Year : 2017

REGULATION OF EFFECTOR VERSUS MEMORY CD8+ T CELL DIFFERENTIATION : THE ROLE OF WNT/TCF-1 SIGNALING

DANILO Maxime

DANILO Maxime, 2017, REGULATION OF EFFECTOR VERSUS MEMORY CD8+ T CELL DIFFERENTIATION : THE ROLE OF WNT/TCF-1 SIGNALING

Originally published at : Thesis, University of Lausanne

Posted at the University of Lausanne Open Archive http://serval.unil.ch
Document URN : urn:nbn:ch:serval-BIB_B9327A7435364

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Département d'Oncologie fondamentale

REGULATION OF EFFECTOR VERSUS MEMORY CD8⁺ T CELL DIFFERENTIATION : THE ROLE OF WNT/TCF-1 SIGNALING

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

Maxime DANILO

Diplôme d'Ingénieur/Master de l'Université de Clermont-Ferrand, France

Jury

Prof. Margot Thome-Miazza, Présidente
Prof. Werner Held, Directeur de thèse
Prof. Fabienne Tacchini-Cottier, expert
Prof. Annette Oxenius, expert

Lausanne 2017

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Ecole Doctorale

Doctorat ès sciences de la vie

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Président·e	Madame	Prof. Margot **Thome Miazza**
Directeur·rice de thèse	Monsieur	Prof. Werner **Held**
Experts·es	Madame	Prof. Fabienne **Tacchini-Cottier**
	Madame	Prof. Annette **Oxenius**

le Conseil de Faculté autorise l'impression de la thèse de

Monsieur Maxime Danilo

Master en Génie Biologique de l' Université de Clermont-Ferrand, France

intitulée

REGULATION OF EFFECTOR VERSUS MEMORY CD8+ T CELL DIFFERENTIATION: THE ROLE OF WNT/TCF-1 SIGNALLING

Lausanne, le 7 avril 2017

pour le Doyen
de la Faculté de biologie et de médecine

Thome - Miazza

Prof. Margot Thome Miazza

Acknowledgments

First of all, I would like to express my special appreciation and thanks to my thesis director, Professor Werner Held. I would like to thank him for encouraging my research and for allowing me to grow as a research scientist. Your advice on my research have been priceless. Lastly, I thank Werner for sharing with me his thorough scientific knowledge throughout the years. It was a great experience overall and a pleasure having Werner's supervision over the years.

Secondly, I would like to thank Caroline, a former post-doctoral researcher in our lab. Caroline taught me most, if not all, the technics I am currently using. She also helped me to set-up my main project and helped me a lot at the beginning of my project to plan and run my experiments. She was also there to discuss results and to help me with analysis. I was also glad to have her company during lunches and on the day to day basis.

I also wish to acknowledge the support received from my PhD colleagues in our group (current and former): Mélanie, Beena, Daniela, Vijay, Imran, Jean-Gaël, Camille, Irene, Elisenda, Laurène, Georgi, Jasmine and Alexandre. Thank you for the stimulating discussions and all the fun we have had in the last four years. I thank especially the ones who helped me out when I had really long experiments.

I also need to thank all my friends with whom I shared so many moments during this PhD. Even though it was sometimes hard to come back to work after our week-ends and/or holydays, they helped me to clear my head during hard periods.

These acknowledgements would not be complete without giving thanks to my parents and my brother. They have always been supportive even when I took the decision to live pretty far away from them for my studies.

Last but certainly not least, I must acknowledge with tremendous and deep thanks my partner Marine. Through her love, patience, support and unwavering belief in me, I have been able to complete this long process. She has taken care of whatever I needed tending to without complaining, just so I could focus on completing my PhD. She has also given me so many happy and beautiful memories throughout this journey. I could not have completed this journey without her by my side.

Résumé large public

REGULATION DE LA DIFFERENTIATION DES LYMPHOCYTES T CD8$^+$ EN CELLULES EFFECTRICES ET MEMOIRES : LE ROLE DE LA VOIE DE SIGNALISATION WNT/TCF-1

L'étude du fonctionnement du système immunitaire permet de développer de nouveaux traitements contre les infections bactériennes et virales, mais aussi contre le cancer. Une des approches prometteuses consiste à renforcer ou induire une réponse immunitaire contre les cellules infectées ou tumorales. Les lymphocytes T sont les cellules immunitaires responsables de la mise en place d'une réponse immunitaire dirigée de manière spécifique contre les cellules infectées ou tumorales. Elles sont capables de tuer ces cellules via la reconnaissance d'antigènes infectieux ou tumoraux grâce à leur récepteur de surface (TCR). Les lymphocytes T peuvent être de type CD8$^+$ ou CD4$^+$. La variabilité de leur TCR leur permet de pouvoir reconnaître une multitude d'antigènes dérivés de protéines et présentés par des cellules spécialisées dans la présentation d'antigène appelées cellules dendritiques (DCs). L'interaction entre les lymphocytes T et les DCs entraîne la perte de l'état naïf des lymphocytes T et induit leur activation, ce qui confère aux lymphocytes T CD8$^+$ la capacité de tuer les cellules infectieuses ou tumorales présentant un antigène spécifique. Le processus de différentiation des lymphocytes T CD8$^+$ est un processus qui a été beaucoup étudié afin de développer des traitements favorisant le développement de lymphocytes T CD8$^+$ avec la capacité de tuer les cellules ciblées ou au contraire pour développer des vaccins favorisant le développement de lymphocytes T CD8$^+$ mémoires conférant une meilleure protection en cas de réinfection.

Le but de mon travail de doctorat a été de caractériser *in vivo* la réponse des lymphocytes T CD8$^+$ en utilisant un modèle basé sur les DCs. Ce système permet de simuler une infection tout en maîtrisant certains paramètres de « l'infection », telle que la présence ou non d'inflammation. En utilisant ce modèle nous avons montré que l'expression de Tcf-1, au pic de la réponse des lymphocytes T dans les lymphocytes T CD8$^+$ était réduite lorsque l'infection avait lieu en présence d'inflammation. Tcf-1 est une protéine impliquée dans la voie de signalisation Wnt. Nous avons aussi pu montrer que l'expression de Tcf-1 bloquait la différentiation des lymphocytes T CD8$^+$ et que la réduction de son expression se produisait via la cytokine IL-12 qui induit l'expression de STAT4. Ainsi, en inhibant l'expression de STAT4 pendant le processus de différentiation des lymphocytes T CD8$^+$ suite à l'administration d'un vaccin, il serait théoriquement possible d'obtenir plus de lymphocytes T mémoires, conférant ainsi une protection supérieure dans le cas d'une réinfection.

Parallèlement, nous avons aussi essayé de déterminer ce qui maintenait l'expression de Tcf-1 dans les lymphocytes T CD8$^+$ naïfs. Nous avons ainsi bloqué la sécrétion de toutes les protéines appartenant à la famille des protéines Wnt. Ces protéines sont responsables de l'activation de la voie de signalisation Wnt, ce qui induit l'expression de Tcf-1. Malheureusement, en supprimant la sécrétion de ces protéines dans plusieurs populations cellulaires, nous n'avons pas pu déterminer si ces protéines étaient réellement responsables de l'expression de Tcf-1 dans les lymphocytes T CD8$^+$ naïfs.

En conclusion, mon travail de doctorat a démontré le rôle de la protéine Tcf-1 dans la différentiation des lymphocytes T CD8$^+$.

Résumé

Maxime DANILO **Department of fundamental Oncology**

REGULATION DE LA DIFFERENTIATION DES LYMPHOCYTES T CD8$^+$ EN CELLULES EFFECTRICES ET MEMOIRES : LE ROLE DE LA VOIE DE SIGNALISATION WNT/TCF-1

L'établissement de nouvelles stratégies thérapeutiques est nécessaire pour contrôler et guérir certaines maladies, telles que les infections chroniques ou le cancer. Une approche prometteuse consiste à augmenter la réponse des lymphocytes T CD8$^+$. Cependant, pour cela, une meilleure compréhension des réponses immunitaires conférant une protection contre une potentielle réinfection par le même pathogène est nécessaire. La voie de signalisation Wnt/Tcf-1 dans les lymphocytes T CD8$^+$ spécifiques pour l'antigène est nécessaire pour la formation de lymphocytes T CD8$^+$ mémoires fonctionnelles. Cette thèse a donc pour but d'étudier comment la voie de signalisation Wnt/Tcf-1 est contrôlée dans les lymphocytes T CD8$^+$ naïves, spécifiques pour l'antigène, mais aussi au cours d'une réaction immunitaire primaire suite à une infection.

Dans la première partie de cette thèse, nous avons établi que l'inflammation systémique était responsable de la régulation négative de la voie de signalisation Wnt/Tcf-1 dans les lymphocytes T CD8$^+$ spécifiques pour l'antigène. Nous avons ensuite montré que l'IL-12 joue un rôle clé et que cette cytokine agit via la protéine STAT4 dans les lymphocytes T CD8$^+$ naïves spécifiques pour l'antigène. Nous avons aussi établi que l'expression du facteur de transcription Tcf-1 empêchait la différentiation des lymphocytes T CD8$^+$ et que la répression de Tcf-1 induite par l'inflammation est importante pour la différentiation des cellules T CD8$^+$ effectrices. En effet, l'absence de Tcf-1 aboutit à la différentiation terminale rapide des lymphocytes T CD8$^+$ et à un défaut dans la formation des lymphocytes mémoires.

Dans un second temps, nous avons vérifié si l'activité de la voie de signalisation Wnt/Tcf-1 dans les lymphocytes T CD8$^+$ naïfs était maintenue par la sécrétion des protéines Wnt. Pour vérifier cette hypothèse, nous avons analysé des souris n'exprimant pas Wntless (Wls) qui a été décrite comme étant une protéine requise pour la sécrétion de toutes les protéines Wnt. Cependant, la délétion de Wls dans les lymphocytes T, le système hématopoïétique ou les cellules stromales des organes lymphoïdes secondaires, n'a pas permis de mettre en évidence que la sécrétion des protéines Wnt permet la maintenance l'activité de la voie de signalisation Wnt/Tcf-1 dans les lymphocytes T CD8$^+$ naïfs.

Ensemble, ces résultats indiquent que l'extinction de la voie de signalisation Wnt/Tcf-1 est importante pour la différentiation des cellules effectrices, au détriment de la différentiation en cellules T CD8$^+$ mémoires. La prévention de la régulation négative de la voie de signalisation Wnt/Tcf-1 par la voie IL-12/STAT4 dans les lymphocytes T CD8$^+$ pendant la vaccination, pourrait donc être une nouvelle approche pour améliorer la formation de cellules T CD8$^+$ mémoires.

Summary

Maxime DANILO **Department of fundamental Oncology**

REGULATION OF EFFECTOR VERSUS MEMORY CD8$^+$ T CELL DIFFERENTIATION: THE ROLE OF WNT/TCF-1 SIGNALING

The design of new therapeutic approaches is needed to control and cure certain diseases such as chronic infections or cancer. One promising approach is to promote CD8$^+$ T cell responses. However, to do so, a better understanding of naturally occurring protective immune responses will be needed. These latter responses are able to induce life-long immune protection from re-infections with the same pathogen. Since Wnt/T cell factor-1 (Tcf-1) signaling in antigen-specific CD8$^+$ T cells is required for the formation of functional CD8$^+$ T cell memory, this thesis addressed how Wnt/Tcf-1 signaling was controlled in antigen-specific naïve CD8$^+$ T cells and during a primary immune response.

In the first part of this thesis, we established that systemic inflammation was responsible for the downregulation of Wnt/Tcf-1 signaling in antigen-specific CD8$^+$ T cells. We further found that interleukin-12 (IL-12) played a key role and that this cytokine acted via STAT4 signaling in antigen-specific CD8$^+$ T cells. We also established that the expression of the transcription factor Tcf-1 normally prevented CD8$^+$ T cell effector differentiation and that inflammation-induced Tcf-1 repression was important for effector differentiation. Indeed, the absence of Tcf-1 resulted in the rapid terminal differentiation of CD8$^+$ T cells and a concomitant the defect in memory formation.

In the second part of this thesis, we addressed whether Wnt/Tcf-1 signaling in naïve CD8$^+$ T cells was maintained via the secretion of Wnt proteins. To address this hypothesis, we analyzed mice lacking Wntless (Wls) which is reportedly required for the secretion of all Wnt proteins. However, Wls deletion in T cells, in all hematopoietic cells or in stromal cells of secondary lymphoid organs did not provide evidence for a role of Wnt protein secretion in the maintenance of Wnt/Tcf-1 pathway activity in naïve CD8$^+$ T cells.

Together, these data indicate that the shutdown of Wnt/Tcf-1 is important for effector differentiation but detrimental for memory CD8$^+$ T cells differentiation. Preventing IL-12/STAT4-mediated Tcf-1 downregulation in CD8$^+$ T cells during vaccination may thus be an approach to improve memory formation.

Abbreviations

Ag: Antigen

AMPK: Adenosine monophosphate-activated protein kinase

APC: Adenomatosis polyposis coli

APCs: Antigen presenting cells

APLs: Altered peptide ligands

B6: C57BL/6

Bcl6: B cell lymphoma 6

Blimp-1: B lymphocyte-induced maturation protein 1

βTrCP: β-transducin-repeat-containing protein

Cl13: Clone 13

CD40L: CD40 ligand

CFU: Colony forming unit

Ck1: Casein kinase 1

CpG: CpG dinucleotides

CTLA-4: Cytotoxic T-lymphocyte-associated protein 4

CTLs: Cytolytic $CD8^+$ T cells

DC: Dendritic cells

DC33: Bone marrow-derived DCs matured with LPS and pulsed with Gp_{33-41} peptide

DP: Double positive

Eomes: Eomesodermin

Fzd: Frizzled

GSK3β: Glycogen-synthase kinase 3 β

Gzm: Granzyme

H3K4me3: Trimethylation of lysine 4 of histone 3

HCV: Hepatitis C virus

HEV: High endothelial venule

HIV: Human immunodeficiency virus

Id2: Inhibitor of DNA binding 2

IFN: Interferon

IFNAR: Interferon α receptor

IL: Interleukin

IRF: Interferon regulatory factor

i.v.: intravenously

JAK2: Janus kinase 2

KLRG1: Killer cell lectin-like receptor G1

LacZ: Bacterial *β-galactosidase* gene

Lag3: Lymphocyte activation gene 3

LCMV: *Lymphocytic choriomeningitis virus*

Lef: Lymphoid enhancer binding factor

L.m.: *Listeria monocytogenes*

LPS: Lipopolysaccharide

LRPs: Lipoprotein receptor-related proteins

MAPK: Mitogen-activated protein kinase

MHC: Major molecular histocompatibility

MPECs: Memory-precursor effector cells

mTOR: Mammalian target of rapamycin

NK: Natural killer

OVA: Ovalbumin

PAMPs: Pathogen-associated molecular patterns

PD-1: Program cell-death 1

PI3K: Phosphoinositide 3 kinase

Poly(I:C): polyinosinic:polycytidylic acid

Porcn: Porcupine

PRRs: Pattern recognition receptors

SD: Standard deviation

SEM: Standard error of the mean

SLECs: Short-lived effector cells

SLOs: Secondary lymphoid organs

STAT4: Signal transducer and activator of transcription 4

T-bet: T-box transcription factor 21

Tcf-1: T-cell factor-1

Tcf-1 p45: Full length Tcf-1 isoform

T_{CM}: Central memory T cells

TCR: T cell receptor

T_{EM}: Effector memory T cells

T_{FH}: Follicular helper T cells

TILs: Tumour infiltrating lymphocytes

TNF: Tumour necrosis factor

T_{RM}: Tissue resident memory T cells

T_{SCM}: Stem cell-like $CD8^+$ T cells

TLRs: Toll-like receptors

TYK2: Tyrosine kinase 2

Wls: Wntless

Wnts: Wnt proteins

WT: Wild type

Table of contents

List of figures

List of tables

INTRODUCTION

The natural course of most viral and intracellular bacterial infections in mammals involve the generation of a protective T cell-mediated immune response, which rapidly and specifically eliminates pathogen-infected cells, and which protects individuals from future infections with the same pathogen. Examples of controlled viral and bacterial infections in humans include Influenza A and measles viruses or bacterial *Listeria monocytogenes* (*L.m.*) [14-16]. Mouse models used to characterize the $CD8^+$ T cell response to acute infections include the *Lymphocytic choriomeningitis virus* (LCMV) Armstrong or WE strains and *Listeria monocytogenes* [15, 17].

However, the immune system sometimes fails to protect from infection. Such instances include impairments or failures in protecting some individuals from Influenza virus infections or infections caused by certain pathogens such as human immunodeficiency virus (HIV) or the hepatitis C virus (HCV) [18-20]. T cell responses in the context of chronic infections are characterized using mouse models such as LCMV clone 13 infection [17]. A prerequisite for understanding why the immune system fails in controlling these infections is to have a clear picture of the molecular and cellular mechanisms that facilitate the generation of protective effector T cell responses and the formation of immune memory in infections that the immune system can control. Since our understanding of the complex mechanisms controlling T cell differentiation is still incomplete, the aim of this thesis is to gain further insights into these processes.

1.1 Innate immune response

The innate immune system provides the first line of immune defense against an infection. This system is relatively non-specific but acts rapidly upon exposure to infectious organisms [21]. Innate immune cells, such as granulocytes, macrophages and dendritic cells (DCs), natural killer (NK) cells and other innate lymphoid cells can recognize microorganisms and induce an innate immune response.

An innate immune response is induced via the engagement of pattern recognition receptors (PRR) expressed on innate immune cells. PRRs recognize specific conserved motifs in pathogens called pathogen-associated molecular patterns (PAMPs) [22, 23]. PAMPs are invariant and are essential pathogen components such as bacterial cell wall components including lipopolysaccharide (LPS), lipopeptides or flagellin but also viral nucleic acids such as bacterial DNA or viral double-stranded RNA.

PRRs include Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-leucine-rich repeat-containing receptors among others [24]. TLRs are transmembrane proteins which are evolutionarily conserved between insects and humans [25]. There exist more than ten TLRs (Table 1) which are expressed by immune cells, notably on dendritic cells and monocytes/macrophages. TRL1, TRL2, TLR4, TRL5, and TRL6 are specialized in the recognition of common components of bacterial cell walls such as LPS, a cell-wall component of Gram-negative bacteria. TLRs triggered in response to a bacterial infection activate NF-κB or the activator protein 1 transcription factors to induce the production of pro-inflammatory and immune stimulatory cytokines such as interleukin (IL)-1β , IL-6, IL-12, IL-18 and tumour necrosis factor α (TNFα) [26]. TLR3, TLR7, TLR8 and TLR9 are expressed in endosomes and allow the detection of viral and bacterial nucleic acids. TLR9 also recognizes bacterial DNA containing unmethylated CpG dinucleotides (CpG). Stimulation of TLR3, TLR7, TRL8 and TLR9 leads to the production of type I interferons (IFNs) via the family of interferon regulatory factor (IRF) transcription factors [22].

Receptor	Ligand	Origin	Localization
TLR1	Bacterial lipoproteins	Bacteria	Cell surface
TLR2	Peptidoglycan	Bacteria	Cell surface
	B. fragilis lipopolysaccharide	Bacteria	
	Heat-shock protein 70	Host	
	Zymosan	Fungi	
TLR3	Double-stranded RNA	Virus	Intracellular
TLR4	*E. coli* LPS	Bacteria	Cell surface
	Heat-shock protein 70	Host	
	Fibrinogen	Host	
TLR5	Flagellin	Bacteria	Cell surface
TLR6	Peptidoglycan	Bacteria	Cell surface
	Zymosan	Fungi	
TLR7	Signle-stranded RNA	Virus	Intracellular
TLR8	Single-stranded RNA	Virus	Intracellular
TLR9	Unmethylated CpG DNA	Bacteria/Virus	Intracellular
TLR10	Not determined		Cell surface
TLR11	Flagellin	Bacteria	Cell surface

TABLE 1 – Toll-like receptors and their typical ligands (adapted from Akira *et al.* [2] and Shah *et al.* [6]).

Tissue resident macrophages and DCs are the first cells to respond to infection by the production of inflammatory cytokines such as IL-1β , IL-6 and TNFα [26]. This leads to the induction of an inflammatory response at the site of infection, which results in the recruitment of additional innate immune cells such as neutrophils and monocytes. Depending on the cytokine milieu, monocytes differentiate into

macrophages or dendritic cells [27]. DCs link the innate immune system with the adaptive immune response [28]. They are the main antigen presenting cells (APCs) and the only immune cell type able to initiate an antigen (Ag)-specific adaptive immune response. Antigen presentation requires antigen uptake from the environment, protein degradation in the cytosol and peptide loading onto major molecular histocompatibility complex (MHC) class I and II molecules on the cell surface of DCs. MHC class I molecules present peptides that are derived from endogenous or from exogenous proteins via cross-presentation. MHC class II molecules acquire short peptides generated by the proteolytic degradation in endosomal compartments. The MHC class II binding peptides are mainly generated from exogenous antigens [29]. DCs are continuously taking up material from the environment. The engagement of TLRs stops further Ag uptake and induces DC maturation i.e. the expression of co-stimulatory ligands (such as CD80 or CD86) and the up-regulation of MHC class I and II molecules on their surface. In addition, it also induces the expression of CCR7 at the surface of DCs, which thus acquire the capacity to migrate to lymph nodes. This is based on CCL19 and CCL21 chemokines, which attract cells expressing CCR7 to lymph nodes. Further TLR engagement triggers the transcription of immune-stimulatory cytokines depending on the type of infection. On one hand, viral infection leads to the production of type I IFNs. On the other hand, bacterial infection leads to the production of IL-12 which is the main cytokine produced by DCs. In secondary lymphoid organs (SLOs), DCs have the unique ability to activate naïve T cells whose T cell receptor (TCR) is specific for peptide/MHC complexes expressed at the surface of DCs.

In order to mimic innate immune responses to infection, specific microbial components are used to stimulate innate immune responses. Agents increasing the antigenic response during vaccination are referred to as adjuvants. For example, CpG-B oligodeoxynucleotide, an unmethylated synthetic single-stranded DNA molecule, directly stimulates B cells, macrophages and DCs via TLR9. DCs are then able to secrete cytokines such as IL-12 and type I IFNs [30-32]. Similarly, polyinosinic:polycytidylic acid (poly(I:C)) and LPS induce the production of type I IFNs and IL-12 via their interaction with TRL3 and TLR4 respectively [33, 34]. Human vaccination is also improved by the addition of microbial components including CpG-B or monophosphoryl lipid A which have also been shown to induce cytokines such as IL-12 and type I IFNs [35, 36].

1.2 Adaptive immune response

Following their development in the thymus and export to the periphery, naïve $CD4^+$ and $CD8^+$ T cells are maintained by IL-7 signaling and interactions of their TCR with self-peptide/MHC complexes [37, 38]. Naïve T cells will permanently circulate between SLOs, the blood and the lymph. Naïve T cells are attracted to LN via the chemokine receptor CCR7. The ligands of CCR7, the chemokines CCL19 and CCL21, are produced by stromal cells in the lymph nodes and attract naïve T cells as well as matured DCs via a gradient of these chemokines. Naïve T cells can enter LNs based on the expression of L-selectin (CD62L) which functions as a homing receptor and allows their entrance via the high endothelial venules (HEV) [39]. Once inside the LNs, T cells reside in the T cell zones while scanning APCs for cognate peptide/MHC complexes (Fig. 1).

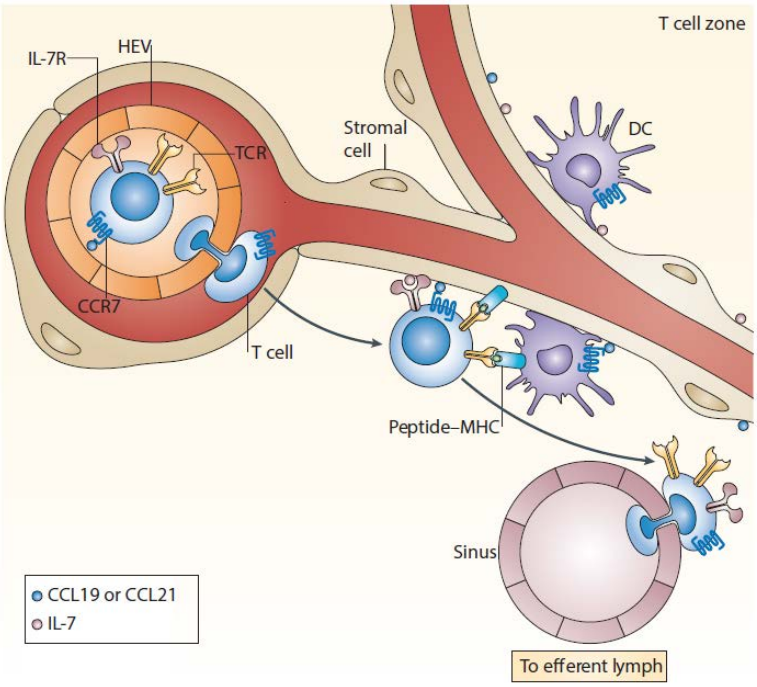

FIGURE 1 - Migration and activation of naïve T cells in lymph nodes (adapted from 2009 Takada and Jameson [8]). Naïve T cells enter into lymph nodes via the high endothelial venule (HEV) being attracted by chemokines, CCL19 and CCL21, produced by stromal cells, via their CCR7 receptor. They then get activated by APCs via the interaction between TCR and peptide/MHC complexes.

The recognition of antigen (i.e. peptide/MHC complexes) expressed by DCs by the very rare T cells expressing the correct T cell receptor leads to the activation, proliferation and differentiation of T lymphocytes. $CD4^+$ T cells differentiate into different subtypes of T helper (T_H) cells, depending on the type of infection and the ensuing cytokine milieu. Viral and bacterial infections, that are characterized by type I IFN or IL-12, mainly induce a T_H1 response which is characterized by the production of IFNγ

by $CD4^+$ T cells. While T_H1 cells have direct antiviral functions, T_H cells also provide help to cytotoxic $CD8^+$ T cells. The helper function depends on their production of IL-2 [40] which, together with other signals improves $CD8^+$ T cell expansion and differentiation into effector cells [41]. Moreover, T_H1 cells express CD40, which can interact with CD40 ligand (CD40L) expressed by DCs. This interaction licenses DCs to stimulate $CD8^+$ T cells via their TCR and co-stimulatory signals (CD27-CD70) [42-44]. Another subset of $CD4^+$ T cells, follicular helper (T_{FH}) cells, are located in B cell follicles of SLOs and trigger the formation of germinal centers through the expression of CD40L and the secretion of IL-21 and IL-4 [45, 46]. By interacting with B cells, T_{FH} cells help B cell to survive and to differentiate into plasma cells able to produce Ag-specific antibodies. They also promote antibody class switch recombination and somatic hypermutation, which helps antibodies to acquire distinct effector functions and improves their affinity for antigen, respectively [47].

$CD8^+$ T cell response to acute infection

Similar to $CD4^+$ T cells, the frequency of $CD8^+$ T cells expressing a TCR specific for a given antigen is very low, ranging between 1 to 100 per million of $CD8^+$ T cells [48]. Following the encounter of peptide/MHC class I complex, specific naïve $CD8^+$ T cells expand massively. A single naïve T cell can undergo more than 15 consecutive divisions and can give rise to more than 30'000 daughter T cells [49, 50]. During the expansion phase, naïve antigen-specific $CD8^+$ T cells differentiate and acquire effector functions. $CD8^+$ T cells acquire the capacity to secrete cytokines such as IFN-γ, TNF-α and TNF-β. IFN-γ can directly inhibit viral replication and induce an increased expression of MHC class I and other molecules involved in antigen presentation in infected cells. This increases target cell recognition by $CD8^+$ T cells. IFN-γ can also activate macrophages. TNF-α and TNF-β can act synergistically with IFN-γ in the process of macrophage activation but they can also kill targets through binding to TNF-R [51].

The main function of effector $CD8^+$ T cells is to kill infected cells by inducing programmed cell death (apoptosis). Upon recognition of antigen on the surface of a target cell, cytolytic $CD8^+$ T cells (CTLs) release specialized lytic granules. Lytic granules release perforin, which can polymerize and create transmembrane pores in target cell membranes and allow the entry of proteases called Granzymes. Granzymes (Gzm) induce apoptosis by inducing programmed caspase-dependent or -independent cell death [52]. In addition, effector $CD8^+$ T cells can kill target cells using the Fas pathway. Indeed, effector

$CD8^+$ T cells (and T_H1 cells) express Fas ligand, which can trigger apoptosis of cells expressing Fas [53].

Most effector cells, representing 90-95% of the $CD8^+$ T cell population, are relatively short-lived as they die after the clearance of the pathogen. They are called short-lived effector cells (SLECs). SLECs express low levels of CD127 (IL-7 receptor subunit-α (IL-7Rα)) and high levels of killer cell lectin-like receptor G1 (KLRG1). However, not all cells with a SLEC phenotype are short lived and cells with a SLEC phenotype are not observed in all infections (e.g. influenza) [54]. Other effector cells display a memory-precursor effector (MPECs) phenotype i.e. they express high levels of CD127 and low levels of KLRG1. Functionally, SLECs produce high amounts of GzmB but low levels of IL-2 whereas MPECs produce lower levels of GzmB and relatively high levels of IL-2. MPECs give rise predominantly to a long-lived population of memory cells that is stably maintained in the absence of antigen [55] (Fig. 2). Memory cells are maintained in the absence of tonic TCR signals but depend on both IL-15 and IL-7 for self-renewal and survival [37, 38].

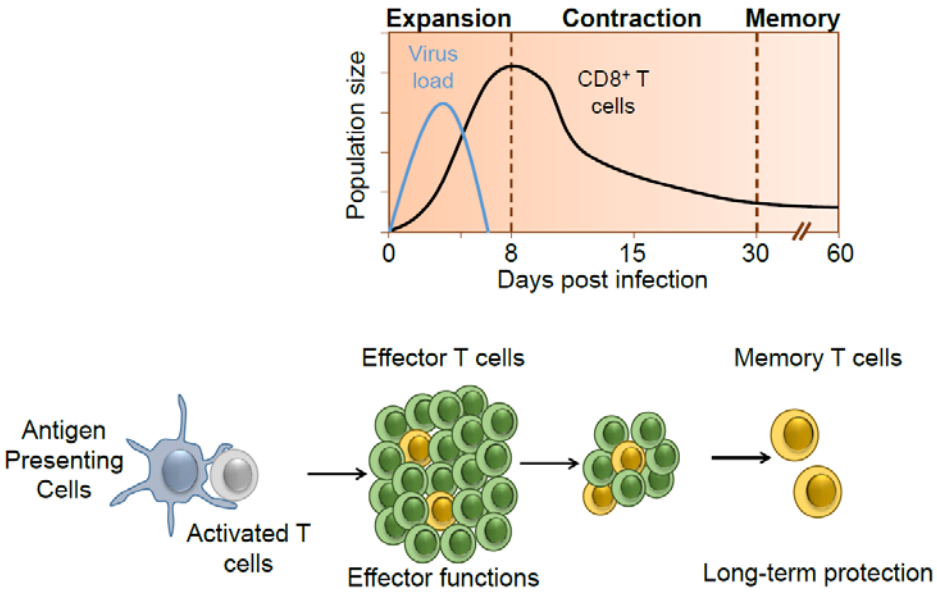

FIGURE 2 – Kinetics of T cell-response and distribution of memory cell potential (adapted from 2012 Kaech and Cui [11]). During an acute viral infection, antigen-specific T cells get activated by antigen presenting cells and rapidly proliferate (during the expansion phase) and differentiate into cytotoxic T lymphocytes that mediate viral clearance. Most of these cells die over the next several weeks during the contraction phase. Only a small percentage of effector T cells (5-10%) survive and further develop into functional mature memory $CD8^+$ T cells. Not all effector T cells have an equal potential to form memory T cells. Some cell surface markers correlate with distinct effector and memory T cell fates: terminal effector T cells (shown in green) are $KLRG1^{hi}$ $CD127^{low}$ and have effector functions and long-lived memory (and memory precursor) cells (shown in yellow) are $KLRG1^{low}$ $CD127^{hi}$ confer the long-term protection. However, other T cell subsets, with intermediate differentiation states, also exist that have mixed phenotypes, longevities and abilities to self-renew.

Similar to effector cells, the memory $CD8^+$ T cell pool is heterogeneous. This heterogeneity is based on differences in migration capacity, self-renewal and recall potential [56]. The first memory $CD8^+$ T cell subpopulations characterized were the effector memory $CD8^+$ T cells (T_{EM} cells) and the central memory $CD8^+$ T cells (T_{CM} cells) by Sallusto and colleagues [57]. T_{EM} cells express high levels of CD127 and CD44 (activation marker) and low levels of CD62L. Absence of CD62L limits their ability to migrate to LNs. T_{EM} cells recirculate and can migrate to peripheral tissues such as the lungs and intestine [58]. They have limited re-expansion potential but have readily available effector functions such as cytotoxicity [11]. On the other hand, T_{CM} express CD62L together with CD127 and CD44. They can self-renew and are thought to be the source of T_{EM} [56]. In addition, as compared to T_{EM}, T_{CM} have a greater capacity to produce IL-2 and to expand following a second encounter with the same antigen [59, 60]. During re-stimulation, they generate both secondary memory cells as well as effector cells.

An additional type of memory $CD8^+$ T cells is characterized by their presence in non-hematopoietic tissues and their failure to recirculate. These tissue resident memory cells (T_{RM} cells) express CD69 (early activation marker) and CD103. CD103 is also known as αEβ7 integrin which is important for T cell homing to the intestinal sites [61]. They are located in mucosal tissues such as lungs, skin or intestines and provide direct local protection upon secondary infection of the tissue of residence and recruit other immune cells in an antigen non-specific fashion [62].

An additional memory $CD8^+$ T cell population with a naïve-like phenotype has been described [63]. These cells express high levels of CD62L and low levels of CD44, similarly to naïve $CD8^+$ T cells but they also express high levels of CD122 (a component of IL-15 receptor) [63, 64]. One marker which permits to discriminate T_{CM} from T_{SCM} is CD45RA in human which was originally known as leukocyte common antigen. Indeed, T_{SCM} express CD45RA whereas T_{CM} do not [65]. These cells undergo homeostatic proliferation in response to IL-7 and IL-15. This subset of memory $CD8^+$ T cells has been termed stem cell-like $CD8^+$ T cells (T_{SCM}). It has the potential to give rise to multiple memory subsets and effector cells.

Thus a productive immune response to acute infection generates diversity of antigen-specific effector and memory cells that allows rapid pathogen control as well as the long-term protection from re-infection.

Memory differentiation is programmed during the primary immune response to infection [66] i.e. dependent on the signals perceived during primary stimulation of naïve T cells.

1.2.1 CD8⁺ T cell response to chronic infection and cancer

Similar to acute infections, chronic infections are accompanied by strong CD8⁺ T cell responses but CD8⁺ T cell differentiation is profoundly altered. These CD8⁺ T cells show strong impairments in effector properties such as reduced ability to produce cytokines (IFN-γ, TNFα and IL-2) together with an increased expression of inhibitory receptors such as program cell death -1 (PD-1) and lymphocyte activation gene 3 (Lag3) [67]. They are also characterized by a reduced proliferative potential (Fig. 3). These cells are commonly referred to as “exhausted” cells [67]. While T cells in chronic infections fail to control viral infection, T cell exhaustion limits T cell mediated immunopathology [13]. It has been thought that exhausted cells are terminally differentiated and that chronic infection prevents the formation of memory [67]. However, exhausted CD8⁺ T cells expand when the inhibitory PD-1 signaling is blocked [68]. Also, transfer of exhausted cells into naïve recipients and re-infection leads to a secondary population expansion [69] suggesting that not all exhausted cells are terminally differentiated.

