{-/-}$ mice [128] or the severity of GVHD [129]. The observation that BTLA binding to HVEM induces NF-κB downstream activation, promoting the survival of T cells *in vitro* [48], revealed a bi-directional signalling pathway of BTLA and HVEM. Indeed, recent reports show that BTLA binding to HVEM expressed on CD8+ T cells is crucial for the survival of effector and memory antigen-specific CD8+ T cells during a vaccinia virus (VV) and a *Listeria monocytogenes* infection [42, 43].

We however made the unexpected finding, that both molecules are dispensable for the T cell response *in vitro* and *in vivo*. Co-culturing of BTLA or HVEM deficient BMDCs with T cells revealed no difference in T cell priming or the cytokine response. In contrast, it was initially proposed that T cell proliferation *in vitro* was inhibited when HVEM on APCs bound to BTLA on T cells [27]. However, while latter used non-professional APCs, namely Chinese hamster ovary cells, we used bone marrow differentiated professional DCs, what might lead to the observed difference.

Besides, the anti-viral CD8+ T cell response and virus clearance *in vivo* were comparable between WT, $BTLA^{-/-}$ and $HVEM^{-/-}$ mice infected with an acute or a chronic dose of LCMV. Furthermore, we did not observe differences in the kinetics or the effector response of virus-specific CD8+ T cells nor in virus clearance between BTLA/HVEM double deficient and control mice, excluding redundancy of the two molecules. Moreover, unpublished data also indicate, that neither of the molecules are required for the control of a murine cytomegalovirus (MCMV) infection by CD8+ T cells (Fig. 12 and data not shown).

Thus, BTLA and HVEM are dispensable for mounting a protective CD8+ T cell response during LCMV and MCMV infection, but are crucial upon infection with VV [42]. Interestingly, also the requirement for CD28 co-stimulation in mounting a protective CD8+ T cell response varies between different virus infections. Whereas CD28 deficient animals infected with LCMV did not show an impaired cytotoxic T cell response or virus control [263, 264], lysis of target cells by CD8+ T cells isolated from vesicular stomatitis virus (VSV) infected animals was highly dependent on CD28 co-stimulation [264]. By comparing the cytotoxic activity of CD8+ T cells from mice infected with a low or control high virulent VV strain, the need for CD28 co-stimulation during an infection that induces a weak signal 1, due to lower virus burden or shorter exposure to virus, was demonstrated [264]. Thus, the requirement of BTLA and HVEM for mounting the anti-viral CD8+ T cell response during a VV but not a LCMV infection may be caused by differences in strength of signal 1. Besides, the cytokines required for mounting the CD8+ T cell response may influence the need for BTLA-HVEM co-stimulation. Whereas the expansion of CD8+ T cells during a LCMV infection depends on type I IFNs, the CD8+ T cell response during a VV infection is independent of this cytokines [265]. Thus, possibly CD8+ T cells primed with a VV epitope rely more on co-stimulation than on cytokines for the subsequent expansion then LCMV epitope primed CD8+ T cells do.

Interestingly, adoptive transfer of WT together with BTLA$^{-/-}$ OT-I cells into WT animals that were subsequently immunized with LPS maturated SIINFEKL-peptide-pulsed BMDCs revealed an advantage of KO cells in the maintenance [83]. However, we did not observed differences in frequencies or numbers of BTLA deficient virus-specific CD8+ T cells over time in the blood or 65 days after infection with an acute dose of LCMV. Thus, the maintenance of specific CD8+ T cells most likely depends on the model system used. Furthermore, an *in vivo* killing assay indicated that BTLA$^{-/-}$ OT-I cells had an increased killing activity after 4h of exposure to target cells [83], which however might have been due to higher numbers of effector T cells 3.5 dpi when target cells were injected. Overall, our data indicate that BTLA and HVEM are dispensable for the anti-viral CD8+ T cell response during an acute and a chronic LCMV infection. Besides, we observed that BTLA is expressed on LCMV-specific CD8+ T cells and gets down-regulated on effector cells. Retroviral overexpression of BTLA on virus-specific CD8+ T cells did not affect the expansion during an acute LCMV infection, however reduced contraction and resulted in increased numbers of BTLA overexpressing memory virus-specific CD8+ T cells. Thus, low expression of BTLA on virus-specific CD8+ T cells limits the formation of memory cells after clearance of LCMV. The observed reduced contraction of BTLA overexpressing virus-specific CD8+ T cells may by caused by an enhance survival or a reduced death of the cells. BTLA downstream signalling is mediated via its immunoreceptor tyrosine-based inhibitory motifs (ITIMs), to which Src homology domain 2-containing protein tyrosine phosphatases SHP-1 and SHP-2 bind [68] and induce a co-inhibitory signal. Thus, BTLA overexpression could intrinsically inhibit apoptosis during contraction. Besides, the cytoplasmic domain of BTLA contains a third motif, proposed to serve as a binding site for Grb2 [68] and the p85 subunit of PI3K, which could potentially induce cell survival [70]. Co-transfer of WT and BTLA deficient CD4+ T cells into Rag$^{-/-}$ animals resulted in reduced numbers of BTLA$^{-/-}$ cells [128], hypothesizing about an intrinsic pro-survival action of BTLA. However, by restoring the ligand but not receptor activity of BTLA, the survival of *in vitro* cultured T cells was increased as well [129]. Thus, if a possible pro-survival effect of BTLA in overexpressing virus-specific CD8+ T cells is mediated cell-intrinsically or extrinsically would require further research. In contrast, during a chronic infection, frequencies of BTLA overexpressing gp33-specific CD8+ T cells were comparable to control cells at all time points analysed. Since the virus was not cleared in OT-I recipients 62 dpi (data not shown), memory was not yet established

with the result that numbers of BTLA overexpressing virus-specific CD8+ T cells were comparable to $P14^{rvGFP}$ control cells 62 dpi. Interestingly, BTLA overexpression on virus-specific CD8+ T cells moderately increased their cytokine production. Since intrinsic BTLA signalling was proposed to be co-inhibitory, the observed phenotype is presumably mediated cell-extrinsically.

Besides BTLA, HVEM was as well down-regulated on virus-specific CD8+ T cells at the peak of the T cell response against LCMV. Retroviral overexpression of HVEM on virus-specific CD8+ T cells strongly reduced the numbers of effector cells 7 dpi during a LCMV infection whereas numbers of memory cells were increased. Thus down-regulation of HVEM ensures expansion of virus-specific CD8+ T cells, but limits the formation of memory cells after clearance of the virus. The reduced numbers of virus-specific CD8+ T cells overexpressing HVEM at the peak of the T cell response may be due to an impaired proliferation or increased death. Since HVEM downstream signalling is proposed to act co-stimulatory [48], triggering of HVEM might induce expression of death associated genes cell intrinsically, whereas inhibition of proliferation is possible rather regulated cell-extrinsically. In contrast, numbers of HVEM overexpressing memory cells are increased during an acute infection. Since binding of BTLA to HVEM has been shown to promote the survival of VV- and a *Listeria monocytogenes*-specific CD8+ T cells [42, 43], the increased numbers of overexpressing memory cells during an acute LCMV infection might be due to a cell-intrinsic pro-survival signal. During a chronic infection, frequencies of HVEM overexpressing virus-specific CD8+ T cells were lower than the one from $P14^{rvGFP}$ cells at all time points analyzed. Since the virus was not cleared in OT-I recipients 62 dpi (data not shown), the memory phase was not yet established, thus numbers of HVEM overexpressing virus-specific CD8+ T cells were still below the ones from control $P14^{rvGFP}$ cells and not enhanced as during the memory phase after an acute LCMV infection.

Besides being indispensable for the long-term survival of VV-specific CD8+ T cells [42], HVEM is crucial for the generation of MPECs, shown during a respiratory VV infection [266]. In contrast, we did not observe a significant difference in frequencies of MPECs between WT and HVEM deficient animals infected with LCMV, though they were slightly reduced in $HVEM^{-/-}$ mice. However, overexpression of HVEM on virus-specific CD8+ T cells significantly increased the frequency of MPECs during a LCMV

infection. Thus, our overexpression data confirm that HVEM promotes MPEC formation but the molecule seems to be play a minor role in LCMV infected WT mice. Interestingly, especially HVEM is also strongly down-regulated on virus-specific CD4+ T cell 8 dpi, whereas BTLA expression is rather increased. Infection of single or double KO mice with an acute dose of LCMV indicated that frequencies of total CD4+ T cells are slightly enhanced compared to controls (data not shown). However, cytokine production of CD4+ T cells upon specific restimulation revealed no difference between knockouts and WT mice (data not shown). Thus, to conclude about the role of BTLA and HVEM on the CD4+ T cell response, kinetics of virus-specific CD4+ T cells in KO animals should be assessed first.

Interestingly, naïve B cells highly expressed BTLA and to a lower degree also HVEM. Upon LCMV infection, expression of BTLA does not change whereas HVEM expression increases. However, absence of either or both of the molecules did not impair the antibody response during a LCMV infection or after VLP immunization. Thus, the function of BTLA and HVEM on B cells is still unclear.

In summary, we could show that BTLA down-regulation on virus-specific CD8+ T cells is important to dampen the cytokine production, whereas HVEM down-regulation is crucial for accumulation of effector virus-specific CD8+ T cells during a LCMV infection. Furthermore, down-regulation of BTLA and HVEM limits the formation of memory virus-specific CD8+ T cells after LCMV clearance.

2. Addendum to Chapter 1

2.1. Material and Methods

Mice

C57/BL6J CD45.1+ mice were bought from The Jackson Laboratory (Maine, USA) and maintained in house. The other mice strain used were described previously (see section 1.3).

Virus and infection

Mice were infected with $2x10^6$ pfu recombinant MCMV lacking m157 (MCMVΔm157) *i.v.*. Virus was kindly provided by Annette Oxenius (ETH Zurich, Switzerland).

Retroviral transfection of bone marrow (BM) cells

Eight days before transfer of overexpressing BM cells, donor mice were injected with 16,6 µl/g mouse weight of 5-Fluorouracil (9 mg/ml in PBS [Sigma-Aldrich]) *i.p.*.

Six days later, bone marrow was flashed out with RPMI (Gibco) containing 10% heat-inactivated FBS (Gibco) from femur and tibia of hind legs of donor mice and lysed with Gey's solution (20 parts stock A [35 g NH_4Cl, 1.85 g KCl, 1.5 g $Na_2HPO_4.2H_2O$, 0.12 g KH_2PO_4, 5 g Glucose, 50 mg Phenol red, bring to 1000 ml], 5 parts stock B [0.42 g $MgCl_2.6H_2O$, 0.14 g $MgSO_4.7H_2O$, 0.34 g $CaCl_2$, bring to 100 ml], 5 parts stock C [2.25 g $NaHCO_3$, bring to 100 ml] and 70 parts distilled sterile water) for 1-2 min at RT followed by centrifugation and counting of cells. Only samples with bone marrow cell numbers between $2\text{-}6x10^6$ cells per mouse were centrifuged and resuspend in BMC medium (RPMI [Gibco], 10% heat-inactivated FBS [Gibco], 2mM L-Glutamine [Sigma-Aldrich], 100 U/ml Penicillin/100 µg/ml Streptomycin [Gibco], 50µM 2-Mercaptoethanol [Gibco], 10 ng/ml rM-SCF [BioLegend], 10 ng/ml rM-IL-3 [PeproTech, London, UK], 10 ng/ml rM-IL-6 [BioLegend]) followed by filtration through a blue cap tube. Subsequently, $3\text{-}5x10^6$ cells were plated in a non-treated Petri dish and incubated overnight at 37°C, 5% CO_2.

The next day, cells were washed off the dish and counted. Concentration was adjusted to $4x10^5$ cells/ml and split into 3 equal parts corresponding to the three retroviral vectors (GFP, BTLA-GFP, HVEM-GFP). Cells were centrifuged, resuspended in the respective retroviral supernatant, which was beforehand concentrated by ultracentrifugation (see section 1.3) and resuspended in BMC medium containing

Polybrene and HEPES buffer, and seeded into wells of a 12-well plate. Subsequently, cells were retrovirally transduced as described previously (see section 1.3), followed by incubation for 2-3h at 37°C, 5% CO_2 in the incubator. Retroviral supernatant was removed and fresh BMC medium was added, followed by overnight incubation at 37°C, 5% CO_2. On the same day, recipient mice were irradiated as described previously (see section 1.3).

The next day, retroviral transduction was repeat. After incubation for 2-3h at 37°C, 5% CO_2, cells were detached from the wells, resuspended in PBS and counted. Eventually, irradiated recipient mice were injected *i.v* with a minimum of $5x10^5$ cells in PBS per mouse.

Determination of MCMV titer

Virus titer in the SG was determined by plaque-forming assay on M2-10B4 fibroblasts as described in [267].

Cell preparation

For the thymus, organ was smashed through a 70 μm cell strainer, centrifuged and resuspended in PBS containing 2% heat-inactivated FBS (Gibco). The obtained single cell suspension was subsequently filtered through a blue cap tube.