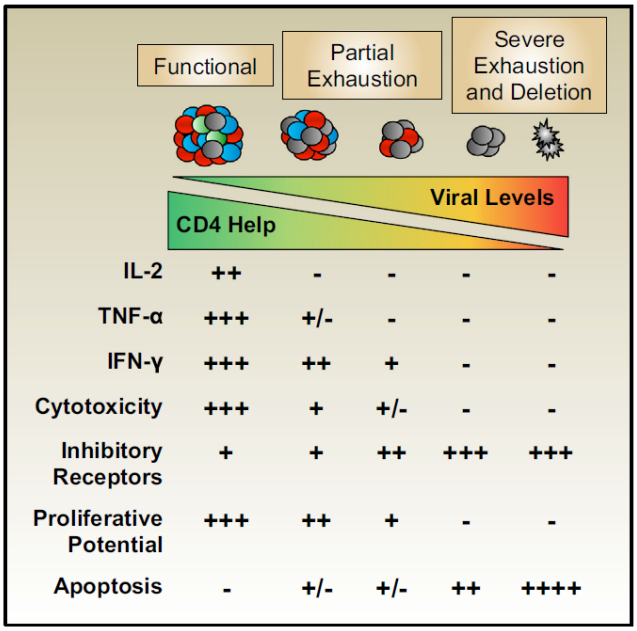

FIGURE 3 – CD8⁺ T cells can adopt a spectrum of exhausted states (2015 Kahan, Wherry and Zajac [13]). CD8⁺ T cell exhaustion is characterized by the step-wise and progressive loss of effector capabilities, the sustained upregulation of inhibitory receptors, and the loss of self-renewal abilities, which compromise viral control. Severely exhausted T cells may undergo apoptosis and become deleted from the chronically infected host.

Recently, a small subpopulation of virus-specific CD8⁺ T cells, which sustains the T cell response during chronic infections, has been identified by us [70] and Im and colleagues [71]. Interestingly, this

subpopulation has characteristics of central memory cells combined with an exhausted phenotype. This population thus qualifies a memory-like $CD8^+$ T cell population in chronic infection. Importantly, this population is needed for T cell expansion in response to inhibitory receptor blockade. These data highlight that the immune response to acute and chronic infection may be governed by similar principles i.e. both generate less differentiated memory(-like) cells and more differentiated effector(-like) cells.

Tumour immune responses

It is now well established that the adaptive immune system can fight against cancer. Patient samples and mouse models revealed a correlation between the abundance of tumour infiltrating lymphocytes (TILs) at the site of tumour and overall prognosis [72]. High levels of $CD8^+$ T cells in TILs are often correlated with a better prognosis in several tumour types including colorectal and ovarian cancers [73, 74]. $CD8^+$ T cells thus seem to play a protective role against cancer development. Indeed, most tumour cells express antigens which can be recognized by $CD8^+$ T cells [75].

However, there are multiple mechanisms that limit the efficacy of $CD8^+$ T cells. Tumours may resist the T cell response by inhibiting the immune cells or by simply blocking entry of immune cells into the tumour. Similar to chronic infections, tumour-specific $CD8^+$ T cells have a reduced ability to secrete effector cytokines and to kill due to the expression of inhibitory receptors such as PD-1 [76]. Antibodies blocking inhibitory receptors, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or PD-1, have shown great effects as therapeutic approaches to treat cancer. It will be important to test whether these responses are based on the presence of memory-like $CD8^+$ T cells.

The above considerations show that T cells can under certain circumstances control chronic infections and cancer. Approaches to induce protective T cell responses (either therapeutically or prophylactically) may thus protect from disease. However, current vaccine approaches are relatively ineffective at inducing T cell responses. This suggests that our understanding of the mechanisms that induce protective T cell responses and memory is still incomplete and needs to be further improved.

1.2.2 Regulation of CD8$^+$ T cell activation and differentiation

Signals necessary for CD8$^+$ T cell differentiation and activation

The magnitude and the quality of the primary CD8$^+$ T cell response to infection depends on several types of signals received by CD8$^+$ T cells.

The recognition of peptide/MHC complex by TCR, which represents signal 1 [77], is sufficient for T cell activation and expansion. However, the resulting T cells are anergic, i.e. they are dysfunctional but they remain alive in a hyporesponsive state [78]. This is one of the major mechanisms for T cell tolerance to antigens [79, 80]. In order to circumvent anergy induction, signal 1 needs to be combined with co-stimulatory signals (signal 2). In addition to ensure functionality, co-stimulation also enhances the survival of activated T cells and thus the magnitude of expansion.

Co-stimulation can be provided by multiple receptor ligand interactions including CD28 with CD80 (B7.1) or CD86 (B7.2). CD40/CD40L has been shown to indirectly contribute co-stimulatory signals to activated CD8$^+$ T cells [42, 81]. Indeed, CD40L is expressed on T_H cells and can bind to CD40 expressed on DCs. This provokes CD70 upregulation on DCs which binds CD27 on CD8$^+$ T cells and improves IL-2 production by CD8$^+$ T cells [82]. Other molecules can also enhance T cell activation, such as ICOSL, expressed at the surface of APCs, which can interact with ICOS on T cells [83]. In contrast to CD28, which is constitutively expressed by naïve CD8$^+$ T cells, ICOS is upregulated upon T cell priming [83].

Inflammatory signals (signal 3) have been extensively studied for their role in effector differentiation. While CD8$^+$ T cells receiving TCR and co-stimulatory signals are activated and proliferate, the acquisition of effector functions is suboptimal [84]. Inflammatory signals mediated by IL-12 and/or type I IFN (IFNα/β) [85, 86] represent a third type of signal that promotes the efficient acquisition of effector functions, which further increases clonal expansion but which also modulate effector versus memory cell fate decisions [87]. IL-12 and type I IFNs are mainly secreted by mature DCs [88] and plasmacytoid dendritic cells respectively [89].

Thus the magnitude and the quality of the primary CD8$^+$ T cell response to infection is determined by signals generated by antigen recognition, co-stimulation as well as the cytokine milieu.

Inflammatory cytokines promote CD8⁺ T cell differentiation

Mouse models of acute infection such as *L.m.* and LCMV have been extensively used to characterize CD8⁺ T cell response to infection. They helped to identify IL-12, mainly produced during *L.m.* infection and type I IFNs induced during LCMV infection [90-92] as the main inflammatory cytokines involved in CD8⁺ T cell differentiation. Inflammatory cytokines induce or repress the expression of specific transcription factors involved in the process of effector and memory CD8⁺ T cell differentiation. Interestingly, it seems that most transcription factors function in pairs that maintain a balance between memory differentiation and terminal differentiation.

Several studies showed that IL-12 can induce SLEC differentiation by acting directly on CD8⁺ T cells [93, 94] but IL-12 also regulates memory CD8⁺ T cell differentiation [95]. IL-12 receptor is composed of IL-12 receptor β1 (IL-12Rβ1), which is associated with tyrosine kinase 2 (TYK2) and IL-12Rβ2, which is associated with Janus kinase 2 (JAK2). IL-12 induces the phosphorylation of signal transducer and activator of transcription 4 (STAT4) via JAK2 which then allows the translocation of STAT4 into the nucleus where it can bind to the promoter site of target genes [1, 96] (Fig. 4). Well established target genes of the pathway are IFNγ, IL-12Rβ2 and IL-18Rβ1 which are involved in the amplification of IL-12

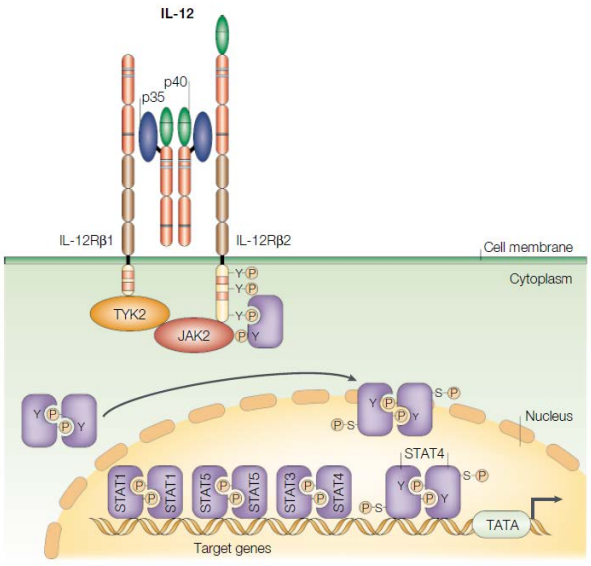

FIGURE 4 – IL-12 receptor and signal transduction (2003, Trinchieri [1]). The IL-12 receptor is composed of two chains, IL-12Rβ1 and IL-12Rβ2. Co-expression of both chains is necessary for the generation of high affinity IL-12-binding sites. The IL-12Rβ2 subunit functions as the signal transducing component. Signal transduction through IL-12R induces phosphorylation of JAK2 and TYK2 which in turn phosphorylate and activate STAT4. This then allow the transcription of IL-12 target genes.

signaling and Th1 differentiation [97-100]. Moreover, IL-12 also promotes expression of IL-2 receptor α (CD25) thereby enhancing T cell proliferation [101].

Like IL-12, type I IFNs have also been shown to promote $CD8^+$ T cell differentiation [102]. Interferons form a large family of cytokines with important biological roles like antiviral, antiproliferative, antitumor and immunomodulatory effects [103, 104]. IFNα and IFNβ bind to the type I IFN receptor formed by two subunits, interferon α receptor 1 (IFNAR1) and 2 (IFNAR2). IFNAR activates JAK1 and TYK2, which then activate STAT 1 and 2 proteins. The nuclear translocation of STAT1 and STAT2 induce expression of certain genes such as *IRF7* or interferon-stimulated genes that have antiviral functions [3, 105, 106] (Fig. 5). Type I IFNs can also provide a third signal to $CD8^+$ T cell via the activation of STAT4 in order to stimulate survival, cytolytic function and production of IFNγ [92, 107].

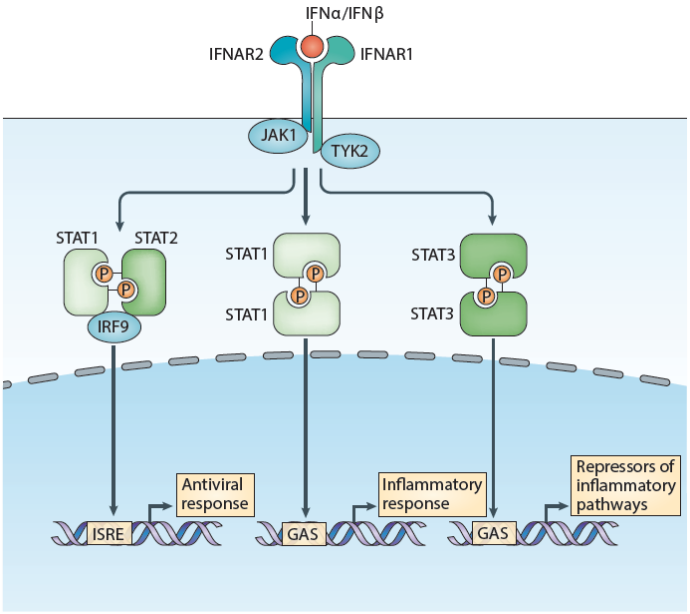

FIGURE 5 – The type I interferon signaling pathway (adapted from 2014, Ivashkiv L. B. and Donlin L. T. [3]). On engagement, the interferon-α receptor, composed of IFNAR1 and IFNAR2 subunits, activates Janus kinase 1 (JAK1) and tyrosine kinase 2 (Tyk2). Phosphorylation of the receptor by these kinases results in the recruitment of signal transducer and activator of transcription (STATs) proteins, phosphorylation, dimerization and nuclear translocation of STATs. The complex composed of STAT1, STAT2 and IFN-regulatory factor 9 (IRF9) binds to IFN-stimulated response elements (ISRE) to activate classical antiviral genes, whereas STAT1 homodimers bind to gamma-activated sequences (GASs) to induce pro-inflammatory genes. STAT3 homodimers indirectly suppress pro-inflammatory gene expression.

In addition to the well characterized IL-12 and type I IFNs, IL-2 also impacts $CD8^+$ T cell differentiation. Indeed, high IL-2 levels promote effector differentiation, while low IL-2 signaling promotes the development of memory $CD8^+$ T cells [59]. Regarding the latter, IL-2 secreted by antigen-specific $CD8^+$ T cells themselves plays a major role. IL-2 signaling is mediated by the IL-2 receptor complex formed by the IL-2Rα (CD25), IL-2Rβ (CD122) and IL-2Rγ (common γ chain) subunits. While IL-2Rβ

and IL-2Rγ chains can bind IL-2 and transduce signals, the IL-2Rα (CD25) increases the affinity for IL-2 [108]. The IL-2 receptor leads to the activation of several different signaling pathways including the JAK1/3-STAT5 pathway, the phosphoinositide 3-kinase (PI3K)-AKT pathway and the mitogen-activated protein kinase (MAPK) pathway. These different pathways induce the transcription of IL-2 target genes such as CD25 itself (Fig. 6).

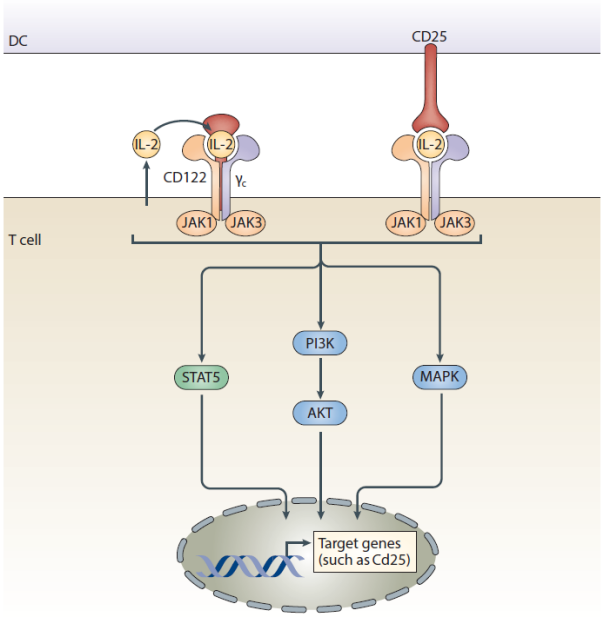

FIGURE 6 – The IL-2 receptor and signaling (adapted from 2012, Boyman and Sprent [9]). IL-2 is secreted as a soluble molecule by activated T cells or dendritic cells (DCs). IL-2 binds to CD25 (IL-2Rα) which then allows the recruitment of CD122 (IL-2Rβ) and the common γ chain (γ_c). IL-2 can also bind to the CD122/γ_c complex directly but with a lower affinity for its receptor in absence of CD25. Once IL-2 binds to its receptor, it induces the transcription of IL-2 target genes such as CD25 through several signaling pathways such as JAK-STAT, phosphoinositide 3-kinase-AKT pathway and the mitogen-activated protein kinase (MAPK) pathway.

Finally, IL-21, a cytokine related to IL-2, has also been shown to enhance the activation and clonal expansion of $CD8^+$ T cells but also to promote their survival in a context of tumor development [109] and during chronic viral infections [110], while IL-21 can also sustain memory $CD8^+$ T cell development [111], possibly via its immunosuppressive role by inducing the production of IL-10, an immunosuppressive cytokine [112-114]. On the other hand, it can also promote the terminal differentiation of $CD8^+$ T cells by promoting the expression of the transcription factor T-bet [115]. So similar to IL-2, IL-21 has a dual role. IL-21 is produced by $CD4^+$ T cells. It binds to its high affinity receptor formed by the IL-21R and the common γ chain. It activates JAK1 and JAK3 which will lead to the activation of STAT1 and STAT3 which in turn activate the transcription of GzmB, Eomes or Bcl6. IL-21 can also trigger the PI3K/AKT and the MAPK pathways [116] (Fig. 7).

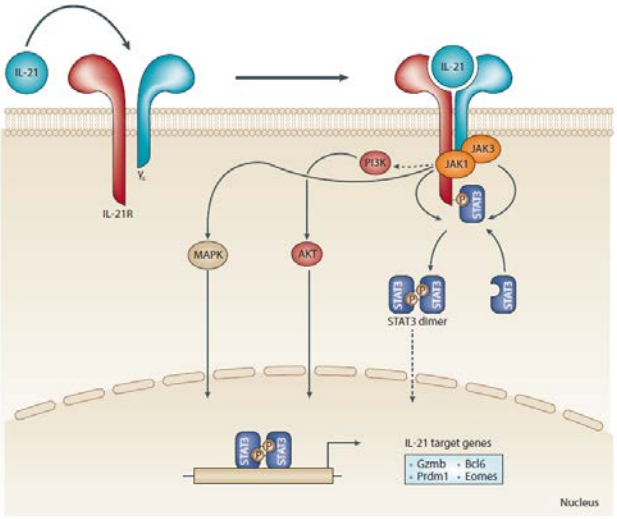

FIGURE 7 – IL-21 signals through IL-21R and utilizes the JAK-STAT, MAPK and PI3K pathways (adapted from 2014, Spolski and Leonard [12]). IL-21 binding stabilizes the complex between the IL-21 receptor (IL-21R) and the common cytokine-γ chain, γ_c. This leads to the activation of Janus kinase 1 (JAK1) and JAK3, which allows the recruitment and phosphorylation of signal transducer and activator of transcription (STAT) proteins (predominantly STAT3, but also STAT1 and STAT5). These STAT proteins dimerize, enter the nucleus and activate a transcription program that includes some of the target gene such as *Prdm1*, *GzmB* or *Eomes*. IL-21 binding can also activate the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways.

Several cytokines including IL-12 and IL-21 can induce T-bet expression. T-bet and Eomes are two T-box transcription factors playing important roles in the differentiation of $CD8^+$ T cells into effector and memory $CD8^+$ T cells [117]. Early during $CD8^+$ T cell activation, T-bet and Eomes induce terminal differentiation of $CD8^+$ T cells by inducing the expression of IFNγ, GzmB and perforin [114]. T-bet is induced by the combination of TCR signaling and IL-12 signaling while Eomes is mainly amplified by IL-2 and repressed by IL-12 [114, 118]. At the memory stage, T-bet and Eomes sustain the homeostatic proliferation of memory cells by inducing the expression of IL-2Rβ, which allows IL-15 signaling [119]. Based on the analysis of T-bet and Eomes-deficient $CD8^+$ T cells, T-bet induces the terminal differentiation of $CD8^+$ T cells whereas Eomes promotes central memory formation [117, 120].

In a similar manner, IL-12 and IL-2 promote the expression of Blimp-1 which promotes terminal differentiation by inducing effector functions such as GzmB and IFNγ expression [121]. But over the time following the clearance of an infection, Blimp-1 expression decreases during memory development [122]. Bcl6 is an antagonist of Blimp-1 activity and its expression inversely correlates with Blimp-1 expression in effector and memory $CD8^+$ T cells, meaning that Bcl6 is highly expressed in central memory $CD8^+$ T cells [123]. Thus Blimp-1 and Bcl6 are a pair of transcription factors with antagonistic effects on cell fate decisions.

Another set of transcription factors regulated by IL-12 are Id2 and Id3. IL-12 promotes the expression of Id2 and represses Id3 expression [124]. Id2 and Id3 are both expressed in effector $CD8^+$ T cells. Id2 is involved in the survival of naïve $CD8^+$ T cells differentiating in terminally differentiated cells [125] whereas Id3 mostly supports the survival of cells differentiating in memory $CD8^+$ T cells [126]. Moreover, Id3 can also be used to identify effector $CD8^+$ T cells starting to acquire a memory-precursor genetic signature [124].

Finally, Tcf-1 is also involved in the $CD8^+$ T cell differentiation process. Contrary to other transcription factors previously described, Tcf-1 is highly expressed in naïve $CD8^+$ T cells, downregulated at the effector stage of the $CD8^+$ T cell response against infection and highly expressed again in memory $CD8^+$ T cells [127]. It has been shown that Tcf-1 was necessary for the formation of functional memory $CD8^+$ T cells able to re-expand upon re-infection [128-130]. The regulation of Tcf-1 expression during T cell differentiation has not been characterized. During T cell development, Tcf-1 can be activated by Notch 1 binding to the *Tcf7* locus [131]

Epigenetic modifications influence $CD8^+$ T cell differentiation

Transient changes in transcription factor expression lead to permanent changes in gene expression patterns and cell fate. The latter is based on epigenetic changes. DNA and histone modifications are the most commonly studied epigenetic changes.

The chromatin structure is dynamic and differs from one gene to another depending on the state of activation of genes. Indeed, there are two basic states of chromatin. It can be in an open state which makes it accessible to DNA-binding proteins such as transcription factors or transcriptional activators or repressors, thus facilitating the transcription. On the other hand, the chromatin can be in a closed state called heterochromatin, which lacks accessibility for the transcriptional machinery and is associated with

gene repression [10]. Several epigenetic markers have been identified and characterized by associating the epigenetic modifications with the chromatin state and the transcription state (Table 2).

Target	Modification	Nucleotide or amino acid	Residue position	Chromatin state	Transcription state
DNA	Methylation	Cytosine (C)	CpG islands	Closed	Repressed
Histones	Acetylation	Lysine (K)	H2AK5, H2BK12, H2BK15, H3K9, H3K14, H3K18, H3K56, H4K5, H4K8, H4K13, H4K16	Open	Active
	Methylation	Arginine (R)	H3R17, H3R23, H4R3	Open	Active
	Methylation	Lysine (K)	H3K4, H3K36, H3K79	Open	Active
			H3K9, H3K27, H4K20	Closed	Repressed
	Phosphorylation	Serine (S) or threonine (T)	H3T3, H3S10, H3S28, H2BS14	Open	Active
	Sumoylation	Lysine (K)	H2AK126, H2BK6, H2BK7	Closed	Repressed
	Ubiquitylation	Lysine (K)	H2AK119	Closed	Repressed
			H2BK120	Open	Active

TABLE 2 – Chemical modifications of DNA and histones and their association with chromatin and transcription states (adapted from 2012 Weng, Araki and Subedi [10]).

Several DNA modifications have been identified that influence gene expression. Among them, covalent modifications of DNA such as cytosine methylation occurs mainly in clusters of CpG dinucleotides called CpG islands and is correlated with closed chromatin, indicating repressed gene expression. Other covalent modifications include the amino-terminal tails of histones (H2A, H2B, H3 and H4). Some of these modifications, such as acetylation of H3 lysine 9 (H3K9), methylation of H3K4, phosphorylation of H3 threonine 3 (H3T3) and ubiquitylation of H2BK120, are associated with an open chromatin. On the opposite, others are associated with a closed chromatin such as methylation of H3K27 or ubiquitylation of H2AK119. For example, high histone acetylation levels have been observed at the promoters of genes encoding cytokines such as IFNγ or effector molecules such as GzmB [132, 133]. Moreover, the induced hyperacetylation or hypoacetylation at these gene loci results in an increase or decrease, respectively, in their expression in $CD8^+$ T cells providing evidence that histone acetylation state regulates memory T cell function [10]. Genome-wide analysis of trimethylation of lysine 4 of histone 3 (H3K4me3) and H3K27 (H3K27me3) combined with gene expression profiling showed a correlation between gene expression and the distribution of histone methylation. Indeed, H3K4me3 positively correlates and H3K27me3 negatively correlates with gene expression [134]. For example, high levels of H3K4me3 and low levels of H3K27me3 are observed in $CD8^+$ memory T cells genes associated with effector functions such as *KLRG1*, *Ifng* and *GzmB [135]* suggesting that histone methylation might regulate gene expression in memory $CD8^+$ T cells.

Further, DNA methylation of CpG islands in genes encoding cytokines and their receptors, effector molecules and their regulators has been studied in memory $CD8^+$ T cells. For example, it has been observed that the *Ifn-γ* and *IL-2* promoters are highly methylated in naïve $CD8^+$ T cells and in memory $CD8^+$ T cells. On the contrary, in effector $CD8^+$ T cells, DNA methylation levels are low and thus protein are highly expressed following their activation [132, 136, 137]. Similar observations have been made at the programmed cell death 1 (*Pdcd1*) gene locus encoding a regulator of cell proliferation and exhaustion called PD-1 in a context of both acute and chronic viral infection. Indeed, Youngblood and colleagues [138] showed that this locus is highly methylated in naïve $CD8^+$ T cells and then demethylated during their differentiation. Moreover, the DNA methylation status being stable and passing from a parental cell to its descendant during memory $CD8^+$ T cell division [136], it is clear that DNA methylation has a key role in the regulation of gene expression. Another piece of evidence indicating that DNA methylation could regulate $CD8^+$ T cell differentiation is the fact that the *Tcf7* locus, gene coding for Tcf-1, is significantly more methylated at day 8 after acute LCMV infection when compared with naïve $CD8^+$ T cells [139].

Epigenetic changes can be induced by pro-inflammatory cytokines since STATs regulate the expression of genes by modulating histone modifications [4, 140], indicating that pro-inflammatory cytokines can induce epigenetic modifications. Transcription factors such as T-bet or Blimp-1 also play a role in the activity of the enzymes responsible for DNA and histone modification [141, 142].

1.3 T cell factor 1 and the canonical Wnt signaling pathway

1.3.1 The canonical Wnt signaling pathway

As previously mentioned, Tcf-1 is necessary for the formation of functional memory $CD8^+$ T cells [128-130]. Tcf-1 is a well-known nuclear effector of the canonical Wnt signaling pathway.

The *Wnt1* gene, originally named *Int-1*, was identified in 1982 as a proto-oncogene virally induced in breast tumors by Nusse and Varmus [143] and encodes for a secreted, cysteine-rich protein. The fly *Wingless* (wg) gene, which controls segment polarity during larval development [144], was later shown to be a homolog of *Wnt1* [145].

The human and mouse genomes contain 19 Wnt genes [146]. Wnt proteins (Wnts) are very insoluble as they are modified with palmitoleic acid. Lipid addition is mediated by porcupine (Porcn) [147, 148].

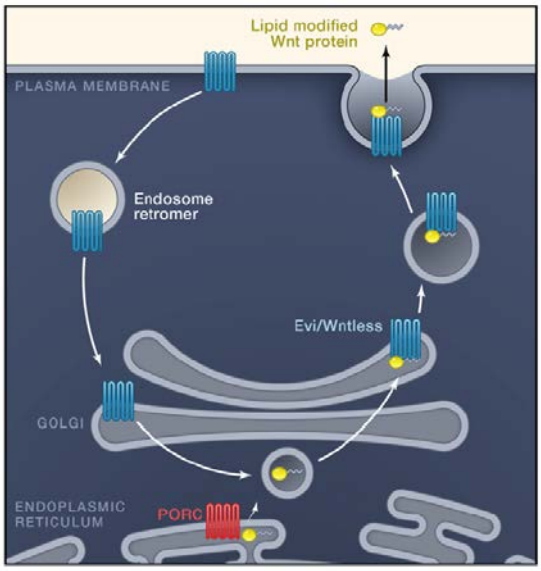

FIGURE 8 – The Wnt secretion machinery (2012 Clevers and Nusse [7]). Wnt proteins become lipid modified in the endoplasmic reticulum by the porcupine enzyme. Further transport and secretion is dependent on the Wntless transmembrane protein which carry Wnt proteins from the Golgi apparatus until the surface of Wnt producing cells.

This lipid modification is important for Wnt secretion which depends on the seven-transmembrane protein Wntless (Wls) protein [149]. Wls transports lipid-modified Wnt proteins from the Golgi apparatus to endosomes and allows their secretion (Fig. 8). Porcn and Wls are essential for Wnt secretion by Wnt-producing cells such as Paneth cells in the gut [7, 150]. Due to lipid modifications, Wnts are supposed to act only at a short distance of Wnt producing cells [151].

Combined observations from *Drosophila* and *Xenopus* identified three conserved signaling pathways commonly referred to as Wnt signaling pathways. Below, I will discuss only the so-called canonical Wnt pathway, which results in transcriptional responses to extracellular Wnt proteins.

Signaling via the canonical Wnt pathway is activated when Wnts interact with heterodimeric receptor complexes composed by Frizzled (Fzd) and lipoprotein receptor-related protein (LRP) 5 or 6. There exist 10 Fzd proteins which are seven-transmembrane domain proteins. The LRP co-receptors are single-pass transmembrane proteins. In the absence of Wnts, the cytoplasmic levels of β-catenin are regulated by the “destruction complex”. This complex is composed of the adenomatosis polyposis coli (APC) and Axin scaffold proteins but also includes the serine-threonine kinases glycogen-synthase kinase 3β (GSK3β), casein kinase 1 (Ck1) and other proteins. β-catenin binds to Axin and APC and is then

phosphorylated by Ck1 and GSK3β. β-catenin phosphorylation leads to its ubiquitination by the β-transducin-repeat-containing protein (βTrCP), which targets it for proteasome-mediated degradation. Upon Wnt binding to Fzd and LRP5/6, the destruction complex is recruited to the Wnt/receptor complex via Disheveled and Axin and this leads to the phosphorylation of LRP5/6 co-receptor by GSK-3β and Ck1. The recruitment of the destruction complex to the Wnt/receptor complex blocks the phosphorylation and ubiquitination of newly synthetized β-catenin. Consequently, β-catenin can accumulate in the cytoplasm and eventually translocate to the nucleus (Fig. 9).

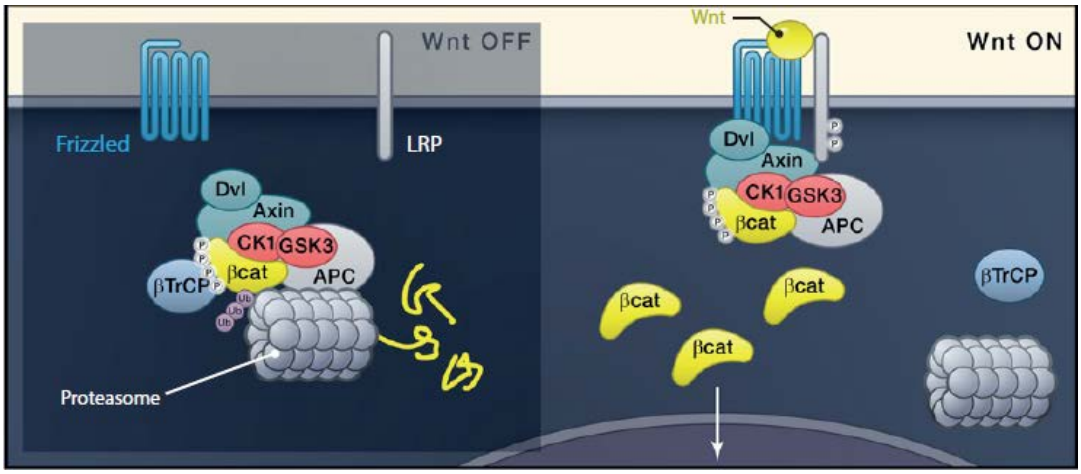

FIGURE 9 - Wnt signaling at the receptor and destruction complex level (adapted from 2012 Clevers and Nusse [7]). In the absence of Wnt, the destruction complex composed by Dishevelled (Dvl), Axin, Casein kinase 1 (Ck1), Glycogen-synthase kinase 3 (GSK3) and Adenomatosis coli (APC), resides in the cytoplasm where it binds, phosphorylates and ubiquitinates β-catenin (βcat) by βTrCP. The proteasome recycles the complex by degrading β-catenin. Wnt induces the association of the intact complex with phosphorylated LRP. After binding to LRP, the destruction complex captures and phosphorylates β-catenin and the ubiquitination of β-catenin by βTrCP is blocked. Newly synthetized β-catenin can accumulate and translocate into the nucleus.

In the nucleus, β-catenin binds to members of Tcf/Lymphoid enhancer binding factor (Lef) transcription factor family i.e. Tcf-1 (encoded by *Tcf7*), Lef-1, Tcf-3 (encoded by *Tcf7L1*) and Tcf-4 (encoded by *Tcf7L2*). In the Wnt "off" state, Tcf/Lef interacts with Groucho/TLE co-repressors, preventing gene transcription. In the Wnt "on" state, the association with β-catenin converts Tcf/Lef into a transcriptional activator of its target genes. Known target genes include *Axin2*, *Tcf7*, *c-myc* or *cyclin D1* [146, 152, 153]. *Axin2* is regarded as a general transcriptional Wnt target and therefore a general indicator of Wnt pathway activity (Fig. 10) [7].

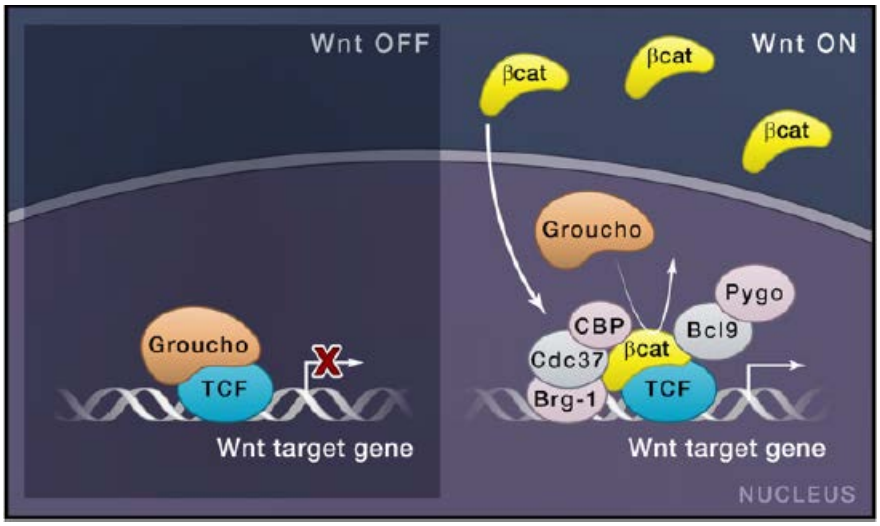

FIGURE 10 – Wnt signaling in the nucleus [7]. In the absence of Wnt signals, Tcf/Lef occupies and represses its target genes, helped by transcriptional co-repressors such as Groucho. Upon Wnt signaling, β-catenin (βcat) replaces Groucho from Tcf/Lef and recruits transcriptional co-activators and histone modifiers such as Brg1, CBP, Cdc47, Bcl9 and Pygopus to drive target gene expression.

Non-canonical activation of the canonical Wnt pathway

While the canonical Wnt signaling pathway can be activated by extracellular Wnts, it is important to note that other factors can also use this signaling pathway. For example, growth factors such as hepatocyte growth factor or insulin-like growth factor can active β-catenin/Tcf-1 signaling. They do so via the PI3K/AKT pathway which can phosphorylate and thus inhibit GSK-3β [154]. The lipid prostaglandin E2 can also activate the Wnt signaling pathway. Indeed, PGE2 stabilizes β-catenin by inactivating GSK-3β after binding to its G-protein-coupled receptor which in turn activates the PI3K-Akt signaling pathway [155]. The complement component C1q can also activate Wnt signaling by binding to the Fzd receptor and by cleaving the LRP6 co-receptor [156]. Following its binding to CD70, CD27 can also induce Wnt signaling by stabilizing β-catenin via TRAF-2 and TNIK [157]. Thus, in addition to extracellular Wnt proteins, multiple additional extrinsic factors can signal via the non-canonical Wnt pathway.

1.3.2 Role of Wnt/Tcf-1 signaling in immune system

Wnt signaling is involved in virtually every aspect of embryonic development. Moreover, it ensures the self-renewal of multiple adult tissues by regulating adult stem cells [158]. Uncontrolled Wnt signaling is responsible for the development of various types of cancers. For example, a mutated, and thus constitutively active, form of β-catenin results in aggressive T cell lymphomas in mice [159]. Increased activity can also be due to mutations in other pathways components. For example, mutations in APC leads to the stabilization of β-catenin and this leads to colorectal cancer development [160, 161].

Wnt/Tcf-1 signaling and its role in T cell development

The first evidence indicating a role of Wnt pathway components in the immune system came from the studies focused on T cell development in the thymus. The absence of Tcf-1 incompletely blocked intrathymic T cell development at multiple discrete stages [162, 163]. Tcf-1 is a Notch target and plays a critical role in early T-lineage specification [164]. Moreover, Tcf-1 ensures thymocyte survival once cells have passed the pre-TCR stage [165]. Tcf-1 is further needed for the transition from the immature single-positive to the $CD4^+/CD8^+$ double-positive (DP) stage [5, 166] and for ensuring DP lymphocytes survival [167]. T cell development is even more severely blocked in the absence of both Tcf-1 and Lef-1 [168]. Moreover, Tcf-1 and Lef-1, together with other transcription factors, promote $CD4^+$ lineage differentiation [166]. They ensure $CD8^+$ T cell identity by repressing $CD4^+$ lineage-associated genes such as *Cd4* or *Foxp3* in $CD8^+$ T cells [5, 166].

In addition to their role in T cell development, Tcf-1 and β-catenin impact the fate of mature peripheral $CD4^+$ T cells. Indeed, they promote T_H2 fate by inducing the upregulation of the transcription factor GATA-3. They inhibit T_H1 and T_H17 fates by repressing IFNγ and by negatively regulating *Il-17* gene respectively [169, 170]. β-catenin also improves the survival of regulatory T cells by upregulating Bcl-X_L [171]. Finally, Tcf-1 and Lef-1 expression increase the differentiation of naïve $CD4^+$ T cells into T_{FH} cells [172]. Tcf-1 promotes Bcl6 expression and inhibits Blimp-1 expression, leading to the initiation of T_{FH} cells [173].

Tcf-1 is not only impacting $CD4^+$ T cell differentiation. Indeed, absence of Tcf-1 in $CD8^+$ T cells did not impair the ability of $CD8^+$ T cells to expand and undergo effector differentiation in response to acute LCMV infection. However, $CD8^+$ T cells lacking Tcf-1 had a reduced capacity to expand upon secondary infection [128]. Thus Tcf-1 is required for memory formation and function in response to acute viral or bacterial infections.

Chronic viral infections were previously thought to preclude the formation of memory [174]. However, terminally differentiated (exhausted) virus-specific T cells persist for extended periods of time in chronic infections. Further, transfer of exhausted cells into naïve secondary hosts and reinfection resulted in secondary expansion of virus-specific cells, indicating that not all cells were terminally differentiated. Indeed, we and others recently identified a memory-like, Tcf-1-expressing, subpopulation of virus-

specific CD8$^+$ T cells which sustains the immune response to chronic infection [70]. In addition to features of central memory cells, these memory-like CD8$^+$ T cells have an “exhausted” phenotype. Importantly these cells are needed for clonal expansion to inhibitory blockade.

Even though Tcf-1 plays a prominent role for memory formation, a role for the canonical Wnt pathway is less well established. We showed that CD8$^+$ T cell differentiation and memory formation and function depended on the presence of a full length Tcf-1 isoform (called Tcf-1 p45). Tcf-1 p45 is able to bind β-catenin and γ-catenin [128]. While Prlic and Bevan did not find a role for β-catenin in memory CD8$^+$ T cell formation [175], re-expansion of memory CD8$^+$ T cell was reduced in chimeras with combined absence of β- and γ-catenin, indicating an essential role for Wnt/Tcf-1 [130].

In agreement with these findings, Gattinoni et al. showed that the enforced of Wnt/Tcf-1 signaling blocked CD8$^+$ T cell differentiation and generated stem cell-like memory cells *in vitro* [176]. Wnt signaling was promoted using a small molecule GSK3-β inhibitor (TWS119). However, TWS119 was subsequently shown to also inhibit the mammalian target of rapamycin (mTOR) pathway in an adenosine monophosphate-activated protein kinase (AMPK)-dependent manner [177]. It is thus not clear whether Wnt signaling was required for the generation of stem cell-like cells.

Notwithstanding, we and others showed that Tcf-1 is highly expressed in naïve CD8$^+$ T cells [127]. We further showed that naïve CD8$^+$ T cells express high levels of phosphorylated LRP6, non-phosphorylated (active) β-catenin and the Wnt/Tcf-1 target Axin2 [129], indicating that the canonical Wnt pathway is constitutively active in naïve CD8$^+$ T cells.

However, it was not known how Tcf-1 expression is maintained in naïve CD8$^+$ T cells. As Tcf-1 itself is a Wnt target, one possibility was that Wnt protein secretion played a role for the maintenance of Tcf-1 expression in naïve CD8$^+$ T cells. If so, we wanted to identify the cellular source of Wnt proteins and to determine the role of Wnt protein secretion for the homeostasis and differentiation of naïve CD8$^+$ T cells.

On the other hand, Tcf-1 was downregulated during a primary immune response and this correlated with effector differentiation. However, the signals responsible for Tcf-1 downregulation and the putative role of Tcf-1 downregulation for effector differentiation were not known. By investigating the control of

Tcf-1 expression, this thesis aimed at gaining novel insights into T cell homeostasis and effector versus memory differentiation.

AIM

Tcf-1 expression and canonical Wnt signal transduction are high in naïve $CD8^+$ T cells, downregulated during effector $CD8^+$ T cell differentiation and high again in memory $CD8^+$ T cell. A first aim of this thesis was to determine whether Wnt protein secretion is responsible for the maintenance of Wnt/Tcf-1 signaling in naïve T cells and to address the role of Wnt/Tcf-1 pathway activity for the homeostasis of $CD8^+$ T cells. A second aim was to determine the basis for the downregulation of Wnt/Tcf-1 signaling during $CD8^+$ T cell differentiation and to identify its role for $CD8^+$ T cell differentiation. The identification of factors regulating Wnt/Tcf-1 pathway activity in $CD8^+$ T cells may be useful to modify T cell effector differentiation and improve memory formation during vaccination.

RESULTS

Part I – The role of Wnt/Tcf-1 expression for effector CD8$^+$ T cell differentiation

Wnt/Tcf-1 is highly expressed in naïve CD8$^+$ T cells whereas it is only present in a subset of CD8$^+$ T cells at the peak of the primary immune response to LCMV infection. In the memory phase, the vast majority of CD8$^+$ T cells express Tcf-1 [129]. We have shown that Wnt/Tcf-1 signaling is important to generate functional memory CD8$^+$ T cells which are able to re-expand upon re-infection [129]. However, the control of Wnt/Tcf-1 signaling and its role for the initial differentiation of naïve CD8$^+$ T cells into effector cells or memory-precursor cells has not been studied. To address this question, we tested the role of proliferation and of signal 1,2 and 3 for the regulation of Wnt/Tcf-1 pathway activity in CD8$^+$ T cells.

Homeostatic proliferation does not impact Wnt pathway activity in CD8$^+$ T cells

Acute infection is associated with extensive proliferation of antigen-specific CD8$^+$ T cells. This raised the possibility that proliferation induced downregulation of Wnt/Tcf-1 signaling. Naïve CD8$^+$ T cells undergo homeostatic proliferation in T cell-deficient hosts, which is due to excessive IL-7 and IL-15 levels plus contact with self-MHC/peptide ligands [178]. This homeostatic proliferation allowed us to test whether Wnt/Tcf-1 signaling downregulation was due to CD8$^+$ T cell proliferation.

In order to follow the Wnt/Tcf-1 signaling transduction in CD8$^+$ T cells at the single cell level, we took advantage of the Axin2 reporter mice. *Axin2* is expressed in response to Wnt signal transduction and is a direct target of Tcf-1 [179]. In Axin2 reporter mice, the bacterial *β-galactosidase* (LacZ) gene has been inserted into the endogenous *Axin2* locus (Axin2^{LacZ}) [180]. β-galactosidase activity is used as a read-out for Axin2 expression and can be measured by flow cytometry using fluorescein di-β-galactopyranoside (FDG) substrate. Axin2^{LacZ} expression was high in naïve Axin2^{LacZ} CD8$^+$ T cells (Fig. 11).

We adoptively transferred 1 x 10^6 naïve $Axin2^{LacZ}$ $CD8^+$ T cells into lymphocyte-deficient RAG-2 γc double knock out mice and analyzed transferred $CD8^+$ T cells 3.5 and 8.5 days later. While transferred $CD8^+$ T cells had expanded to around $3x10^6$ cells at day 8.5 (only 10% of cells transferred intravenously (i.v.) can be found in the spleen after transfer [181, 182]), they did not downregulate Axin2 expression (Fig. 11). These data suggest that $CD8^+$ T cell downregulation of Wnt/Tcf-1 signaling is not simply due to $CD8^+$ T cell proliferation.

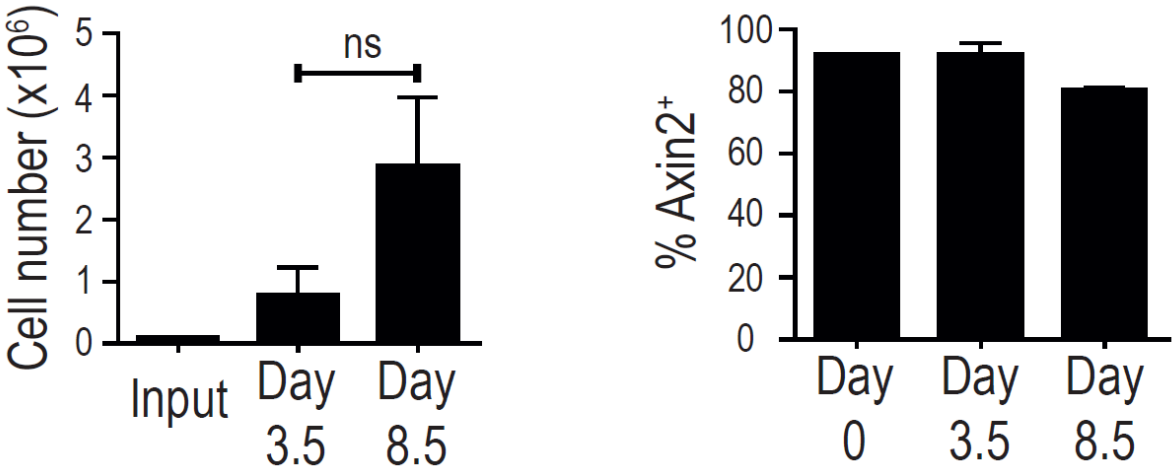

FIGURE 11 – Wnt/Tcf-1 signaling in transferred $CD8^+$ T cells after homeostatic proliferation. 10^6 naïve $CD8^+$ T cells were transferred into RAG-2 γc double knock out mice and analyzed at day 3.5 and 8.5 post transfer. Bar graph on the left shows the number of transferred $CD8^+$ T cells in the spleen at day 0 (10% of input), 3.5 and 8. Bar graph on the right indicates the percentage of transferred $Axin2^+$ $CD8^+$ T cells at day 0 (before injection) and at days 3.5 and 8.5 days after transfer. These results are representative of two independent experiments. Data are shown as mean ± standard deviation (SD) (n=1-3). Unpaired *t*-test was used for statistical analyses and significant differences between groups are in the graphs (n=5). ns stands for not significant.

TCR signal strength regulates expansion of $CD8^+$ T cells but not Wnt/Tcf-1 downregulation

Following acute LCMV infection, Wnt/Tcf-1 downregulation has been observed in antigen-specific $CD8^+$ T cells. We hypothesized that this reduction was related to the TCR signal strength (signal 1) received by antigen-specific $CD8^+$ T cells during their priming.

In order to test this hypothesis, we used $CD8^+$ T cells from OT-1 TCR transgenic mice. These $CD8^+$ T cells express a TCR specific for the ovalbumin (OVA) peptide SIINFEKL (called N4). In addition to the native peptide (N4), there exist altered peptide ligands (APLs) called T4 and V4. In the T4 APL, the asparagine from the N4 peptide has been replaced by a threonine which reduces its affinity for the OT-

1 TCR. In the V4 peptide, the asparagine has been replaced by a valine, which further reduces the affinity. The APLs bind equally well to MHC-I molecules but they differ in their potency to stimulate OT-1 cells. These APLs have been introduced into recombinant *L.m.* [183] which can be used to assess the role of TCR signal strength in the $CD8^+$ T cell response.

OT-1 mice were crossed to $Axin2^{LacZ}$ mice. OT-1 $Axin2^{LacZ}$ $CD8^+$ T cells ($CD45.1^+$) were transferred into wild type (WT) $CD45.2^+$ C57BL/6 (B6) recipient mice, which were then infected with *L.m.* Seven days later, we confirmed that *L.m.* expressing the wild-type (N4) peptide induced a strong expansion of OT-1 cells. The expansion was progressively decreased when *L.m.* expressed T4 or V4 APLs respectively (Fig. 12A). Regardless of the TCR affinity for the peptide, OT-1 cells were able to differentiate into SLECs ($CD127^-$ $KLRG1^+$) even though the abundance of SLECs was decreased with decreasing affinity (Fig. 12B) in agreement with published data [183]. We next confirmed that $Axin2^{LacZ}$ expression was high in naïve OT-1 $Axin2^{LacZ}$ cells (Fig. 12C). At day 7 post infection with *L.m.* expressing N4 peptide,

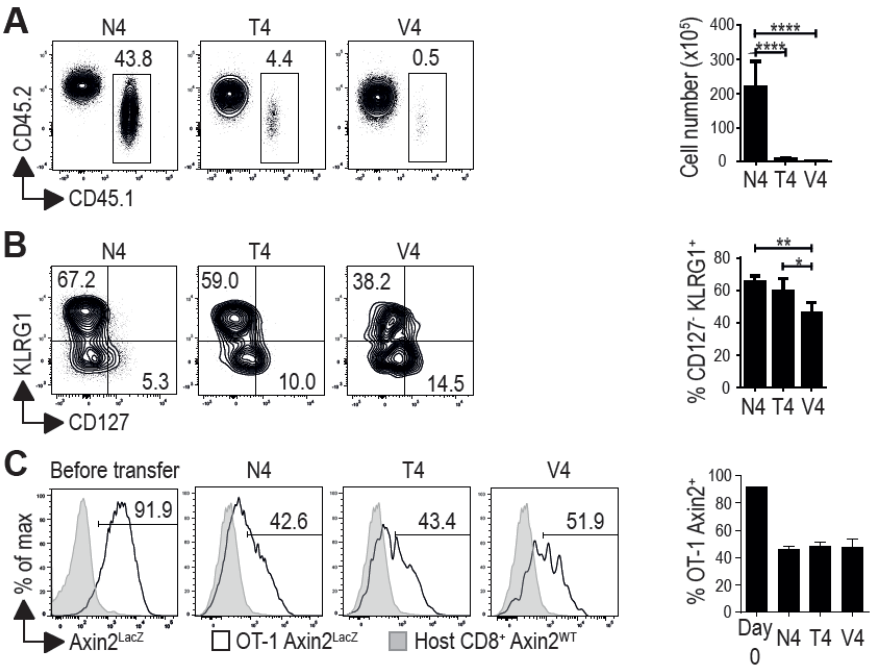

FIGURE 12 – Wnt/Tcf-1 pathway activity in OT-1 cells stimulated with APLs with different TCR affinities. (**A**) Dot plots show the percentages of OT-1 $CD8^+$ T cells ($CD45.1^+$ $CD45.2^-$) in the spleen at day 7 after infection with *L.m.* expressing either N4, T4 or V4 peptide ligand. Numbers indicate the percentage of cells in the respective gate. Bar graph shows the numbers of OT-1 cells in the spleen. (**B**) Dot plots indicate the percentage of SLECs ($CD127^-$ $KLRG1^+$) and MPECs ($CD127^+$ $KLRG1^-$) in the OT-1 $CD8^+$ T cells at day 7 after *L.m.* infection. Numbers indicate the percentage of cells in the respective quadrant. Bar graph shows the percentage of SLECs in the OT-1 $CD8^+$ T cell population. (**C**) Histograms show the expression of $Axin2^{LacZ}$ in OT-1 $CD8^+$ T cells (before transfer) or at day 7 after *L.m.* infection (N4, T4 or V4) in the spleen. Numbers indicate the percentage of cells expressing Axin2. Bar graphs show the percentage of OT-1 cells expressing $Axin2^{LacZ}$. Data are shown as mean ± SD. One-way ANOVA test was used for statistical analyses and significant differences between significant groups are shown (n=5). ****$p < 0.0001$; **$p < 0.05$; *$p < 0.05$.

Axin2^{LacZ} was downregulated (Fig. 12C) [129]. The weaker TCR ligands T4 and V4 induced comparable reductions in Axin2^{LacZ} expression (Fig. 12C). Corresponding data were obtained when analyzing Tcf-1 expression (not shown). Thus, in the context of acute infection, the downregulation of Wnt/Tcf-1 signaling occurs independent of the strength of TCR for peptide/MHC-I.

Systemic inflammation downregulates Wnt/Tcf-1 signaling in primed CD8$^+$ T cells

To gain further insights into the downregulation of Tcf-1 expression during a primary CD8$^+$ T cell response, we next addressed the importance of antigen and co-stimulation (signal 1 and 2) versus inflammation (signal 3). To this end, we adopted a vaccination protocol using dendritic cell immunization as schematically shown in Fig. 13A. This model was established by Badovinac et al. [184]. A small number (10^4) of naïve P14 CD8$^+$ T cells (CD45.2$^+$) was transferred into CD45.1$^+$ B6 mice. P14 CD8$^+$ T cells express a TCR specific for the LCMV-specific Gp_{33-41} epitope (KAVYNFATC). Recipient mice were immunized using bone marrow-derived DC, which were matured using the TLR4 ligand LPS and pulsed with LCMV Gp_{33-41} peptide (termed DC33). DC vaccination was combined with systemic exposure of mice to the TLR9 ligand CpG-B (termed DC33/CpG) [93, 184].

P14 CD8$^+$ T cells were comparably abundant at day 7 post DC33 and DC33/CpG vaccination (Fig. 13B), but were below detection in the absence of vaccination (data not shown) in agreement with Cui et al. [93]. Most P14 cells expressed high levels of Tcf-1 after DC33 but reduced levels following DC33/CpG vaccination (Fig. 13C). Tcf-1 downregulation did not occur with CpG alone (data not shown) (note that these mice received a large dose of P14 cells (10^6 cells) in these experiments). Thus systemic inflammation in the context of priming reduces Tcf-1 expression in CD8$^+$ T cells.

P14 cells primed with DC33 efficiently produced IL-2 upon peptide re-stimulation *in vitro*, expressed low levels of GzmB and were mostly KLRG1$^-$ CD127$^+$, which corresponds to a MPEC phenotype (Fig. 13D-F). In contrast, P14 cells primed with DC33/CpG produced little IL-2, expressed high levels of GzmB and predominantly had a KLRG1$^+$ CD127$^-$ phenotype, which corresponds to a SLEC phenotype (Fig. 13D-F). On the other hand, IFNγ production was comparable between P14 cells stimulated with DC33 and DC33/CpG (Fig. 13C). Under both stimulation conditions, Tcf-1 downregulation was predominantly

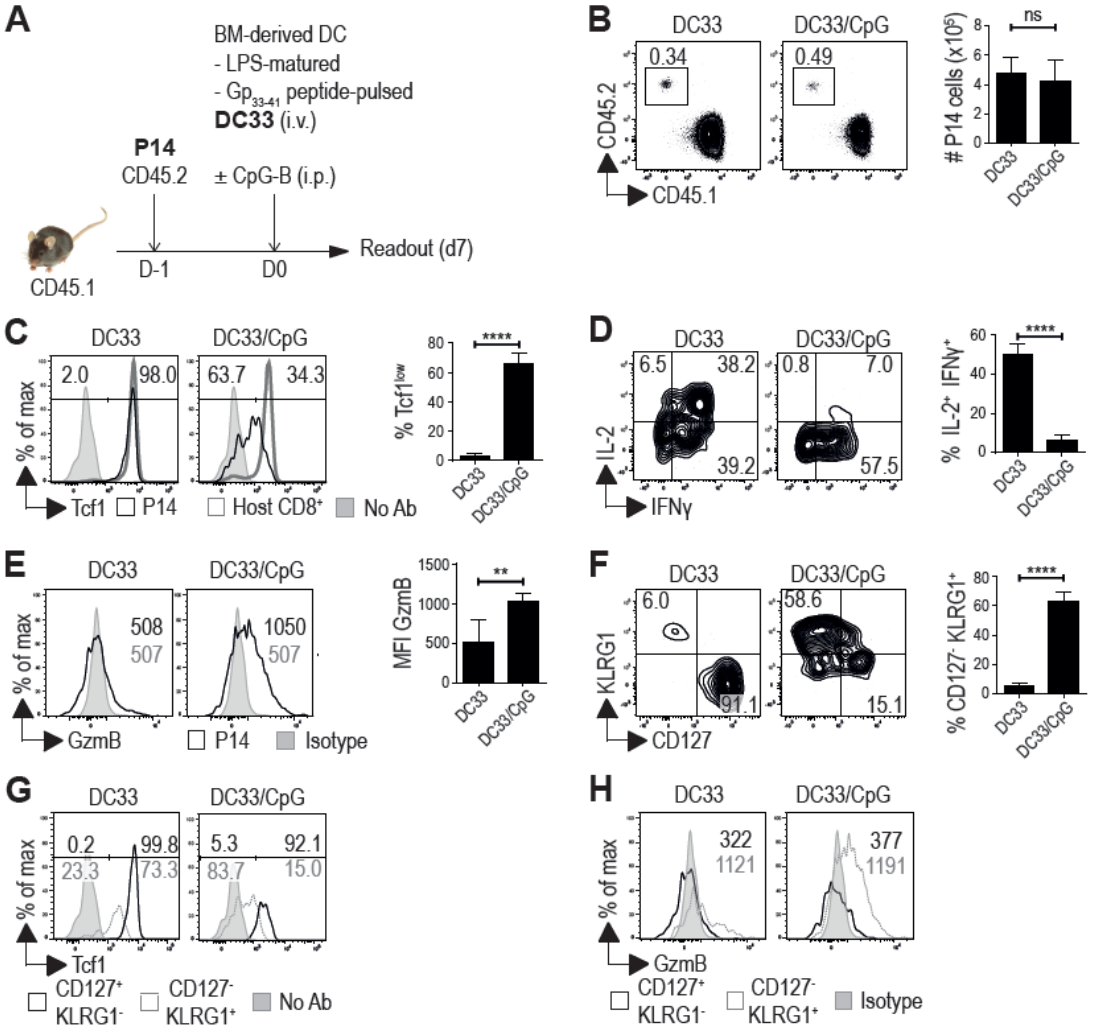

FIGURE 13 – Tcf-1 expression and effector differentiation is regulated by inflammation (**A**) The scheme shows the experimental approach. Small numbers (1-2 x 10^4) of naïve $CD8^+$ T cells ($CD45.2^+$), specific for the LCMV-derived Gp_{33-41} epitope presented by H-$2D^b$ (P14 cells), were transferred into $CD45.1^+$ C57BL/6 (B6) recipients. The day after, bone marrow-derived dendritic cells matured with LPS and pulsed with Gp_{33-41} peptide were transferred. Seven days later, recipient mice were sacrificed and analyzed. (**B-H**) Wild-type (WT) P14 cells ($CD45.2^+$) were transferred into recipient mice ($CD45.1^+$), which were vaccinated with DC33 or DC33/CpG. (**B**) Recipient spleens were analyzed for the abundance of P14 cells ($CD45.2^+$ $CD45.1^-$) at day 7 post vaccination. The corresponding bar graph depicts mean numbers (± SD) of P14 cells present in spleens. (**C-H**) Gated P14 cells were analyzed for (**C**) the percentage of cells expressing intracellular Tcf-1 (percentage of host $CD8^+$ is always >95%) (**D**) the percentage of cells producing IL-2^+ IFNγ$^+$ following re-stimulation with Gp_{33-41} peptide, (**E**) the mean fluorescence intensity (MFI) of granzyme B (GzmB) expression, (**F**) the percentage of $CD127^+$ $KLRG1^-$ (MPECs) and $CD127^-$ $KLRG1^+$ (SLECs) cells, (**G**) the percentage of Tcf-1-expressing cells in gated MPECs and SLECs subsets and (**H**) the MFI of GzmB expression in gated MPECs and SLECs. Bar graphs show the mean ± standard error of the mean (SEM). (**B**) or ± SD (**C-F**). Results are representative of at least two independent experiments. Unpaired *t*-tests were used for statistical analyses and significant differences between groups are shown in the graphs (n=5). ****$p < 0.0001$; **$p < 0.05$; ns stands for not significant.

observed in $KLRG1^+$ $CD127^-$ (SLECs) but not in $KLRG1^-$ $CD127^+$ (MPECs) (Fig. 13G) and this inversely correlated with GzmB expression (Fig. 13H). Similar to Tcf-1 downregulation, the expression of the Wnt/Tcf-1 target Axin2 was also downregulated in response to DC33/CpG (Fig. 14). Thus Wnt/Tcf-1 signaling in primed $CD8^+$ T cells is negatively regulated by systemic inflammation and this correlates with effector differentiation.

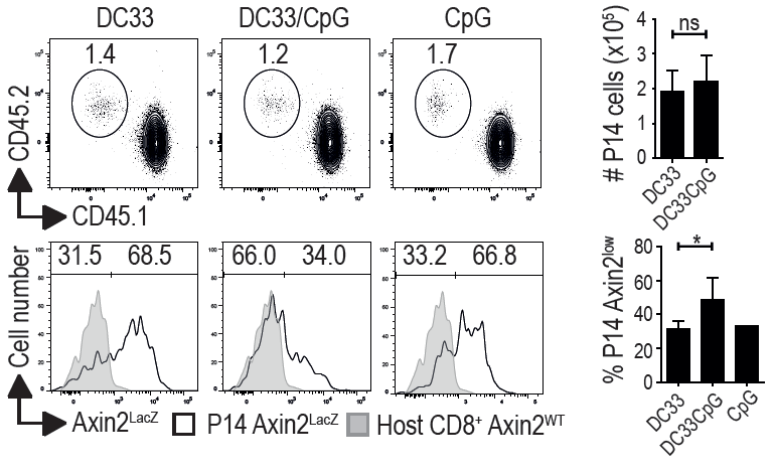

FIGURE 14 – Regulation of Axin2 expression by inflammation. Wild-type (WT) and Axin2$^{LacZ/+}$ P14 cells (CD45.2$^+$) were transferred into recipient mice (CD45.1$^+$), which were then vaccinated with DC33, DC33/CpG or CpG alone. Dot plots show the percentage of P14 cells (CD45.2$^+$ CD45.1$^-$) among total CD8$^+$ T cells. Histograms show Axin2 expression in P14 cells. Corresponding bar graphs depicts mean values (± SEM for cell numbers and ± SD for percentages) derived from n=3-6 mice of a representative experiment of at least two independent experiments. Unpaired *t*-test was used for statistical analyses. *p < 0.05; ns stands for not significant.

Tcf-1 downregulation following DC33/CpG vaccination depends on IL-12 p40 but is not impacted by IL-2

Our data so far show that TLR9 signaling plays a key role for suppressing Wnt/Tcf-1 signaling in primed CD8$^+$ T cells. It has been shown that TLR9 expression by the immunizing DCs or the responding CD8$^+$ T cells is not essential for effector differentiation [185] indicating that effector differentiation depends on soluble factors produced by TLR9$^+$ cells of the host. Indeed DC vaccination in the presence of CpG-B increased serum levels of IL-1β, IL-6, IL-10, IL-12, IFNγ [185, 186] and type I IFN (IFNα, β) [187]. Of particular interest, the signal 3 cytokines IL-12 and type I IFN, together with IL-2, are known to promote the expansion as well as the acquisition of effector functions of CD8$^+$ T cells *in vivo* [60, 117, 188-191]. We thus tested whether these cytokines were involved in Tcf-1 downregulation induced by DC33/CpG vaccination. IL-12 (p40) antibody blockade almost completely prevented the downregulation of Tcf-1 (Fig. 15A), while interferon-α/β receptor (IFNAR) blockade had no effect (Fig. 15A). The combination of IL-12 (p40) and IFNAR blockade had some additional effects (not significant) on Tcf-1 expression in

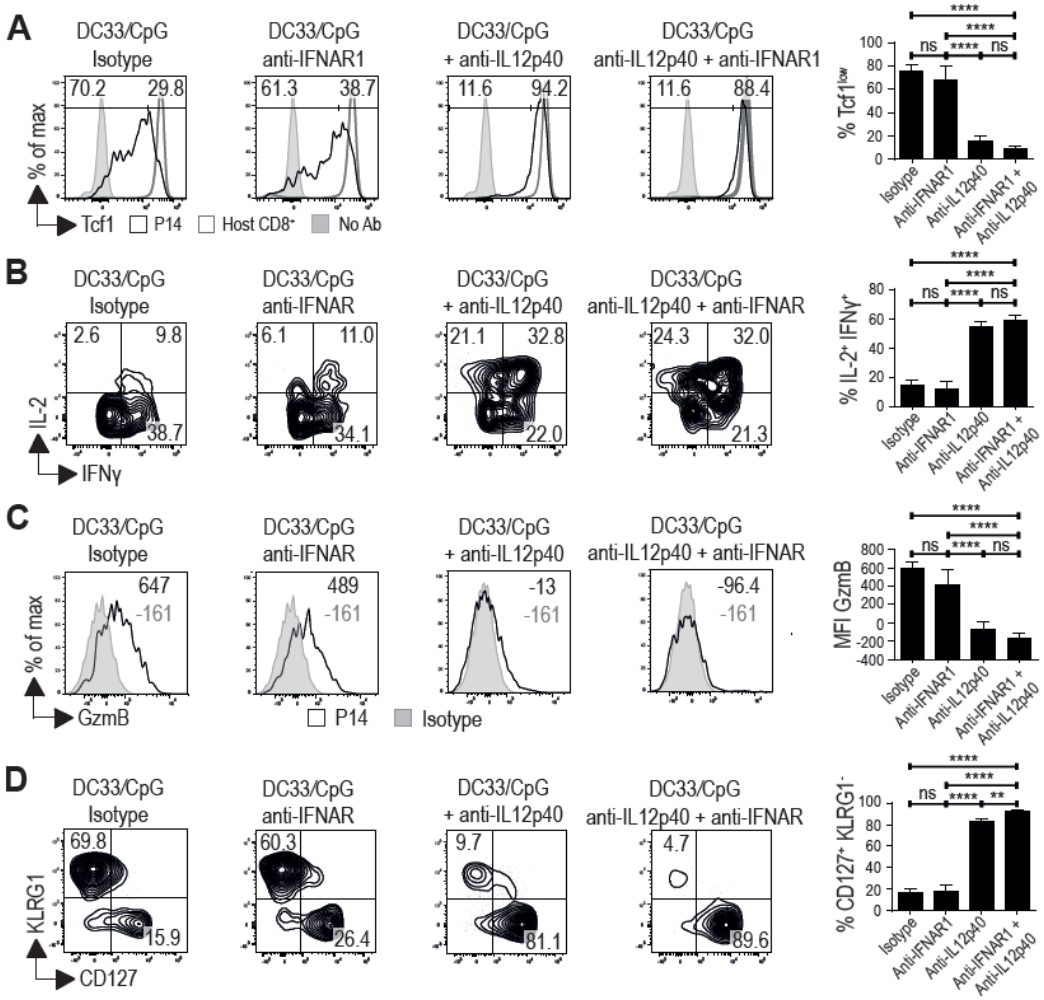

FIGURE 15 – IL-12 (p40) blockade prevents inflammation-induced Tcf-1 loss. WT P14 cells (CD45.2$^+$) were transferred into recipient mice (CD45.1$^+$), which were vaccinated with DC33/CpG. Mice were treated twice with anti-IL-12 (p40) mAb on the day of DC vaccination, once with anti-IFNAR mAb on the day of CD8$^+$ T cell injection or with isotype control mAbs. At day 7 post vaccination, recipient spleens were harvested and gated P14 cells (CD45.2$^+$ CD45.1$^-$) were analyzed for (**A**) the percentage of cells expressing intracellular Tcf-1, (**B**) the percentage of cells producing IL-2, (**C**) the MFI GzmB expression and (**D**) the percentage of CD127$^+$ KLRG1$^-$ (MPECs) and CD127$^-$ KLRG1$^+$ (SLECs) cells. Corresponding bar graphs depict mean values (± SD) derived from n=5 mice of a representative experiment of two independent experiments performed. Error bars indicate SD. Statistical significance was determined with one-way ANOVA test. ****p < 0.0001; ***p < 0.001; **p < 0.01; *p < 0.05; ns stands for not significant.

P14 cells (Fig. 15A). IL-12 (p40) blockade improved IL-2 production, reduced GzmB expression and prevented SLEC differentiation (Fig. 15B-D). Combined IL-12 (p40) and IFNAR blockade further reduced effector differentiation (Fig. 15D), while IFNAR blockade had no effect (Fig. 15B-D).

Similar to IFNAR blockade, IL-2 blockade did not significantly affect Tcf-1 expression and effector differentiation (Fig. 16A-C). IL-2 administration (complexed with an antibody) did also not alter Tcf-1 expression (Fig. 16A) or effector differentiation (Fig. 16B). Administration of IL-2 complexes did result in

an increased expansion of CD8⁺ both in presence and in absence of inflammation (Fig. 16C). We conclude that Tcf-1 repression in primed CD8⁺ T cells is chiefly mediated by IL-12 (p40).

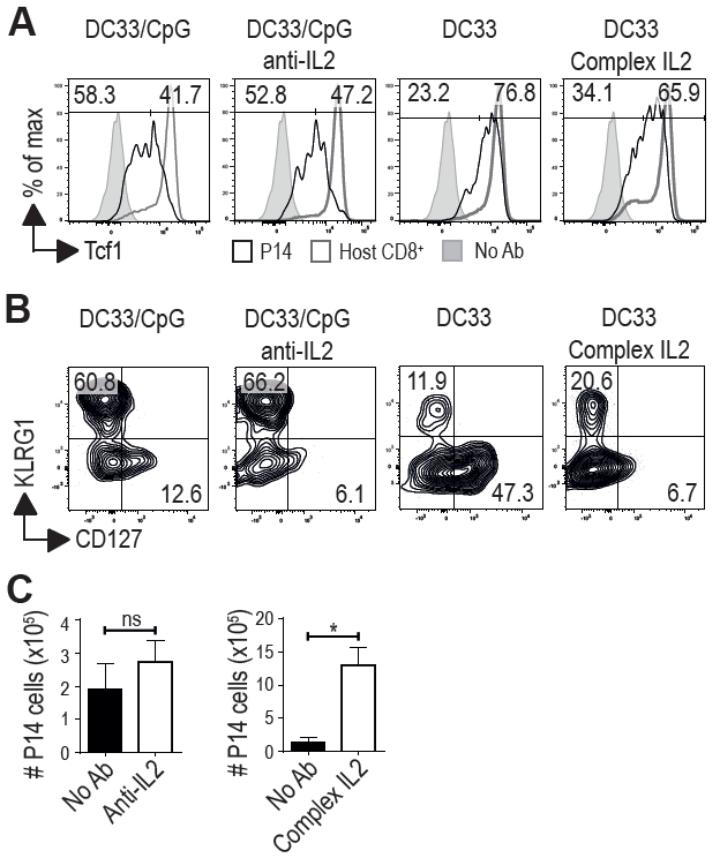

FIGURE 16 – IL-2 blockade or IL-2 complex administration does not alter Tcf-1 downregulation. 1-2x10^4 P14 CD45.2$^+$ cells were transferred into naïve CD45.1$^+$ recipient mice. Recipient mice were then vaccinated with DC33 or DC33/CpG. Anti-IL-2 S4B6 antibody complexed with IL-2, which increases the half-life of IL-2 *in vivo*, or JES6, a blocking anti-IL-2 antibody, were injected in order to induce or block IL-2 signaling respectively 4.5 and 6 days later. At day 7 post DC vaccination, recipient mice were killed and spleens were analyzed. (**A-B**) Gated P14 cells were analyzed for (**A**) the percentage of cells expressing intracellular Tcf-1 and (**B**) the percentage of CD127$^+$ KLRG1$^-$ (MPECs) and CD127$^-$ KLRG1$^+$ (SLECs) cells. (**C**) The bar graphs depict mean numbers (± SEM) of P14 cells present in the spleen. Plots are representative examples from n=2-4 mice from both experiments performed. Unpaired *t*-test was used for statistical analyses. *p < 0.05; ns stands for not significant.

Tcf-1 is suppressed by direct and indirect effects of IL-12 (p40) and by STAT4 signaling in CD8⁺ T cells

The blockade of IL-12 using Abs revealed an important role for IL-12 in Tcf-1 downregulation. Thus we hypothesized that IL-12 acts redundantly with IL-23. To address this possibility, we stimulated purified CD8⁺ T cells *in vitro* with anti-CD3/CD28 antibodies and low levels of IL-2 to sustain the proliferation of CD8⁺ T cells. Tcf-1 expression was maintained at high levels in anti-CD3/CD28-activated cells as compared to IL-2 alone, in agreement with our observation that DC33 vaccination does not suppress Tcf-1 protein *in vivo*. Addition of IL-12 significantly reduced Tcf-1 expression in an IL-12Rβ2-dependent

fashion (Fig. 17A), indicating that IL-12 can directly repress Tcf-1 in activated CD8⁺ T cells. On the other hand, IL-23, which shares the p40 subunit with IL-12, had no effect and the combination of IL-23 and IL-12 had no additional effect on Tcf-1 expression (Fig. 17A and not shown). These data show that IL-12 can act directly on CD8⁺ T cells and that IL-23 plays no role.