For the MCMV experiments, first the spleen was removed, mice were perfused with PBS and the salivary glands (SG) were removed. Half of the spleen and one SG were used for determination of virus titers, the other half of the spleen and the other SG were used for FACS staining. The portion of the spleen used for FACS staining was processed as described previously (see section 1.3). The SG used for staining was minced and digested in IMDM + GlutaMAX-I (Gibco), 100 U/ml Penicillin/100 µg/ml Streptomycin (Gibco) containing 600 U/ml Collagenase IV (Worthington, New Jersey, USA) and 200 U/ml DNase I (Sigma-Aldrich) for 30 min at 37°C and 130 rpm. Subsequently, the tissue was homogenized and smashed through a 70 μm cell strainer. Last, the single cell suspension of the spleen was filtered through a blue cap tube once, whereas the one from the SG was filtered three times.

Restimulation

For analysis of the cytokine production, cells from the SG were stimulated with 2 μg/ml Monensin (Sigma-Aldrich), 0.1 μg/ml PMA (Sigma-Aldrich), 1 μg/ml Ionomycin (Brunschwig, Basel, Switzerland) in IMDM (1X) + GlutaMAX-I (Gibco) containing 10% heat-inactivated FBS (Gibco), 100 U/ml Penicillin/100 µg/ml Streptomycin (Gibco) and 50 µM 2-Mercaptoethanol (Gibco) for 3-4h at 37°C.

Reagents

M38 and M45 PE- and APC-conjugated MHC class I tetramers were kindly provided by Annette Oxenius (ETH Zurich, Switzerland). For exclusion of dead cells, efluor780 (eBioscience, Massachusetts, USA) or ZombieAqua (BioLegend) was used. Antibodies used were as follows: CD45.1-PE (A20; BioLegend), CD45.2-BV785 (104; BioLegend), BTLA-APC (8F5; BioLegend), HVEM-APC (LH1; eBioscience), CD8a-FITC (53-6.7;eBioscience), CD8a-APC-Cy7 (53-6.7; BioLegend), CD8a-PE (53-6.7; BD Bioscience), CD8a-Percp-Cy5.5 (53-6.7; BioLegend), CD8a-PE-cy7 (53-6.7; BioLegend), CD4-BV650 (RM4-5; BioLegend), CD4-APC-Cy7 (GK1.5; BioLegend), TNFα-FITC (MP6-XT22; BioLegend), IFNγ-APC (XMG1.2; BioLegend).

Flow cytometry

Cell staining was performed as described previously (see section 1.3).

2.2. Results

BTLA and HVEM are dispensable for the T cell development in the thymus

Preliminary data indicated that BTLA and HVEM are expressed after positive/negative selection on double positive (DP) CD69+ cells from the thymus of WT and P14 tg mice (data not shown). Surprisingly, neither $BTLA^{-/-}$ nor $HVEM^{-/-}$ or $BTLA^{-/-}/HVEM^{-/-}$ animals showed big differences in frequencies or numbers of DP or single positive (SP) thymocyte subsets (Fig. 9).

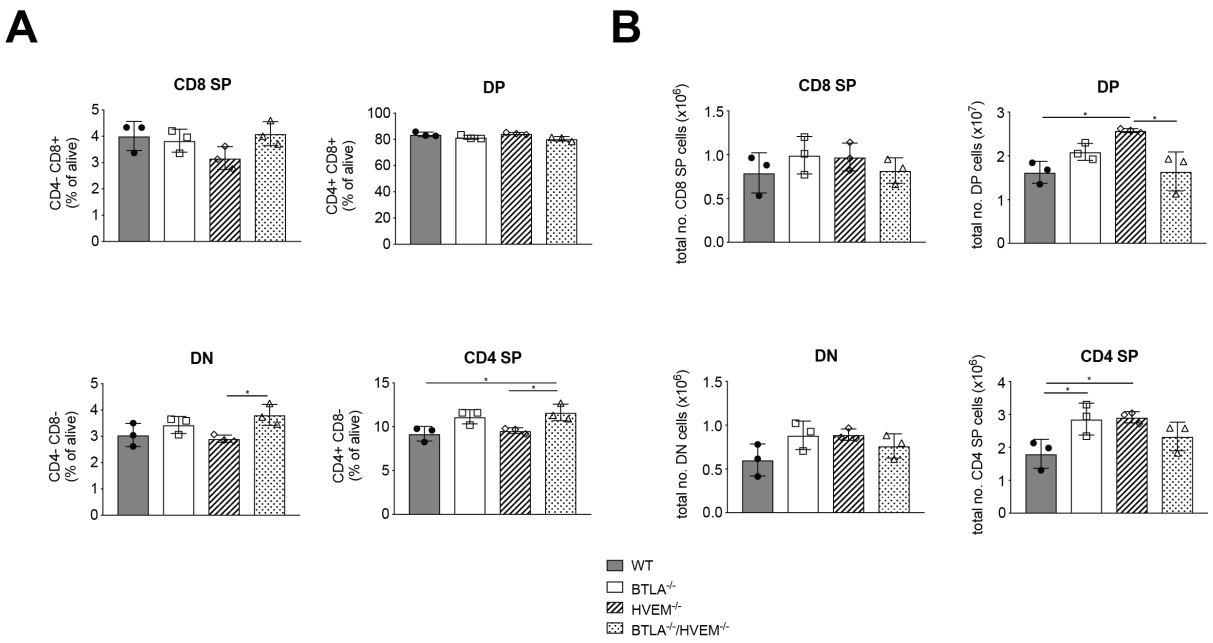

Fig. 9: Absence of BTLA, HVEM or both molecules does not affect thymocyte subset distribution. (A) Frequency of WT, BTLA$^{-/-}$, HVEM$^{-/-}$ and BTLA$^{-/-}$/HVEM$^{-/-}$ CD4 and CD8 thymocyte subsets. (B) Numbers of WT, BTLA$^{-/-}$, HVEM$^{-/-}$ and BTLA$^{-/-}$/HVEM$^{-/-}$ CD4 and CD8 thymocyte subsets. * $p \leq 0.05$, (one-way ANOVA, multiple-comparison). Symbols represent individual mice, bars represent means ± SD. Three mice per group were used. Data are representative of two experiments.

To answer the question if low expression of BTLA and HVEM before positive/negative selection is crucial for the accumulation of DP and SP cells in the thymus, we transferred bone marrow cells from WT or P14 tg mice retrovirally transduced with a BTLA-GFP, HVEM-GFP or control GFP vector into irradiated animals and assessed the frequency and number of the different thymocyte subsets around 50 days post reconstitution.

In irradiated WT CD45.1+ mice receiving overexpressing WT CD45.2+ bone marrow cells, only few thymocytes overexpressed BTLA (Fig. 10, A, left) respectively HVEM (Fig. 10, A, right) 50 days post reconstitution. However, staining of bone marrow cells prior to transfer already revealed that the retroviral transduction was not as efficient (data not shown). Interestingly, overexpression of BTLA or HVEM on WT CD45.2+ thymocytes did not affect subset distribution (Fig. 10, B, C).

In irradiated WT mice receiving retrovirally transduced P14 tg bone marrow cells, overexpressing BTLA (Fig. 10, D, left) and HVEM (Fig. 10, D, right) thymocytes could

be detected 45 days post reconstitution. Whereas overexpression of BTLA or HVEM on P14 tg thymocytes reduced the frequency of SP CD8+ T cells (Fig. 10, E), numbers of any thymocyte subset was not changed (Fig. 10, F).

Thus, BTLA and HVEM seem to be dispensable for the T cell development in the thymus.

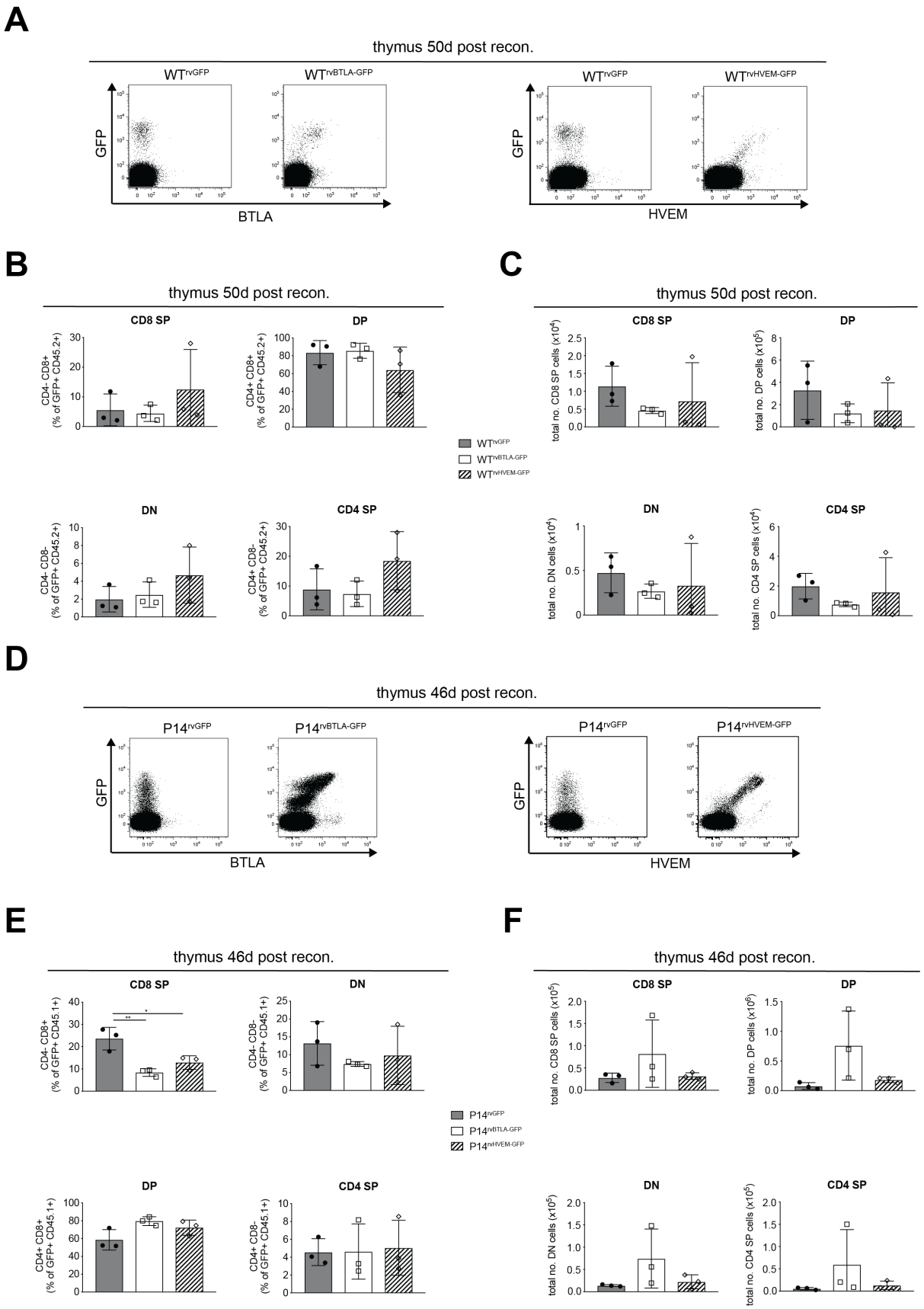

Fig. 10: Continuous expression of BTLA or HVEM on WT or P14 tg thymocytes does not interfere with subset distribution. (A - C) WT CD45.2+ bone marrow cells were

transduced with a GFP, BTLA-GFP or HVEM-GFP retrovirus and subsequently transferred into lethally irradiated WT CD45.1+ mice. 50 days post reconstitution, the thymus was analysed. (A) Representative FACS plots of the expression of BTLA and GFP (left) and HVEM and GFP (right) on WT CD45.2+ thymocytes. Cells were pregated on alive, CD45.2+. Frequency (B) and numbers (C) of $WT^{rvBTLA\text{-}GFP}$, $WT^{rvHVEM\text{-}GFP}$ or control WT^{rvGFP} CD4 and CD8 thymocyte subsets. (D - F) P14 CD45.1+ bone marrow cells were transduced with a GFP, BTLA-GFP or HVEM-GFP retrovirus and subsequently transferred into lethally irradiated WT CD45.2+ mice. 46 days post reconstitution, the thymus was analysed. (D) Representative FACS plots of the expression of BTLA and GFP (left) and HVEM and GFP (right) on P14 CD45.1+ thymocytes. Pregated on alive, CD45.1+. Frequency (E) and numbers (F) of $P14^{rvBTLA\text{-}GFP}$, $P14^{rvHVEM\text{-}GFP}$ or control $P14^{rvGFP}$ CD4 and CD8 thymocyte subsets. * $p \leq 0.05$, ** $p \leq 0.01$ (one-way ANOVA, multiple-comparison). (B, C, E, F) Symbols represent individual mice, bars represent means ± SD. Three mice per group were used. (A - C) Data are representative of one experiment. (D - F) Data are representative of three independent experiments.

HVEM is highly expressed on inflationary M38-specific CD8+ T cells from the salivary gland (SG) of chronic mouse cytomegalovirus (MCMV) infected mice

MCMV establishes a latent infection in mice, characterized by sporadic recurrence of virus particles [268]. The initially viral burst during the acute phase elicits a prominent CD8+ T cell response mainly mediated by conventional CD8+ T cells, which will contract after virus clearance and form a stable pool of memory cells. However, CD8+ T cells with certain epitope specificities do not follow this classical pathway, but rather continuously increase over time after the acute phase. These termed inflationary T cells are involved in the control of the reactivated virus during latency.