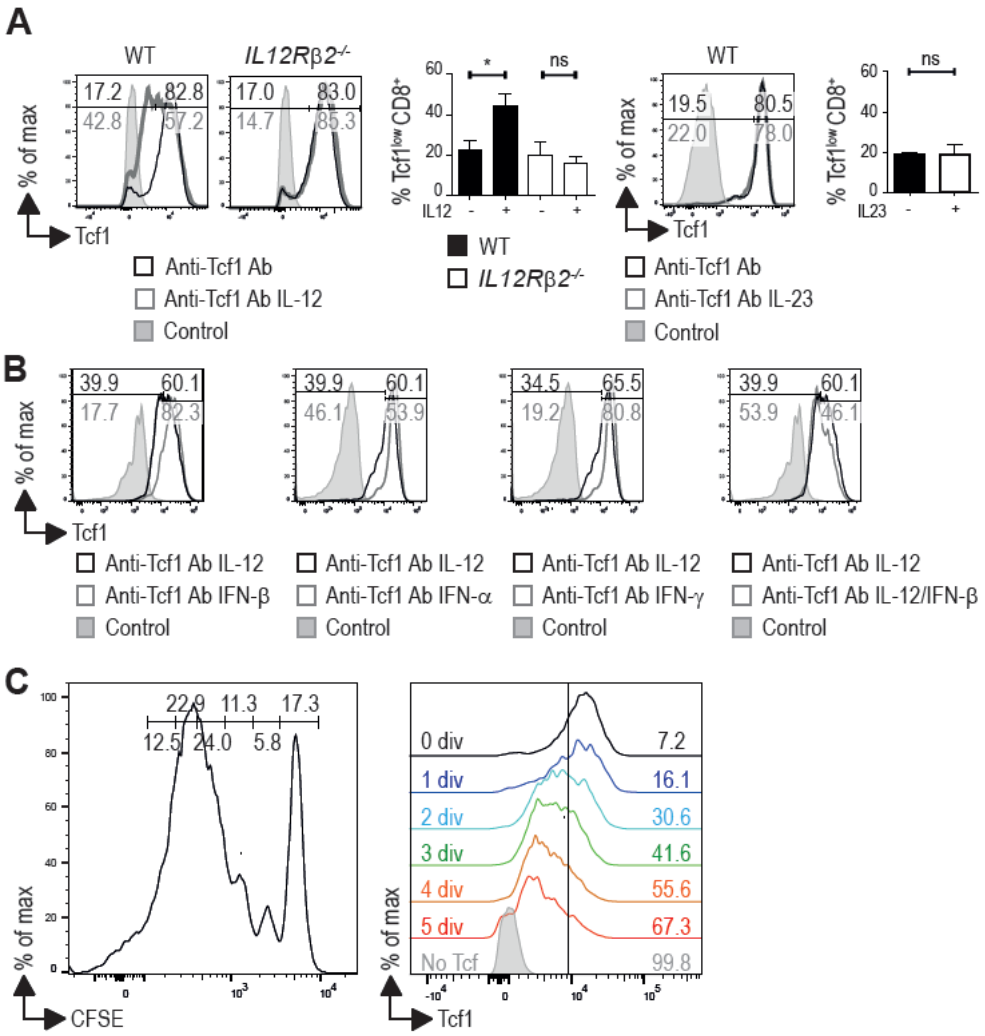

FIGURE 17 – Tcf-1 expression is reduced by direct IL-12 signaling in CD8⁺ T cells. Purified CD8⁺ T cells were activated *in vitro* with anti-CD3/CD28 mAbs and were analyzed 72h later. (**A**) Purified CD8⁺ T cells were stimulated *in vitro* in presence (open grey histogram) or not (open black histogram) of recombinant IL-12 or IL-23 and analyzed for the percentage of CD8⁺ T cells expressing Tcf-1. Bar graphs depict mean values (± SD) from n=2-3 wells from two independent experiments performed. Statistical significance was determined with unpaired *t*-tests. *p < 0.05; ns stands for not significant. (**B**) Purified CD8⁺ T cells were stimulated *in vitro* in presence of IL-12 (open black histogram) or various IFNs (IFN-β, IFN-α, IFN-γ or IL-12/IFN-β) (open grey histogram) and analyzed for the percentage of cells expressing Tcf-1. (**C**) Purified CD8⁺ T cells were loaded with CFSE and stimulated with recombinant IL-12 in presence of anti-CD3/CD28 mAbs. Proliferation of CD8⁺ T cells was assessed by analyzing CFSE dilution and proliferating cells were analyzed for the percentage of cells expressing Tcf-1.

We next attempted to identify additional cytokines that suppressed Tcf-1. Addition of IFN-α, IFN-β or IFN-γ had no significant effect on Tcf-1 expression (Fig. 17B). However, IFN-α or IFN-β greatly reduced T cell cycling (data not shown), which may be of importance, since significant Tcf-1 downregulation *in*

vitro was only observed when cells that had divided more than 2 times (Fig. 17C). The combination of IFNβ with IL-12 restored cycling but did not result in a significant additional Tcf-1 downregulation as compared to IL-12 alone (Fig. 17B). Thus, so far only IL-12 can reduce Tcf-1 expression in activated $CD8^+$ T cells.

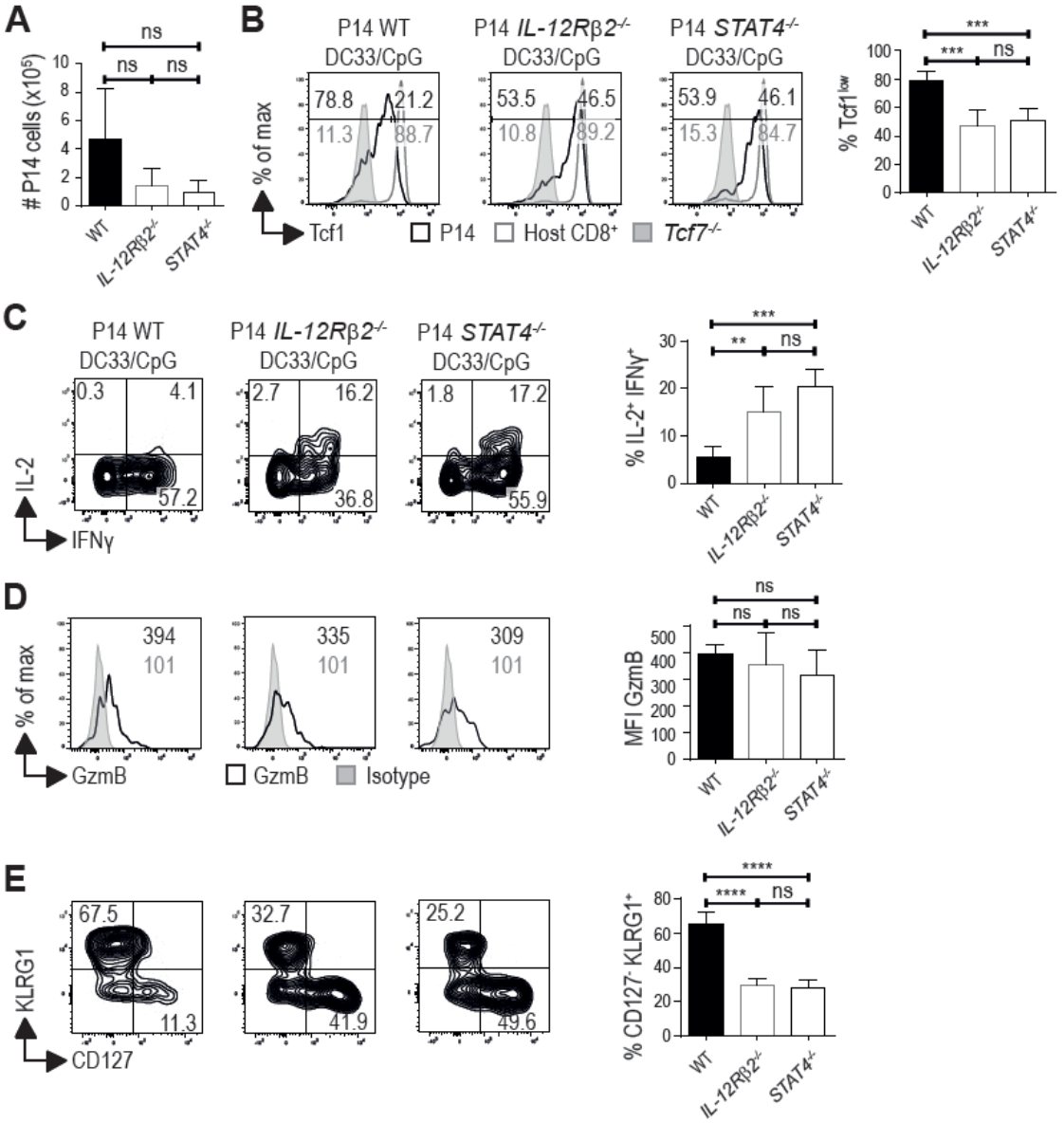

FIGURE 18 – Absence of STAT4 in $CD8^+$ T cells reduces Tcf-1 downregulation. WT, *IL-12Rβ2*$^{-/-}$ or *STAT4*$^{-/-}$ P14 cells ($CD45.2^+$) were transferred into recipient mice ($CD45.1^+$), which were then vaccinated with DC33/CpG. (**A**) Recipient spleens were analyzed seven days later for the abundance of WT, *IL-12Rβ2*$^{-/-}$ or *STAT4*$^{-/-}$ P14 cells. The corresponding bar graph depicts mean numbers (± SD) of P14 cells present in spleens. (**B-E**) Gated WT, *IL-12Rβ2*$^{-/-}$ or *STAT4*$^{-/-}$ P14 cells were analyzed for (**B**) the percentage of cells expressing intracellular Tcf-1, (**C**) the percentage of cells producing IL-2, (**D**) the MFI GzmB expression and (**E**) the percentage of $CD127^-$ $KLRG1^+$ (SLECs). Corresponding bar graphs depict mean values (± SD) derived from n=5 mice of a representative experiment of two independent experiments performed. Statistical significance was determined with unpaired *t*-tests. ****p<0.0001; ***p<0.001; **p < 0.01 and ns stands for not significant.

Our *in vitro* data showed that IL-12 can act directly on CD8$^+$ T cells. To exclude the possibility that IL-12 acts indirectly, we next used *IL-12Rβ2*$^{-/-}$ P14 CD8$^+$ T cells *in vivo* using our DC33 vaccination system. Since IL-12 activates STAT4 [96, 192], we also tested whether STAT4 signaling in CD8$^+$ T cells played a role for Tcf-1 downregulation. Following DC33/CpG vaccination, both WT, *IL-12Rβ2*$^{-/-}$ and *STAT4*$^{-/-}$ P14 cells expanded equally well (Fig. 18A). We observed that Tcf-1 downregulation was significantly less prominent in *IL-12Rβ2*$^{-/-}$ and *STAT4*$^{-/-}$ as compared to WT P14 cells (Fig. 18B). Accordingly, IL-2 production was improved and SLEC differentiation was reduced (Fig. 18C and E). Despite reduced SLEC differentiation, GzmB expression was not reduced in the absence of IL-12Rβ2 or STAT4 (Fig. 18D). Overall, these data suggest that IL-12/STAT4 signaling in CD8$^+$ T cells downregulates Tcf-1 in response to DC vaccination.

Tcf-1 downregulation following *Listeria monocytogenes* infection depends on IL-12Rβ2 and STAT4 expression by CD8$^+$ T cells

It has been reported that effector differentiation in response to *Listeria monocytogenes* (*L.m.*) infection was mainly driven by IL-12 and that it was largely reduced when CD8$^+$ T cells lacked IL-12Rβ2 [190]. We thus verified the regulation of Tcf-1 expression in CD8$^+$ T cells responding to *L.m.* infection. P14 cell proliferation was reduced in the absence of STAT4 but not in the absence of IL-12Rβ2 (Fig. 19A). Similar to DC33/CpG vaccination, infection with *L.m.* expressing Gp$_{33\text{-}41}$ (*L.m.-Gp$_{33\text{-}41}$* including Ova T4) resulted in Tcf-1 downregulation in a significant fraction of P14 cells (Fig. 19B). When P14 cells lacked IL-12Rβ2 or STAT4, Tcf-1 downregulation was considerably less prominent (Fig. 19B). This was associated with increased IL-2 production and a reduced differentiation into SLECs (Fig. 19C and E), in agreement with Keppler et al. [193]. Despite reduced SLEC differentiation, GzmB was not reduced (Fig. 19D). Thus IL-12Rβ2-STAT4 signaling in CD8$^+$ T cells contributes to Tcf-1 downregulation and effector differentiation in response to bacterial infection.

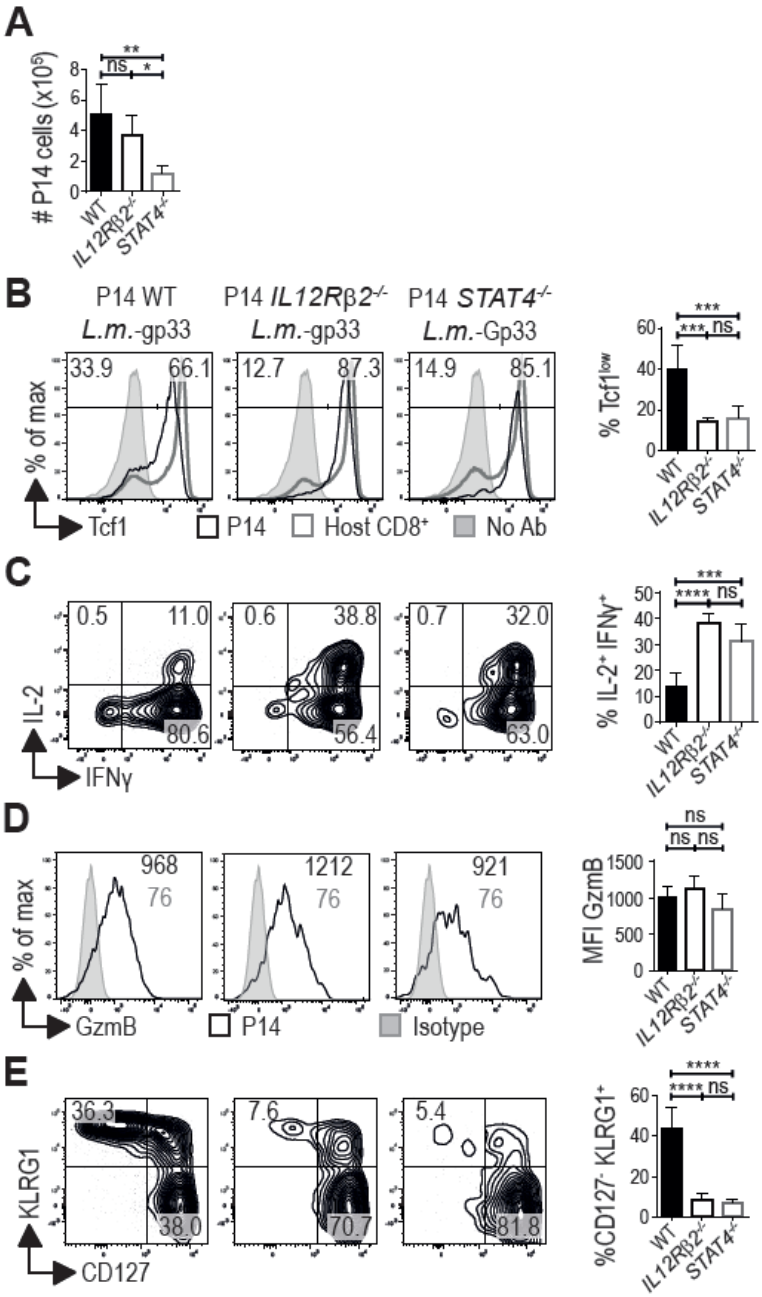

FIGURE 19 – Absence of IL-12Rβ2 or STAT4 in CD8⁺ T cells reduces Tcf-1 downregulation induced by *L.m.* infection. WT, *IL-12Rβ2*$^{-/-}$ or *STAT4*$^{-/-}$ P14 cells (CD45.2⁺) were transferred into recipient mice (CD45.1⁺), which were then infected with *L.m.*-Gp$_{33-41}$. (**A**) Recipient spleens were analyzed for the abundance of WT, *IL-12Rβ2*$^{-/-}$ or *STAT4*$^{-/-}$ P14 cells at day 8 post infection. The corresponding bar graph depicts mean numbers (± SD) of P14 cells. (**B-E**) Gated WT, *IL-12Rβ2*$^{-/-}$ or *STAT4*$^{-/-}$ P14 cells were analyzed for (**B**) the percentage of cells expressing intracellular Tcf-1, (**C**) the percentage of cells producing IL-2 and IFNγ, (**D**) the MFI of GzmB expression and (**E**) the percentage of CD127⁻ KLRG1⁺ (SLECs) cells. Corresponding bar graphs depict mean values (± SD) derived from n=5 mice of a representative experiment of two independent experiments performed. Statistical significance was determined with ordinary one-way ANOVA tests. ****p < 0.0001; ***p < 0.001; **p < 0.01; *p < 0.05 and ns stands for not significant.

Absence of Tcf-1 leads to effector CD8$^+$ T cell differentiation in the absence of inflammation

Our data so far show that inflammation negatively regulated Tcf-1 expression and promoted effector differentiation. This raised the question regarding the causal relationship between these events. As schematically depicted in Fig. 20A, inflammation-dependent suppression of Tcf-1 may be a prerequisite for effector differentiation (Fig. 20A, left). Conversely, inflammation may induce effector differentiation and this leads to Tcf-1 suppression (Fig. 20A, middle). Finally, inflammation may independently promote effector differentiation and inhibit Tcf-1 expression (Fig. 20A, right). To begin to discriminate between these possibilities we used P14 cells lacking Tcf-1 which have a reduced ability to transduce Wnt signals [129]. If Wnt/Tcf-1 signaling suppressed effector differentiation, *Tcf7*$^{-/-}$ P14 cells should differentiate more efficiently as compared to WT P14 cells.

Indeed, in response to DC33/CpG, *Tcf7*$^{-/-}$ P14 cells expressed more GzmB, less IL-2 and differentiated significantly more efficiently into KLRG1$^+$ CD127$^-$ SLECs as compared to WT P14 cells (Fig. 20B-D). The absence of Tcf-1 did not impact the expansion of P14 cells (Fig. 20E). Interestingly, considerable effector differentiation of *Tcf7*$^{-/-}$ P14 cells was observed with DC33 vaccination (Fig. 20B-D). Thus, the absence of Tcf-1 results in efficient effector differentiation even in the absence of systemic inflammation. This indicates that Tcf-1 expression counteracted effector differentiation and that inflammation-induced Tcf-1 suppression facilitated effector differentiation.

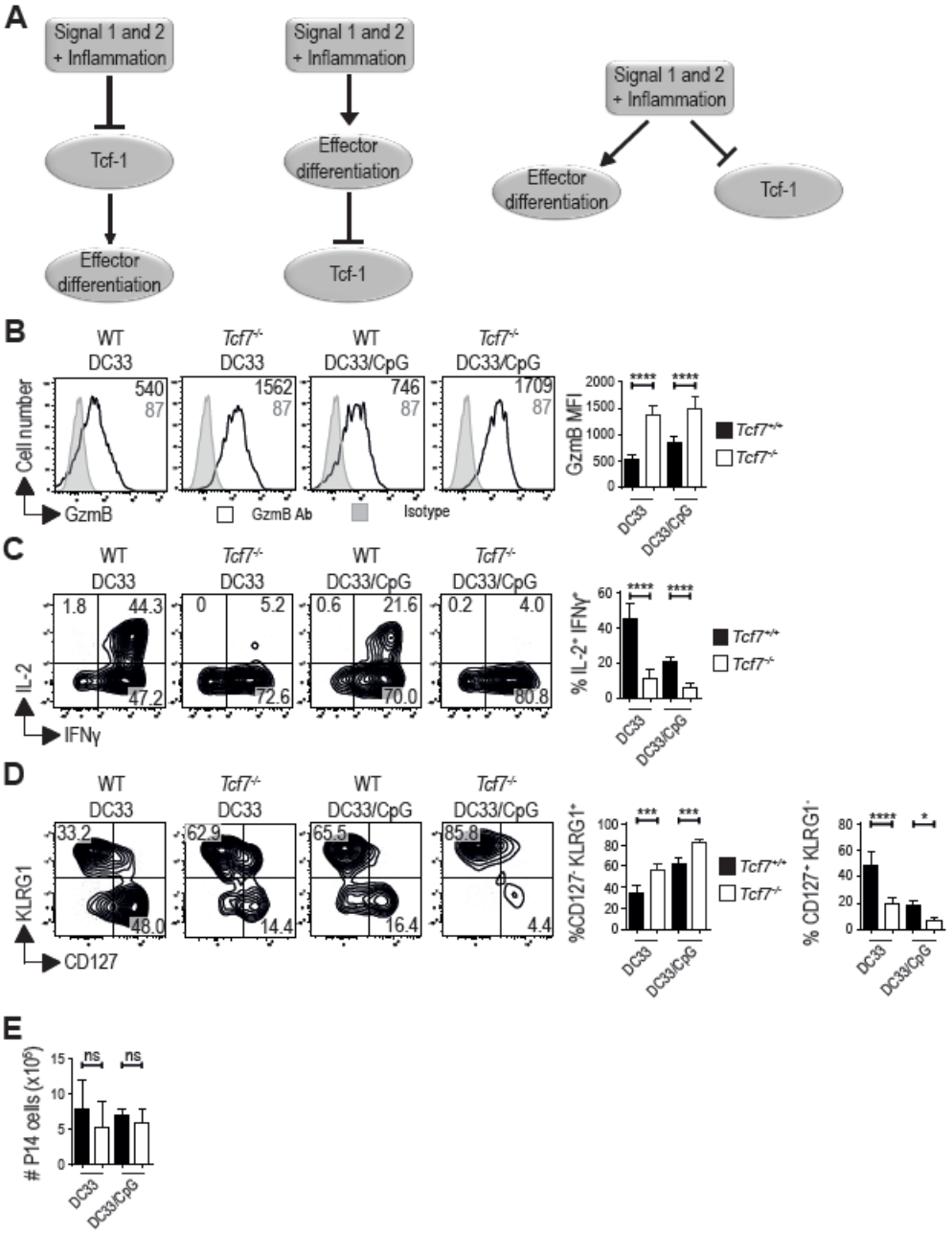

FIGURE 20 – Absence of Tcf-1 facilitates CD8⁺ T cell effector differentiation in the absence of systemic inflammation. (**A**) Hypothetical relationships between inflammation, Tcf-1 expression and effector differentiation of primed CD8⁺ T cells. Inflammation-dependent suppression of Tcf-1 may be a prerequisite for effector differentiation (left). Conversely, inflammation may induce effector differentiation and this leads to Tcf-1 suppression (middle) or may independently promote effector differentiation and suppress Tcf-1 (right). (**B-E**) P14 WT or *Tcf7*$^{-/-}$ cells were transferred into recipient mice (CD45.1⁺) that were then vaccinated the day after with DC33 or DC33/CpG. (**B-D**) Gated WT and *Tcf7*$^{-/-}$ P14 cells were analyzed for (**B**) the MFI of GzmB expression, (**C**) the percentage of cells producing IL-2 and IFNγ and (**D**) the percentage of CD127⁺ KLRG1⁻ (MPECs) and CD127⁻ KLRG1⁺ (SLECs) cells. Corresponding bar graphs depict mean values (± SD). Plots are representative example from n=5 mice of a representative experiment of two experiments performed. (**E**) Recipient spleens were analyzed for the abundance of WT and *Tcf7*$^{-/-}$ P14 cells 7 days later. The corresponding bar graph depicts mean numbers (±SD) of P14 cells present in the spleens. Unpaired *t*-test was used for statistical analyses. ****p < 0.0001; ***p < 0.001; **p < 0.05; *p < 0.05; ns stands for not significant.

The above experiments were performed with P14 cells from mice with germ-line *Tcf7* deletion, in which T cell development is impaired [162, 167]. We thus ensured that increased effector differentiation in the absence of Tcf-1 was independent of altered T cell development. To this end, we generated conditional

Tcf7$^{lox/lox}$ mice harboring a Rosa26 driven lox stop lox EYFP (Rosa-lsl-EYPF) cassette and a P14 TCR transgene (Fig. 21A). Exposure to Tat-Cre fusion protein *in vitro* induced EYFP expression in a considerable fraction of naïve P14 cells. Importantly, the vast majority of EYFP$^+$ cells had deleted the floxed portion of the *Tcf7* gene and lost Tcf-1 protein expression, while most EYFP$^-$ cells were Tcf-1$^+$ (Fig. 21B). Thus, EYFP induction can be used to track cells, in which Tcf-1 has been deleted.

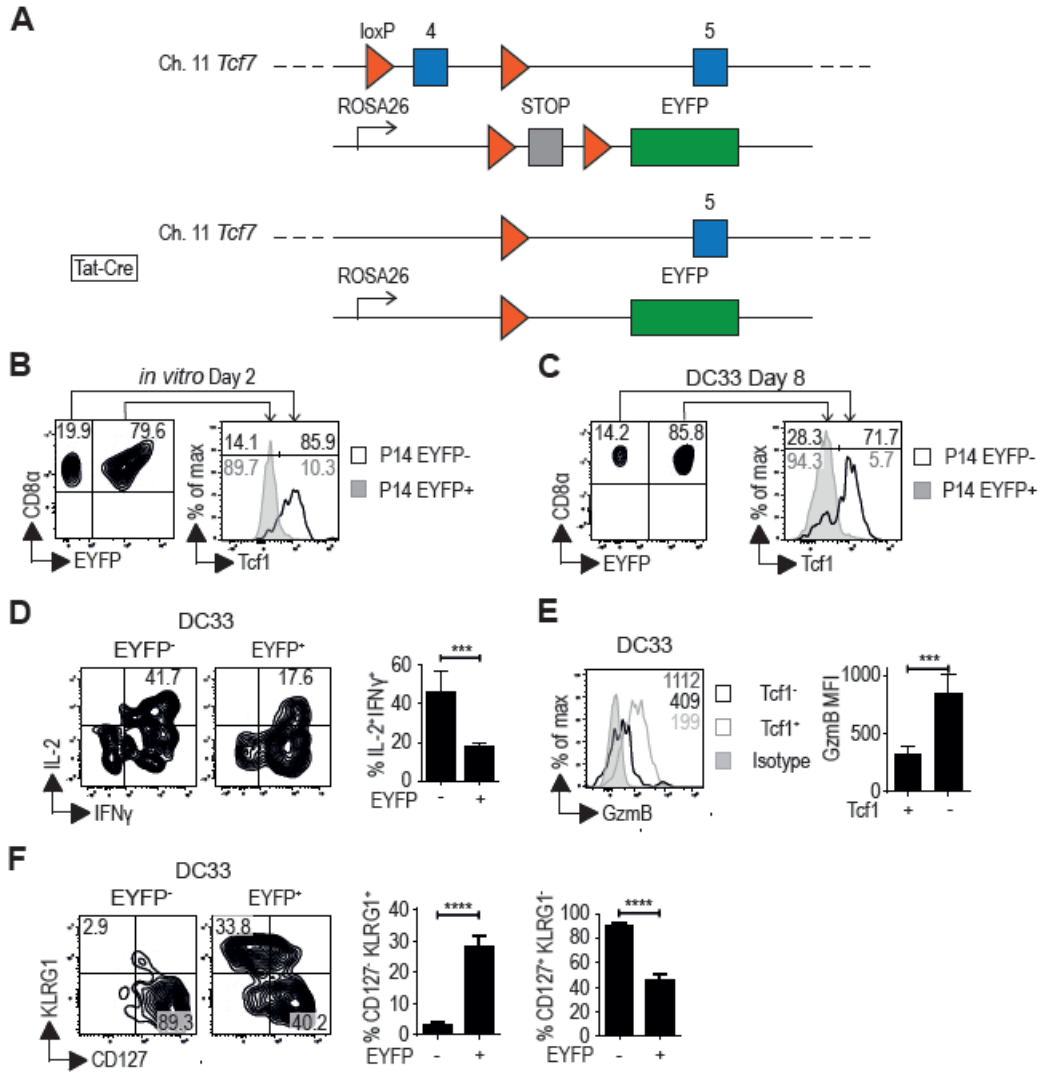

FIGURE 21 – *Tcf7*-deletion in naive CD8$^+$ T cells facilitates effector differentiation after DC33 vaccination. (**A**) Scheme represents the genomic locus of *Tcf7*^{LoxLlox} *Rosa26Sor*$^{tm1(EYFP)}$ before and after treatment with Tat-Cre *in vitro*. (**B-C**) *Tcf7*$^{Lox/Lox}$ *Rosa26Sor*$^{tm(1EYFP)}$ P14 cells (CD45.2$^+$) were treated with Tat-Cre *in vitro* and the frequency of EYFP$^+$ P14 cells (*Tcf7*-deleted) was determined (**B**) after 48h of culture *in vitro* and (**C**) at day 7 post vaccination with DC33. (**D-F**) Gated EYFP$^+$ (*Tcf7*-deleted) and EYFP$^-$ (non-deleted) P14 cells (CD45.2$^+$) were analyzed for (**D**) the percentage of cells producing IL-2 and IFNγ, (**E**) the MFI of GzmB expression and (**F**) the percentage of CD127$^+$ KLRG1$^-$ (MPECs) and CD127$^-$ KLRG1$^+$ (SLECs) cells. Corresponding bar graphs depict mean values (± SD) derived from n=5 mice of a representative experiment of two independent experiments performed. Statistical significance was determined with unpaired t-tests. ****p < 0.0001; ***p < 0.001; ns stands for not significant.

Next, Tat-Cre treated, naïve P14 *Tcf7*$^{Lox/Lox}$ Rosa-lsl-EYFP cells (CD45.2$^+$) were adoptively transferred into CD45.1$^+$ recipient mice, which were then vaccinated with DC33. The frequency of EYFP$^+$ P14 cells (*Tcf7* deleted) seven days later corresponded to that of input (Fig. 21B-C), indicating comparable expansion of EYFP$^+$ and EYFP$^-$ cells. We confirmed that EYFP$^+$ cells were mostly Tcf-1$^-$ and EYFP$^-$ cells were predominantly Tcf-1$^+$ (Fig. 21C). As expected, EYFP$^-$ P14 cells (Tcf-1$^+$) were IL-2$^+$, GzmB$^-$ and predominantly KLRG1$^-$ CD127$^+$ MPEC cells. In contrast, EYFP$^+$ (*Tcf7*-deleted) P14 cells were IL-2$^-$, GzmB$^+$ and predominantly KLRG1$^+$ CD127$^-$ SLECs (Fig. 21D-F). More efficient effector differentiation of EYFP$^+$ (*Tcf7*-deleted) as compared to EYFP$^-$ P14 cells was similarly observed following DC33/CpG vaccination (Fig. 22). Thus enhanced effector differentiation of CD8$^+$ T cells lacking *Tcf7* is not due to a developmental defect as it is readily observed when Tcf-1 is deleted in naïve CD8$^+$ T cells. Collectively, these data show that Tcf-1 counteracts effector differentiation and that inflammation-dependent signals downregulate Tcf-1 in primed CD8$^+$ T cells to allow efficient effector differentiation.

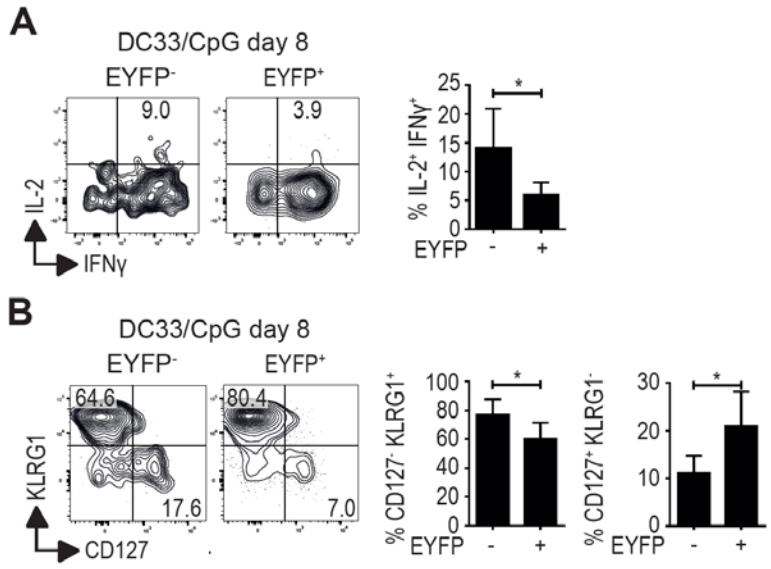

FIGURE 22 – *Tcf7*-deletion in naive CD8$^+$ T cells facilitates effector differentiation after DC33/CpG vaccination. Gated EYFP$^+$ (*Tcf7*-deleted) and EYFP$^-$ (*Tcf7* non-deleted) P14 cells (CD45.2$^+$) were analyzed for (**A**) the percentage of cells producing IL-2 and IFNγ, (**B**) the percentage of CD127$^+$ KLRG1$^-$ (MPECs) and CD127$^-$ KLRG1$^+$ (SLECs) cells. Corresponding bar graphs depict mean values (± SD) derived from n=5 mice of a representative experiment of two independent experiments performed. Statistical significance was determined with unpaired t-tests. *p < 0.05; ns stands for not significant.

Tcf-1 expression counteracts effector differentiation

We showed that lack of Tcf-1 expression enhanced effector differentiation. We next addressed which domain in Tcf-1 was needed to repress effector differentiation. To answer this question, we used transgenic mice expressing the p33 or the p45 Tcf-1 isoform. The former mediates only repressor function while the latter can additionally mediate signal transduction via its association with co-activators (Fig. 23A).

In response to DC33 vaccination, p33 Tg expression by *Tcf7*$^{-/-}$ P14 cells showed enhanced effector differentiation (data not shown), indicating that the repressive short Tcf-1 isoform p33 does not play a role in CD8^{+} T cell differentiation. On the other hand, p45 Tg expression in *Tcf7*$^{-/-}$ P14 cells reduced effector differentiation as compared with *Tcf7*$^{-/-}$ P14 cells based on IL-2 production, GzmB expression and CD127/KLRG1 phenotype (Fig. 23B-D). We ensured that the p45 Tg was still expressed after DC33 activation (Fig. 23E). These data indicate that the N-terminus of Tcf-1 is needed to counteract effector differentiation.

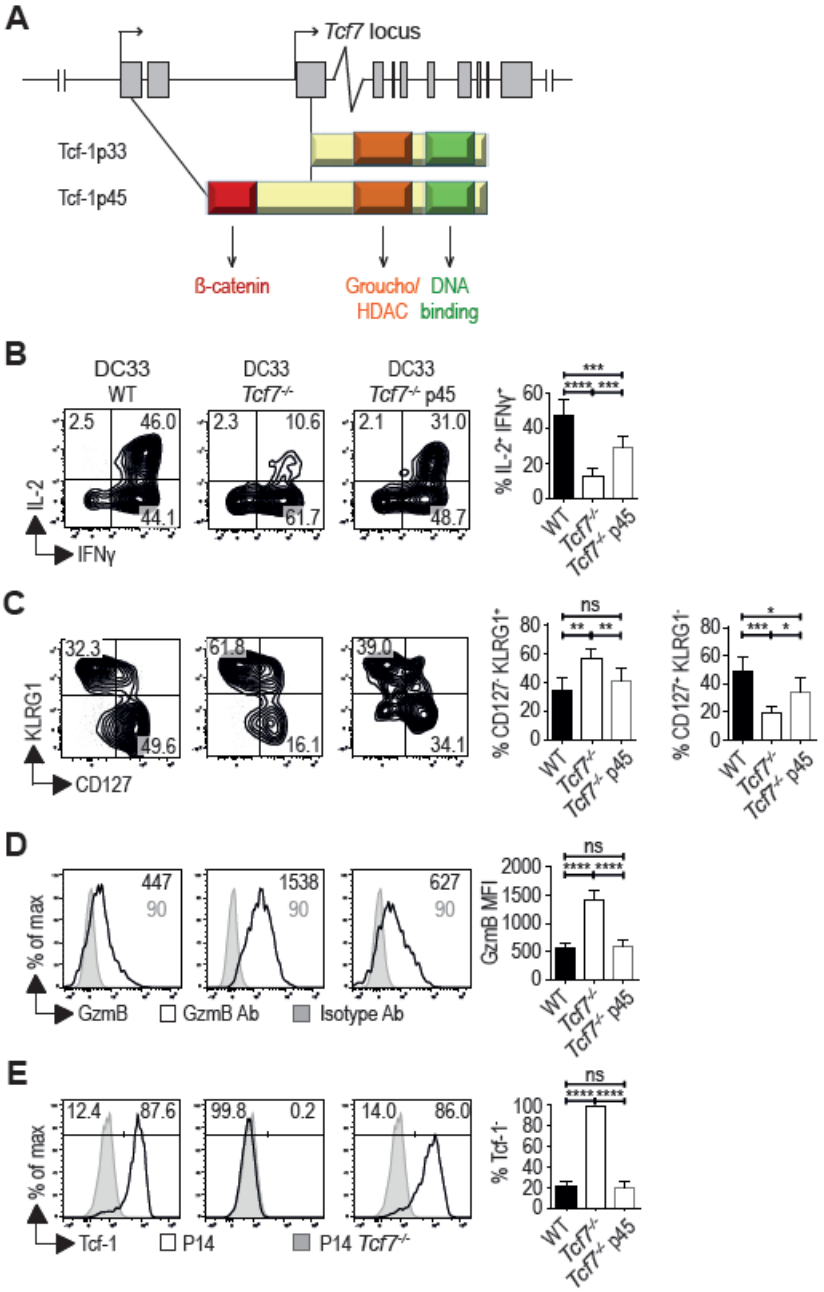

FIGURE 23 – Enforced Tcf-1 expression represses CD8⁺ T cell differentiation in absence of systemic inflammation. (**A**) Scheme represents Tcf-1 isoforms expressed by mice. Tcf-1 p33 isoform is a short isoform lacking the β-catenin domain and Tcf-1 p45 is the full length isoform. (**B-E**) WT, *Tcf7*$^{-/-}$ and *Tcf7*$^{-/-}$ p45 P14 cells (CD45.2⁺) were transferred into CD45.1⁺ recipient mice which were then vaccinated with DC33. Gated P14 cells were analyzed at day 7 post vaccination for (**B**) the percentage of cells secreting IL-2 and IFNγ after re-stimulation with Gp_{33-41} peptide, (**C**) the percentage of CD127⁺ KLRG1⁻ (MPECs) and CD127⁻ KLRG1⁺ (SLECs) cells, (**D**) the MFI of GzmB expression and (**E**) the percentage of cells expressing Tcf-1. Corresponding bar graphs depict mean values (± SD) derived from n=5 mice of a representative experiment of two independent experiments performed. Statistical significance was determined with one-way ANOVA tests. ****p < 0.0001; ***p < 0.001; **p < 0.05; *p < 0.05; ns stands for not significant.