In the spleen, the initially virus burst is controlled within the first two weeks and the virus subsequently establishes latency [269]. In the salivary gland (SG), the virus persists up to 30 dpi but is eventually cleared [269], followed by virus latency.

We observed that virus-specific CD8+ T cells down-regulated BTLA and HVEM at the peak of the T cell response to an LCMV infection (Fig. 5). Thus, we were wondering if expression of BTLA and HVEM on inflationary MCMV-specific CD8+ T cells, exhibiting a effector-like phenotype in the spleen [270] and the salivary gland (data not shown), is similar as on effector LCMV-specific CD8+ T cells and if the expression of the two

molecules are regulated comparably on non-inflationary MCMV-specific CD8+ T cells as on LCMV-specific CD8+ T cells.

In the spleen, BTLA can be detected on non-inflationary M45-specific CD8+ T cells 30 dpi, exhibiting a memory phenotype [270], but the expression is very low on cells 58 dpi (Fig. 11, A, left). On inflationary M38-specific CD8+ T cells from the spleen, BTLA is not expressed at either of the two time points (Fig. 11, A, right). Thus as expected, BTLA is generally higher expressed on memory-like M45-specific T cells, than on effector-like M38-specific CD8+ T cells. In the SG, both M38-specific and M45-specific CD8+ T cells do not express BTLA 30 or 58 dpi (Fig. 11, A).

In the spleen, HVEM expression is lower on M38-specific CD8+ T cells (Fig. 11, B, right) than on M45-specific CD8+ T cells (Fig. 11, B, left) at 30 and 58 dpi. In the SG, M45-specific CD8+ T cells highly expressed HVEM (Fig. 11, B, left), which was especially 30 dpi even greater than on splenic non-inflationary CD8+ T cells. Most strikingly, the effector-like M38-specific CD8+ T cells from the SG do express HVEM at high levels at both time points (Fig. 11, B, right). In contrast, HVEM on LCMV-specific effector CD8+ T cells 8 dpi is almost absent (Fig. 5).

Thus, whereas BTLA is similarly expressed on effector-like inflationary and memory-like non-inflationary CD8+ T cells during a MCMV infection as on effector respectively memory cells during a LCMV infection, HVEM is not, as expected, expressed at low levels on inflationary M38-specific CD8+ T cells 30 and 58 dpi.

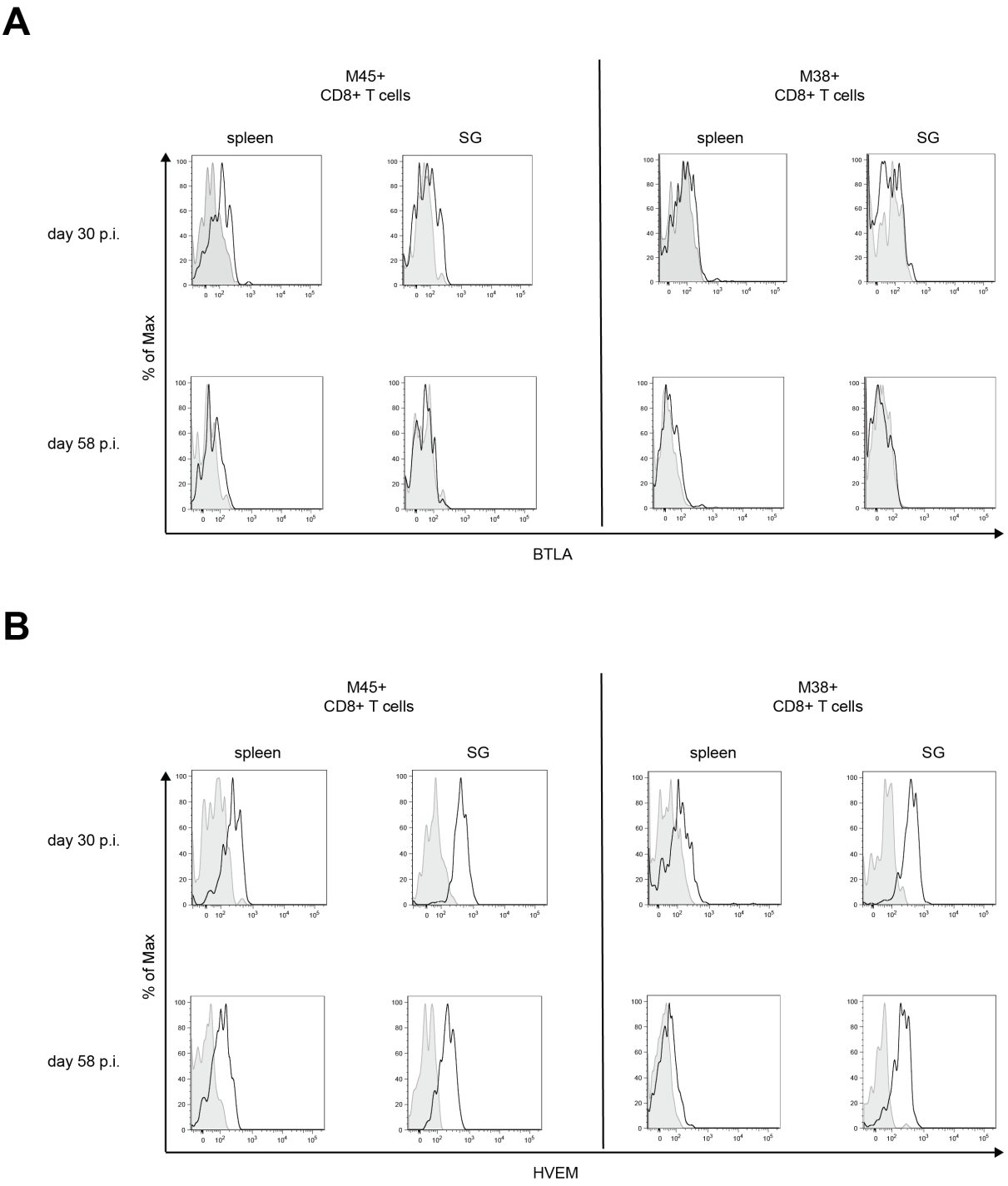

Fig. 11: Expression of BTLA and HVEM on non-inflationary M45-specific and on inflationary M38-specific CD8+ T cells from the spleen and the SG of chronically MCVM infected WT mice. WT and BTLA$^{-/-}$/HVEM$^{-/-}$ mice were infected with 2x10^6 pfu MCMVΔm157 *i.v.* Expression of BTLA (A, black line) and HVEM (B, black line) was assessed on endogenous M45-specific and M38-specific CD8+ T cells in the spleen and the SG of WT mice 30 and 58 dpi. As a control (grey), expression of BTLA and HVEM on endogenous M45-specific and M38-specific CD8+ T cells from infected BTLA$^{-/-}$/HVEM$^{-/-}$ mice at indicated time points was

used. Cells were pregated on alive, CD8+ and M38+ resp. M45+. Data are representative of three independent experiments.

Comparable CD8+ T cell response in the SG of HVEM$^{-/-}$ and control mice infected with MCMV

Since HVEM is highly expressed on virus-specific CD8+ T cells in the SG of chronically MCMV infected animals, we were wondering if the molecule is crucial for virus control. Surprisingly, frequency of M38-specific and M45-specific CD8+ T cells 30 and 58 dpi in the SG of MCMV infected HVEM$^{-/-}$ mice were comparable to infected WT controls (Fig. 12, B). Furthermore, frequency of total CD8+ T cells were not changed in the absence of HVEM (Fig. 12, A). Additionally, we did not observe differences in numbers of total CD8+, M38- and M45-specific CD8+ T cells between WT and HVEM$^{-/-}$ mice (data not shown). Furthermore, absence of HVEM did not impair the frequency of IFNγ or TNFα producing CD8+ T cells from the SG at both time points analysed (Fig. 12, C). Moreover, viral titers in the SG were comparable between MCMV infected HVEM$^{-/-}$ and WT mice (Fig. 12, D), suggesting that also the CD4+ T cell response, shown to be indispensable for virus control in the organ [271, 272], were not affected in the absence of HVEM.

Thus, even though HVEM is highly expressed on MCMV-specific CD8+ T cell from the SG, it seems to be dispensable for their response.

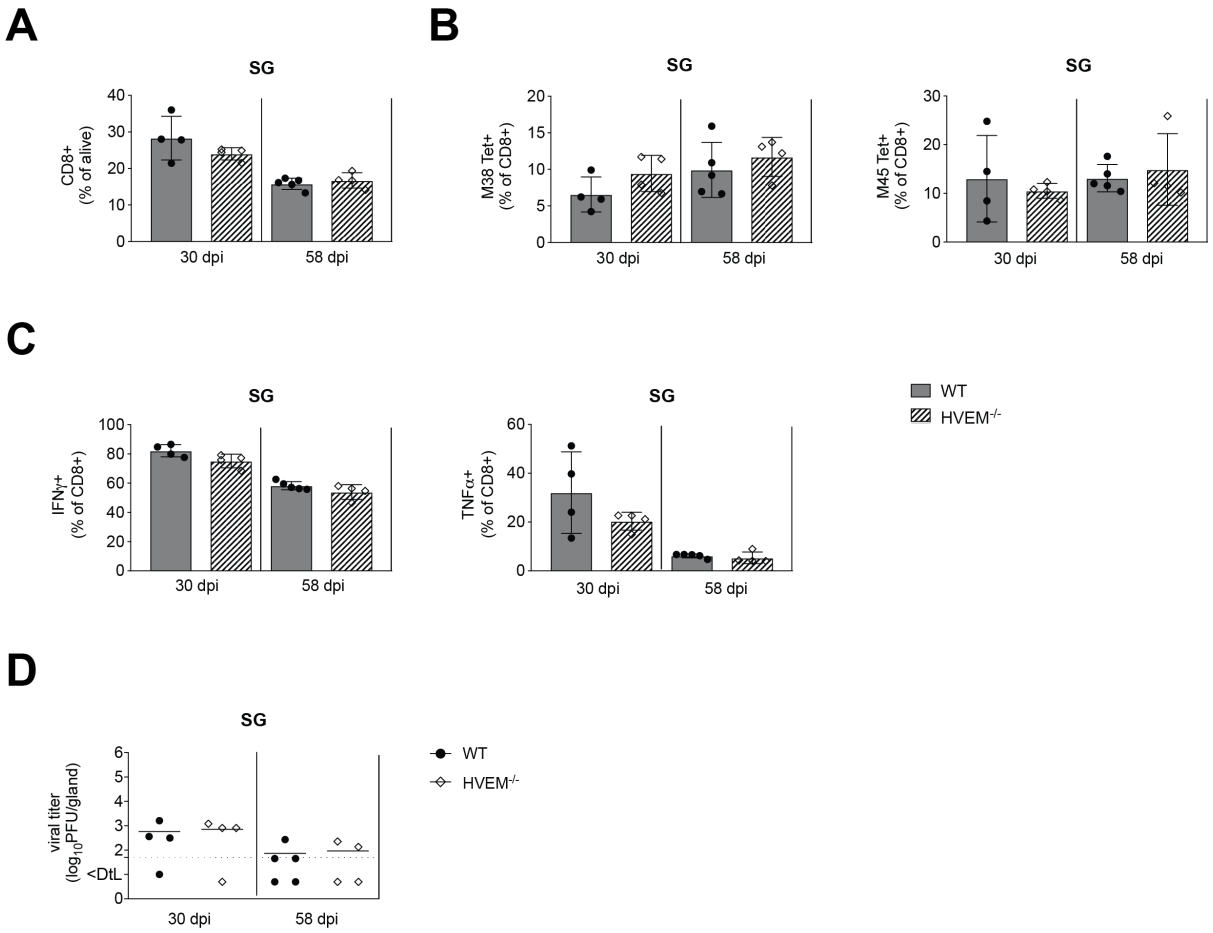

Fig. 12: HVEM is dispensable for the CD8+ T cell response in the SG to a chronic MCMV infection. Salivary glands of WT and HVEM$^{-/-}$ mice infected with 2x10^6 pfu MCMVΔm157 *i.v.*. were analysed at 30 and 58 dpi. (A) Frequency of CD8+ T cells in WT and KO mice at indicated time points. (B) Frequency of M38-specific (left) and M45-specific (right) CD8+ T cells in HVEM$^{-/-}$ and control WT mice at indicated time points. (D) Frequency of IFNγ+ (left) and TNFα (right) producing CD8+ T cells from the SG of infected animals 30 and 58 dpi after in vitro restimulation with PIM, followed by intracellular cytokine staining. (D) Viral titers in the SG at indicated time points in WT and HVEM KO mice. Symbols represent individual mice, bars represent means $\pm$ SD. Dotted line represents detection limit (DtL). Four mice per group were used. Data are from one experiment.

3. Combined absence of the IL-2Rα and the IL-21R impairs maintenance and the sustained effector response of CD8+ T cells during an acute viral infection

Patrizia Diethelm[1], Franziska Ampenberger[1], Luigi Tortola[1], and Manfred Kopf[1]

[1] Institute of Molecular Health Sciences, Department of Biology, ETH Zurich, 8093, Zurich, Switzerland

Key words: Interleukin-2, interleukin-21, LCMV, CTL

3.1. Abstract

Maintenance of functional pathogen-specific memory CD8+ T cells is crucial to mount a rapid secondary immune response upon a re-infection. Whereas the long-term persistence of memory CD8+ T cells is strongly dependent on IL-7 and IL-15, absence of IL-2 or IL-21 signals, two other common receptor γ-chain (γc) family cytokines, does not greatly impair maintenance during an acute virus infection. Through the generation of mixed bone chimeras, to avoid the adverse effects seen in IL-2-deficient mice, we could now show that IL-2 and IL-21 act partial compensatory to ensure the long-term persistence of virus-specific CD8+ T cell during an acute/resolved infection. Interestingly, IL-2Rα$^{-/-}$/IL-21R$^{-/-}$ virus-specific CD8+ T cells preferentially exhibited a central memory phenotype when compared to WT cells, while formation of effector memory cells was not affected. Furthermore, the sustained effector response of CD8+ T cells was strongly impaired in the absence of the IL-2Rα together with the IL-21R. In this study we revealed a partial compensatory action of the two cytokines IL-2 and IL-21 in the long-term persistence and the sustained cytokine response of CD8+ T cells during a resolved infection.

3.2. Introduction

Following an acute virus infection, CD8+ T cells undergo dynamic changes as they expand, contract and generate long-lasting memory T cells (reviewed in [273]). Effector CD8+ T cells produce large amounts of pro-inflammatory cytokines such as IFNγ and TNFα and contribute to efficient killing of infected cells. While most T cells die following clearance of the pathogen, a small subset of self-renewing memory T cells form, persisting for long-term and mounting a rapid secondary immune response upon re-infection.

Cytokines play a key role during the T cell response to an infection. While the generation of effector CD8+ T cells is dependent on IL-12 and type 1 IFNs [29-31, 274], memory CD8+ T cell rely on IL-7 and IL-15 for their maintenance [275-279]. Besides the two latter, both members of the common receptor gamma c (γc) chain family cytokines (reviewed in [158]), also the members IL-2 and IL-21 have shown to be crucial during CD8+ T cell responses. IL-2 was the first cytokine described in the family and has initially been identified *in vitro* as a T cell growth factor [162, 163] and

later as an inducer of apoptosis [280]. IL-2 binds to the low-affinity dimeric IL-2 receptor, expressed at low levels on naïve and at high levels on memory CD8+ T cells, consisting of the IL-2Rβ (CD122) and the γc subunit (CD132), or to the high-affinity trimeric receptor, where the IL-2Rα (CD25) subunit, induced upon TCR activation, additionally associates with the receptor dimer [179]. *In vivo*, deficiency in IL-2 decreases cytotoxic activity of CD8+ T cells [281-283] and impairs secondary T cell responses [281, 282]. However, absence of IL-2 in mice causes sever autoimmunity driven by excessive lymphocyte proliferation [175-177, 284] due to the lack of regulatory T cells [173], making it difficult to study the immune response to infections in IL-2 deficient animals.

Il-21 is the most recently described member of the γc cytokine family. Its receptor, expressed on naïve CD8+ T cells and upregulated upon activation [192], consists of the IL-21R and the γc subunit [285]. *In vitro*, addition of IL-21 to anti-CD3 activated lymphocytes induces their proliferation [286].

During a virus infection, IL-2 and IL-21 have shown to be mostly dispensable for the expansion and long-term maintenance of CD8+ T cells during an acute infection [5, 208, 209] and IL-21 additionally being dispensable for mounting a secondary CD8+ T cell response [209]. However, the re-expansion of antigen-experienced cells upon a re-challenge is strongly impaired when the IL-2 signal is lost [5, 6]. Contrary to an acute infection, both IL-2 and IL-21 are indispensable for sustained primary anti-viral CD8+ T cell responses when antigen persists [6, 208-210].

Since the possibility of a compensatory action of IL-2 and IL-21 in mounting the anti-viral CD8+ T cell response during an acute infection has not been considered, we aimed to investigate the interplay of the two molecules during an acute/resolved infection with lymphocytic choriomeningitis virus (LCMV) in a mixed bone marrow chimera system by using double deficient bone marrow donors. We propose a partial compensatory action of IL-2 and IL-21 for the maintenance and the sustained effector response of virus-specific CD8+ T cells during an acute LCMV infection.

3.3. Material and Methods

Mice

C57/BL6J CD45.1+ mice were bought from Charles River (Massachusetts, USA) and maintained in house. C57/BL6 CD45.1+ CD45.2+ mice were generated by crossing

C57/BL6J CD45.1+ with C57/BL6J CD45.2+ animals. IL-2Rα$^{-/-}$/ IL-21R$^{-/-}$ mice were generated by crossing in-house bred IL-21R$^{-/-}$ with IL-2R$^{-/-}$ animals. WT CD45.2+, IL-2Rα$^{-/-}$, IL-21R$^{-/-}$ and IL-2Rα$^{-/-}$/ L-21R$^{-/-}$ were bred as littermates. All animals were housed in individually ventilated cages under specific pathogen-free conditions at the ETH Phenomics Center (EPIC, Zurich, Switzerland). For experiments, sex-matched mice were used at an age of 6-8 weeks for CD45.2+ donors, 10 weeks for CD45.1+ animals and 6-10 weeks for bone marrow recipients. Mice were euthanized with CO_2. All experiments were performed according to institutional guidelines and the Swiss animal protection law (Swiss Animal Protection Ordinance [TschV and TschG] Switzerland) and had been approved by local animal ethics committee of Kantonales Veterinäramt in Zurich, Switzerland (permission no. ZH135/15).

Bone marrow chimeras

One day prior to bone marrow transfer, recipient mice were lethally irradiated with a total dose of 950 RAD using a RS 2000 X-ray irradiator (Rad Source Technologies, Georgia, USA). On day of transfer, bone marrow was flush out from femur and tibia of hind legs of donor mice and lysed with ACK buffer (155mM NH_4Cl [Sigma Aldrich, Missouri, USA], 10mM $KHCO_3$ [Sigma Aldrich], 0.1mM EDTA [Sigma Aldrich] dissolve in ddH_2O) followed by T cell depletion with CD90.2 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) by MACS. Congenically marked CD45.1+ WT bone marrow cells were mixed in a 1:1 ratio with the respective CD45.2+ cells and reconstituted into CD45.1+CD45.2+ recipient mice by intravenous (*i.v.*) injection at a concentration of $3x10^6$ cells. Recipient mice were treated with antibiotics (0.024% Borgal, MSD Animal Health, New Jersey, USA) in drinking water for 5 weeks.

Infection

The LCMV strain WE was originally provided by Rolf Zinkernagel (University of Zurich, Zurich, Switzerland) and was propagated on L929 cells.

For LCMV production, cell line was grown until confluency in RPMI medium (Gibco, Massachusetts, USA) containing 5% heat-inactivated FBS (Gibco), 100 U/ml Penicillin/100 µg/ml Streptomycin (Gibco) and 2mM L-Glutamine (Sigma-Aldrich). Cells were counted in the morning (cells per one tissue culture flask represents 100% confluency) and appropriate numbers were seeded to get 70-80% confluency. In the

afternoon, virus in 5 ml medium/flask at a MOI of 0.01 was added to the adhered cells and incubated for 1h at room temperature. Tissue culture flasks were taped every 15 min. Subsequently, 15 ml of fresh medium was added to the cells and incubated for 48h at 37°C, 5% CO_2. Medium was harvested and centrifuged. The supernatant was aliquoted and frozen at -80°C. 20 ml of fresh medium was added to the cells and incubated for an additional day to receive a second batch of virus. The virus titer of the supernatants was determined by plaque assay [259].
Mice were infected *i.v.* with 500 pfu LCMV WE after 12 weeks of reconstitution.

Cell preparation

Blood samples were collected in Heparin (Liquemin, 25 000 U.I./5 ml, Drossa Pharm, Basel, Switzerland) and subsequently treated twice with ACK buffer for 5 min at 4°C to remove erythrocytes. Single cell suspension of the spleen was obtained by smashing organs through a 70 μm cell strainer followed by lysis of erythrocytes with ACK buffer for 5 min at 4°C. Liver was minced and smashed through a 70 μm cell strainer. After centrifugation, cell pellet was resuspended in 5ml of pure PBS (Life Technologies, California, USA) and centrifuge at 20g for 5 min at RT to exclude hepatocytes. Upper layer was transferred into a new tube, underlaid with 5ml of Lympholyte-M (Cedarlane, Ontario, Canada) solution and centrifuged at RT, 836g for 20 min without break. Intermediate phase was washed with PBS containing 2% heat-inactivated FBS (Gibco). Before FACS staining, spleen and liver cells were filtrated through a 35 μm cell strainer.

Reagents

The gp33-41 peptide (KAVYNFATM) and the gp61-80 (GLNGPDIYKGVYQFKSVEFD) peptides were bought from Mimotopes (Victoria, Australia). The PE-conjugated gp33-MHC class I tetramer (H-2Db/gp33-41) was kindly provided by the National Institute of Health tetramer core facility (Bethesda, Maryland, USA). Antibodies used were as follows: CD8a-FITC (53-6.7; eBioscience, Massachusetts, USA), CD45.1-PE (A20; BioLegend, California, USA), CD45.2-Pe-Cy7 (104; BioLegend), CD62L-FITC (MEL-14; eBioscience), CD8-PercP (53-6.7; BioLegend), CD45.1-APC (A20; BioLegend), CD127-BV421 (A7R34; BioLegend), KLRG-1-biotin (2F1/KLRG1; BioLegend), Streptavidin-BV711 (BD Bioscience,

California, USA), TNFα-FITC (MP6-XT22; BioLegend), IFNγ-APC (XMG1.2; BioLegend).

Flow cytometry

All stainings were performed in FACS buffer (PBS + 0.5% heat-inactivated FBS) except stated otherwise. First, cells were incubated for 5 min at 4°C with anti-CD16/CD32 mAb (2.4G2, homemade) to block FcγIII/II receptors. Cells were stained with gp33-tetramer for 15 min at room temperature followed by staining of surface markers for 20 min at 4°C. Before acquisition, samples were fixed with 4% Formalin (Sigma-Aldrich) at room temperature for 5 min.

For analysis of the cytokine production, spleen and liver cells were stimulated with 2 μg/ml Monensin (Sigma-Aldrich), 1 μM gp33 and 1μM gp61 peptide for 4h at 37°C. Before staining of surface markers for 20 min at 4°C, FcγR was blocked as described above. Following surface staining, cells were fixed with 4% Formalin for 5 min at room temperature followed by permeabilization with permeabilization buffer (PBS + 0.5% heat-inactivated FBS + 0.5% saponin [Sigma-Aldrich]) for 5 min at room temperature. Intracellular staining was performed for 20 min at 4°C with antibodies diluted in permeabilization buffer. Cells were washed twice with permeabilization buffer and once with FACS buffer.

Data were acquired on a BD FACSCanto II (BD Bioscience) or LSRFortessa (BD Bioscience) and analysed with FlowJo software version 9.9 (TreeStar; Oregon, USA).

Statistics

Data are shown as average ±SD. Ratios of individual mice were normalized to the mean ratio of the WT:WT group. For statistical analysis ordinary one-way ANOVA (multiple comparisons) was performed using Prism 7 software (GraphPad software, California, USA). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Author Contributions

P.D. and F.A. performed the experiments. P.D., L.T. and M.K. designed the experiments. P.D. and M.K. wrote the manuscript.

3.4. Results

Leukocyte development in the absence of IL-2Rα and IL-21R in mixed bone marrow chimeras

In order to bypass the adverse effect seen in IL-2Rα knockout (KO) mice [175-177, 284, 287] and to study the mutual contribution of IL-2Rα and IL-21R in mounting a protective CD8+ T cell response, mixed bone marrow chimeras were generated. Congenically marked WT, IL-2Rα$^{-/-}$, IL-21R$^{-/-}$ or IL-2Rα$^{-/-}$/IL-21R$^{-/-}$ donor bone marrow (CD45.2+) was mixed with WT bone marrow cells (CD45.1+) at a 1:1 ratio and transferred into lethally irradiated WT recipients (CD45.1+ CD45.2+). After 6 weeks, leukocyte reconstitution was assessed, followed by the infection of mice with an acute dose of LCMV, strain WE, six weeks later (Fig. 1, A).

To determine the efficacy of the reconstitution, the proportion of CD45.2+ to CD45.1+ cells was assessed in the blood of recipients six week post bone marrow transfer (Fig. 1, B, C). For simplification, ratios of individual mice were normalized to the mean ratio of the WT:WT group. We observed that the overall CD45.2+ pool of IL21R$^{-/-}$ single knockouts (SKO) and IL-2Rα$^{-/-}$/IL-21R$^{-/-}$ double knockout (DKO) cells had a slight disadvantage over the WT (CD45.1+) cells to develop in the periphery (Fig. 1, C), which was however evident only in one out of three experiments performed (data not shown). Though, the development of CD8+ T cells was not affected in the absence of either or both of the receptors (Fig. 1, D), confirmed by two additional experiments.