Absence of Tcf-1 prevents default central memory formation

DC immunization in the absence of systemic inflammation accelerates CD8$^+$ T cell memory formation [184]. As we observed enhanced effector differentiation of DC33-stimulated *Tcf7*$^{-/-}$ CD8$^+$ T cells, we next asked whether the default memory formation depended on Tcf-1 expression. WT and *Tcf7*$^{-/-}$ P14 cells were readily detected at day 40 after DC33 vaccination, although *Tcf7*$^{-/-}$ P14 cells were somewhat reduced (p=0.08) (Fig. 24A). However, cells with a CD127$^+$ CD62L$^+$ central memory phenotype were severely reduced (8 fold) among *Tcf7*$^{-/-}$ P14 cells (Fig. 24B). Functionally, *Tcf7*$^{-/-}$ P14 memory cells showed a greatly reduced ability to produce IL-2 and a significantly increased expression of GzmB (Fig. 24C-D). Finally, equal numbers of flow-sorted memory P14 WT and *Tcf7*$^{-/-}$ cells were transferred into naïve secondary recipients, which were then challenged with LCMV clone 13 (cl13) infection. While WT

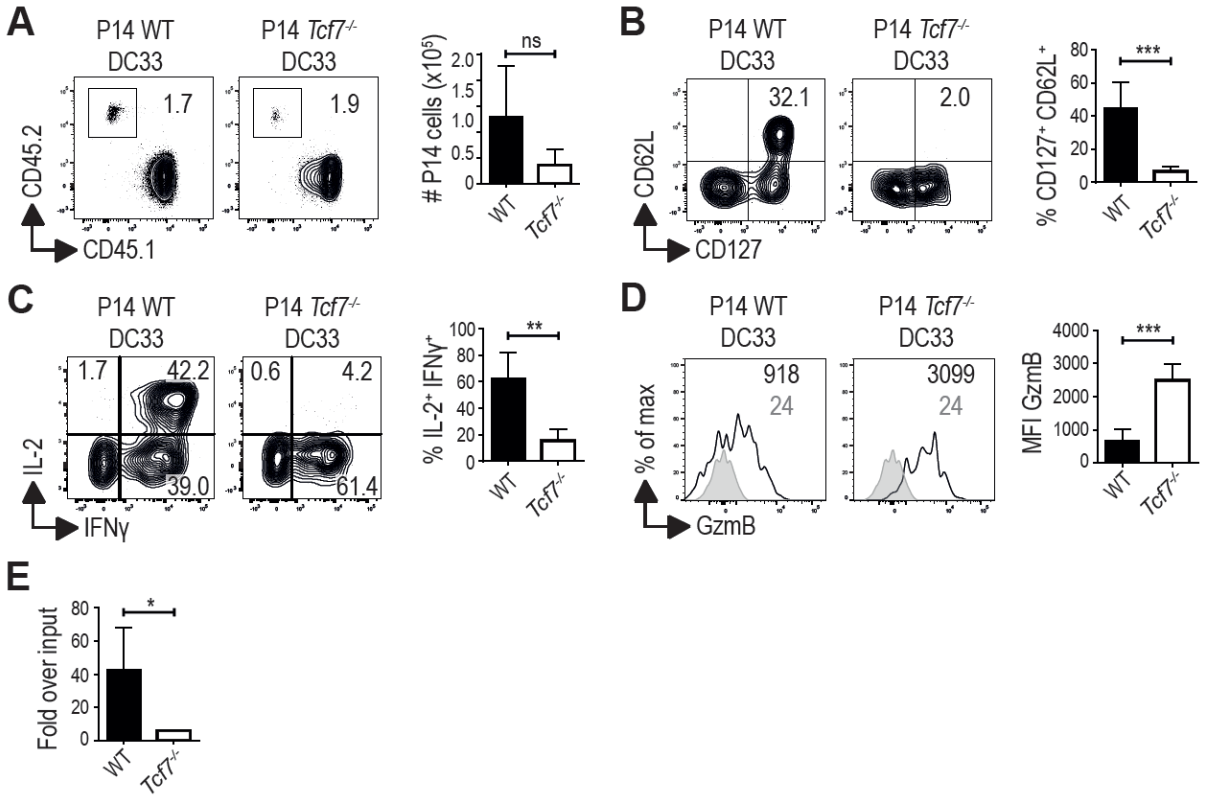

FIGURE 24 – Tcf-1 expression is essential for default central memory formation in response to DC33 vaccination. WT or *Tcf7*$^{-/-}$ P14 cells (CD45.2$^+$) were transferred into recipient mice (CD45.1$^+$) which were then vaccinated with DC33. (**A**) Recipient spleens were analyzed for the presence of WT or *Tcf7*$^{-/-}$ P14 cells at day 40 post vaccination. The corresponding bar graph depicts mean numbers (± SD) of P14 cells present in the spleen. (**B-D**) Gated P14 cells were analyzed for (**B**) the percentage of CD127$^+$ CD62L$^+$ central memory cells, (**C**) the percentage of cells producing IL-2, and (**D**) the MFI of GzmB expression. Corresponding bar graphs depict mean values (± SD) derived from n= 3 (**E**) WT and *Tcf7*$^{-/-}$ P14 cells (CD45.2$^+$) were flow-sorted and equal numbers were transferred into secondary recipients (CD45.1$^+$). Secondary recipients were infected with LCMV cl13 and the recall expansion of P14 cells was determined 8 days later. Bar graphs depicts mean values (± SD) derived from n= 2-8 mice of a representative experiment of two independent experiments performed. Statistical significance was determined with unpaired *t*-tests. ***p < 0.001; **p < 0.01; *p < 0.05; ns stands for not significant.

P14 cells memory cells expanded efficiently, recall expansion of *Tcf7*$^{-/-}$ P14 cells was greatly reduced (Fig. 24E). Corresponding results were obtained when mice had been vaccinated with DC33/CpG (Fig. 25).

Thus, the formation of default central memory cells, but not effector memory cells, is stringently dependent on Tcf-1. Deficient central memory formation in the absence of Tcf-1 may be the direct consequence of the increased effector differentiation during the primary immune response.

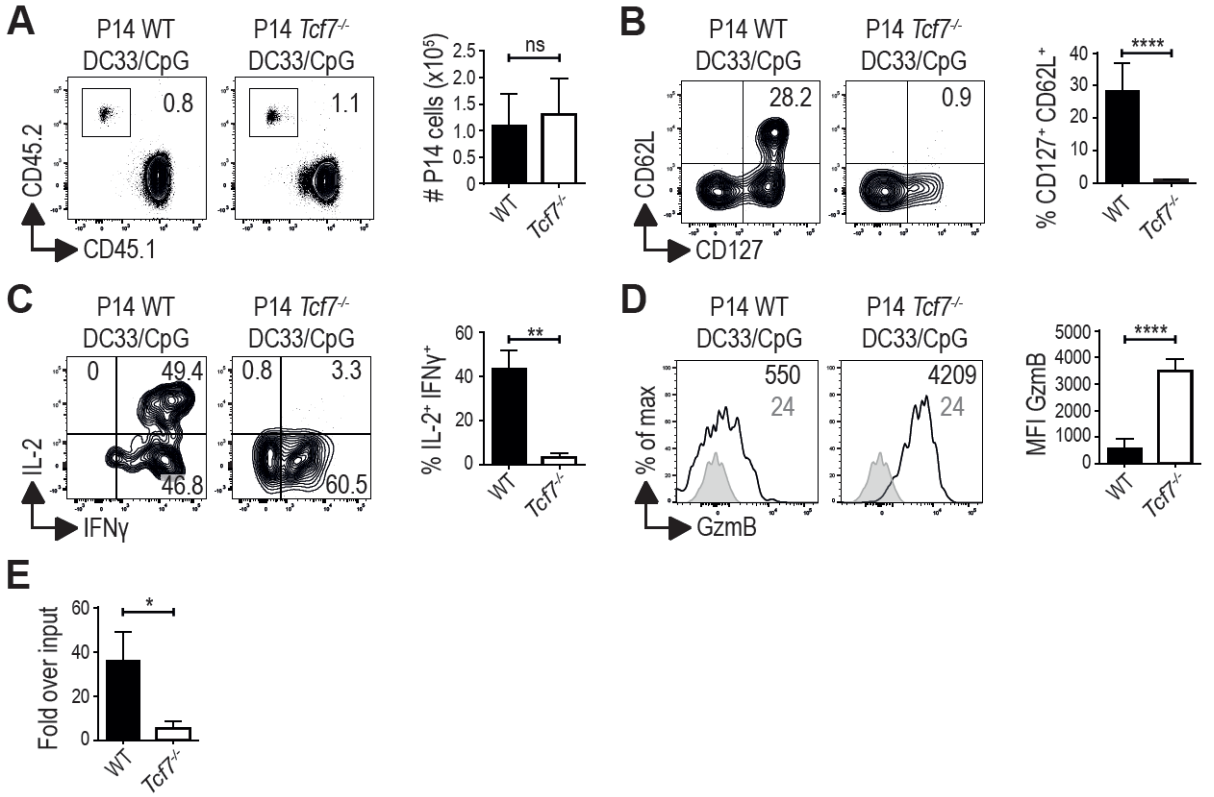

FIGURE 25 - Tcf-1 expression is essential for default central memory formation in response to DC33/CpG vaccination. WT or *Tcf7*$^{-/-}$ P14 cells (CD45.2^{+}) were transferred into recipient mice (CD45.1^{+}) which were then vaccinated with DC33/CpG. (**A**) Recipient spleens were analyzed for the presence of WT or *Tcf7*$^{-/-}$ P14 cells at day 40 post vaccination. The corresponding bar graph depicts mean numbers (± SD) of P14 cells derived from n=5 mice of a single experiment performed. (**B-D**) Gated P14 cells were analyzed for (**B**) the percentage of cells producing IL-2 and IFNγ, (**C**) the percentage of CD127^{+} CD62L^{+} central memory cells and (**D**) the MFI of GzmB expression. Corresponding bar graph depicts mean numbers (± SD) of P14 cells derived from n=5 mice of a single experiment performed. (**E**) WT and *Tcf7*$^{-/-}$ P14 cells (CD45.2^{+}) were flow-sorted and equal numbers were transferred into secondary recipients (CD45.1^{+}). Secondary recipients were infected with LCMV cl13 and the recall expansion of P14 cells was determined 8 days later. Bar graph depicts mean values (± SD) derived from n= 3 mice of a single experiment performed. Statistical significance was determined with unpaired *t*-tests. ****p < 0.0001; **p < 0.01; *p < 0.05; ns stands for not significant.

Tcf-1 represses IL-12Rβ2 expression and establishes a threshold for effector differentiation

Although STATs were discovered as activators of gene expression, our data suggest that STAT4 represses *Tcf7*. We wondered whether STAT4 could directly regulate Tcf-1 expression. Inspection and analysis of publically available STAT4 CHIPSeq data from T_H1 cells [4] revealed that STAT4 was bound to the *Tcf7* locus and this correlated with the presence of repressive H3K27me3 marks (Fig. 26A). In the absence of STAT4, activating H3K4me3 marks and *Tcf7* expression are moderately increased based on gene array data (2.25 fold) [4], suggesting that STAT4 binding has a direct and repressive effect on *Tcf7*. Conversely, it is well known that *IL-12Rβ2* expression is induced by STAT4 [99, 194-196]. Indeed, STAT4 is bound to the *IL-12Rβ2* loci in T_H1 cells and this correlates with the presence of activating H3K4me3 marks (Fig. 26B). In the absence of STAT4, repressive H3K27me3 marks are increased at *IL-12Rβ2* locus and *IL-12Rβ2* expression is strongly reduced (8.6 fold) [4]. Thus STAT4 plays a role for *Tcf7* repression and *IL-12Rβ2* induction in T_H1 cells. The latter improves IL-12 responsiveness, which leads to further and reinforced *Tcf7* repression and effector differentiation. To prevent unwanted or excessive effector differentiation, we postulated that Tcf-1 inhibits *IL-12Rβ2* expression. To address this possibility, we compared *IL-12Rβ2* levels in WT and *Tcf7*$^{-/-}$ P14 cells. *IL-12Rβ2* mRNA expression was significantly increased when MPECs or SLECs lacked Tcf-1 whereas there was no difference in naïve $CD8^+$ T cells (Fig. 27A). Consistent with a direct effect, inspection of publically available CHIPSeq data [5] revealed that Tcf-1 is associated with the *IL-12Rβ2* locus in $CD8^+$

thymocytes (Fig. 26C). Thus, Tcf-1 seems to directly suppress *IL-12Rβ2* expression. This suppression may be overcome when IL-12 induces STAT4-dependent *Tcf7* repression.

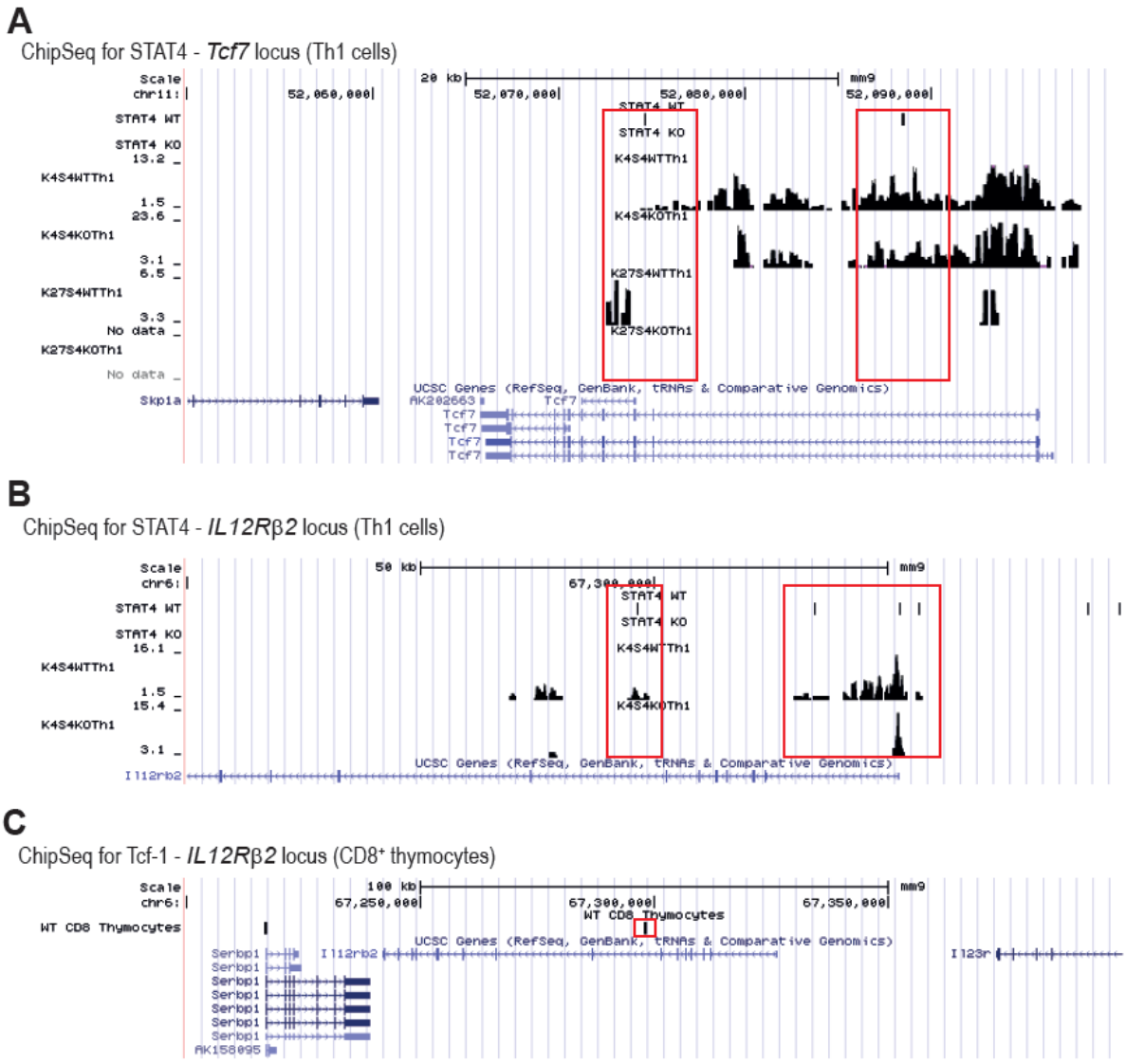

FIGURE 26 – STAT4 positively regulates IL-12Rβ2 expression and inhibits Tcf-1 expression which can inhibit IL-12Rβ2 expression. (**A**-**B**) CHIP-Seq of STAT4 was reported by Wei et al. [4] and (**C**) CHIP-Seq of Tcf-1 in $CD8^+$ thymocytes was reported by Xing et al. [5]. The CHIP-Seq track wiggle files were uploaded to the UCSC genome browser for visualization of enriched binding by the indicated transcription factors. For the select gene locus, the transcription start site and orientation are marked by arrows. The vertical bars over STAT4 or Tcf-1 tracks indicate the enriched binding peaks. (**A**-**B**) Red squares mark the enriched binding sites. (**A**) Pictures show enriched binding of STAT4 at the Tcf7 and *IL-12Rβ2* locus and these data are correlated with activating H3K4me3 and inhibitory H3K27me3 marks. (**B**) Picture shows enriched binding of Tcf-1 at the *IL-12Rβ2* loci. (**C**) Pictures show enriched binding of Tcf-1 at the *IL-12Rβ2* locus.

Finally, to gain insights into T cell differentiation in the absence of Tcf-1, we assessed the expression of key transcription factors involved in effector or memory differentiation. Among transcription factors involved in memory differentiation, *Tcf7*$^{-/-}$ and WT MPECs expressed comparable levels of *Bcl6*, while *Eomes* was increased in *Tcf7*$^{-/-}$ MPECs (Fig. 27B). These transcription factors were either expressed at the same level in naïve CD8^{+} T cells WT and *Tcf7*$^{-/-}$ (*Bcl6*) or reduced in *Tcf7*$^{-/-}$ as compared to WT naïve CD8^{+} T cells (*Eomes*) (Fig. 27B). These expression patterns did not provide a straightforward explanation for deficient memory formation in the absence of Tcf-1.

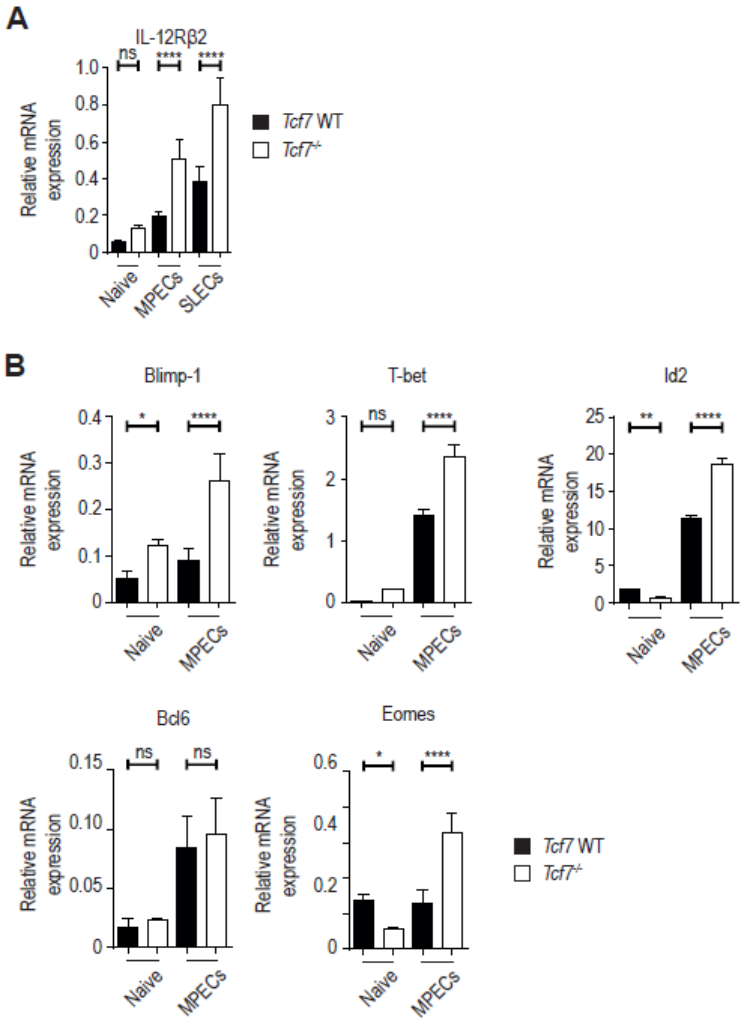

FIGURE 27 – Tcf-1 inhibits *IL-12Rβ2* expression and regulates the expression of several transcription factors involved in CD8^{+} T cell differentiation. (**A**) *IL-12Rβ2* expression was analyzed in flow-sorted naïve CD8^{+} T cells (CD44^{-} CD62L^{+}) and in MPECs (CD127^{+} KLRG1^{-}) or SLECs (CD127^{-} KLRG1^{+}) at day 8 post DC33 vaccination in P14 WT or Tcf-1-deficient cells. (**B**) mRNA expression levels of indicated transcription factors were analyzed in naïve CD8^{+} T cells (CD44^{-} CD62L^{+}) and in MPECs (at day 8 post DC33 vaccination in P14 WT or Tcf-1-deficient cells. (**A**-**B**) Bar graphs depict the relative mRNA expression (± SD) of indicated genes in the indicated population relative to *HPRT* housekeeping gene expression. Statistical significance was determined with unpaired *t*-tests. ****p < 0.0001; **p < 0.05; *p < 0.05; ns stands for not significant.

On the other hand, *Tcf7*$^{-/-}$ MPECs overexpressed several transcription factors implicated in effector differentiation i.e. *Id2*, *Blimp-1* and *Tbx21* (Fig. 27B). *Tcf7*$^{-/-}$ MPECs expressed these transcription factors at equal or even higher levels than those observed in WT SLECs (data not shown). Thus the upregulation of several transcription factors implicated in effector differentiation in MPECs can account for the default effector differentiation of primed *Tcf7*$^{-/-}$ CD8^{+} T cells. We concluded that Tcf-1 was blocking CD8^{+} T cell differentiation by repressing the expression of several transcription factors involved this differentiation process.

Part II – Role of Wnt protein secretion for Wnt pathway activity in naïve CD8$^+$ T cells, for CD8$^+$ T cell homeostasis and differentiation in response to infection

Wnt/Tcf-1 signaling is constitutively active in naïve CD8$^+$ T cells based on the expression of the Wnt target genes *Axin2* and *Tcf7*, the presence of phosphorylated LRP6 and the presence of active, non-phosphorylated β-catenin [128, 129]. We hypothesized that the constitutive Wnt pathway activity in naïve CD8$^+$ T cells depended on the exposure of CD8$^+$ T cells to Wnt proteins.

There exist 19 different Wnt genes which prevents a simple loss of function approach to study the role of Wnt proteins for the homeostasis of naïve CD8$^+$ T cells. To overcome the possible problem of *in vivo* redundancy of Wnt proteins, we took advantage of mice lacking Wntless, which is required for the secretion of all Wnt proteins [149]. *Wls* deletion is embryonic lethal but a conditional allele of *Wls* has been generated by K. Basler *[197]*. We thus used cell type-specific *Wls* deletion to address the role of Wnt protein secretion for Wnt pathway activity in naive CD8$^+$ T cells. In addition, we addressed whether *Wls* deletion impacted the abundance, the activation status and the function of CD8$^+$ T cells.

Wnt secretion by T cells is not needed to maintain Wnt/Tcf-1 signaling pathway in naïve CD8$^+$ T cells

To delete *Wls* in T cells, we crossed *Wls*$^{flox/flox}$ mice to mice expressing the Cre recombinase under the control of the CD4 promoter [198]. The *Wls*$^{flox/flox}$ locus is depicted in Fig. 28A with the different primers used to verify the deletion of *Wls*. *Wls* deletion was assessed using PCR. Complete deletion of *Wls* was observed in CD4-Cre *Wls*$^{flox/flox}$ mice (Fig. 28B). *Wls* deletion completely prevented *Wls* gene expression in T cells, based on two distinct primer pairs, one of which did not include the deleted region. Unexpectedly, we observed that *Wls* expression was already strongly reduced (10 fold) in *Wls*$^{flox/flox}$ mice

i.e. in the absence of Cre recombinase (Fig. 28C). We thus included WT mice as controls in our experiments.

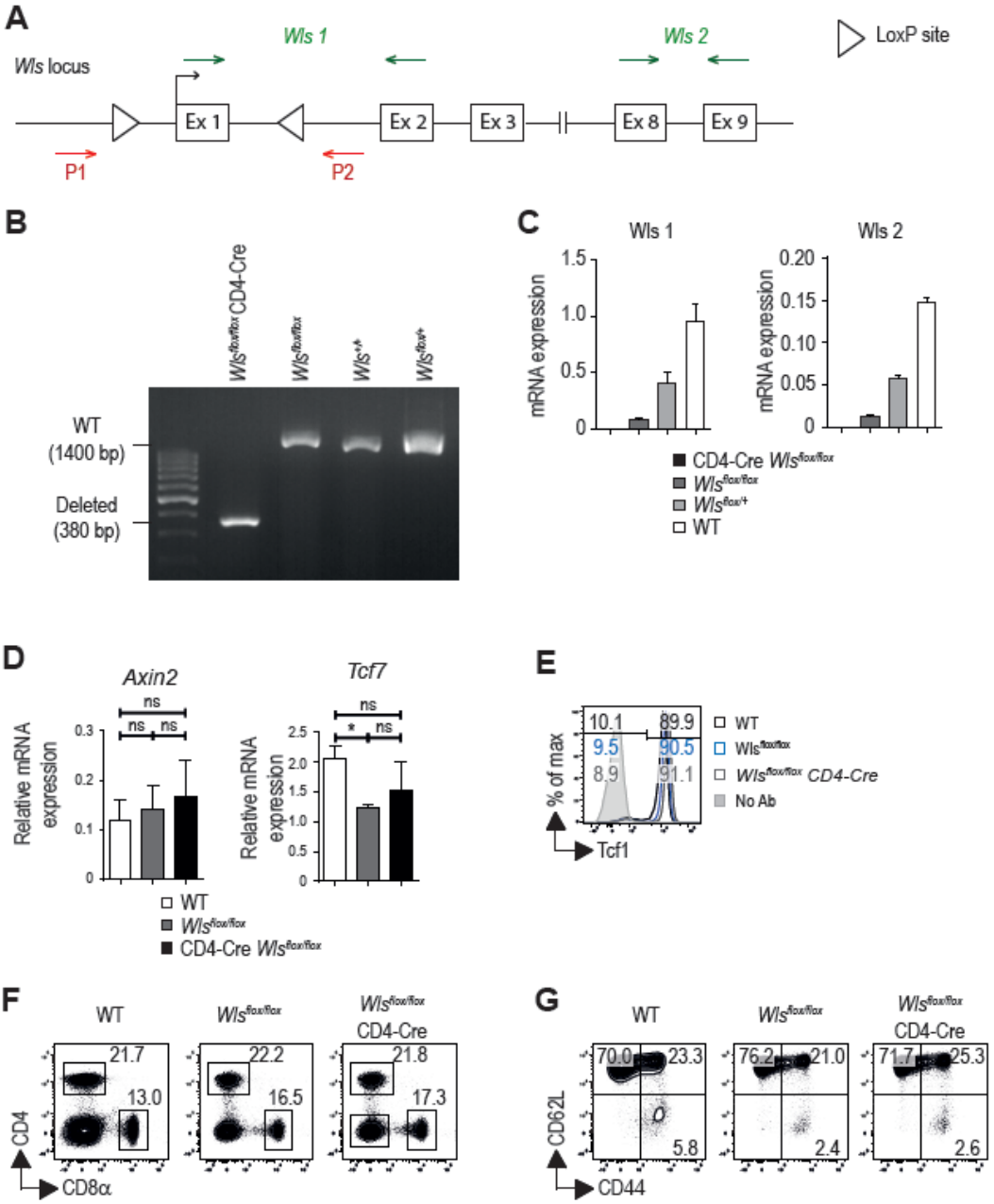

FIGURE 28 – Wnt pathway activity in CD8$^+$ T cells in *Wls*-deleted T cells. (**A**) Scheme represents the *Wls* locus of *Wls*$^{flox/flox}$ mice and the different primers used for either PCR (P1 and P2 in red) or qPCR (Wls 1 and Wls 2 in green). (**B-C**) Naïve CD8$^+$ T cells from either *Wls*$^{flox/flox}$ CD4-Cre, *Wls*$^{flox/flox}$, *Wls*$^{flox/+}$ or *Wls*$^{+/+}$ (WT) mice were FACS-sorted and DNA and RNA were extracted from sorted cells. (**B**) Image shows *Wls* deletion after a PCR using P1 and P2 primers allowing to detect both the WT (1400bp) and the deleted *Wls* band (380bp) at the same time. (**C**) Bar graphs show the mRNA expression (± SD) of *Wls* gene using the two different pairs of primers Wls 1 and Wls 2, assessed by qPCR relative to *HPRT* housekeeping gene (CD4-Cre *Wls*$^{flox/flox}$ in black, *Wls*$^{flox/flox}$ in dark grey, *Wls*$^{flox/+}$ in light grey and WT in white). (**D**) Bar graphs show the mRNA expression (± SD) of Wnt reporter genes, *Axin2* and *Tcf7,* assessed by qPCR relative to *HPRT* housekeeping gene in CD8$^+$ T cells WT (in white), *Wls*$^{flox/flox}$ (in dark grey) and CD4-Cre *Wls*$^{flox/flox}$ (in black). Statistical significance was determined with one-way ANOVA tests. *p < 0.05 and ns stands for not significant. (**E**) Histogram shows the Tcf-1 protein expression in WT, *Wls*$^{flox/flox}$ and *Wls*$^{flox/flox}$ CD4-Cre CD8$^+$ T cells freshly isolated from the spleen. Numbers indicate the percentage of Tcf-1$^+$ and Tcf-1$^-$ cells in the corresponding gates. (**F**) Dot plots show the percentages of both CD4$^+$ and CD8α$^+$ cells among total splenocytes in either WT or *Wls*$^{flox/flox}$ CD4-Cre splenocytes. Numbers indicate the percentage of cells in the corresponding gate. (**G**) Contour plots show the percentage of WT, *Wls*$^{flox/flox}$ or CD4-Cre *Wls*$^{flox/flox}$ naïve CD8$^+$ T cells (CD44$^-$ CD62L$^+$) or activated CD8$^+$ T cells (CD44$^+$ CD62L$^-$) among CD8$^+$ T cells. Data are representative from at least 2 independent experiments (n=2-3).

In order to determine whether Wls deficiency in T cells impacted Wnt/Tcf-1 pathway activity in naive $CD8^+$ T cells, we measured the expression of two Wnt target genes using quantitative PCR. The expression of *Axin2* was not altered by the absence of Wls in naïve $CD8^+$ T cells whereas *Tcf7* expression was reduced in *Wls*$^{flox/flox}$ $CD8^+$ T cells but not in *Wls*$^{flox/flox}$ CD4-Cre $CD8^+$ T cells (Fig. 28D). We further measured Tcf-1 protein expression by flow cytometry. In absence of Wls in T cells, Tcf-1 was highly expressed in naïve $CD8^+$ T cells (Fig. 28E). Finally, the absence of Wls in T cells did not impact the abundance of both $CD4^+$ and $CD8^+$ T cells (Fig. 28F) and did not modify the activation status of $CD8^+$ T cells (Fig. 28G). Together, these data indicate that Wls, i.e. Wnt protein secretion, in T cells is not responsible for the Wnt/Tcf-1 pathway activity in naïve $CD8^+$ T cells.

Hematopoietic Wls is not necessary for the maintenance of Wnt/Tcf-1 pathway activity in naïve $CD8^+$ T cells

Since the deletion of Wls in T cells did not impact Wnt/Tcf-1 pathway activity in $CD8^+$ T cells, we extended the deletion of *Wls* to the entire hematopoietic system by crossing *Wls*$^{flox/flox}$ mice with Vav-Cre mice [199]. *Wls* expression was assessed by qPCR in flow sorted naïve $CD8^+$ T cells ($CD44^-$ $CD62L^+$) and B cells from spleens of *Wls*$^{flox/flox}$ Vav-Cre, *Wls*$^{flox/flox}$ and WT mice. *Wls* expression was absent in both $CD8^+$ T cells and B cells from Vav-cre *Wls*$^{flox/flox}$ mice (Fig. 29A). This confirmed the deletion of *Wls* in the hematopoietic system.

In order to determine whether Wls deficiency in hematopoietic cells impacted Wnt/Tcf-1 pathway activity in $CD8^+$ T cells, we measured the expression of Wnt target genes in naïve $CD8^+$ T cells using semi-quantitative PCR.

The expression of *Axin2* was significantly reduced in both *Wls*$^{flox/flox}$ and in Vav-Cre *Wls*$^{flox/flox}$ CD8^{+} T cells as compared with WT CD8^{+} T cells. However, there was no difference between *Wls*$^{flox/flox}$ and Vav-Cre *Wls*$^{flox/flox}$ CD8^{+} T cells (Fig. 29B). Moreover, *Tcf7* expression was significantly reduced in CD8^{+} T cells in absence of Wls in hematopoietic cells whereas *Lef-1* expression was increased in the *Wls*$^{flox/flox}$ CD8^{+} T cells (Fig. 29B). However, the expression of Wnt target genes was highly variable. Indeed, out of 4 experiments, *Axin2* expression was increased once, reduced in another experiment and was similar between *Wls*$^{flox/flox}$ and WT CD8^{+} T cells in two experiments.