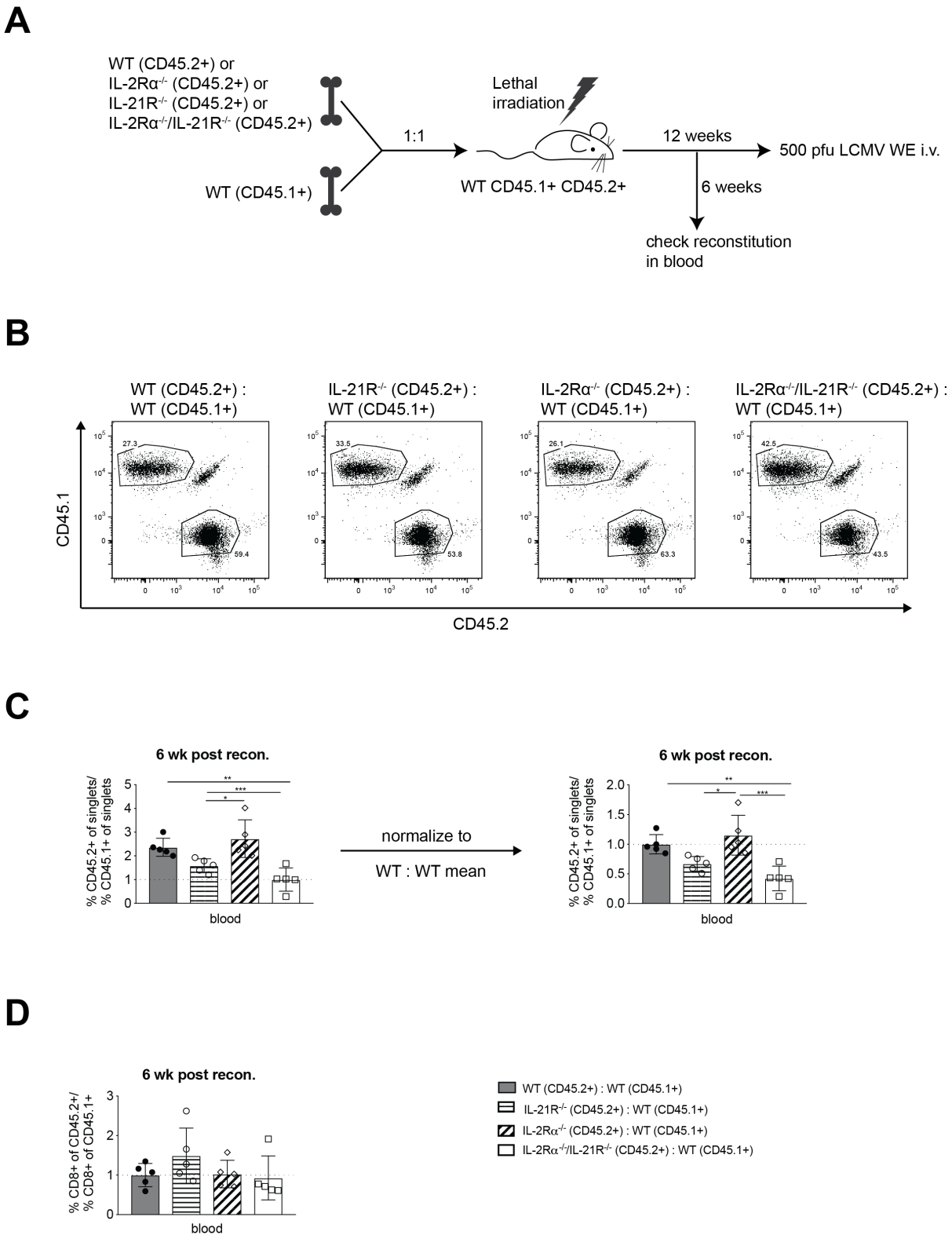

Fig. 1: Generation and characterization of WT:WT, IL-21R$^{-/-}$:WT, IL-2Rα$^{-/-}$:WT and IL-2Rα$^{-/-}$/IL-21R$^{-/-}$:WT mixed bone marrow chimeras. A 1:1 mix of WT (CD45.2+) with WT (CD45.1+), IL-21R$^{-/-}$ (CD45.2+) with WT (CD45.1+), IL-2Rα$^{-/-}$ (CD45.2+) with WT (CD45.1+) or IL-2Rα$^{-/-}$/IL-21R$^{-/-}$ (CD45.2+) with WT (CD45.1+) of total 3x10^6 bone marrow cells was injected *i.v.* into lethally irradiated WT mice (CD45.1+ CD45.2+). 6 weeks post transfer,

reconstitution was assessed in the blood. (A) Experimental setup. (B) Representative FACS plots of CD45.1+ and CD45.2+ staining 6 weeks post reconstitution in the blood of chimeric mice. Pre-gated on singlets. (C) Ratios of CD45.2+ to CD45.1+ cells 6 weeks post reconstitution in the blood of chimeric mice. Graph on the right shows the normalized ratios. (D) Ratios of WT to WT, single knockout (SKO) to WT or double knockout (DKO) to WT CD8+ T cells in the blood of recipient mice 6 weeks post bone marrow transfer. Ratios of individual chimeric mice in (C, right) and (D) were normalized to the mean ratio of the WT:WT group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (one-way ANOVA, multiple-comparison). Dots represent ratios of individual mice, bars represent means ± SD of five mice per group. Data from (D) are representative of three independent experiments and (C) from one.

Absence of either of the receptors can be partially compensated by the other to ensure maintenance of virus-specific CD8+ T cells

To assess the contribution of IL-2Rα and IL-21R on the CD8+ T cell response during an acute viral infection, chimeric mice were infected with 500 pfu LCMV WE 12 weeks post reconstitution (Fig. 1, A). Frequencies of total CD8+ and virus-specific CD8+ T cells from the CD45.2+ and the CD45.1+ compartment were analysed (Fig. 2, A) during different phases of the T cell response to infection: At the peak of the expansion 8 days post infection (dpi), during the contraction phase 14 dpi and when memory is established 44 dpi.

Interestingly, the ratio of IL-2R$\alpha^{-/-}$/IL-21R$^{-/-}$ to WT CD8+ T cells was reduced as early as 8 dpi when compared to WT:WT chimeras, whereas lack of either of the two receptors only slightly impaired maintenance but did not change the expansion of CD8+ T cells (Fig. 2, B, C). In line, also ratios of IL-2R$\alpha^{-/-}$/IL-21R$^{-/-}$ to WT gp33-specific CD8+ T cells were reduced 8 dpi (Fig. 2, D), with ratios further decreasing 14 dpi, however absence of either of the receptors did not greatly change the ratios of gp33-specific CD8+ T cells. At 44 dpi, the ratio between CD45.2+ IL-2R$\alpha^{-/-}$/IL-21R$^{-/-}$ and CD45.1+ WT virus-specific CD8+ T cells in the spleen was below 1:5, whereas lack of either of the two receptors reduced the ratio only by two fold (Fig. 2, E). Besides, DKO gp33-specific CD8+ T cell in the liver were also significantly reduced, however not as strongly as in the spleen (Fig. 2, E).

Taken together, these results indicate that the cytokines can partially compensate absence of the other to ensure long-term maintenance of virus-specific CD8+ T cells during an acute LCMV infection.

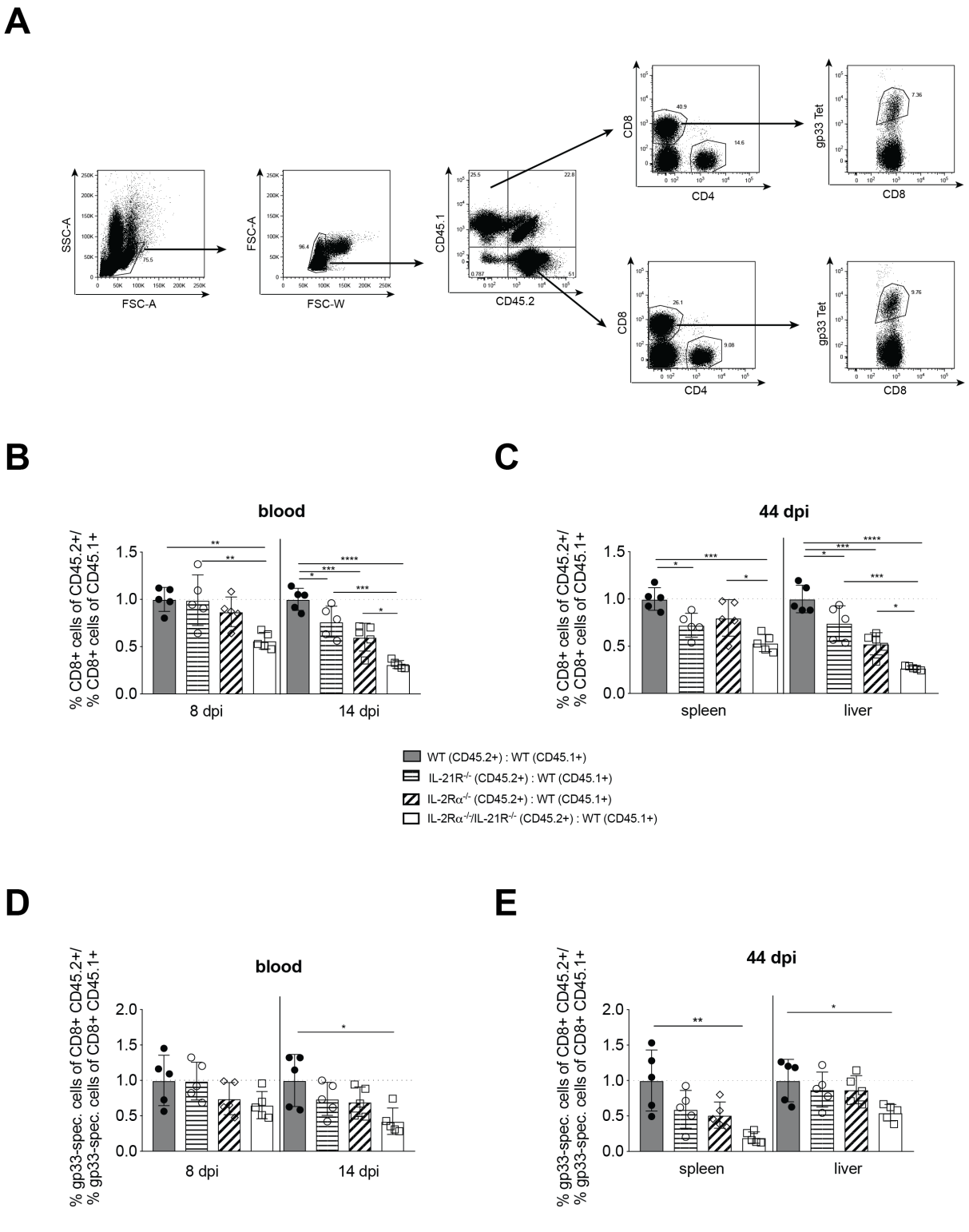

Fig. 2: Decreased ratios of gp33-specific CD8+ T cells in the absence of the IL-2Rα and the IL-21R during the memory phase. Mixed chimeric mice were infected 12 weeks post reconstitution with 500 pfu LCMV WE *i.v.*. (A) Gating strategy shown on blood cells isolated 8 dpi from a WT:WT chimeric mouse. Ratios of CD45.2+ CD8+ T cells to CD45.1+ CD8+ T cells

in the blood 8 and 14 dpi (B) and 44 dpi in the spleen and liver (C) of chimeric mice. Ratios of CD45.2+ gp33-specific CD8+ T cells to CD45.1+ gp33-specific CD8+ T cells in the blood 8 and 14 dpi (D) and 44 dpi in the spleen and the liver (E). Ratios of individual chimeric mice in (B) to (E) were normalized to the mean ratio of the WT:WT group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ (one-way ANOVA, multiple-comparison). Dots represent ratios of individual mice, bars represent means $\pm$ SD of five mice per group. Data are representative of two independent experiments.

Combined signals from the IL-2Rα and IL-21R are required for sustaining the cytokine production of CD8+ T cells

To investigate if IL-2 and IL-21 are required for the effector function, the IFNγ and TNFα production of CD8+ T cells isolated from the spleen (Fig. 3, A) and the liver of chimeras was determined.

Although the ratio of cytokine producing IL-2Rα and IL-21R SKO to WT CD8+ T cells in the spleen was already decreased by half, which was however not significant, ratios of IFNγ and TNFα producing CD8+ T cells were reduced by tenfold in IL-2Rα$^{-/-}$/IL-21R$^{-/-}$:WT chimeras (Fig. 3, B). Whereas ratios of IFNγ producing CD8+ T cells in the liver of DKO:WT mice were also significantly reduced (Fig. 3, C, left), however less strong as in the spleen, the TNFα producing cells were not affected in the absence of both receptors (Fig. 3, C, right). When comparing data from two additional experiments, also ratios of IFNγ+ IL-2Rα$^{-/-}$/IL-21R$^{-/-}$ to WT CD8+ T cells from the liver were not changed in DKO:WT chimeras, thus the observed difference in ratios of IFNγ producing CD8+ T cells the liver might be experiment dependent.

Together these results indicate that signalling through IL-2Rα and IL-21R contribute additively to the sustained cytokine production of splenic CD8+ T cells after resolution of an acute LCMV infection.

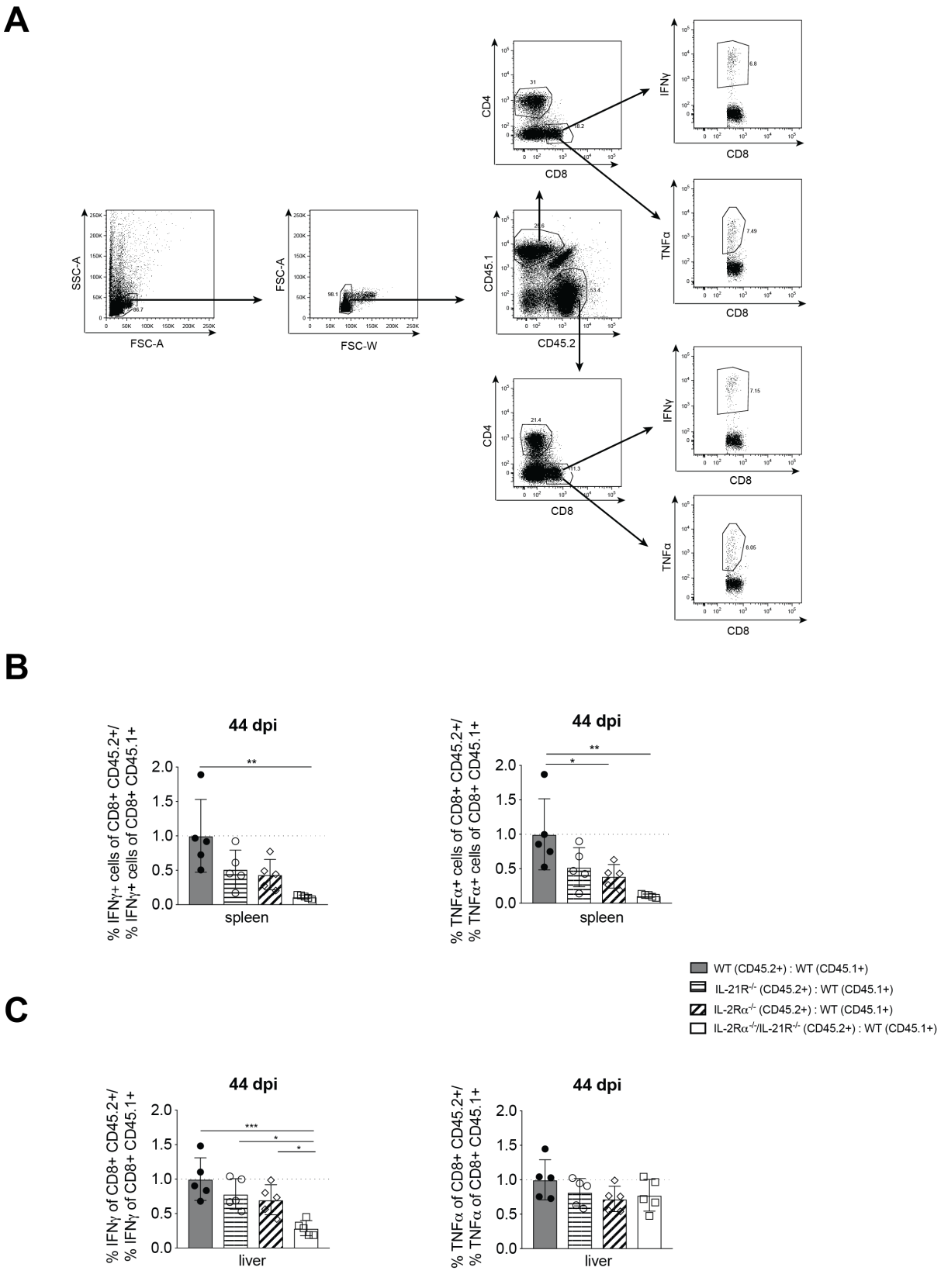

Fig. 3: Impaired cytokine response of CD8+ T cells lacking the IL-2Rα together with the IL-21R. Mixed chimeric mice were infected 12 weeks post reconstitution with 500 pfu LCMV WE i.v. and killed 44 dpi to analyse the cytokine response of cells. (A) Gating strategy shown

on splenocytes from a WT:WT chimeric mouse isolated 44 dpi. IFNγ (left) and TNFα (right) production of CD8+ T cells 44 dpi from the spleen (B) and the liver (C) after in vitro restimulation with gp33 peptide, followed by intracellular cytokine staining. Ratios of individual chimeric mice in (B) and (C) were normalized to the mean ratio of the WT:WT group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (one-way ANOVA, multiple-comparison). Dots represent ratios of individual mice, bars represent means ± SD of five mice per group. Data from (B) are representative of two independent experiments and (C) from one.

IL-2Rα together with the IL-21R regulate the accumulation of memory precursor effector cells (MPEC)

Differences in the expression of killer cell lectin-like receptor G 1 (KLRG-1) and the IL-7Rα (CD127) predicts the fate of an effector CD8+ T cell after antigen clearance [135, 136]. While the majority of terminally differentiated KLRG-1+ CD127- short-lived effector cells (SLEC) die, most KLRG-1- CD127+ memory precursor effector cells (MPEC) persist and give rise to long-lasting memory cells.

Since we observed a reduction in ratios of IL-2Rα$^{-/-}$/IL-21R$^{-/-}$ to WT virus-specific CD8+ T cells in the blood from 8 to 14 dpi (Fig. 2, D), which was further decreased in peripheral tissues 44 dpi (Fig. 2, E), we were wondering if the combined absence of IL-2Rα and L-21R diminishes the formation of long-lasting MPEC. Surprisingly, ratios of MPECs in the blood of DKO:WT chimeras were not reduced 8 dpi (Fig. 4, A, right). Interestingly, 14 dpi ratios of IL-2Rα$^{-/-}$/IL-21R$^{-/-}$ to WT MPECs were rather slightly enhanced when compared to control chimeras and even further increased in the spleen 44 dpi (Fig. 4, A, B, right). On the other hand, ratios of DKO to WT SLECs decreased over time and were significantly reduced 44 dpi in the spleen when compared to control chimeras (Fig. 4, A, B, left). In the liver (Fig. 4, C), differences in SLECs and MPECs between IL-2Rα$^{-/-}$/IL-21R$^{-/-}$ and WT chimeras were not as strong as in the spleen. Deficiency in the IL-2Rα alone increased the accumulation of MPECs in the spleen 44 dpi as well (Fig. 4, B, right), however not as strongly as by the additional absence of the IL-21R.

Thus, the continued reduction of IL-2Rα$^{-/-}$/IL-21R$^{-/-}$ virus-specific CD8+ T cells is not due to a defect in the formation of long-lasting memory cells. In contrast, absence of both receptors rather increases MPEC formation.

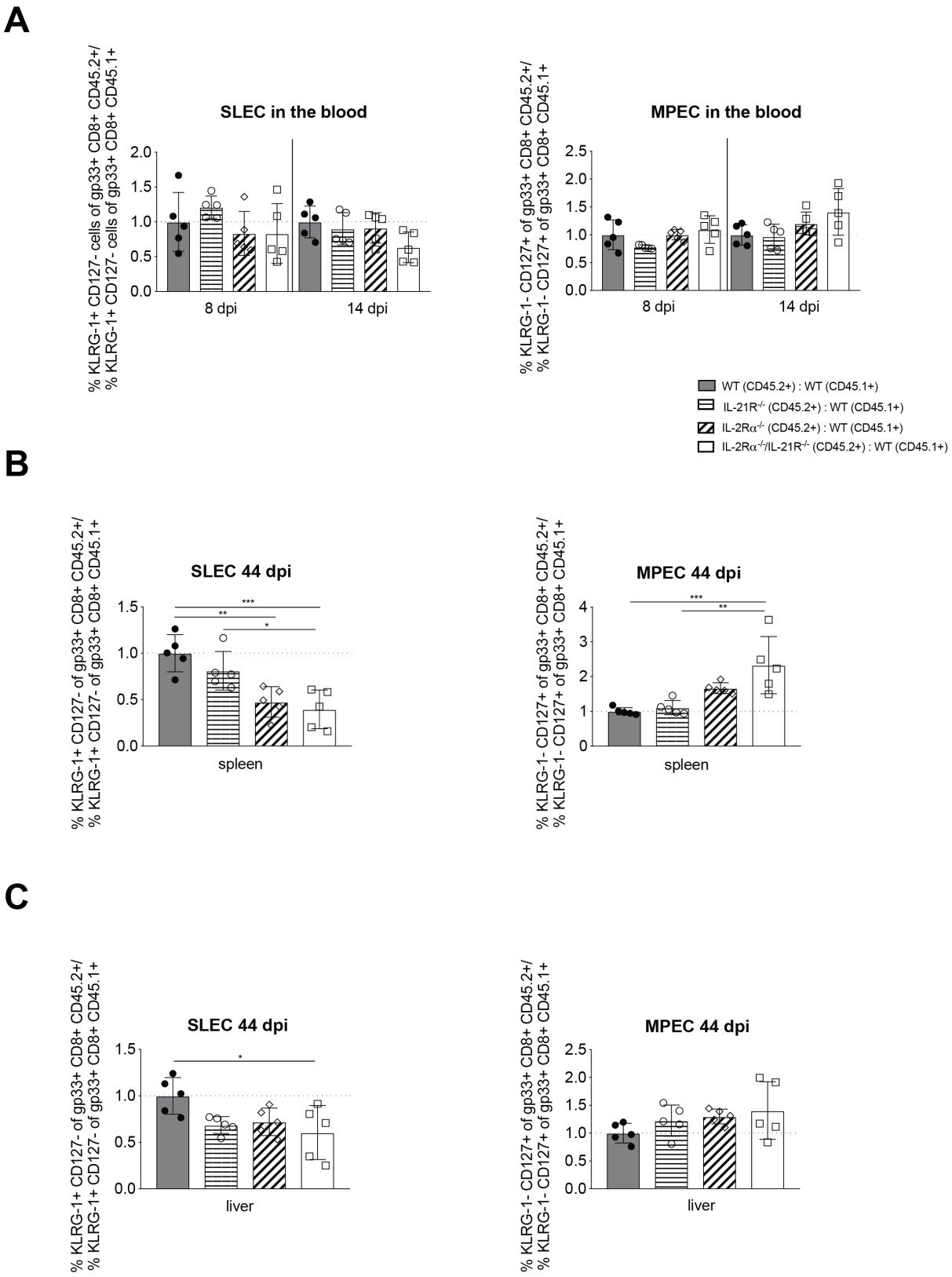

Fig. 4: Combined absence of IL-2Rα and IL-21R enhances accumulation of memory precursor effector cells (MPEC). Blood cells isolated 8 and 14 dpi and splenocytes or liver cells 44 dpi from mixed chimeric mice infected with 500 pfu LCMV WE i.v. 12 weeks post reconstitution were stained with KLRG-1 and CD127 antibodies to distinguish effector cell

subsets. Ratios of CD45.2+ to CD45.1+ virus-specific CD8+ KLRG-1+ CD127- short-lived effector cells (SLEC) and KLRG-1- CD127+ memory precursor effector cells (MPEC) in the blood 8 and 14 dpi (A) and in the spleen (B) or in the liver (C) 44 dpi from indicated groups. Ratios of individual chimeric mice in (A) to (C) were normalized to the mean ratio of the WT:WT group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (one-way ANOVA, multiple-comparison). Dots represent ratios of individual mice, bars represent means ± SD of five mice per group. Data are representative of two independent experiments.

Absence of the IL-2Rα together with the IL-21R favours the accumulation of central memory virus-specific CD8+ T cells

Memory CD8+ T cells form a heterogeneous population, consisting of CD62L+ CD127+ central memory cells (T_{CM}) residing mostly in LNs, are able to produce IL-2 and show a higher protective capacity due to an increased proliferative ability and CD62L- CD127+ effector memory T cells (T_{EM}) that can enter peripheral tissues thus mediating protection against local infections [144-147].

As early as 8 dpi, absence of the IL-2Rα led to a higher proportion of virus-specific CD8+ central memory cells however only minimally interfering with T_{EM} formation when compared to WT chimeras (Fig. 5, A). In contrast, absence of the IL-21R did not interfere with memory subset formation (Fig. 5, A). Interestingly, the combined absence of IL-2Rα and IL-21R further enhanced the ratio of central memory gp33-specific CD8+ T cells from DKO to WT controls 14 dpi in the blood (Fig. 5, A) and 44 dpi in the spleen and the liver (Fig. 5, B, C) when compared to IL-2Rα$^{-/-}$:WT chimera. Overall, these data imply that IL-21R signalling limits further accumulation of central memory cells caused by the absence of the IL-2Rα.