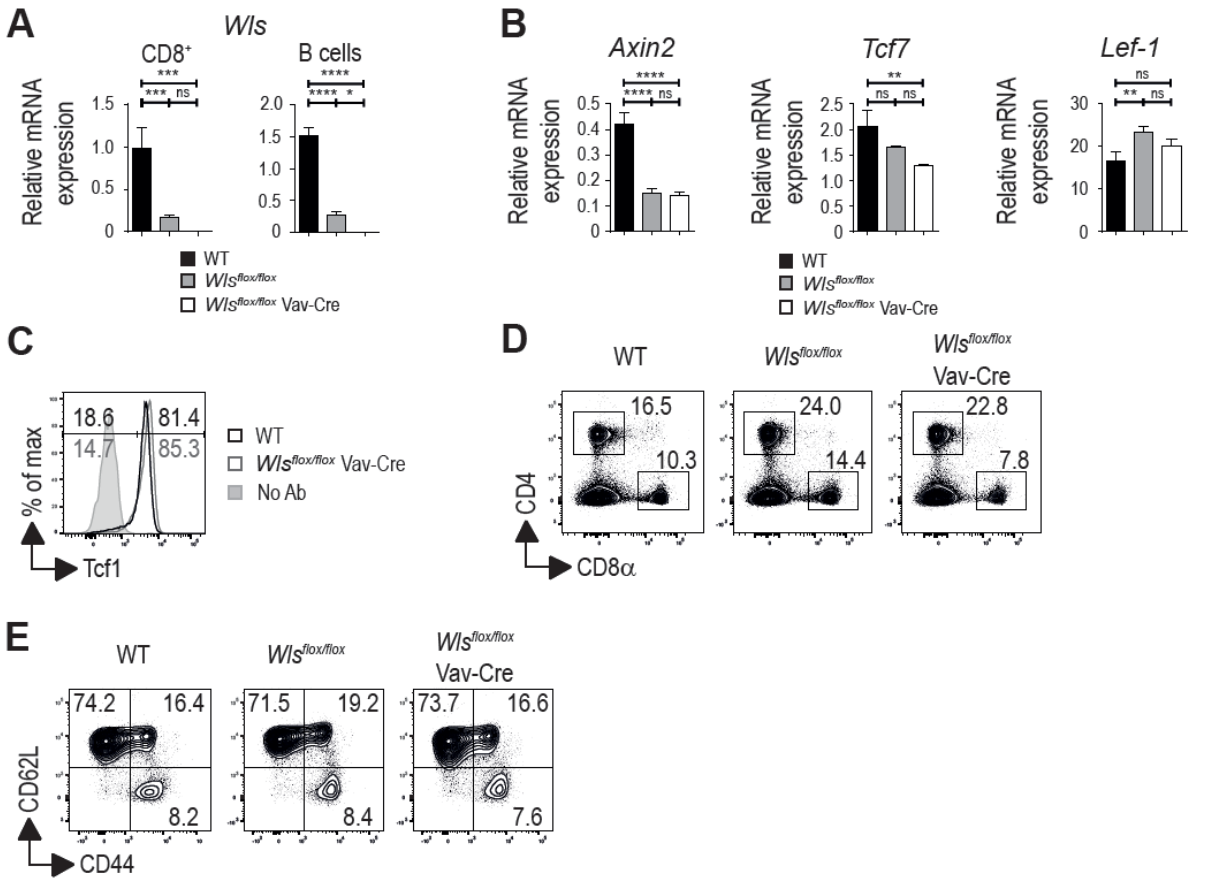

FIGURE 29 – Wnt pathway activity in CD8^{+} T cells is not impacted by the absence of Wls in hematopoietic cells. (**A-B**) Naïve CD8^{+} T cells and B cells from either WT (in black), *Wls*$^{flox/flox}$ (in grey) or *Wls*$^{flox/flox}$ Vav-Cre (in white) were FACS-sorted and RNA was extracted from sorted cells. (**A**) Bar graphs depict the mRNA expression of *Wls* (± SD), assessed by qPCR, in sorted CD8^{+} T cells and B cells relative to *HPRT*. (**B**) Bar graphs depict the mRNA expression (± SD) of Wnt target genes, *Axin2, Tcf7* and *Lef-1,* assessed by qPCR in sorted naïve CD8^{+} T cells relative to *HPRT*. (**C**) Histogram shows the protein expression level of Tcf-1 in WT, *Wls*$^{flox/flox}$ and *Wls*$^{flox/flox}$ Vav-Cre CD8^{+} T cells freshly isolated from the spleen. Numbers indicate the percentage of Tcf-1^{+} and Tcf-1^{-} cells in the indicated gates. (**D**) Contour plots show the percentage of both CD4^{+} and CD8α^{+} cells among total splenocytes from either WT, *Wls*$^{flox/flox}$ or *Wls*$^{flox/flox}$ Vav-Cre mice. (**E**) Contour plots show the expression in CD8^{+} T cells of CD44 and CD62L in the indicated mice. Numbers indicate the percentage of cells in the corresponding gate. Statistical significance was determined with one-way ANOVA tests. ****p < 0.0001, ***p < 0.001; **p < 0.05; *p < 0.05; ns stands for not significant.

Even though *Tcf7* mRNA levels were reduced when Wls was deleted in the hematopoietic system, this did not impact Tcf-1 protein expression (Fig. 29C). Thus these data indicate that Wls in the hematopoietic system does not regulate Wnt target gene expression in naïve $CD8^+$ T cells.

The absence of Wls in the hematopoietic system did not impact the abundance of both $CD8^+$ and $CD4^+$ T cells in the spleen (Fig. 29D) and $CD8^+$ T cells remained mainly in a naïve state ($CD44^-$ $CD62L^+$) even in the absence of Wls in the hematopoietic system (Fig. 29E). Together, these data indicate a normal naïve $CD8^+$ T cell compartment even in the absence of Wls in the entire hematopoietic system.

Stromal Wls is not necessary for the maintenance of Wnt/Tcf-1 pathway activity in naïve $CD8^+$ T cells

We next investigated a role of stromal Wls for Wnt/Tcf-1 pathway activity in $CD8^+$ T cells. Stromal cells have been shown to produce Wnt proteins [200]. We took advantage of mice expressing Cre recombinase under the control of the CCL19 promoter, which is active in stromal cells of secondary lymphoid organs [201]. We verified the absence of *Wls* expression in the $CD45^-$ fraction of flow-sorted from $Wls^{flox/flox}$ CCL19-Cre splenocytes (Fig. 30A). We next determined the expression of Wnt target genes in naïve $CD8^+$ T cells. We observed a reduction of *Axin2* in $CD8^+$ T cells in absence of stromal Wls in secondary lymphoid organs in some but not all mice, similarly to what has been observed above. We did not observe differences in *Tcf7* and *Lef-1 expression* (Fig. 30B). Finally, the abundance and the activation status of both $CD4^+$ and $CD8^+$ T cells was not different in absence of stromal *Wls* (Fig. 30C, D). Stromal Wls is thus not necessary for the maintenance of Wnt/Tcf-1 pathway activity in naïve $CD8^+$ T cells or for the maintenance of a normal T cell compartment.

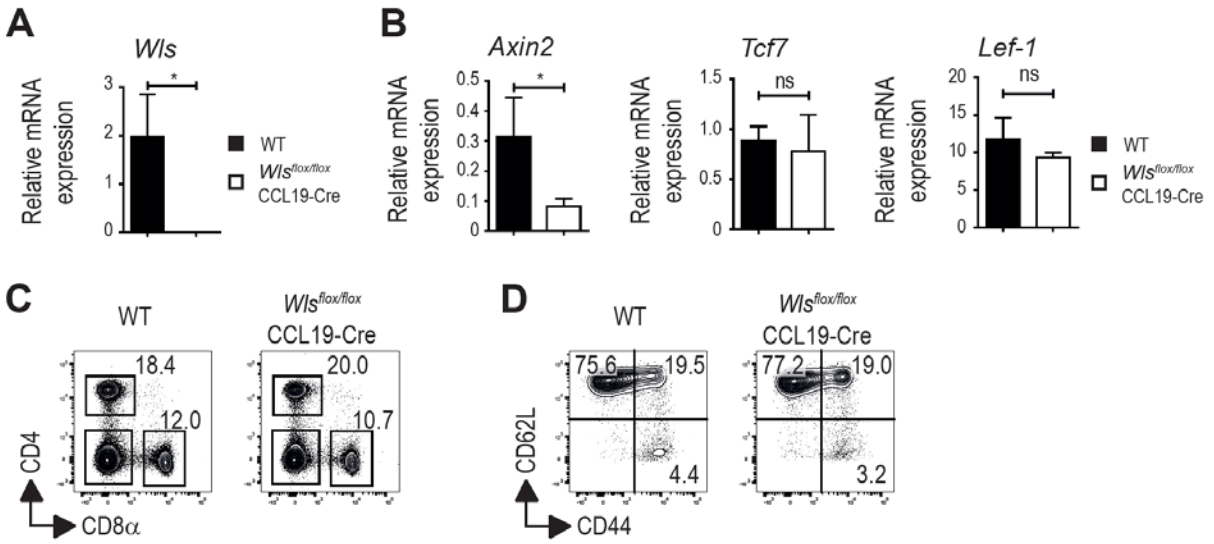

FIGURE 30 – Absence of stromal Wls does not impact Wnt/Tcf-1 signaling in CD8$^+$ T cells. (**A**) Wls expression was analyzed in flow-sorted CD45$^-$ splenocytes from WT (in black) and Wls$^{flox/flox}$ CCL19-Cre (in white) mice. Bar graph depicts the relative mRNA expression of *Wls* in CD45$^-$ splenocytes from indicated mice relative to *HPRT* housekeeping gene. (**B**) Bar graphs show the mRNA expression of *Axin2*, *Tcf7* and *Lef-1* genes in flow-sorted naïve WT (in black) or *Wls$^{flox/flox}$* CCL19-Cre (in white) CD8$^+$ T cells relative to *HPRT*. Indicated values are shown as mean ± SD. (**C**) Contour plots show the percentages of both CD4$^+$ and CD8α$^+$ cells among total splenocytes in either WT or *Wls$^{flox/flox}$* CCL19-Cre splenocytes. (**D**) Contour plots show the percentage of naïve CD8$^+$ T cells (CD44$^-$ CD62L$^+$) and activated CD8$^+$ T cells (CD44$^+$ CD62L$^-$). Statistical significance was determined with unpaired *t*-tests. *p < 0.05; ns stands for not significant.

Wls deletion in T cells or in stromal cells does not impact the CD8$^+$ T cell response to acute LCMV infection

We next wanted to determine whether Wls expressed by T cells played a role for the CD8$^+$ T cell response to infection with LCMV WE strain, which leads to an acute resolved infection in WT mice. We followed the virus specific CD8$^+$ T cell response over time using a tetramer specific for the LCMV epitope (Gp_{33-41}). Eight days after LCMV infection, Gp33-specific CD8$^+$ T cells in the blood were equally abundant in Wls$^{flox/flox}$ CD4-Cre, *Wls$^{flox/flox}$* and WT mice (Fig. 31A). In addition, the proportions of SLECs and MPECs were not different in these mouse strains (Fig. 31B). At day 29 after LCMV infection, the abundance of antigen-specific CD8$^+$ T cells was also similar (Fig. 31C) and the percentage of central memory cells (CD127$^+$ CD62L$^+$) was also not affected by the absence of Wls protein in T cells (Fig. 31D). Similarly, Gp_{33-41}-specific CD8$^+$ T cells expressed same levels of KLRG1 and CD44 even in absence of Wls in T cells (data not shown). Similar results were obtained in the spleen when infected mice were sacrificed at day 42 post infection (data not shown). Thus, there is so far no evidence that Wls in T cells plays a role for the CD8$^+$ T cell response to acute LCMV infection.

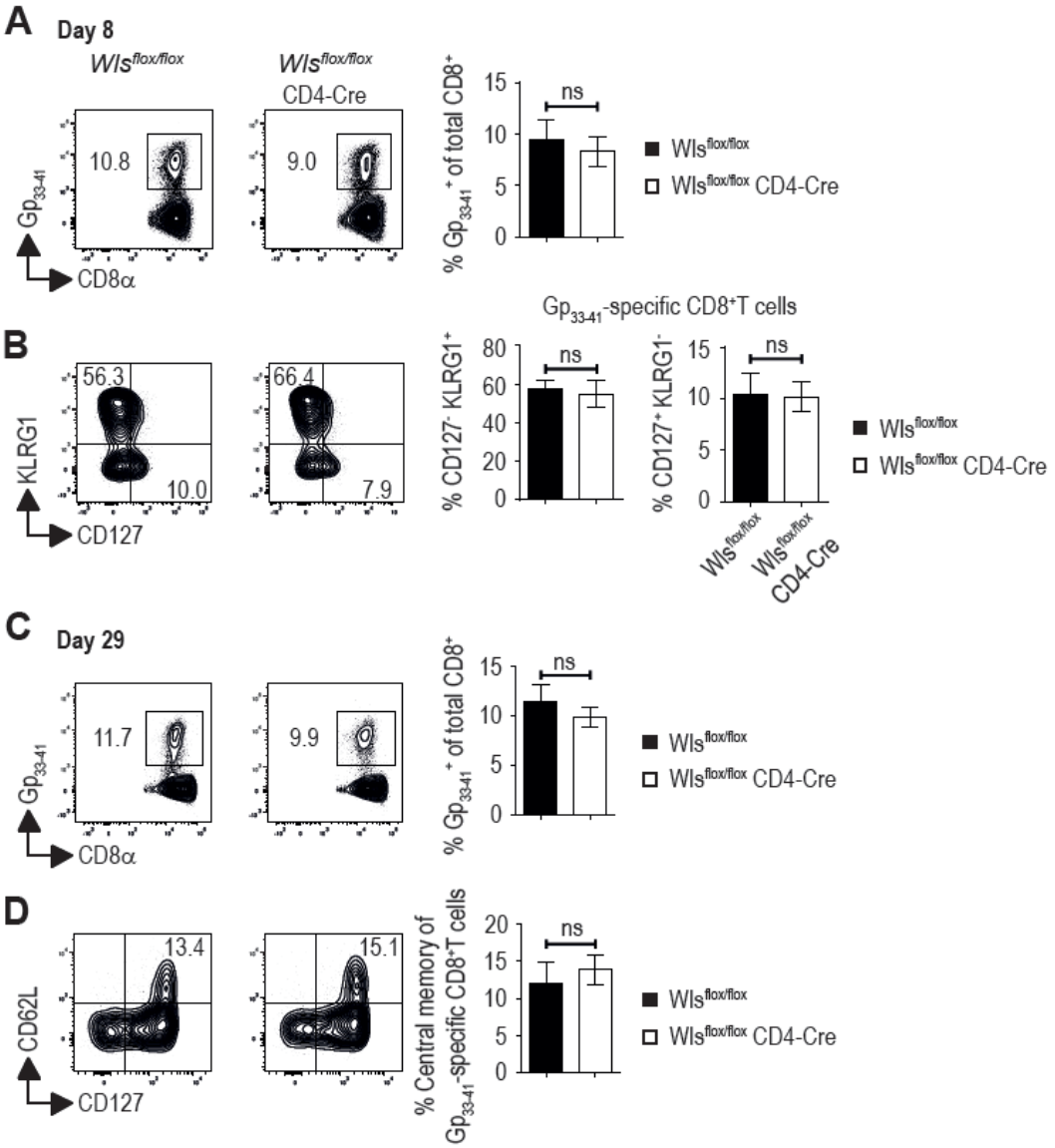

FIGURE 31 – Absence of Wls in T cells does not impact CD8$^+$ T cell response during acute LCMV infection. (**A**-**B**) *Wls*$^{flox/flox}$ and *Wls*$^{flox/flox}$ CD4-Cre mice were bled at day 8 after acute LCMV infection and were analyzed by flow cytometry. (**A**) Contour plots show the percentage of LCMV Gp$_{33-41}$-specific CD8$^+$ T cells among total CD8$^+$ T cells. Bar graph shows the mean percentage (± SD) of Gp$_{33-41}$-specific CD8$^+$ T cells among total CD8$^+$ T cells (*Wls*$^{flox/flox}$ in black and *Wls*$^{flox/flox}$ CD4-Cre in white). (**B**) Contour plots show the percentage of SLECs (CD127$^-$ KLRG1$^+$) and MPECs (CD127$^+$ KLRG1$^-$) among Gp$_{33-41}$-specific cells. Bar graph shows the mean percentage of SLECs and MPECs (± SD) among Gp$_{33-41}$-specific CD8$^+$ T cells (*Wls*$^{flox/flox}$ in black and *Wls*$^{flox/flox}$ CD4-Cre in white). (**C**-**D**) *Wls*$^{flox/flox}$ and *Wls*$^{flox/flox}$ CD4-Cre mice were bled at day 29 after acute LCMV infection and were analyzed by flow cytometry. (**C**) Contour plots show the percentage of LCMV Gp$_{33-41}$-specific CD8$^+$ T cells among total CD8$^+$ T cells. Bar graph depicts the mean percentage (± SD) of Gp$_{33-41}$-specific CD8$^+$ T cells among total CD8$^+$ T cells (*Wls*$^{flox/flox}$ in black and *Wls*$^{flox/flox}$ CD4-Cre in white). (**D**) Contour plots show the percentage of central memory cells (CD62L$^+$ CD127$^+$) among Gp$_{33-41}$-specific CD8$^+$ T cells. Bar graph shows the mean percentage of central memory cells (± SD) among Gp$_{33-41}$-specific CD8$^+$ T cells (*Wls*$^{flox/flox}$ in black and *Wls*$^{flox/flox}$ CD4-Cre in white). Results derived from n= 2-3 mice per group of a single experiment performed. Statistical significance was determined with *t*-tests. ns stands for not significant.

We also addressed whether Wls in stromal cells of secondary lymphoid organs is important for the CD8$^+$ T cell response to acute LCMV infection. Since we observed no difference between WT and *Wls*$^{flox/flox}$

mice, we only used *Wls*$^{flox/flox}$ mice as controls to verify whether Wls in stromal cells was important for the CD8$^+$ T cell response to acute infection. At day 8, Gp$_{33}$-specific CD8$^+$ T cells were equally abundant in the blood of Wls$^{flox/flox}$ CCL19-Cre and *Wls*$^{flox/flox}$ mice (Fig. 32A) and these cells were equally differentiated into SLECs and MPECs (Fig. 32B). Moreover, at day 26 post LCMV infection, in the early memory phase, the abundance of antigen-specific in the blood was similar between *Wls*$^{flox/flox}$ CCL19-Cre and *Wls*$^{flox/flox}$ mice (Fig. 32C). The percentage of central memory cells (CD127$^+$ CD62L$^+$) was also not impacted by the absence of Wls protein in stromal cells (Fig. 32D). Similar results were obtained in

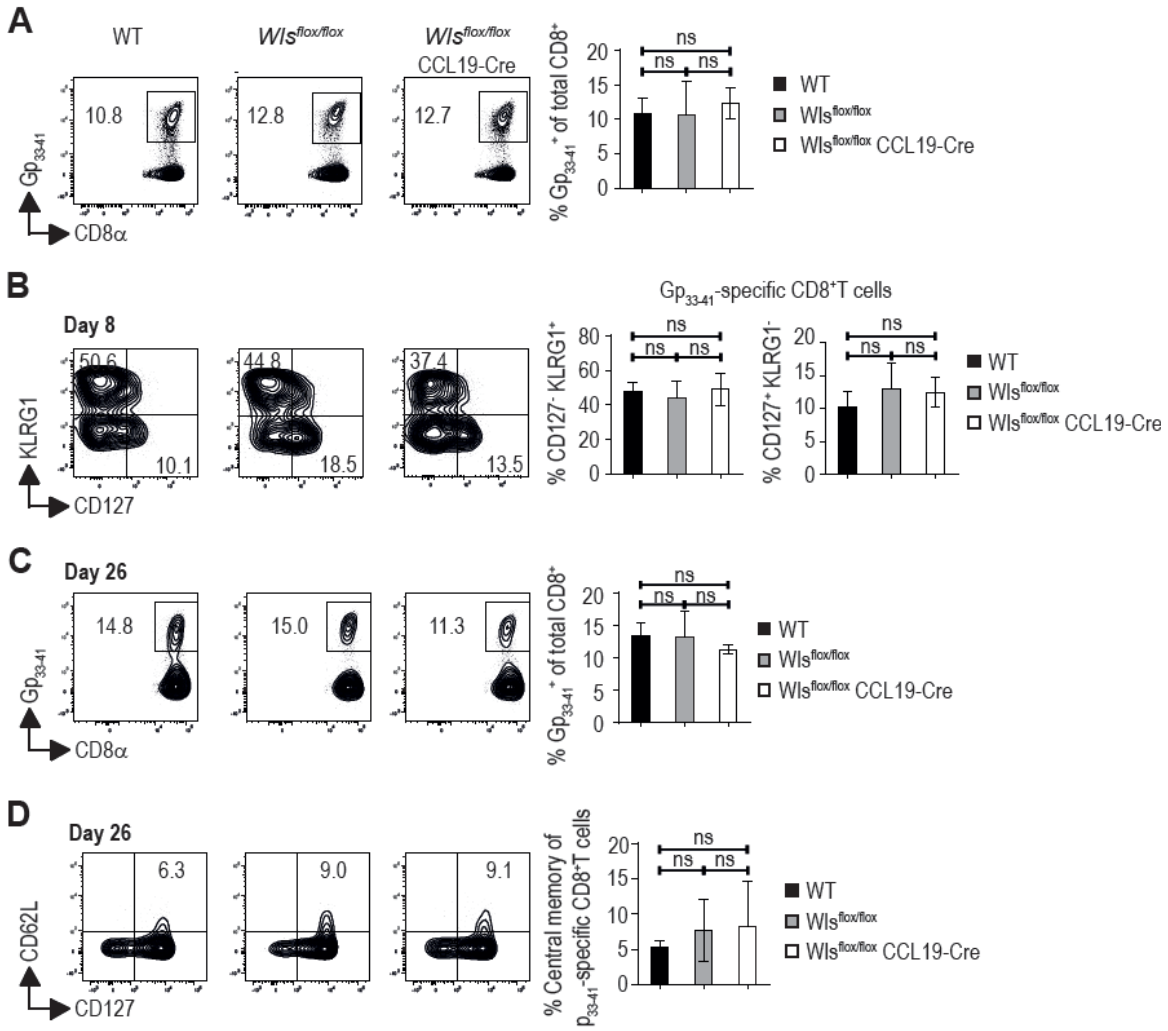

FIGURE 32 – Absence of stromal Wls does not impact CD8$^+$ T cell response during acute LCMV infection. (**A**-**B**) WT, *Wls*$^{flox/flox}$ and *Wls*$^{flox/flox}$ CCL19-Cre mice were bled at day 8 after acute LCMV infection and were analyzed by flow cytometry. (**A**) Contour plots show the percentage of LCMV Gp$_{33-41}$-specific CD8$^+$ T cells among total CD8$^+$ T cells. Bar graph shows the mean percentage (± SD) of Gp$_{33-41}$-specific CD8$^+$ T cells among total CD8$^+$ T cells. (**B**) Contour plots show the percentage of cells of SLECs (CD127$^-$ KLRG1$^+$) and MPECs (CD127$^+$ KLRG1$^-$). Bar graph shows the mean percentage of SLECs and MPECs (± SD) among Gp$_{33-41}$-specific CD8$^+$ T cells. (**C**-**D**) WT (in black), *Wls*$^{flox/flox}$ (in grey) and *Wls*$^{flox/flox}$ CCL19-Cre (in white) mice were bled at day 26 after acute LCMV infection and were analyzed by flow cytometry. (**C**) Contour plots show the percentage of LCMV Gp$_{33-41}$-specific CD8$^+$ T cells among total CD8$^+$ T cells. Bar graph shows the mean percentage (± SD) of Gp$_{33-41}$-specific CD8$^+$ T cells among total CD8$^+$ T cells. (**D**) Contour plots show the percentage of cells with central memory phenotype (CD62L$^+$ CD127$^+$) among Gp$_{33-41}$-specific CD8$^+$ T cells. Bar graph shows the mean percentage of central memory cells (± SD) among Gp$_{33-41}$-specific CD8$^+$ T cells. Results derived from n= 2-3 mice per group of a single experiment performed. Statistical significance was determined with unpaired *t*-tests. ns stands for not significant.

the spleen when infected mice were sacrificed at day 38 post infection (data not shown). We conclude that Wls in stromal cells was also not important for CD8$^+$ T cell response to an acute LCMV infection.

Wls deletion in either T cells or stromal cells does not impact the CD8$^+$ T cell response to chronic LCMV infection

Finally, we tested whether Wls played a role for the CD8$^+$ T cell response to chronic LCMV infection. To this end, we infected WT, *Wls*$^{flox/flox}$ and *Wls*$^{flox/flox}$ CD4-Cre mice with LCMV cl13 strain, which leads to chronic viral infection in WT mice. We followed the virus-specific CD8$^+$ T cell response over time using tetramers specific for the LCMV epitopes, Gp_{33-41} and Gp_{276}. At day 28 post infection, Gp_{276}- and Gp_{33-41}-specific CD8$^+$ T cells were equally abundant in WT, *Wls*$^{flox/flox}$ and *Wls*$^{flox/flox}$ CD4-Cre mice (Fig. 33A

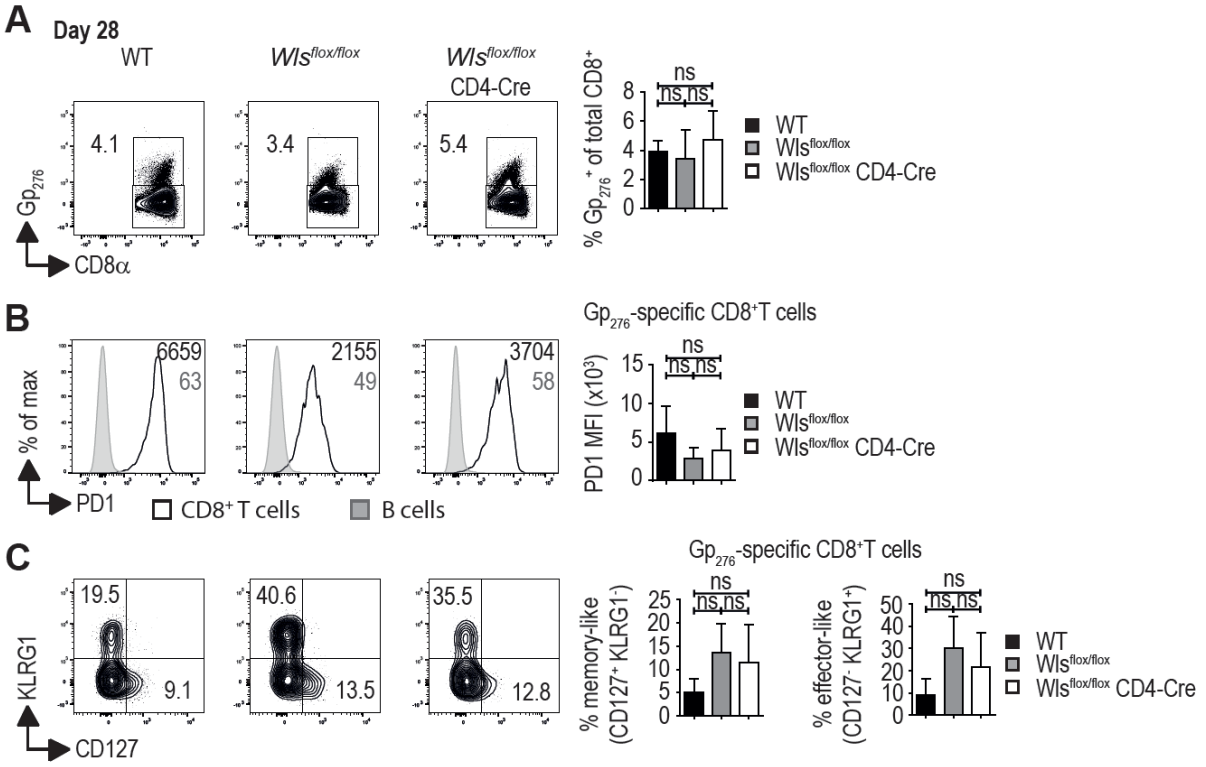

FIGURE 33 – Absence of Wls in T cells does not impact memory-like CD8$^+$ T cell formation in LCMV cl13 infection. (**A**-**C**) WT, Wls$^{flox/flox}$ and Wls$^{flox/flox}$ CD4-Cre mice were analyzed at day 28 after LCMV cl13 infection and splenocytes were analyzed by flow cytometry. (**A**) Contour plots show the percentage of LCMV Gp_{276}-specific peptide versus CD8$^+$ T cells among total CD8$^+$ T cells. Numbers indicate the percentage of Gp_{276}-specific CD8$^+$ T cells among total CD8$^+$ T cells. Bar graph shows the mean percentages (± SD) of Gp_{276}-specific CD8$^+$ T cells among total CD8$^+$ T cells. (**B**) Histograms show the expression of PD1 in Gp_{276}-specific CD8$^+$ T cells. Numbers indicate the PD1 MFI in the indicated populations. Bar graphs shows the mean PD1 MFI (± SD) in Gp_{276}-specific CD8$^+$ T cells. (**C**) Contour plots show the expression of CD127 and KLRG1 in Gp_{276}-specific CD8$^+$ T cells. Numbers indicate the percentage of memory-like (CD127$^+$ KLRG1$^-$) and effector-like (CD127$^-$ KLRG1$^+$) cells among Gp_{276}-specific CD8$^+$ T cells. Bar graph shows the mean percentages (± SD) of memory-like and effector-like cells among Gp_{276}-specific CD8$^+$ T cells. Results derived from n= 4-7 mice per group of a representative experiment of two independent experiments performed. Statistical significance was determined with one-way ANOVA tests. ns stands for not significant.

and not shown). These cells were all expressing high levels of PD1 indicating that LCMV infection was chronic and that the cells were exhausted (Fig. 33B).

We have recently identified a memory-like $CD8^+$ T cells subset among virus specific $CD8^+$ T cells after LCMV cl13 infection. While this subset is defined by Tcf-1 expression, a subset of these cells can also be identified by low levels of KLRG1 and expression of CD127. We then looked at CD127 and KLRG1 expression in Gp_{276}- and Gp_{33-41}-specific $CD8^+$ T cells. We did not observe any significant difference in the percentage of memory-like $CD8^+$ T cells ($CD127^+$ $KLRG1^-$) or effector-like $CD8^+$ T cells ($CD127^-$ $KLRG1^+$) between WT, *Wls*$^{flox/flox}$ and *Wls*$^{flox/flox}$ CD4-Cre $CD8^+$ T cells, (Fig. 33C and not shown). These data indicate that Wls expression in T cells does not have a role for mounting a $CD8^+$ T cell response against chronic LCMV infection. This indicates that either Wnt protein secretion in other cell population plays a role for $CD8^+$ T cell response or that Wls is not involved at all in this process.

DISCUSSION

Part I – The role of Wnt/Tcf-1 expression for effector CD8$^+$ T cell differentiation

In my thesis, we aimed at better understanding the process of CD8$^+$ T cell differentiation. Since Wnt/Tcf-1 signaling in antigen-specific CD8$^+$ T cells is required for the formation of functional CD8$^+$ T cell memory, we addressed how Wnt/Tcf-1 signaling was controlled during a primary immune response.

Here, we found that the downregulation of Wnt/Tcf-1 signaling was driven by systemic inflammation and that Tcf-1 repression was necessary to allow CD8$^+$ T cell differentiation. We then identified IL-12 as the main pro-inflammatory cytokine responsible for Tcf-1 downregulation. Moreover, we also showed that STAT4, acting downstream of IL-12, regulated Tcf-1 expression. Tcf-1 expression counteracted CD8$^+$ T cell differentiation by repressing several transcription factors involved in CD8$^+$ T cell differentiation such as Blimp-1 or T-bet. Finally, we observed that Tcf-1 was necessary to form central memory CD8$^+$ T cells and thus to protect against a re-infection since memory CD8$^+$ T cells lacking Tcf-1 expression presented a default in their capacity to re-expand.

IL-12

Antibody blockade revealed an important role of IL-12 for CD8$^+$ T cell differentiation and Tcf-1 downregulation. The role of IL-12 for CD8$^+$ T cell differentiation in response to DC vaccination in presence of CpG is actually somewhat controversial [202]. Pham et al. [203] used OT-1 cells deficient for IL-12Rβ1 and showed that IL-12 signals were not necessary for the differentiation of CD8$^+$ T cells. On the other hand, Cui et al. [93] showed that IL-12 signaling in P14 cells deficient for IL-12Rβ2 promoted effector differentiation of responding CD8$^+$ T cells.

The discrepancies between Cui et al. [93] and Pham et al. [203] might be explained by the fact that they used different experimental protocols. Cui et al. [93] used P14 cells whereas Pham et al. [203] used OT-1 cells. Then, while Pham et al. [203] were purifying splenic DC from B6 mice which have been subcutaneously injected with B16 cells expressing Flt3L, Cui. et al. [93] generated bone-marrow derived DCs. These DCs might be phenotypically different and may, depending on the way they have been purified, secrete or induce different types and/or levels of pro-inflammatory cytokines and this could

explain the discrepancies observed between our results and results from other groups. Moreover, the dose of CpG used to induce systemic inflammation influences SLEC differentiation [203] which might also explain the discrepancy between Cui et al. [93] and Pham et al. [203] because Cui et al. used twice as less CpG than Pham et al. Finally, if Pham et al. used $CD8^+$ T cells deficient for IL-12Rβ1, Cui et al. used $CD8^+$ T cells deficient for IL-12Rβ2. While both IL-12R subunits have been shown to be important for IL-12 signaling, IL-12Rβ1 subunit is constitutively expressed whereas IL-12Rβ2 subunit is induced following infection. This could further explain the differences between the data obtained by those two groups.

We also used P14 *IL-12Rβ2*$^{-/-}$ cells in our DC vaccination system. We observed that the absence of IL-12Rβ2 in antigen-specific $CD8^+$ T cells blocked Tcf-1 downregulation and $CD8^+$ T cell differentiation. In the *L.m.* context, the absence of IL-12Rβ2 on $CD8^+$ T cells also led to a maintenance of Tcf-1 expression and a blocking of $CD8^+$ T cell differentiation in a reproducible manner. Thus, our data actually fit both groups since the neutralizing mAbs block IL-12 signaling in all cell types and not only in responding $CD8^+$ T cells led to a complete blocking of Tcf-1 neutralization whereas IL-12Rβ2 deficiency in responding $CD8^+$ T cells led to a strong but only partial blocking of Tcf-1 downregulation. So we concluded that IL-12 is necessary for $CD8^+$ T cell differentiation, which is in accordance with Cui et al. [93] and that IL-12 acted directly on $CD8^+$ T cells, in accordance with what Pham et al. [185], but acted also indirectly.

We also tested whether the administration of recombinant IL-12 in combination with DC33 vaccination was sufficient to downregulate Tcf-1. Tcf-1 expression and SLEC differentiation were not impacted by the injection of IL-12 contrary to what Cui et al. published [93]. This led us to the conclusion that IL-12 was necessary for Tcf-1 downregulation but that it was not sufficient (data not shown). However, we cannot exclude the possibilities that a single injection of IL-12 was not sufficient to induce the downregulation of Tcf-1 *in vivo*, that IL-12 needs to act in conjunction with other factors to repress Tcf-1 or that IL-12 needs to be trans-presented as described for IL-15 [204] to be active.

Type I IFNs

The role of type I IFNs in $CD8^+$ T cell differentiation remains controversial. Type I IFNs have been shown to be critical for SLEC differentiation [205] whereas others showed that they were only important for

expansion of CD8$^+$ T cells [203]. However, when we blocked type I IFN signaling using IFNAR blocking Abs, we did not observe any effect on Tcf-1 expression or SLEC differentiation. When combined with IL-12 blockade, IFNAR blockade led to a minor further reduction of Tcf-1 downregulation. Moreover, the addition of type I IFNs *in vitro* did not provoke the downregulation of Tcf-1. We concluded that type I IFNs were not necessary for Tcf-1 downregulation and SLEC differentiation during DC vaccination. However, since we did not observe any effect of IFNAR blockade, we cannot exclude the possibility that the blockade was not efficient. To rule out a role of type I IFN, we could use P14 cells lacking IFNAR in our DC33 vaccination system.

IL-2

Since a high dose of IL-2 induced SLEC differentiation [114], we also verified a possible role of IL-2 for controlled Tcf-1 expression. We did not find any changes in Tcf-1 expression. However, contrary to published data [206], increased availability of IL-2 (using IL-2/anti-IL-2 complexes) did not induce SLEC differentiation. Blocking of IL-2 did also not change Tcf-1 expression. Again, contrary to published data [206], IL-2 blockade did not reduce SLEC differentiation and CD8$^+$T cell proliferation.

There are several differences in the respective protocol that may explain these differences. To increase the bioavailability of IL-2 *in vivo*, we injected IL-2 complexed with an anti-IL-2 mAb 4.5 days and 6 days after dendritic cell vaccination in order to increase the half-life of IL-2 *in vivo*. With this approach, we observed an increased proliferation of CD8$^+$ T cells in accordance with previously published data from Boyman et al. [207]. Boulet et al. injected recombinant murine IL-2 twice daily for 7 days. The protocol adopted by Boulet et al. may induce vascular leak syndrome, which is associated with liver cell damage [9] due to an over stimulation of NK cells and the release of pro-inflammatory cytokines. Thus, enhanced SLEC differentiation may be explained by the release of pro-inflammatory cytokines. IL-2 complexes mostly expand CD8$^+$ T cells with limited stimulation of T_{reg} cells and lower incidence of VLS as compared to soluble IL-2 injection to mice [9].