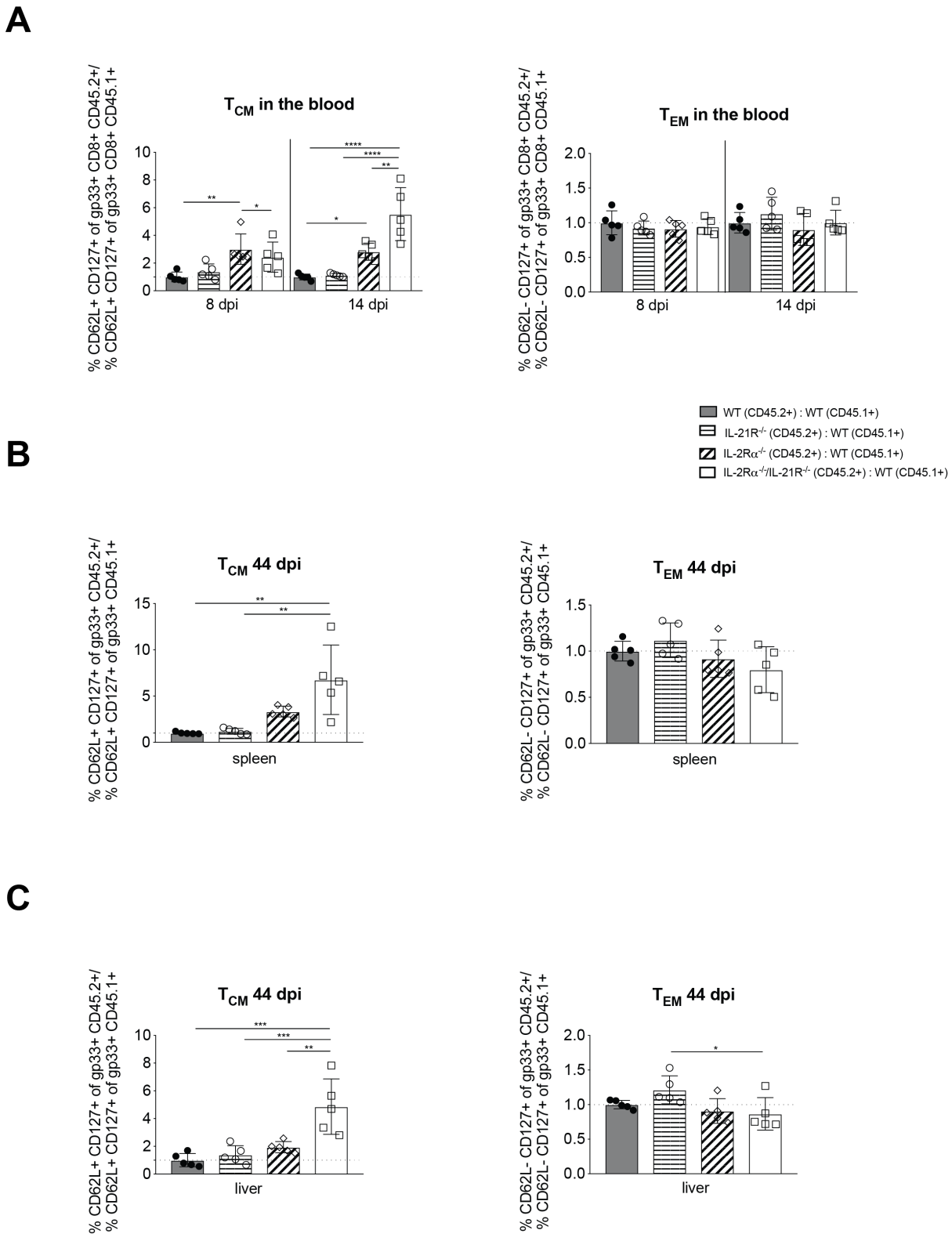

Fig. 5: The central memory cell subset is increased upon combined absence of the IL-2Rα and the IL-21R. Blood cells isolated 8 and 14 dpi and splenocytes or liver cells 44 dpi from mixed chimeric mice infected with 500 pfu LCMV i.v. 12 weeks post reconstitution were stained with CD62L and CD127 to distinguish different memory subsets. Ratios of CD45.2+

over CD45.1+ virus-specific CD8+ CD62L+ CD127+ central memory cells (T_{CM}) or CD62L- CD127+ effector memory cells (T_{EM}) in the blood 8 and 14 dpi (A) or in the spleen (B) or in the liver (C) 44 dpi from indicated groups. Ratios of individual chimeric mice in (A) to (C) were normalized to the mean ratio of the WT:WT group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ (one-way ANOVA, multiple-comparison). Dots represent individual mice, bars represent means ± SD of five mice per group. Data are representative of two independent experiments.

3.5. Discussion

Absence of the IL-2Rα or the IL-21R has been shown to only marginally impair the CD8+ T cell response during an acute LCMV infection in a mixed chimeric system [5, 208]. Here we reported that absence of both receptors together impaired the maintenance of virus-specific CD8+ T cells after antigen clearance in mixed chimeras. We propose a partial compensatory action of IL-2 and IL-21 signals in ensuring the long-term persistence of virus-specific CD8+ T cells during an acute LCMV infection.

As shown previously, absence of the IL-21R in a mixed chimeric system slightly decreased the frequency of virus-specific CD8+ T cells during a LCMV infection [208], which is in line with our data. However in the context of a fully IL-21R deficient mouse, infection with an acute low dose of LCMV DO impaired the maintenance of virus-specific CD8+ T cells, whereas infection with the WE strain did not [209]. Thus, in a mixed chimeric system absence of IL-21R affects, if only mildly, the maintenance of virus-specific CD8+ T cells during a LCMV WE infection, whereas in fully IL-21R deficient mice the long-term persistence of antigen-experienced CD8+ T cells depends on the virulence of the LCMV strain used. In contrast, during a chronic LCMV infection deficiency in the receptor strongly impaired the maintenance of anti-viral CD8+ T cell [208-210], due to a reduced proliferation of IL-21R$^{-/-}$ CD8+ T cells [209]. Thus, our observation of a slight impairment in the long-term maintenance of virus-specific CD8+ T cells in the absence of the IL-21R might be due to a decreased homeostatic proliferation. If the additional lack of the IL-2Rα exacerbate the homeostatic proliferation or if it impairs the survival of memory virus-specific CD8+ T cells would need to be assessed.

Whereas the IL-2Rα has shown to be indispensable for the maintenance of virus-specific CD8+ T cells during a chronic infection [6], our results indicated that lack of

the IL-2Rα alone did only slightly reduce the frequency of virus-specific CD8+ T cells during an acute infection with LCMV WE. Indeed, also infection of IL-2Rα$^{-/-}$:WT chimeras with an acute dose of LCMV Arm only marginally impaired the expansion and maintenance of virus-specific CD8+ T cells [5]. However, one group reported a fivefold reduction of IL-2Rα deficient compared to WT virus-specific CD8+ T cells 8 dpi, with the difference maintained over time [6]. By comparing the setup of the experiments, the latter group reconstituted irradiated recipients with a 4:1 mix of IL-2Rα$^{-/-}$ to WT bone marrow cells, whereas the prior group [5], and also we, used a 1:1 mixture. Furthermore, mice reconstitute with a 2:1 mixture of KO to WT bone marrow cells also showed a significantly reduced frequency of IL-2Rα$^{-/-}$ antigen-specific CD8+ T cells after a *Listeria monocytogenes* or Vaccinia virus infection at the peak of the immune response [164]. Thus, the higher the proportion of total CD45 IL-2Rα deficient to WT cells injected into irradiated animals is, the fewer IL-2Rα$^{-/-}$ antigen-specific CD8+ T cells accumulate at the peak of T cells response during an infection. Our data indicate that deficiency in the IL-2 signal can be partially compensated by IL-21. The main producers of latter cytokine during a LCMV infection are CD4+ T cells [208]. Absence of the IL-2 signal reduces the accumulation of functional polarized Th1 cells [183] (data not shown), the main subset that forms during a virus infection. Thus possibly, by increasing the amount of KO bone marrow cells, the proportion of dysfunctional IL-2Rα$^{-/-}$ CD4+ helper T cells during a LCMV infection increases, what might lead to a reduction in IL-21 and thus to a decrease in virus-specific CD8+ T cells due to the limited compensatory action of the cytokine. Hence, the levels of IL-21 and the frequency of IL-21+ CD4+ T cells in the 1:1 and the 4:1 IL-2Rα$^{-/-}$:WT chimeras should be assessed. In case of a reduction in cytokine levels in the latter system, it would be interesting to assess if administration of IL-21 in the 4:1 chimeras could rescue the poor accumulation of virus-specific CD8+ T cells at the peak of the immune response.

Besides difference in the kinetics of IL-2Rα$^{-/-}$/IL-21R$^{-/-}$ virus-specific CD8+ T cells, we could show that DKO cells preferentially, and even to a higher degree as IL-2Rα deficient ones, accumulated as central memory cells 44 dpi. Previous studies indicate that IL-2Rα$^{-/-}$ virus-specific CD8+ T cells preferentially acquire a central memory phenotype [5, 6]. The induction of the transcriptional repressor Blimp-1 in T cells is dependent on IL-2 [182]. Besides ensuring formation of terminally differentiated

effector cells [181], Blimp-1 also regulates the distribution of CD8+ T cells into the different memory subsets, where absence of the transcription factor leads to an increase in central but not effector memory cells. A direct target of the repressor Blimp-1 is Bcl6. Overexpression of Bcl6 increased the number of central memory cells while not interfering with effector memory formation [288]. Bcl6 is elevated in IL-2Rα deficient cells [289]. Thus, the observed increase in T_{CM} in the absence of the IL-2Rα could be due to lower levels of Blimp-1 and thus increased levels of Bcl6. If IL-21 would be able to induce Blimp-1 or directly dampen Bcl6 expression thus limiting central memory cell formation, the increased ratio of T_{CM} in IL-2Rα$^{-/-}$/IL-21R$^{-/-}$:WT chimeras could be explained.

Since memory cells develop mostly from the KLRG-1^{lo} population [136] and we observe an increase in T_{CM}, it is not surprising that ratios of KLRG-1^{-} CD127^{+} MPECs are increased 14 dpi in the blood and 44 dpi in the spleen of IL-2Rα$^{-/-}$/IL-21R$^{-/-}$:WT compared to control chimeras. However, we did not observe differences in ratios of MPECs between IL-2Rα$^{-/-}$/IL-21R$^{-/-}$:WT and WT:WT chimeras 8 dpi, possibly since ratios of T_{CM} between the two groups are only slightly, but not yet significantly, enhanced at this time point. In the liver, there is only a trend towards higher ratios of MPECs in IL-2Rα$^{-/-}$/IL-21R$^{-/-}$:WT chimeras even though ratios of T_{CM} are significantly increased when compared to control chimeras. Thus, why we could only observe a slight change in MPEC ratios in DKO:WT chimeras would need to be determined.

Lastly, we could show that the effector response of virus-specific CD8+ T cells is dependent on the additive signals from the IL-2Rα and the IL-21R. Whereas deficiency in either of the receptors reduced the ratio of KO to WT cytokine producing CD8+ T cells in the spleen by half, which was however not significant and no phenotype was visible in the liver, IFNγ and TNFα producing IL-2Rα$^{-/-}$/IL-21R$^{-/-}$ CD8+ T cells were strongly reduced in the spleen of DKO:WT chimeras. Previous reports indicated that in the absence of IL-2Rα, the frequency of IFNγ producing CD8+ T cells isolated during the memory phase were indeed slightly decreased [5], however absence of the IL-21R did not greatly change the frequency of cytokine producing CD8+ T cells in mice infected with a low virulent virus strain [208, 209]. While in latter experiments, full KO mice were used, we assessed the role of the IL-21R on the cytokine production of CD8+ T cells in a mixed chimeric system, thus the variation might be caused by differences in the experimental setup. The underlaying mechanism causing the

massive reduction in cytokine producing CD8+ T cells upon absence of the IL-2Rα and the IL-21R would require further research, especially since overexpression of Bcl6, which is increased in IL-2Rα deficient CD8+ T cells [289] and possibly even higher expressed in DKO cells, decreased the Granzyme B production [290] while not changing the IFNγ production of CD8+ T cells [288, 290]. Interestingly, during a chronic infection, the cytokine response in the absence of the IL-2Rα or the IL-21R is strongly reduced ([6] and [208-210]) and goes a long with an increased viral burden [208-210]. Thus, when antigen persists, the signal from IL-2 and IL-21 are crucial to mount a protective effector response, whereas during a resolved infection, cytokine production of CD8+ T cells can be mostly sustained by either of the two cytokines.

Overall, we could show that the receptors for IL-2 and IL-21 act in a partial compensatory way to ensure maintenance and cytokine production of virus-specific CD8+ T cells during an acute LCMV infection while limiting the formation of central memory CD8+ T cells.

VI. GENERAL DISCUSSION

Co-signalling molecules and cytokines are key in shaping the T cell response induced upon pathogen encounter. In the thesis presented, we aimed to elucidate the role of the two co-signalling molecules BTLA and HVEM and the interplay of the two cytokines IL-2 and IL-21 in the CD8+ T cell response to a LCMV infection.

Initially, HVEM has been identified as a ligand for BTLA, impairing the proliferation of T cells *in vitro* [27]. We however made the unexpected finding, that culturing of BTLA, HVEM or double deficient APCs with T cells did not affect T cell priming. Though, while in the former experiment Chinese hamster ovary cells were used as APCs, we cultured T cells with bone marrow-derived professional APCs, possibly explaining the observed difference.

Despite previous reports revealing a pivotal role of BTLA and HVEM in the survival of effector and memory virus- and bacterial-specific CD8+ T cells in the spleen [42, 43], we observed that absence of either or both of the two molecules did not affect the different stage of the CD8+ T cell response nor virus clearance during an acute or a chronic LCMV infection in mice. Besides, we showed that the two molecules are also dispensable for mounting the protective CD8+ T cell response against MCMV. Thus, previous results together with our data propose that the requirement for the BTLA-HVEM system in mounting a CD8+ T cell response highly depends on the pathogen. Indeed, also the requirement for CD28 co-stimulation was shown to vary between different virus infections [263, 264], indicating that the strength of signal 1 determines the need for co-stimulation. Thus possibly, while infection with LCMV or MCMV elicits a strong signal 1, thereby the CD8+ T cell response depends less on the BTLA-HVEM system, the response against VV requires co-stimulation due to a weaker signal 1. Furthermore, the reliance on different cytokines for mounting the T cell response may influence the need for co-stimulation. While during a LCMV infection, type I IFNs are crucial, the T response mounted during a VV infection is independent of these cytokines [265]. Thus possibly, CD8+ T cells primed with a VV epitope rely more on co-stimulation than on cytokines, opposed to LCMV-primed CD8+ T cells.