Further, Boulet et al. stimulated antigen-specific CD8$^+$ T cells *in vivo* in the absence of inflammatory signals. We tested IL-2 blockade in presence of systemic inflammation to establish whether IL-2 signaling was critical for SLEC differentiation. In the absence of systemic inflammatory signals, IL-2

might contribute to limited SLEC differentiation. However, in the presence of systemic inflammatory signals, IL-2 signaling was not a main inducer of SLEC.

Another difference between our study and the one performed by Boulet et al. is the antigen-specific $CD8^+$ T cells used. Indeed, we used P14 $CD8^+$ T cells whereas they used OT-1 $CD8^+$ T cells. Systemic inflammation does not improve the expansion of P14 cells while that of OT-1 cells is improved via the maintenance of high-affinity IL-2 signaling [208]. Thus, IL-2 might play a different role on P14 and OT-1 cells.

Irrespectively, we found no difference in Tcf-1 expression when reducing or increasing the availability of IL-2 during the primary response of $CD8^+$ T cells.

The optimal re-expansion of memory $CD8^+$ T cells depends on the presence of IL-2 during the primary response [209]. The source of IL-2 was thought to be $CD4^+$ T_H cells. But Williams et al. [60] and more recently Feau et al. [59] indicated an essential role of autocrine IL-2. We observed that DC33-primed *Tcf7*$^{-/-}$ P14 cells did not produce IL-2 and were unable to give rise to central memory $CD8^+$ T cells. Moreover, memory *Tcf7*$^{-/-}$ P14 cells showed a clear defect in their re-expansion capacity. This could be explained by their reduced ability to produce IL-2. However, naïve P14 *Tcf7*$^{-/-}$ cells produced IL-2 normally in response to stimulation by anti-CD3 and anti-CD28 antibodies *in vitro* (Laurène Pousse data, unpublished). Thus, the absence of IL-2 production following DC33 priming is likely a phenotype acquired during priming rather than an intrinsic defect of *Tcf7*$^{-/-}$ P14 cells to produce IL-2.

Other cytokines regulating Tcf-1 expression

We established a critical role of IL-12 for Tcf-1 downregulation. It seems unlikely that IL-12 is the only inflammatory cytokine capable of reducing Tcf-1 expression. Since IFNγ promotes naïve $CD8^+$ T cell differentiation and that the differentiation is further supported by addition of type I IFNs [210], we tested whether IFNγ could regulate Tcf-1 expression. However, the addition of recombinant IFNγ *in vitro* did not lead to the downregulation of Tcf-1. Thus, we concluded that IFNγ was not necessary for Tcf-1 downregulation during $CD8^+$ T cell differentiation.

Moreover, members of the IL-1 family of cytokines which includes IL-1, IL-18 and IL-33, have been shown to influence anti-viral $CD8^+$ T cell responses [211]. *In vitro* priming of $CD8^+$ T cells has shown that IL-12 and IL-33 synergistically increase the effector differentiation of $CD8^+$ T cells (production of IFNγ) by enforcing the expression of transcription factors T-bet and Blimp-1 and repressing Eomes, Lef-1 and Tcf-1 [212]. Moreover, *in vivo*, it has been demonstrated that IL-33 signaling is necessary to induce $CD8^+$ T cell differentiation and proliferation based on the reduced SLEC population observed at day 8 after LCMV infection in absence of IL-1RL1 or IL-33 itself [213]. We thus tested IL-33 alone or in combination with IL-12 in using our *in vitro* system in a preliminary experiment. However, IL-33 did not provoke the downregulation of Tcf-1 protein. The other members of the IL-1 family could also be tested in our DC vaccination system in order to gain further insight into $CD8^+$ T cell differentiation process.

IL-12 signaling

Since IL-12 mainly signals via STAT4, we further studied the role of STAT4 for Tcf-1 downregulation and $CD8^+$ T cell differentiation. Although STATs were originally characterized as activators of gene transcription, there have been indications that STATs can also function as transcriptional repressors. Indeed, STAT5 directly represses Bcl6 in various types of cancers [214, 215]. Along the same lines, STAT4-dependent repressive histone marks in T_H1 cells have been identified on a small number of genes that are usually expressed in T_H2 cells, suggesting a role for STAT4 as a transcriptional repressor [216]. We showed that STAT4-deficiency in P14 cells abolished Tcf-1 downregulation and $CD8^+$ T cell differentiation, suggesting that STAT4 represses *Tcf7* expression. As mentioned earlier, the analysis of publically available STAT4 CHIPSeq data from T_H1 cells [4] revealed that STAT4 is bound to the *Tcf7* locus and that this correlates with the presence of repressive H3K27me3 marks. In the absence of STAT4, activating H3K4me3 marks and *Tcf7* expression are also moderately increased in T_H1 cells [4]. These data suggest that STAT4 binding has a direct and repressive effect on *Tcf7* expression in $CD4^+$ T cells. It will be of interest to verify whether STAT4 can also bind to *Tcf7* locus in antigen-specific $CD8^+$ T cells activated in the context of DC vaccination.

Conversely, it is well known that *IL-12Rβ2* expression is induced by STAT4 [99, 194-196]. Indeed, STAT4 is bound to the *IL-12Rβ2* loci in T_H1 cells and this correlates with the presence of activating H3K4me3 marks. In the absence of STAT4, repressive H3K27me3 marks are increased and *IL-12Rβ2*

expression is strongly reduced [4]. Moreover, the IL-12R is undetectable on most resting T cells but the activation through the TCR and co-stimulatory interactions induces the transcription and expression of both chains of the IL12R [1, 217]. However, activated T_H1 cells further upregulate the IL-12Rβ2 chain in response to IL-12 [218]. Thus IL-12 is thought to further improve IL-12 signaling and at the same time repress Tcf-1, which will collectively promote effector differentiation. As excessive effector differentiation seems to impair memory formation, we hypothesized that Tcf-1 may counteract this IL-12 – effector differentiation loop. Indeed, we observed that Tcf-1 reduced *IL-12Rβ2* expression in MPECs responding to DC33 vaccination. Thus Tcf-1 dampens IL-12R expression to prevent excessive or premature effector differentiation.

Default effector differentiation in the absence of Tcf-1

Absence of Tcf-1 resulted in increased expression of transcription factors in MPECs that mediate effector differentiation such as *Prdm1*(Blimp-1) and *Tbx21* (T-bet) expression. This indicates that the absence of Tcf-1 results in effector differentiation by allowing the expression of transcription factors responsible for $CD8^+$ T cell differentiation. To follow up on these data, it would be of interest to demonstrate that the absence of STAT4 in P14 cells leads to a reduced IL-12Rβ2 expression in MPECs and to reduced expression of transcription factors such as Blimp-1 or T-bet. This would confirm that STAT4 is responsible for the repression of Tcf-1 and is necessary for effector $CD8^+$ T cell differentiation. Thus the default effector differentiation induced by the absence of Tcf-1 may directly impair central memory formation.

Cytokines maintaining Tcf-1 expression

We have been mainly looking for inflammatory signals responsible for Tcf-1 downregulation and thus for SLEC differentiation based on the view that the memory $CD8^+$ T cell differentiation was a default pathway counteracted by the encounter of inflammatory cytokines [185]. However, MPEC differentiation is also dependent on specific cytokines produced during infection. For example, IL-10, which is known to limit pro-inflammatory responses, is required for optimal $CD8^+$ T cell memory development [219]. Moreover, while IL-2 promotes proliferation of effector $CD8^+$ T cells, IL-21, which is closely related to IL-2, promotes memory $CD8^+$ T cell formation and function [220, 221]. IL-21 acts in combination with IL-10 to trigger STAT3 activation [123]. These cytokines may help to maintain Tcf-1 in antigen-primed $CD8^+$

T cells [222]. Indeed, Cui et al. [186] compared different adjuvants in a DC vaccination system and suggested that IL-10 at high levels counteracted the action of IL-12. So a different strategy to investigate the regulation of Tcf-1 during $CD8^+$ T cell response is to investigate the role of IL-10 and IL-21 in $CD8^+$ T cell differentiation and Tcf-1 expression in our DC vaccination system.

Conclusion and perspectives

An adaptive $CD8^+$ T cell response normally controls infections with intracellular pathogens and forms a long-lived pool of memory $CD8^+$ T cells that is able to protect from re-infection. Knowledge regarding protective immune responses to natural infections is essential for understanding why the immune system fails to control certain pathogens such as HIV or HCV, which lead to chronic infections and for the generation of vaccines that can induce protective T cell responses. Although vaccines against smallpox or yellow fever have been developed, the mechanism of action of these vaccines is not completely understood [223]. Indeed, while the humoral response has been shown to be important to mediate protection again yellow fever virus, $CD8^+$ T cells are also important for the control of yellow fever virus replication [224]. These $CD8^+$ T cells induce a long-lasting stem cell-like memory $CD8^+$ T cell population [225]. Moreover, DC vaccination in mice has been shown to induce memory $CD8^+$ T cells which mediate protection against chronic infection such as LCMV cl13 [186] and $CD8^+$ T cell depletion after LCMV cl13 infection led to an increased virus titer [226]. Together, these data indicate that $CD8^+$ T cells plays an important role in the vaccination process and that a better understanding of their mechanism of action is necessary to develop new vaccines.

Moreover, some newly developed vaccines and T-cell based therapies generate terminally differentiated $CD8^+$ T cells [227-229]. Thus, certain vaccines might not be effective because they generate effector $CD8^+$ T cells rather than memory $CD8^+$ T cells that are more protective in both viral infections and tumor models [230, 231]. Since Wnt/Tcf-1 signaling is essential for the formation of functional $CD8^+$ T cell memory, we studied the precise regulation of Wnt/Tcf-1 signaling and its role during a primary immune response.

We showed the IL-12/STAT4 axis is involved in Tcf-1 downregulation in the course of a $CD8^+$ T cell response, which reduces memory formation. Our data thus identify a potential new target to increase memory $CD8^+$ T cell formation: STAT4. It may be of interest to reassess specific inhibitors of STAT4

such as lisofylline. This inhibitor was actually used in a clinical trial in order to verify its efficacy to prevent type I diabetes [232], likely by inhibiting effector differentiation. However, this study has been terminated because the lack of participants. Based on our findings, it would be interesting to address whether STAT4 blockade by lisofylline improved memory formation during vaccination.

Part II – Role of Wnt protein secretion for Wnt pathway activity in naïve CD8$^+$ T cells, for CD8$^+$ T cell homeostasis and differentiation in response to infection

Wnt/Tcf-1 signaling pathway is extensively studied for its role in stem cells and cancer development [158]. However, Wnt/Tcf-1 signaling also pays roles in immune responses [127-129]. We and others established that Wnt/Tcf-1 signaling was highly active in naïve CD8$^+$ T cells [129] but the role in CD8$^+$ T cell homeostasis and the source of these Wnt signals have not been defined.

Prior studies investigated the role of a single Wnt protein [233, 234] but since 19 different Wnt proteins have been identified [146] there is a high chance of redundancy between them. In order to avoid this problem, we exploited the discovery of the Wntless protein [149] which is involved in the secretion of all Wnt proteins [7]. The use of Wntless-deficient mice allowed us to study the role of Wnt secretion for Wnt/Tcf-1 signaling in naïve CD8$^+$ T cells. However, it has been shown that Wls is necessary for the proper embryonic development of mice [235] Consequently, we used a conditional *Wls* deletion by crossing *Wls$^{flox/flox}$* mice with mice expression a cell- or tissue-specific Cre [197].

We then analyzed Wnt/Tcf-1 signaling in naïve CD8$^+$ T cells after the deletion of *Wls* in various cell populations. We separately deleted *Wls* in T cells, in the entire hematopoietic compartment or in stromal cells. After verifying the deletion of *Wls* in the expected tissue, we concluded that Wls expression in T cells, the hematopoietic system or stromal cells played a role for the maintenance of Wnt/Tcf-1 signaling in naïve CD8$^+$T cells. We found no evidence that the T cell compartments were altered. Moreover, since we only targeted Wls and not the secretion of Wnts directly, it might be of interest to either target another molecule involved in the secretion of Wnts such as porcupine or to study the role of one or several Wnt proteins.

We further verified whether Wls in T cells or stromal cells played a role for the CD8$^+$ T cell response to an acute or chronic LCMV infection. We did not observe any difference in the respective responses when *Wls* was deleted in T cells or in stromal cells.

However, the results we obtained had two major caveats. First when analyzing the phenotype of *Wls*$^{flox/flox}$ mice in absence of Cre recombinase, we noticed that *Wls* expression of *Wls* was reduced 10 fold in *Wls*$^{flox/flox}$ as compared to WT mice. Since this reduction was not lethal and not associated with any phenotype, we concluded that the remaining level of Wls were enough to allow Wnt protein secretion. This mRNA level reduction can be explained by the fact that one of the loxP sites has been inserted into the promoter region of *Wls* gene.

The second caveat is that we did not verify whether Wnt protein secretion was reduced. Even though we can conclude that the absence of *Wls* in T cells, stromal cells or in the hematopoietic system does not lead to a reduction of Wnt/Tcf-1 signaling activity in naïve CD8$^+$, we cannot exclude the possibility that Wnt proteins can be secreted using another mechanism.

Together these data indicate that Wls expression in T cells, hematopoietic cells or stromal cells of secondary lymphoid organs is not necessary for Wnt/Tcf-1 pathway activity in naïve CD8$^+$ T cells. Perhaps Wls expression and thus Wnt secretion by another cell type, such as endothelial cells, is essential for the maintenance of Wnt/Tcf-1 pathway activity in naïve CD8$^+$ T cells.

Alternatively, Wls and thus Wnt protein secretion does not play a role for maintaining Wnt/Tcf-1 signaling in naïve CD8$^+$ T cells and signaling is ensured by other factors. Prostaglandin E2 can induce Wnt/Tcf-1 by inhibiting GSK-3 [236]. C1q, a protein of the complement, can activate Wnt/Tcf-1 signaling by cleaving LRP co-receptor [156] or hepatocyte growth factor which can directly activate β-catenin [237]. Further work will be needed to address these possibilities.

EXPERIMENTAL PROCEDURES

Mice

B6 mice (CD45.2$^+$) were obtained from Charles River. *Rag2$^{-/-}$γc$^{-/-}$* mice were bred locally. P14 TCR Tg (line 237) (B6) mice were provided by A. Oxenius [238], Tcf-1 knockout (*Tcf7$^{-/-}$*) (B6 backcross >10) was provided by H. Clevers [162]. *STAT4$^{-/-}$* mice were provided by M. Löhning [239]. *Wls*$^{flox/flox}$ mice were provided by K. Basler [197]. Ccl19-Cre were provided by B. Ludewig [240]. CD4-Cre transgenic [198], Mx-Cre transgenic [241] and Vav-cre transgenic [199] mice have been described. The following mouse strains were purchased from Jackson Laboratories: CD45.1$^+$ congenic B6 mice used as recipients and bred in house, *Tcf7$^{-/-}$* (p45 isoform) transgenic (Tg) and *Tcf7$^{-/-}$* (p33 isoform) Tg (B6 backcross >10) [167], OT-1 TCR Tg [242], Axin2^{LacZ} (B6) [180], *IL-12Rβ2$^{-/-}$* (B6) [243] . P14 cells are CD8$^+$ T cells expressing a LCMV Gp$_{33-41}$-specific transgenic T cell receptor. P14 *Tcf7$^{-/-}$*, P14 Axin2$^{LacZ/+}$, P14 *Tcf7$^{-/-}$* p45, P14 *Tcf7$^{-/-}$* p33, P14 *IL-12Rβ2$^{-/-}$*, P14 *STAT4$^{-/-}$*, OT-1 Axin2^{LacZ}, CD4-Cre Wls$^{flox/flox}$, Mx-Cre Wls$^{flox/flox}$, Vav-cre Wls$^{flox/flox}$ and Ccl19-Cre Wls$^{Flox/Flox}$ mice were obtained by breeding. As controls for *Tcf7$^{-/-}$* mice, we used B6 mice.

P14 *Tcf7$^{Lox/Lox}$ ROSA26Sor$^{tm1(EYFP)}$* were generated by crossing commercially available *ROSA26Sor$^{tm1(EYFP)}$* (Jackson Laboratory) mice with P14 mice and *Tcf7$^{tm1a(EUCOMM)Wtsi/+}$* founder mice from the EUCOMM consortium which have the exon 4 from the *Tcf7* gene flanked by two loxP sites.

Mice were bred and maintained in the SPF facility, vaccinated and injected in the conventional animal facility of the university of Lausanne. Experiments were performed in 6 to 12 weeks old mice in compliance with the University of Lausanne Institutional regulations and were approved by the veterinarian authorities of the Canton de Vaud.

Purification of mouse T cells and adoptive cell transfers

Single cell splenocyte suspensions were obtained by mashing total spleens through a 40µm nylon cell strainer (BD Falcon). Red blood cells were lysed with a hypotonic ACK buffer. CD8$^+$ T cells were isolated using mouse CD8$^+$ T-cell enrichment kits (StemCell Technologies). One-2 x 10^4 WT, *Tcf7$^{-/-}$*, *Tcf7$^{-/-}$* p33, *Tcf7$^{-/-}$* p45, Axin2^{LacZ}, *IL-12Rβ2$^{-/-}$*, *STAT4$^{-/-}$* CD45.2$^+$ P14 cells were transferred into naïve CD45.1$^+$ B6 mice. One-2 x 10^4 OT-1 Axin2^{LacZ} CD45.1$^+$ cells were transferred into naïve CD45.2$^+$ B6 mice.

In the experiments in which we addressed memory development and functionality after peptide DC vaccination, WT and *Tcf7*$^{-/-}$ CD45.2^{+} P14 cells were enriched from DC vaccinated mice by staining total splenocytes with anti-CD8α (53.6.7, eBioscience), anti-CD45.2 (104, eBioscience) and anti-CD45.1 (A20.1, eBioscience) fluorescent mAbs. CD8^{+} CD45.2^{+} CD45.1^{-} cells were isolated by fluorescence activated cell sorting. The purity of sorted cells was greater than 99%. Six-10 x 10^3 sorted WT and *Tcf7*$^{-/-}$ CD45.2^{+} P14 cells were then transferred into new naïve CD45.1^{+} B6 recipients. The fold expansion of WT and *Tcf7*$^{-/-}$ P14 cells in secondary hosts was determined relative to an estimated 10% "take" of transferred input cells [182].

In the experiments in which we transferred P14 Axin2^{LacZ} cells into *Rag2*$^{-/-}$*γc*$^{-/-}$ mice to induce homeostatic proliferation, 1 x 10^6 P14 cells were transferred.

Surface and intracellular staining and fluorescent activated cell sorting of mouse cells

Splenocytes, thymocytes, bone-marrow cells and blood cells were incubated with anti-CD16/332 (2.4G2) hybridoma supernatant before staining for 15 minutes at 4°C for multicolor flow cytometry with fluorescent mAbs to CD4 (GK1.5), CD8α (53-6.7), CD19 (ID3), CD44 (IM.781), CD45.1 (A20.1), CD45.2 (104), B220 (RA3-6B2), CD62L (Mel14), CD127 (A7R34), and KLRG1 (2F1) from eBioscience, fluorescent mAbs to CD11c (N418), CD80 (16-10A1), CD86 (GL-1), Iab (KH74) and PD-1 (RPM1-30) from Biolegend and fluorescent mAb to Vα2 (B20.1) from BD Pharmingen. PE-labeled or APC-labeled D^b Gp_{33-41} (KAVYNFATC), D^b Gp_{276} (SGVENPGGYCL), D^b Np_{396} (FQPQNGQFI) tetramers were purchased from TCmetrix and incubated for 30 minutes at room temperature with splenocytes or blood cells. Zombie Aqua Fixable Viability Kit (Biolegend) was used for the exclusion of dead cells.

For intracellular cytokine staining, splenocytes were re-stimulated *in vitro* with Gp_{33-41} peptide (5mM) for 5h in the presence of Brefeldin A (7 µg/mL) for the last 4.5h. They were then fixed and permeabilized using the intracellular fixation and permeabilization buffer kit from eBioscience and stained with mAbs for IFNγ (XMG1.2), TNFα (MP6-XT22) and IL-2 (JES6-5H4) (all from eBioscience).

Transcription factors and Granzyme B were detected using the FoxP3 transcription factor staining kit from eBioscience and stained with mAbs for Granzyme B (GB11, ThermoFisher Scientific) and Tcf-1 (C63D9, Cell signaling) followed by anti-rabbit IgG PE (eBioscience) to detect Tcf-1 mAbs.

β-galactosidase (Axin2^{LacZ}) activity assay

Purified CD8$^+$ T cells or splenocytes were resuspended at 20 x 10^6 cells/mL in HBSS medium containing 5% FCS. 100µL of cells pre-warmed at 37°C were incubated with 100µL of FDG at 2mM (Thermo Fisher Scientific) also pre-warmed at 37°C for exactly 1min at 37°C. After incubation with FDG at 37°C, cells were transferred into 2mL of ice-cold HBSS medium and kept on ice for 1.5 h. Cells were then washed in HBSS medium and surface stained as previously described.

In vivo treatments

Mice were injected intraperitoneally with 500µg of rat anti-mouse IL-12 p40 (C17.8) or with rat IgG2a isotype control (2A3) once at the time of DC vaccination and a second time 5-6h later. Mice were injected intraperitoneally with 2.5mg of mouse anti-mouse IFNAR-1 (MAR1-5A3) or mouse IgG1 isotype control (MOPC-21) once following CD8$^+$ T cell injection. Mice were injected intraperitoneally with 250µg of rat anti-mouse IL-2 (JES6-1A12) or with a complex formed by 10µg of rat anti-mouse IL-2 (S4B6-1) and 300ng of human IL-2 (Glaxo IMB) once 4.5 days after DC vaccination and a second time 6 days after DC vaccination. All antibodies were purchased from BioXCell.

Lymphocytic choriomeningitis virus infection

The LCMV cl13 and WE strains were propagated in baby hamster kidney cells and titrated on Vero African green monkey kidney cells according to an established protocol [244]. Frozen stocks were diluted in PBS. For LCMV infections, WT, CD4-Cre Wls$^{Flox/Flox}$, or Ccl19-Cre Wls$^{Flox/Flox}$ mice were injected intravenously with 2 x 10^6 PFUs of LCMV cl13 or 2x10^2 PFUs of LCMV WE. Alternatively, sorted WT, *Tcf7*$^{-/-}$ CD45.2$^+$ P14 cells were transferred into naïve CD45.1$^+$ B6 hosts one day prior to infection with LCMV cl13. The immune response to LCMV was analyzed with flow cytometry and peptide MHC tetramer specific for endogenous CD8$^+$ T cells specific for LCMV or by identifying P14 cells using congenic markers. Responses were analyzed as indicated in the text.

Listeria monocytogenes bacterial infection

Recombinant *Listeria monocytogenes* strains stably expressing chicken OVA that contain H2-K^b/OVA-derived native ligand $SIINFEKL_{257-264}$ (N4) and altered peptide ligands SIITFEKL (T4) or SIIVFEKL (V4) were previously described [183]. Alternatively, we used another *L.m.* strain co-expressing the altered peptide-ligand T4 and the Gp_{33-41} peptide (KAVYNFATC) [245]. All *L.m.* strains were a gift from D. Zehn. *L.m.* strains were grown in brain heart infusion broth (Beckton Dickinson) to mid-log phase. Then, bacterial numbers were determined by measuring the OD at 600nm, and diluted stocks were injected intravenously in PBS. Naïve $CD45.1^+$ or $CD45.2^+$ mice received 2 x 10^3 colony-forming units (CFU) one day after WT OT-1, WT P14, *Tcf7*$^{-/-}$ P14, *IL-12Rβ2*$^{-/-}$P14 *or STAT4*$^{-/-}$ P14 cells transfer.

Generation of bone marrow-derived dendritic cells and DC vaccination

Bone marrow-derived $CD11c^+$ dendritic cells were generated after 6 days of culture with GM-CSF and IL-4 as described [246]. Lipopolysaccharide (100ng/mL; Sigma) was then added overnight in order to induce maturation of DCs. Matured DCs were pulsed with Gp_{33-41} (1µg/mL) (DC33) for 2h, washed with PBS. The resulting cell population consisted of 50-80% $CD11c^+$ cells and were also positive for H2-IAb, CD80 and CD86. Based on percentage of $CD11c^+$ cells (determined before injection), 1 x 10^6 DC33 were injected intravenously one day after transfer of WT, *Tcf7*$^{-/-}$, *Tcf7*$^{-/-}$ p33, *Tcf7*$^{-/-}$ p45, *IL-12Rβ2*$^{-/-}$, or *STAT4*$^{-/-}$ P14 cells transfer. Simultaneously, DC33 vaccinated mice were injected intraperitoneally with CpG-B 1826 ODNs [184] (50µg).

In vitro deletion with Tat-cre fusion protein

ROSA26EYFP *Tcf7*$^{Lox/Lox}$ $CD45.2^+$ $CD8^+$ T cells were purified, as described above, washed two times with 2% FCS RPMI 1640 medium. 0.5 x 10^6 purified cells were treated *in vitro* in round bottom wells from a 96-well plate. A total of 0.5 x 10^6 cells was incubated in wells in presence of 25µg/mL of Tat-Cre recombinase (Labgene) for 1h at 37°C. After the treatment, cells were washed twice with 10% FCS RPMI 1640. One-2 x 10^4 Tat-Cre treated cells were then transferred into naïve $CD45.1^+$ mice which were vaccinated with DC33, as described above, the day after. Alternatively, 0.5 x 10^6 Tat-Cre treated

cells were cultured *in vitro* for 48h at 37°C in V-bottom 96-well in presence of 50ng/mL of IL-2. Cells were harvested 48h later, washed and stained to check the efficiency of Tat-Cre treatment.

In vitro assay/CFSE labelling

Naïve P14 $CD8^+$ T cells were purified, as described above, and stimulated for 3 days *in vitro* in flat-bottom wells from a 96-well plate coated with 2µg/mL of purified anti-CD3 (eBioscience, 145-2C11) and 1 µg/mL of soluble purified anti-CD28 (eBioscience, 37.51) in presence of IL-2 at 20ng/mL A total of 2 x 10^5 cells in 0.2 mL of RPMI 1640 medium were placed into wells. Where indicated, cultures were supplemented with 10ng/mL of rIL-12 (Peprotech), IL-23 (Peprotech) and/or IFNγ (Peprotech), 5 x 10^2 IU/mL of IFN-α (Millipore) or IFN-β (Millipore). Cells were harvested at the end of day 3, washed and stained for Tcf-1 as described above.

Before their simulation with anti-CD3 and anti-CD28 mAbs, purified $CD8^+$ T cells were pelleted and resuspended at a concentration of 10 x10^6 cells/mL in phosphate-buffered saline (PBS). Cells were labelled with 0.2µM of CFSE (Thermo Fisher Scientific) in PBS for 5-8 min at 37°C. Cells were washed 3 times in medium containing 10% FCS and were subsequently resuspended in culture medium.

Q-PCR

Cells were sorted on a FACS Aria (BD Biosciences) and RNA was extracted in Trizol LS reagent (Life Technologies) and reverse-transcribed using SuperScript III First-Strand Synthesis System (ThermoFisher Scientific). Relative quantification real-time PCR (qRT-PCR) was performed with KAPA SYBR FAST qPCR Kit Master Mix (2X) Universal (KAPABIOSYSTEMS) on a LightCycler 480 Instrument (Roche). Primer pairs used for detection are as follows: IL-12Rβ2 (forward 5'-GTGGACCAAACAATCTGACCTG -3'; reverse, 5'- AACACGGACTATGAACCTGGA -3'), Blimp-1 (forward, 5'- CATGGAGGACGCTGATATGAC -3'; reverse, 5'- ATGCCTCGGCTTGAACAGAAG -3'), T-bet (forward, 5'- AGCAAGGACGGCGAATGTT -3'; reverse, 5'- GGGTGGACATATAAGCGGTTC -3'), Id2 (forward, 5'- ATGAAAGCCTTCAGTCCGGTG -3'; reverse, 5'- AGCAGACTCATCGGGTCGT -3'), Bcl6 (forward, 5'- AAAGGCCGGACACCAGTTTT -3'; reverse, 5'- CCGGAGGCGATTAAGGTTGA -3'), Eomes (forward, 5'- GCGCATGTTTCCTTTCTTGAG -3'; reverse, 5'- GGTCGGCCAGAACCACTTC -3'), Axin2 (forward, 5'- TGACTCTCCTTCCAGATCCCA -3'; reverse, 5'- TGCCCACACTAGGCTGACA

-3'), Tcf-1 (forward, 5'- AGCTTTCTCCACTCTACGAACA -3'; reverse, 5'- AATCCAGAGAGATCGGGGGTC -3'), Lef-1 (forward, 5'- CTGGTCAGCGCGAGACAATTA -3'; reverse, 5'- CTTTGCACGTTGGGAAGGA -3'), Wls1 (forward, 5'- CCCCCTTTCCCTCTCGGTTCC -3'; reverse, 5'- GGCGGCATGGAAGCCAAGGGC -3'), Wls2 (forward, 5'- ACAGATGTTGGAACAGAACTGGC -3'; reverse, 5'- GGCTAGACTGCTTCCCACTG -3') and HPRT as internal control (forward, 5'- GATTCAACTTGCGCTCATCTTAGGC -3'; reverse, 5'- AGGTCGGTGTGAACGGATTTG -3').

Data analyses

Flow cytometry measurements of cells were performed on an LSR-II or LSR-Fortessa II flow cytometer (BD). For cell sorting, living cells were stained in 10% FCS DMEM media and sorted on a FACS Aria (Beckton Fickinson). All data were analyzed using FlowJo X (TreeStar). Graphs were prepared with GraphPad Prism 6.0. Bar graphs depict the mean ± SEM or ± SD as indicated. Statistical analyses were performed using Prism 6.0 (Graphpad Software). Paired and non-paired t tests (two-tailed) or one-way ANOVA were used according to the type of experiments. P-values ≤ 0.05 were considered significant (*: $p<0.05$; **: $p<0.001$; ***: $p<0.0001$); p-values >0.05; non-significant (ns).

References

1. Trinchieri, G., *Interleukin-12 and the regulation of innate resistance and adaptive immunity.* Nat Rev Immunol, 2003. **3**(2): p. 133-46.
2. Akira, S. and K. Takeda, *Toll-like receptor signalling.* Nat Rev Immunol, 2004. **4**(7): p. 499-511.
3. Ivashkiv, L.B. and L.T. Donlin, *Regulation of type I interferon responses.* Nat Rev Immunol, 2014. **14**(1): p. 36-49.
4. Wei, L., et al., *Discrete roles of STAT4 and STAT6 transcription factors in tuning epigenetic modifications and transcription during T helper cell differentiation.* Immunity, 2010. **32**(6): p. 840-51.
5. Xing, S., et al., *Tcf1 and Lef1 transcription factors establish CD8(+) T cell identity through intrinsic HDAC activity.* Nat Immunol, 2016. **17**(6): p. 695-703.
6. Shah, M., et al., *Toll-like receptors: A double edge sword.* Journal of Interdisciplinary Dentistry, 2013. **3**(2): p. 57.
7. Clevers, H. and R. Nusse, *Wnt/beta-catenin signaling and disease.* Cell, 2012. **149**(6): p. 1192-205.
8. Takada, K. and S.C. Jameson, *Naive T cell homeostasis: from awareness of space to a sense of place.* Nat Rev Immunol, 2009. **9**(12): p. 823-32.
9. Boyman, O. and J. Sprent, *The role of interleukin-2 during homeostasis and activation of the immune system.* Nat Rev Immunol, 2012. **12**(3): p. 180-90.
10. Weng, N.P., Y. Araki, and K. Subedi, *The molecular basis of the memory T cell response: differential gene expression and its epigenetic regulation.* Nat Rev Immunol, 2012. **12**(4): p. 306-15.
11. Kaech, S.M. and W. Cui, *Transcriptional control of effector and memory CD8+ T cell differentiation.* Nat Rev Immunol, 2012. **12**(11): p. 749-61.
12. Spolski, R. and W.J. Leonard, *Interleukin-21: a double-edged sword with therapeutic potential.* Nat Rev Drug Discov, 2014. **13**(5): p. 379-95.
13. Kahan, S.M., E.J. Wherry, and A.J. Zajac, *T cell exhaustion during persistent viral infections.* Virology, 2015. **479-480**: p. 180-93.
14. Topham, D.J., R.A. Tripp, and P.C. Doherty, *CD8+ T cells clear influenza virus by perforin or Fas-dependent processes.* J Immunol, 1997. **159**(11): p. 5197-200.
15. Ladel, C.H., et al., *Studies with Mhc-Deficient Knock-out Mice Reveal Impact of Both Mhc I-Dependent and Mhc Ii-Dependent T-Cell Responses on Listeria-Monocytogenes Infection.* Journal of Immunology, 1994. **153**(7): p. 3116-3122.
16. Permar, S.R., et al., *Role of CD8+ Lymphocytes in Control and Clearance of Measles Virus Infection of Rhesus Monkeys.* Journal of Virology, 2003. **77**(7): p. 4396-4400.
17. Zhou, X., et al., *Role of lymphocytic choriomeningitis virus (LCMV) in understanding viral immunology: past, present and future.* Viruses, 2012. **4**(11): p. 2650-69.
18. Miedema, F., et al., *Immunological abnormalities in human immunodeficiency virus (HIV)-infected asymptomatic homosexual men. HIV affects the immune system before CD4+ T helper cell depletion occurs.* J Clin Invest, 1988. **82**(6): p. 1908-14.
19. Rehermann, B., *Interaction between the hepatitis C virus and the immune system.* Seminars in Liver Disease, 2000. **20**(2): p. 127-141.
20. Perez-Padilla, R., et al., *Pneumonia and respiratory failure from swine-origin influenza A (H1N1) in Mexico.* N Engl J Med, 2009. **361**(7): p. 680-9.
21. Murphy, K., *Janeway's Immunobiology*. 2011, Garland Science.
22. O'Neill, L.A., D. Golenbock, and A.G. Bowie, *The history of Toll-like receptors - redefining innate immunity.* Nat Rev Immunol, 2013. **13**(6): p. 453-60.
23. Beutler, B.A., *TLRs and innate immunity.* Blood, 2009. **113**(7): p. 1399-407.
24. Iwasaki, A. and R. Medzhitov, *Control of adaptive immunity by the innate immune system.* Nat Immunol, 2015. **16**(4): p. 343-53.