Besides, while HVEM is down-regulated on pathogen-specific CD8+ T cells during a VV and a *Listeria monocytogenes* infection [42, 43] but BTLA expression is rather increased on VV-specific cells [42], we showed that both molecules are down-

regulated on virus-specific CD8+ T cells at the peak of the T cell response upon a LCMV infection. Thus, even though, in case of HVEM, the molecule is similarly regulated during a VV, *Listeria monocytogenes* and LCMV infection, its early presence on VV- and bacterial-specific CD8+ T cells seems to be important for the effector response and the survival of the cells, but being dispensable for the response of LCMV-specific CD8+ T cells.

Additionally, previous studies revealed that maintenance of VV-specific CD8+ T cells and formation of MPECs in the lung strongly depended on the expression of HVEM on T cells [266]. We however did not observed an impaired formation of memory virus-specific CD8+ T cells in the absence of the molecule during a LCMV infection. Though, overexpression of HVEM on virus-specific CD8+ T cells significantly increased the frequency of MPECs. Thus, we confirmed that HVEM is involved in MPEC formation, however it seems to play a minor role during a LCMV infection.

Through retroviral overexpression of BTLA and HVEM we could show that down-regulation of the two molecules on LCMV-specific CD8+ T cells is crucial for the control of the anti-viral T cell response. While retroviral overexpression of BTLA on virus-specific CD8+ T cells moderately increased the cytokine production, overexpression of HVEM impaired the initially expansion. The subsequent contraction of virus-specific CD8+ T cells was however reduced when HVEM or BTLA were overexpressed resulting in increased numbers of memory cells. Thus taken together, while during the effector stage BTLA down-regulation is important to dampen the cytokine response of virus-specific CD8+ T cells, HVEM down-regulation is crucial to ensure the expansion of virus-specific CD8+ cells during a LCMV infection. However, sustained low expression of either of the two molecules on virus-specific CD8+ T cells impairs formation of memory cells.

In summary, we revealed a so far unknown requirement for the down-regulation of the co-signalling molecules BTLA and HVEM to mount the anti-viral CD8+ T cell response against LCMV.

Besides co-signalling molecules, also cytokines regulate the different stages of the CD8+ T cell response. Previous reports demonstrated that absence of either of the two γ_c family cytokines IL-2 and IL-21 only marginally impaired the CD8+ T cell response during an acute infection with low virulent strains of LCMV [5, 6, 208, 209],

whereas the two cytokines were indispensable during a chronic LCMV infection [6, 208-210]. Our aim was to assess if during an acute LCMV infection, the absence of one cytokine can be compensated by the other. Indeed, we revealed that in the absence of one, the long-term maintenance of virus-specific CD8+ T cells can be partially compensated by the other in a mixed chimeric system. However, we observed that persistence of virus-specific CD8+ T cells after virus clearance in the absence of the IL-2Rα or the IL-21R was also marginally impaired. Whereas it was previously proposed that the frequency of virus-specific CD8+ T cells is also slightly reduced in the absence of IL-21R upon LCMV infection in a mixed chimeric system [208], the long-term maintenance of the cells in fully deficient mice depended on the virulence of the LCMV strain used. Whereas infection of IL-21R KO mice with a low dose of LCMV DO impaired the persistence of virus-specific CD8+ T cells, infection with LCMV WE did not [209]. Thus, in a competitive system, as in mixed chimeras, IL-21R deficient CD8+ T cells have a slight disadvantageous in the persistence. If the cytokine is required for the homeostatic proliferation or for promoting the survival of virus-specific memory CD8+ T cells in our experiments remains to be determined.

Besides, our results indicate that absence of the IL-2Rα also slightly, but not significantly, reduced the frequency of virus-specific CD8+ T cells during a LCMV WE infection. Indeed, also infection of IL-2R$\alpha^{-/-}$:WT chimeras with an acute dose of LCMV Arm marginally impaired the long-term persistence of virus-specific CD8+ T cells [5]. However, one group reported a strong reduction in the expansion of the cells upon a LCMV WE infection, with the difference kept over time when compared to controls [6]. While latter group reconstitute recipient mice with a 4:1 mix of IL-2R$\alpha^{-/-}$ to WT bone marrow, the prior group and also we reconstitute animals with a 1:1 mix. Furthermore, recipient mice reconstitute with a 2:1 mix of KO to WT bone marrow cells showed a significant reduction in Listeria- and VV-specific CD8+ T cells at the peak of the T cell response [164]. Our data indicate that absence of the IL-2Rα can be partially compensated by IL-21. The main producers of the latter cytokine are CD4+ T cells [208], with the Th1 subset mainly forming during a virus infection but being reduced upon loss of the IL-2 signal [183]. Thus, by increasing the amount of IL-2Rα deficient bone marrow cells, the amount of dysfunctional Th1 cells upon a LCMV infection increases, possibly leading to lower amounts of IL-21 and thus to a decrease in IL-2Rα deficient virus-specific CD8+ T cells. However, if the frequency of IL-21 producing

CD4+ T cells in 4:1 IL-2R$\alpha^{-/-}$ to WT bone marrow chimeras is really reduced when compared to 1:1 recipients would need to be assessed.

Furthermore, we could show that the combined absence of the IL-2Rα and the IL-21R directed virus-specific memory CD8+ T cells towards a central memory phenotype, which was even greater than in the absence of the IL-2Rα alone, with deficiency in only latter cytokine already being proposed to increase the accumulation of T_{CM} [5, 6]. Since in the absence of the IL-2Rα, levels of the transcription factor Bcl6 are increased [289], which is shown to promote the formation of central memory cells upon overexpression [288], and its repressor Blimp-1 being a direct target of IL-2 signalling [182], increasing the frequency of T_{CM} upon loss of former [181], the elevated frequency of central memory virus-specific CD8+ T cells upon additional absence of the IL-21R could indicated the IL-21 signalling might regulate the action of Blimp-1 and/or Bcl6. However this would require further research.

Besides, the sustained cytokine response of CD8+ T cells was slightly impaired upon absence of the IL-2Rα or the IL-21R and strongly reduced in the absence of both receptors. Previous reports indicated that whereas deficiency in the IL-2Rα alone did slightly decrease the frequency of IFNγ producing CD8+ T cells [5], complete absence of the IL-21R did not greatly change the frequency of cytokine producing CD8+ T cells in mice infected with low virulent LCMV strains [208, 209]. Thus, during a resolved infection, the cytokine production by CD8+ T cells can be mostly sustained by either of the two cytokines in a mixed chimeric system. The identification of the cell-intrinsic factors induced upon IL-2 and IL-21 signalling regulating the cytokine response of virus-specific CD8+ T cells requires further research.

Overall, the results presented in this thesis highlight the importance of regulating the expression of the co-signalling molecules BTLA and HVEM and revealed a partial compensatory action of the two cytokines IL-2 and IL-21 in the long-term persistence and the sustained cytokine response of virus-specific CD8+ T cells during an acute LCMV infection.

VII. REFERENCES

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VIII. APPENDIX

1. Abbreviations

γc	gamma chain
ACK	Ammonium-Chloride-Potassium
ANOVA	analysis of variance
-AP	alkaline phosphatase
APC	antigen presenting cell
APL	altered peptide ligand
Arm	Armstrong
BAL	bronchoalveolar lavage
Bcl6	B cell lymphoma 6
BCR	B cell receptor
Blimp-1	B lymphocyte-induced maturation protein 1
BM	bone marrow
BMDC	bone marrow-derived dendritic cells
BTLA	B and T lymphocyte attenuator
CCR	CC receptor
CD	cluster of differentiation
cDNA	complementary Deoxyribonucleic acid
Cl13	Clone 13
ConA	Concanavalin A
CRD	cysteine rich domain
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte-associated antigen 4
DARC	gD and BTLA binding site on the TNFR HVEM in CRD1
DC	dendritic cell
ddH_2O	double-distilled water
DKO	double knockout
DO	Docile
DP	double positive
dpi	days post infection
DtL	detection limit
EAE	experimental autoimmune encephalomyelitis

EEC	early effector cell
ELISA	enzyme-linked immunosorbent assay
EOMES	Eomesodermin
FACS	fluorescence-activated cell sorting
FADD	Fas associated death domain
FBS	Fetal Bovine Serum
Fc	fragment crystallizable
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage-colony stimulating factor
GVHD	graft-versus-host-disease
g	gravity of earth
gD	glycoprotein D
gp/GP	glycoprotein
GPI	glycosylphosphatidylinositol
h	hour(s)
hi	high
HSV	human simplex virus
HVEM	Herpes virus entry mediator
Hz	Hertz
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.p.	intraperitoneal
ITIM	immunoreceptor tyrosine-based inhibitory motifs
ITSM	immunoreceptor tyrosine-based switch motif
i.v.	intravenous
JAK	Janus kinase
KLRG-1	killer cell lectin-like receptor G 1
KO	knockout
L	ligand
LCMV	lymphocytic choriomeningitis virus
LIGHT	lymphotoxin-like, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes

LM	Listeria monocytogenes
LN	lymph node
lo	low
LTα	Lymphotoxin alpha
LTβR	Lymphotoxin beta receptor
mAb	monoclonal antibody
MACS	magnetic-activated cell sorting
MAPK	mitogen-activated protein kinase
MCMV	murine cytomegalovirus
MFI	mean fluorescent intensity
MHC	major histocompatibility complex
$MgCl_2$	magnesium chloride
MOG	myelin oligodendrocyte glycoprotein
MOI	multiplicity of infection
MPEC	memory precursor effector cell
Na_2HPO_4 $2H_2O$	Disodium phosphate dihydrate
NK cell	Natural killer cell
NKT cell	natural killer T cell
OPD	o-Phenylenediamine dihydrochloride
OVA	ovalbumin
PAMPs	pathogen associated molecular patterns
PBL	peripheral blood leukocytes
PCR	polymerase chain reaction
p.i.	post infection
PD-1	Programmed cell death 1
pfu	plaque forming units
PI3K	phosphoinositide 3-kinase
PRRs	pattern recognition receptors
pNPP	p-nitrophenyl phosphate
R	receptor
RAD	radiation absorbed dose
RAG	recombination activating gene
recon.	reconstitution

RT	room temperature
rpm	revolutions per minute
SD	standard deviation
SG	salivary gland
SHP	Src homology domain 2-containing protein tyrosine phosphatases
SKO	single knockout
SLEC	short-lived effector cell
SP	single positive
STAT	signal transducer and activator of transcription
T_{CM}	central memory T cell
TCR	T cell receptor
T_{EM}	effector memory T cell
Tfh cell	T follicular helper cell
tg	transgenic
TGFβ	transforming growth factor beta
Th	T helper
TIM	TRAF-interacting motif
TLR	Toll like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAF	TNF-receptor associated factor
Treg cell	regulator T cell
T_{RM}	tissue resident memory T cells
VLP	virus like particle
VSV	vesicular stomatitis virus
VV	vaccinia virus
wk	week
WT	wildtype

2. Acknowledgement

I would like to thank the following people:

Manfred Kopf for giving me the opportunity to do my PhD in his lab. For his support and guidance throughout the past few years and for always having an open door to discuss recent data.

My PhD committee members **Annette Oxenius** and **Burkhard Ludewig** for their input during committee meetings and there encouragement over the past years. I am thankful for their time and effort discussing my projects during committee meetings.

Nicolas Baumann and **Josua Oderbolz** for their help with the MCMV experiments. **Isabel Barnstorf** for providing protocols and reagents.

Franziska Ampenberger for her help with mouse experiments, taking care of my animals during holidays and in general for the great time.

Esther Rosenwald for helping me with cloning and together with **Irini Vgenopoulou** and **Maren Roche** for keeping the lab running.

Jan Kisielow for his help with cloning and together with **Luigi Tortola** for always being willing to discuss data and providing suggestions for further experiments during but also besides lab meetings.

Peter Nielsen for his time to discuss my project and his suggestions for further experiments.

Fengqi Li for the great time during our “Zäh putzä” sessions and sharing the ups and downs of the daily lab routine.

Katarzyna Okreglicka, **Lea Pohlmeier**, **Feng Qian**, **Sofia Braverman**, **Jonathan Muri**, **Federica Piattini**, **Franz Obermair** and **Merve Kayhan** for the great time and there help in the past few years.

Jasmin Spörri and **Ekaterina Stepanova** for being great and motivated students.

Mai Matsushita, **Sebastian Heer**, **Samuel Nobs** and **Mareike Schmid** for providing helpful advice during the first years of my PhD.

Anette Schütz, **Malgorzata Kisielow** and **Hongying Teh** for their support during sorting and FACS analysis.

The animal care takers **Theresia Heuberger, Manuela Tanner, Aretussa Apladas, Dominique Flota**, **André Mund** and **Paul Hauswirth** as well as the rest of the EPIC team for keeping the mouse house running.

Zuletzt an meine Eltern Claudia und Peter Diethelm sowie meinen Bruder Stefan Diethelm für ihre Unterstützung und Ermutigung während dieser langen Zeit.