25. Anderson, K.V., *Toll signaling pathways in the innate immune response.* Curr Opin Immunol, 2000. **12**(1): p. 13-9.
26. Arango Duque, G. and A. Descoteaux, *Macrophage cytokines: involvement in immunity and infectious diseases.* Front Immunol, 2014. **5**: p. 491.
27. Geissmann, F., et al., *Development of monocytes, macrophages, and dendritic cells.* Science, 2010. **327**(5966): p. 656-61.
28. Clark, R. and T. Kupper, *Old meets new: the interaction between innate and adaptive immunity.* J Invest Dermatol, 2005. **125**(4): p. 629-37.
29. Villadangos, J.A. and P. Schnorrer, *Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo.* Nat Rev Immunol, 2007. **7**(7): p. 543-55.
30. Akira, S., K. Takeda, and T. Kaisho, *Toll-like receptors: critical proteins linking innate and acquired immunity.* Nat Immunol, 2001. **2**(8): p. 675-80.
31. Behboudi, S., et al., *The effects of DNA containing CpG motifs on dendritic cells.* Immunology, 2000. **99**: p. 361-366.
32. Liu, Y.C., et al., *CpG-B oligodeoxynucleotides inhibit TLR-dependent and -independent induction of type I IFN in dendritic cells.* J Immunol, 2010. **184**(7): p. 3367-76.
33. Möller, I., et al., *Dendritic cell maturation with poly(I:C)-based versus PGE2-based cytokine combinations results in differential functional characteristics relevant to clinical application.* Journal of Immunotherapy, 2000. **31**(5).
34. Miggin, S.M. and L.A. O'Neill, *New insights into the regulation of TLR signaling.* J Leukoc Biol, 2006. **80**(2): p. 220-6.
35. van Haren, S.D., et al., *In vitro cytokine induction by TLR-activating vaccine adjuvants in human blood varies by age and adjuvant.* Cytokine, 2016. **83**: p. 99-109.
36. Yam, K.K., et al., *AS03-Adjuvanted, Very-Low-Dose Influenza Vaccines Induce Distinctive Immune Responses Compared to Unadjuvanted High-Dose Vaccines in BALB/c Mice.* Front Immunol, 2015. **6**: p. 207.
37. Surh, C.D. and J. Sprent, *Regulation of mature T cell homeostasis.* Semin Immunol, 2005. **17**(3): p. 183-91.
38. Surh, C.D. and J. Sprent, *Homeostasis of naive and memory T cells.* Immunity, 2008. **29**(6): p. 848-62.
39. Brinkman, C.C., et al., *Peripheral tissue homing receptors enable T cell entry into lymph nodes and affect the anatomical distribution of memory cells.* J Immunol, 2013. **191**(5): p. 2412-25.
40. Keene, J.-A. and J. Forman, *Helper activity is required for the in vivo generation of cytotoxic T lymphocytes.* Journal of Experimental Medicine, 1982. **155**: p. 768-782.
41. Cho, J.H., et al., *Unique features of naive CD8+ T cell activation by IL-2.* J Immunol, 2013. **191**(11): p. 5559-73.
42. Bennett, S.R.M., et al., *Help for cytotoxic-T-cell responses is mediated by CD40 signalling.* Nature, 1998. **393**(6684): p. 478-480.
43. Ridge, J.P., F. Di Rosa, and P. Matzinger, *A conditioned dendritic cell can be a temporal bridge between a CD4(+) T-helper and a T-killer cell.* Nature, 1998. **393**(6684): p. 474-478.
44. Schoenberger, S.P., et al., *T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions.* Nature, 1998. **393**(6684): p. 480-483.
45. Seo, G.Y., J. Youn, and P.H. Kim, *IL-21 ensures TGF-beta 1-induced IgA isotype expression in mouse Peyer's patches.* J Leukoc Biol, 2009. **85**(5): p. 744-50.
46. Glatman Zaretsky, A., et al., *T follicular helper cells differentiate from Th2 cells in response to helminth antigens.* J Exp Med, 2009. **206**(5): p. 991-9.
47. McHeyzer-Williams, M., et al., *Molecular programming of B cell memory.* Nat Rev Immunol, 2011. **12**(1): p. 24-34.
48. Jenkins, M.K. and J.J. Moon, *The role of naive T cell precursor frequency and recruitment in dictating immune response magnitude.* J Immunol, 2012. **188**(9): p. 4135-40.
49. Obst, R., *The Timing of T Cell Priming and Cycling.* Front Immunol, 2015. **6**: p. 563.

50. Buchholz, V.R., T.N. Schumacher, and D.H. Busch, *T Cell Fate at the Single-Cell Level.* Annu Rev Immunol, 2016. **34**: p. 65-92.
51. Janeway, C.A.J., et al., *Immunobiology, 5th edition The Immune System in Health and Disease*, G. Science, Editor. 2001.
52. Hiebert, P.R. and D.J. Granville, *Granzyme B in injury, inflammation, and repair.* Trends Mol Med, 2012. **18**(12): p. 732-41.
53. Harty, J.T., A.R. Tvinnereim, and D.W. White, *CD8$^+$ T cell effector mechanisms in resistance to infection.* Annu Rev Immunol, 2000. **18**: p. 275-308.
54. Rutigliano, J.A., et al., *Highly pathological influenza A virus infection is associated with augmented expression of PD-1 by functionally compromised virus-specific CD8+ T cells.* J Virol, 2014. **88**(3): p. 1636-51.
55. Kaech, S.M., et al., *Molecular and functional profiling of memory CD8 T cell differentiation.* Cell, 2002. **111**(6): p. 837-51.
56. Youngblood, B., J.S. Hale, and R. Ahmed, *Memory CD8 T cell transcriptional plasticity.* F1000Prime Rep, 2015. **7**: p. 38.
57. Sallusto, F., et al., *Two subsets of memory T lymphocytes with distinct homing potentials and effector functions.* Nature, 1999. **401**(6754): p. 708-12.
58. Kalia, V., S. Sarkar, and R. Ahmed. *CD8 T-cell memory differentiation during acute and chronic viral infections*. 2000-2013 [cited 2016 September 9 2016]; Available from: http://www.ncbi.nlm.nih.gov/books/NBK28489/.
59. Feau, S., et al., *Autocrine IL-2 is required for secondary population expansion of CD8(+) memory T cells.* Nat Immunol, 2011. **12**(9): p. 908-13.
60. Williams, M.A., A.J. Tyznik, and M.J. Bevan, *Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells.* Nature, 2006. **441**(7095): p. 890-3.
61. Agace, W.W., et al., *T-lymphocyte-epithelial-cell interactions: integrin α_E(CD103)β_7, LEEP-CAM and chemokines.* Current Opinion in Immunology, 2000. **12**: p. 563-568.
62. Laidlaw, B.J., J.E. Craft, and S.M. Kaech, *The multifaceted role of CD4(+) T cells in CD8(+) T cell memory.* Nat Rev Immunol, 2016. **16**(2): p. 102-11.
63. Gattinoni, L., et al., *A human memory T cell subset with stem cell-like properties.* Nat Med, 2011. **17**(10): p. 1290-7.
64. Papatriantafyllou, M., *T cell memory: the stem of T cell memory.* Nat Rev Immunol, 2011. **11**(11): p. 716.
65. Flynn, J.K. and P.R. Gorry, *Stem memory T cells (TSCM)-their role in cancer and HIV immunotherapies.* Clin Transl Immunology, 2014. **3**(7): p. e20.
66. Ballesteros-Tato, A., et al., *Epitope-specific regulation of memory programming by differential duration of antigen presentation to influenza-specific CD8(+) T cells.* Immunity, 2014. **41**(1): p. 127-40.
67. Wherry, E.J., *T cell exhaustion.* Nature Immunology, 2011. **131**(6): p. 492-499.
68. Barber, D.L., et al., *Restoring function in exhausted CD8 T cells during chronic viral infection.* Nature, 2006. **439**(7077): p. 682-7.
69. Utzschneider, D.T., et al., *T cells maintain an exhausted phenotype after antigen withdrawal and population reexpansion.* Nat Immunol, 2013. **14**(6): p. 603-10.
70. Utzschneider, D.T., et al., *T Cell Factor 1-Expressing Memory-like CD8(+) T Cells Sustain the Immune Response to Chronic Viral Infections.* Immunity, 2016. **45**(2): p. 415-27.
71. Im, S.J., et al., *Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy.* Nature, 2016.
72. Reeves, E. and E. James, *Antigen processing and immune regulation in the response to tumours.* Immunology, 2016.
73. Sokol, L., et al., *Loss of tapasin correaltes with diminished CD8$^+$ T-cell immunity and prognosis in colorectal cancer.* J. Transl Med, 2015. **13**.
74. Leffers, N., et al., *Prognostic significance of tumor-infiltrating T-lymphocytes in primary and metastatic lesions of advanced stage ovarian cancer.* Cancer Immunol Immunother, 2009. **58**(3): p. 449-59.

75. Gajewski, T.F., H. Schreiber, and Y.X. Fu, *Innate and adaptive immune cells in the tumor microenvironment.* Nat Immunol, 2013. **14**(10): p. 1014-22.
76. Speiser, D.E., et al., *T cell differentiation in chronic infection and cancer: functional adaptation or exhaustion?* Nat Rev Immunol, 2014. **14**(11): p. 768-74.
77. Mescher, M.F., et al., *Signals required for programming effector and memory development by CD8+ T cells.* Immunol Rev, 2006. **211**: p. 81-92.
78. Schwartz, R.H., *T cell anergy.* Annu Rev Immunol, 2003. **21**: p. 305-34.
79. Appleman, L.J. and V.A. Boussiotis, *T cell anergy and costimulation.* Immunol Rev, 2003. **192**: p. 161-80.
80. Yamamoto, T., M. Hattori, and T. Yoshida, *Induction of T-cell activation or anergy determined by the combination of intensity and duration of T-cell receptor stimulation, and sequential induction in an individual cell.* Immunology, 2007. **121**(3): p. 383-91.
81. Schoenberger, S.P., et al., *T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions.* Nature, 1998. **393**: p. 480-483.
82. van de Ven, K. and J. Borst, *Targeting the T-cell co-stimulatory CD27/CD70 pathway in cancer immunotherapy: rationale and potential.* Immunotherapy, 2015. **7**(6): p. 655-67.
83. Beier, K.C., et al., *Induction, binding specificity and function of human ICOS.* Eur J Immunol, 2000. **30**(12): p. 3707-17.
84. Curtsinger, J.M., D.C. Lins, and M.F. Mescher, *Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function.* J Exp Med, 2003. **197**(9): p. 1141-51.
85. Curtsinger, J.M., et al., *Inflammatory cytokines provide a third signal for activation of naive CD4$^+$ and CD8$^+$ T cells.* Journal of Immunology, 1999. **162**: p. 3256-3262.
86. Curtsinger, J.M. and M.F. Mescher, *Inflammatory cytokines as a third signal for T cell activation.* Curr Opin Immunol, 2010. **22**(3): p. 333-40.
87. Cui, W.G. and S.M. Kaech, *Generation of effector CD8+T cells and their conversion to memory T cells.* Immunological Reviews, 2010. **236**: p. 151-166.
88. Hochrein, H., et al., *Differential Production of IL-12, IFN- , and IFN- by Mouse Dendritic Cell Subsets.* The Journal of Immunology, 2001. **166**(9): p. 5448-5455.
89. Swiecki, M. and M. Colonna, *The multifaceted biology of plasmacytoid dendritic cells.* Nat Rev Immunol, 2015. **15**(8): p. 471-85.
90. Obar, J.J., et al., *Pathogen-induced inflammatory environment controls effector and memory CD8+ T cell differentiation.* J Immunol, 2011. **187**(10): p. 4967-78.
91. Kolumam, G.A., et al., *Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection.* J Exp Med, 2005. **202**(5): p. 637-50.
92. Curtsinger, J.M., et al., *Cutting Edge: Type I IFNs Provide a Third Signal to CD8 T Cells to Stimulate Clonal Expansion and Differentiation.* The Journal of Immunology, 2005. **174**(8): p. 4465-4469.
93. Cui, W., et al., *Effects of Signal 3 during CD8 T cell priming: Bystander production of IL-12 enhances effector T cell expansion but promotes terminal differentiation.* Vaccine, 2009. **27**(15): p. 2177-87.
94. Aichele, P., et al., *Cutting Edge: CD8 T Cells Specific for Lymphocytic Choriomeningitis Virus Require Type I IFN Receptor for Clonal Expansion.* The Journal of Immunology, 2006. **176**(8): p. 4525-4529.
95. Pearce, E.L. and H. Shen, *Generation of CD8 T Cell Memory Is Regulated by IL-12.* The Journal of Immunology, 2007. **179**(4): p. 2074-2081.
96. Jacobson, N.G., et al., *Interleukin 12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (Stat)3 and Stat4.* J Exp Med, 1995. **181**(5): p. 1755-62.
97. Yoshimoto, T., et al., *IL-12 up-regulates IL-18 recptor expression on T cells, TH1 cells, and B cells: Synergism with IL-18 for IFN-g production.* The Journal of Immunology, 1998.

98. Nakahira, M., et al., *An Absolute Requirement for STAT4 and a Role for IFN- as an Amplifying Factor in IL-12 Induction of the Functional IL-18 Receptor Complex.* The Journal of Immunology, 2001. **167**(3): p. 1306-1312.
99. Letimier, F.A., et al., *Chromatin remodeling by the SWI/SNF-like BAF complex and STAT4 activation synergistically induce IL-12Rb2 expression during human Th1 cell differentiation.* The EMBO journal, 2007.
100. Becskei, A. and M.J. Grusby, *Contribution of IL-12R mediated feedback loop to Th1 cell differentiation.* FEBS Lett, 2007. **581**(27): p. 5199-206.
101. Yanagida, T., et al., *Second signal activity of IL-12 on the proliferation and IL-2R expression of T helper cell-1 clone.* J Immunol, 1994. **152**(10): p. 4919-28.
102. Wiesel, M., et al., *Type-I IFN drives the differentiation of short-lived effector CD8+ T cells in vivo.* Eur J Immunol, 2012. **42**(2): p. 320-9.
103. Parmar, S. and L.C. Platanias, *Interferons: mechanisms of action and clinical applications.* Curr Opin Oncol, 2003. **15**(6): p. 431-9.
104. Platanias, L.C., *Mechanisms of type-I- and type-II-interferon-mediated signalling.* Nat Rev Immunol, 2005. **5**(5): p. 375-86.
105. Levy, D.E., et al., *Enhancement and diversification of IFN induction by IRF-7-mediated positive feedback.* J Interferon Cytokine Res, 2002. **22**(1): p. 87-93.
106. Schoggins, J.W. and C.M. Rice, *Interferon-stimulated genes and their antiviral effector functions.* Curr Opin Virol, 2011. **1**(6): p. 519-25.
107. van Boxel-Dezaire, A.H., M.R. Rani, and G.R. Stark, *Complex modulation of cell type-specific signaling in response to type I interferons.* Immunity, 2006. **25**(3): p. 361-72.
108. Cacalno, N.A. and J.A. Johnston, *Interleukin-2 signaling and inherited immunodeficiency.* The American Journal of Human Genetics, 1999.
109. Moroz, A., et al., *IL-21 Enhances and Sustains CD8+ T Cell Responses to Achieve Durable Tumor Immunity: Comparative Evaluation of IL-2, IL-15, and IL-21.* The Journal of Immunology, 2004. **173**(2): p. 900-909.
110. Xin, G., et al., *A Critical Role of IL-21-Induced BATF in Sustaining CD8-T-Cell-Mediated Chronic Viral Control.* Cell Rep, 2015. **13**(6): p. 1118-24.
111. Novy, P., et al., *Intrinsic IL-21 signaling is critical for CD8 T cell survival and memory formation in response to vaccinia viral infection.* J Immunol, 2011. **186**(5): p. 2729-38.
112. Spolski, R., et al., *IL-21 mediates suppressive effects via its induction of IL-10.* J Immunol, 2009. **182**(5): p. 2859-67.
113. Obar, J.J., et al., *CD4+ T cell regulation of CD25 expression controls development of short-lived effector CD8+ T cells in primary and secondary responses.* Proc Natl Acad Sci U S A, 2010. **107**(1): p. 193-8.
114. Pipkin, M.E., et al., *Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells.* Immunity, 2010. **32**(1): p. 79-90.
115. Sutherland, A.P., et al., *IL-21 promotes CD8+ CTL activity via the transcription factor T-bet.* J Immunol, 2013. **190**(8): p. 3977-84.
116. Croce, M., V. Rigo, and S. Ferrini, *IL-21: a pleiotropic cytokine with potential applications in oncology.* J Immunol Res, 2015. **2015**: p. 696578.
117. Joshi, N.S., et al., *Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor.* Immunity, 2007. **27**(2): p. 281-95.
118. Takemoto, N., et al., *Cutting Edge: IL-12 Inversely Regulates T-bet and Eomesodermin Expression during Pathogen-Induced CD8+ T Cell Differentiation.* The Journal of Immunology, 2006. **177**(11): p. 7515-7519.
119. Intlekofer, A.M., et al., *Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin.* Nat Immunol, 2005. **6**(12): p. 1236-44.
120. Banerjee, A., et al., *Cutting edge: The transcription factor eomesodermin enables CD8+ T cells to compete for the memory cell niche.* J Immunol, 2010. **185**(9): p. 4988-92.

121. Kallies, A., et al., *Blimp-1 transcription factor is required for the differentiation of effector CD8(+) T cells and memory responses.* Immunity, 2009. **31**(2): p. 283-95.
122. Rutishauser, R.L., et al., *Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties.* Immunity, 2009. **31**(2): p. 296-308.
123. Cui, W., et al., *An interleukin-21-interleukin-10-STAT3 pathway is critical for functional maturation of memory CD8+ T cells.* Immunity, 2011. **35**(5): p. 792-805.
124. Yang, C.Y., et al., *The transcriptional regulators Id2 and Id3 control the formation of distinct memory CD8+ T cell subsets.* Nat Immunol, 2011. **12**(12): p. 1221-9.
125. Cannarile, M.A., et al., *Transcriptional regulator Id2 mediates CD8+ T cell immunity.* Nat Immunol, 2006. **7**(12): p. 1317-25.
126. Ji, Y., et al., *Repression of the DNA-binding inhibitor Id3 by Blimp-1 limits the formation of memory CD8+ T cells.* Nat Immunol, 2011. **12**(12): p. 1230-7.
127. Willinger, T., et al., *Human Naive CD8 T Cells Down-Regulate Expression of the WNT Pathway Transcription Factors Lymphoid Enhancer Binding Factor 1 and Transcription Factor 7 (T Cell Factor-1) following Antigen Encounter In Vitro and In Vivo.* The Journal of Immunology, 2006. **176**(3): p. 1439-1446.
128. Jeannet, G., et al., *Essential role of the Wnt pathway effector Tcf-1 for the establishment of functional CD8 T cell memory.* Proc Natl Acad Sci U S A, 2010. **107**(21): p. 9777-82.
129. Boudousquie, C., et al., *Differences in the transduction of canonical Wnt signals demarcate effector and memory CD8 T cells with distinct recall proliferation capacity.* J Immunol, 2014. **193**(6): p. 2784-91.
130. Zhou, X., et al., *Differentiation and persistence of memory CD8(+) T cells depend on T cell factor 1.* Immunity, 2010. **33**(2): p. 229-40.
131. Germar, K., et al., *T-cell factor 1 is a gatekeeper for T-cell specification in response to Notch signaling.* Proceedings of the National Academy of Sciences of the United States of America, 2011. **108**(50): p. 20060-20065.
132. Northrop, J.K., et al., *Epigenetic Remodeling of the IL-2 and IFN- Loci in Memory CD8 T Cells Is Influenced by CD4 T Cells.* The Journal of Immunology, 2006. **177**(2): p. 1062-1069.
133. Araki, Y., et al., *Histone Acetylation Facilitates Rapid and Robust Memory CD8 T Cell Response through Differential Expression of Effector Molecules (Eomesodermin and Its Targets: Perforin and Granzyme B).* The Journal of Immunology, 2008. **180**(12): p. 8102-8108.
134. Araki, Y., et al., *Genome-wide analysis of histone methylation reveals chromatin state-based regulation of gene transcription and function of memory CD8+ T cells.* Immunity, 2009. **30**(6): p. 912-25.
135. Zediak, V.P., et al., *Cutting edge: persistently open chromatin at effector gene loci in resting memory CD8+ T cells independent of transcriptional status.* J Immunol, 2011. **186**(5): p. 2705-9.
136. Fitzpatrick, D.R., K.M. Shirley, and A. Kelso, *Cutting edge: Stable epigenetic inheritance of regional IFNg promoter demethylation in $CD44^{high}CD8^{+}$ T lymphocytes.* The Journal of Immunology, 1999. **162**: p. 5053-5057.
137. Kersh, E.N., et al., *Rapid Demethylation of the IFN- Gene Occurs in Memory but Not Naive CD8 T Cells.* The Journal of Immunology, 2006. **176**(7): p. 4083-4093.
138. Youngblood, B., et al., *Chronic virus infection enforces demethylation of the locus that encodes PD-1 in antigen-specific CD8(+) T cells.* Immunity, 2011. **35**(3): p. 400-12.
139. Scharer, C.D., et al., *Global DNA methylation remodeling accompanies CD8 T cell effector function.* J Immunol, 2013. **191**(6): p. 3419-29.
140. Yu, Q., V.T. Thieu, and M.H. Kaplan, *Stat4 limits DNA methyltransferase recruitment and DNA methylation of the IL-18Ra gene during Th1 differentiation.* The EMBO journal, 2007. **26**: p. 2052-2060.
141. Lewis, M.D., et al., *T-bet's ability to regulate individual target genes requires the conserved T-box domain to recruit histone methyltransferase activity and a separate*

family member-specific transactivation domain. Mol Cell Biol, 2007. **27**(24): p. 8510-21.
142. Shin, H.M., et al., *Epigenetic modifications induced by Blimp-1 Regulate CD8(+) T cell memory progression during acute virus infection.* Immunity, 2013. **39**(4): p. 661-75.
143. Nusse, R. and H.E. Varmus, *Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome.* Cell, 1982. **31**(1): p. 99-109.
144. Nüsslein-Volhard, C. and E. Wieschaus, *Mutations affecting segement number and polarity in Drosophila.* Nature, 1980. **287**: p. 795-801.
145. Rijsewijk, F., et al., *The drosophilaa homolog of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless.* Cell, 1987. **50**: p. 649-657.
146. Nusse, R. and X. Lim. *The Wnt Homepage*. 2016 [cited 2016 September 14th]; Available from: http://www.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes.
147. Willert, K., et al., *Wnt proteins are lipid-modified and can act as stem cell growth factors.* Nature, 2003. **423**(6938): p. 448-452.
148. Takada, R., et al., *Monounsaturated fatty acid modification of Wnt protein: its role in Wnt secretion.* Dev Cell, 2006. **11**(6): p. 791-801.
149. Banziger, C., et al., *Wntless, a conserved membrane protein dedicated to the secretion of Wnt proteins from signaling cells.* Cell, 2006. **125**(3): p. 509-22.
150. Sato, T., et al., *Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts.* Nature, 2011. **469**(7330): p. 415-8.
151. Farin, H.F., et al., *Visualization of a short-range Wnt gradient in the intestinal stem-cell niche.* Nature, 2016. **530**(7590): p. 340-3.
152. Kikuchi, A., S. Kishida, and H. Yamamoto, *Regulation of Wnt signaling by protein-protein interaction and post-translational modifications.* Exp Mol Med, 2006. **38**(1): p. 1-10.
153. Gattinoni, L., Y. Ji, and N.P. Restifo, *Wnt/beta-catenin signaling in T-cell immunity and cancer immunotherapy.* Clin Cancer Res, 2010. **16**(19): p. 4695-701.
154. Lu, Z. and T. Hunter, *Wnt-independent b-catenin transactivation in tumor development.* Cell cycle, 2004: p. 571-573.
155. Castellone, M.D., et al., *Prostaglandin E2 promotes colon cancer cell growth through a Gs-axin-beta-catenin signaling axis.* Science, 2005. **310**(5753): p. 1504-10.
156. Naito, A.T., et al., *Complement C1q activates canonical Wnt signaling and promotes aging-related phenotypes.* Cell, 2012. **149**(6): p. 1298-313.
157. Schurch, C., et al., *CD27 signaling on chronic myelogenous leukemia stem cells activates Wnt target genes and promotes disease progression.* J Clin Invest, 2012. **122**(2): p. 624-38.
158. Clevers, H., *Wnt/beta-catenin signaling in development and disease.* Cell, 2006. **127**(3): p. 469-80.
159. Guo, Z., et al., *B-catenin stabilization stalls the transition from double-positive to single-positive stage and predisposes thymocytes to malignant transformation.* Blood, 2007. **109**(12): p. 5463-5472.
160. Clements, W.M., A.M. Lowy, and J. Groden, *Adenomatous polyposis coli/beta-catenin interaction and downstream targets: altered gene expression in gastrointestinal tumors.* Clin Colorectal Cancer, 2003. **3**(2): p. 113-20.
161. Lammi, L., et al., *Mutations in AXIN2 cause familial tooth agenesis and predispose to colorectal cancer.* Am J Hum Genet, 2004. **74**(5): p. 1043-50.
162. Verbeek, S., et al., *An HMG-box-containing T-cell factor required for thymocyte differentiation.* Nature, 1995. **374**(6517): p. 70-4.
163. Staal, F.J. and H. Clevers, *Tcf/Lef transcription factors during T-cell development: unique and overlapping functions.* Hematol J, 2000. **1**(1): p. 3-6.
164. Weber, B.N., et al., *A critical role for TCF-1 in T-lineage specification and differentiation.* Nature, 2011. **476**(7358): p. 63-8.
165. Goux, D., et al., *Cooperating pre-T-cell receptor and TCF-1-dependent signals ensure thymocyte survival.* Blood, 2005. **106**(5): p. 1726-33.

166. Steinke, F.C., et al., *TCF-1 and LEF-1 act upstream of Th-POK to promote the CD4(+) T cell fate and interact with Runx3 to silence Cd4 in CD8(+) T cells.* Nat Immunol, 2014. **15**(7): p. 646-56.
167. Ioannidis, V., et al., *The β-catenin-TCF-1 pathway ensures CD4$^+$CD8$^+$ thymocyte survival.* Nature Immunology, 2001. **2**(8): p. 691-697.
168. Okamura, R.M., et al., *Redundant regulation of T cell differentiation and TCRa gene expression by the transcription factors LEF-1 and TCF-1.* Immunity, 1998. **8**: p. 11-20.
169. Yu, Q., et al., *T cell factor 1 initiates the T helper type 2 fate by inducing the transcription factor GATA-3 and repressing interferon-gamma.* Nat Immunol, 2009. **10**(9): p. 992-9.
170. Ma, J., et al., *Critical role of TCF-1 in repression of the IL-17 gene.* PLoS One, 2011. **6**(9): p. e24768.
171. Ding, Y., et al., *Beta-catenin stabilization extends regulatory T cell survival and induces anergy in nonregulatory T cells.* Nat Med, 2008. **14**(2): p. 162-9.
172. Choi, Y.S., et al., *LEF-1 and TCF-1 orchestrate T(FH) differentiation by regulating differentiation circuits upstream of the transcriptional repressor Bcl6.* Nat Immunol, 2015. **16**(9): p. 980-90.
173. Xu, L., et al., *The transcription factor TCF-1 initiates the differentiation of T(FH) cells during acute viral infection.* Nat Immunol, 2015. **16**(9): p. 991-9.
174. Wherry, E.J., et al., *Antigen-independent memory CD8 T cells do not develop during chronic viral infection.* Proc Natl Acad Sci U S A, 2004. **101**(45): p. 16004-9.
175. Prlic, M. and M.J. Bevan, *Cutting edge: beta-catenin is dispensable for T cell effector differentiation, memory formation, and recall responses.* J Immunol, 2011. **187**(4): p. 1542-6.
176. Gattinoni, L., et al., *Wnt signaling arrests effector T cell differentiation and generates CD8+ memory stem cells.* Nat Med, 2009. **15**(7): p. 808-13.
177. Inoki, K., et al., *TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth.* Cell, 2006. **126**(5): p. 955-68.
178. Tan, J.T., et al., *IL-7 is critical for homeostatic proliferation and survival of naive T cells.* Proc Natl Acad Sci U S A, 2001. **98**(15): p. 8732-7.
179. Jho, E.h., et al., *Wnt/ -Catenin/Tcf Signaling Induces the Transcription of Axin2, a Negative Regulator of the Signaling Pathway.* Molecular and Cellular Biology, 2002. **22**(4): p. 1172-1183.
180. Lustig, B., et al., *Negative Feedback Loop of Wnt Signaling through Upregulation of Conductin/Axin2 in Colorectal and Liver Tumors.* Molecular and Cellular Biology, 2002. **22**(4): p. 1184-1193.
181. Butz, E.A. and M.J. Bevan, *Massive expansion of antigen-specific CD8$^+$ T cells during an acute virus infection.* Immunity, 1998. **8**(2): p. 167-75.
182. Blattman, J.N., et al., *Estimating the precursor frequency of naive antigen-specific CD8 T cells.* J Exp Med, 2002. **195**(5): p. 657-64.
183. Zehn, D., S.Y. Lee, and M.J. Bevan, *Complete but curtailed T-cell response to very low-affinity antigen.* Nature, 2009. **458**(7235): p. 211-4.
184. Badovinac, V.P., et al., *Accelerated CD8+ T-cell memory and prime-boost response after dendritic-cell vaccination.* Nat Med, 2005. **11**(7): p. 748-56.
185. Pham, N.L., V.P. Badovinac, and J.T. Harty, *A default pathway of memory CD8 T cell differentiation after dendritic cell immunization is deflected by encounter with inflammatory cytokines during antigen-driven proliferation.* J Immunol, 2009. **183**(4): p. 2337-48.
186. Cui, W., et al., *TLR4 ligands lipopolysaccharide and monophosphoryl lipid a differentially regulate effector and memory CD8+ T Cell differentiation.* J Immunol, 2014. **192**(9): p. 4221-32.
187. Gray, R.C., J. Kuchtey, and C.V. Harding, *CpG-B ODNs potently induce low levels of IFN-alphabeta and induce IFN-alphabeta-dependent MHC-I cross-presentation in DCs as effectively as CpG-A and CpG-C ODNs.* J Leukoc Biol, 2007. **81**(4): p. 1075-85.

188. Xiao, Z., et al., *Programming for CD8 T cell memory development requires IL-12 or type I IFN.* J Immunol, 2009. **182**(5): p. 2786-94.
189. Le Bon, A., et al., *Direct Stimulation of T Cells by Type I IFN Enhances the CD8+ T Cell Response during Cross-Priming.* The Journal of Immunology, 2006. **176**(8): p. 4682-4689.
190. Keppler, S.J., et al., *Signal 3 cytokines as modulators of primary immune responses during infections: the interplay of type I IFN and IL-12 in CD8 T cell responses.* PLoS One, 2012. **7**(7): p. e40865.
191. Kalia, V., et al., *Prolonged interleukin-2Ralpha expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo.* Immunity, 2010. **32**(1): p. 91-103.
192. Nguyen, K.B., et al., *Critical role for STAT4 activation by type 1 interferons in the interferon-gamma response to viral infection.* Science, 2002. **297**(5589): p. 2063-6.
193. Keppler, S.J., et al., *Effector T-cell differentiation during viral and bacterial infections: Role of direct IL-12 signals for cell fate decision of CD8(+) T cells.* Eur J Immunol, 2009. **39**(7): p. 1774-83.
194. O'Sullivan, A., et al., *STAT4 is required for interleukin-12-induced chromatin remodeling of the CD25 locus.* J Biol Chem, 2004. **279**(8): p. 7339-45.
195. Pesu, M., et al., *Proprotein convertase furin is preferentially expressed in T helper 1 cells and regulates interferon gamma.* Blood, 2006. **108**(3): p. 983-5.
196. Thieu, V.T., et al., *Signal transducer and activator of transcription 4 is required for the transcription factor T-bet to promote T helper 1 cell-fate determination.* Immunity, 2008. **29**(5): p. 679-90.
197. Gay, M.H., et al., *Distinct adhesion-independent functions of beta-catenin control stage-specific sensory neurogenesis and proliferation.* BMC Biol, 2015. **13**: p. 24.
198. Lee, P.P., et al., *A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival.* Immunity, 2001. **15**(5): p. 763-74.
199. Georgiades, P., et al., *VavCre transgenic mice: a tool for mutagenesis in hematopoietic and endothelial lineages.* Genesis, 2002. **34**(4): p. 251-6.
200. Yamane, T., et al., *Wnt Signaling Regulates Hemopoiesis Through Stromal Cells.* The Journal of Immunology, 2001. **167**(2): p. 765-772.
201. Luther, S.A., et al., *Coexpression of the chemokines ELC and SLC by T zone stromal cells and deletion of the ELC gene in the plt/plt mouse.* Proc Natl Acad Sci U S A, 2000. **97**(23): p. 12694-9.
202. Krieg, A.M., *CpG motifs in bacterial DNA and their immune effects.* Annu Rev Immunol, 2002. **20**: p. 709-60.
203. Pham, N.L., V.P. Badovinac, and J.T. Harty, *Differential role of "Signal 3" inflammatory cytokines in regulating CD8 T cell expansion and differentiation in vivo.* Front Immunol, 2011. **2**: p. 4.
204. Stonier, S.W. and K.S. Schluns, *Trans-presentation: a novel mechanism regulating IL-15 delivery and responses.* Immunol Lett, 2010. **127**(2): p. 85-92.
205. Kim, M.T. and J.T. Harty, *Impact of Inflammatory Cytokines on Effector and Memory CD8+ T Cells.* Front Immunol, 2014. **5**: p. 295.
206. Boulet, S., J.F. Daudelin, and N. Labrecque, *IL-2 induction of Blimp-1 is a key in vivo signal for CD8+ short-lived effector T cell differentiation.* J Immunol, 2014. **193**(4): p. 1847-54.
207. Boyman, O., et al., *Selective stimulation of T cell subsets with antibody-cytokine immune complexes.* Science, 2006. **311**(5769): p. 1924-7.
208. Starbeck-Miller, G.R., H.H. Xue, and J.T. Harty, *IL-12 and type I interferon prolong the division of activated CD8 T cells by maintaining high-affinity IL-2 signaling in vivo.* J Exp Med, 2014. **211**(1): p. 105-20.
209. Boyman, O., J.H. Cho, and J. Sprent, *The role of interleukin-2 in memory CD8 cell differentiation.* Adv Exp Med Biol, 2010. **684**: p. 28-41.
210. Curtsinger, J.M., et al., *Autocrine IFN-gamma promotes naive CD8 T cell differentiation and synergizes with IFN-alpha to stimulate strong function.* J Immunol, 2012. **189**(2): p. 659-68.

211. Cox, M.A., S.M. Kahan, and A.J. Zajac, *Anti-viral CD8 T cells and the cytokines that they love.* Virology, 2013. **435**(1): p. 157-69.
212. Yang, Q., et al., *IL-33 synergizes with TCR and IL-12 signaling to promote the effector function of CD8+ T cells.* Eur J Immunol, 2011. **41**(11): p. 3351-60.
213. Bonilla, W.V., et al., *The alarmin interleukin-33 drives protective antiviral CD8(+) T cell responses.* Science, 2012. **335**(6071): p. 984-9.
214. Walker, S.R., E.A. Nelson, and D.A. Frank, *STAT5 represses BCL6 expression by binding to a regulatory region frequently mutated in lymphomas.* Oncogene, 2007. **26**(2): p. 224-33.
215. Tran, T.H., et al., *Prolactin inhibits BCL6 expression in breast cancer through a Stat5a-dependent mechanism.* Cancer Res, 2010. **70**(4): p. 1711-21.
216. O'Shea, J.J., et al., *Genomic views of STAT function in CD4+ T helper cell differentiation.* Nat Rev Immunol, 2011. **11**(4): p. 239-50.
217. Presky, D.H., et al., *A functional interleukin 12 receptor complex is composed f two b-type cytokine receptor subunits.* PNAS, 1996. **93**: p. 14002-14007.
218. Szabo, S.J., et al., *Regulation of the interleukin (IL)-12R b2 subunit expression in developing T helper 1 (Th1) and Th2 cells.* J Exp Med, 1997. **185**(5): p. 817-824.
219. Foulds, K.E., M.J. Rotte, and R.A. Seder, *IL-10 Is Required for Optimal CD8 T Cell Memory following Listeria monocytogenes Infection.* The Journal of Immunology, 2006. **177**(4): p. 2565-2574.
220. Allard, E.L., et al., *Overexpression of IL-21 promotes massive CD8$^+$ memory T cell accumulation.* Eur J Immunol, 2007. **37**(11): p. 3069-77.
221. Yi, J.S., M. Du, and A.J. Zajac, *A vital role for interleukin-21 in the control of a chronic viral infection.* Science, 2009. **324**(5934): p. 1572-6.
222. Hinrichs, C.S., et al., *IL-2 and IL-21 confer opposing differentiation programs to CD8+ T cells for adoptive immunotherapy.* Blood, 2008. **111**(11): p. 5326-33.
223. Poland, G. and A. Barrett, *The old and the new: successful vaccines of the 20th century and approaches to making vaccines for the important diseases of the 21st century.* Curr Opin Immunol, 2009. **21**(3): p. 305-7.
224. Bassi, M.R., et al., *CD8+ T cells complement antibodies in protecting against yellow fever virus.* J Immunol, 2015. **194**(3): p. 1141-53.
225. Fuertes Marraco, S.A.F., et al., *Long-lasting stem cell-like memory CD8$^+$ T cells with a naive-like profile upon yellow fever vaccination.* Science Translational Medicine, 2015. **7**(282).
226. Jin, X., et al., *Dramatic rise in plasma viremia after CD8 T cell depletion in simian immunodefiency virus-infected macaques.* J Exp Med, 1999. **189**(6).
227. Klebanoff, C.A., L. Gattinoni, and N.P. Restifo, *CD8$^+$ T-cell memory in tumor immunology and immunotherapy.* Immunol Rev, 2006. **211**: p. 214-24.
228. Mutua, G., et al., *Broad HIV-1 inhibition in vitro by vaccine-elicited CD8(+) T cells in African adults.* Mol Ther Methods Clin Dev, 2016. **3**: p. 16061.
229. Therapeutics, A. *Cancer vaccine*. Therapeutic cancer vaccines 30th of October 2016]; Available from: http://www.amaltherapeutics.com/site/en/research-a-development/cancer-vaccine/.
230. Wherry, E.J., et al., *Lineage relationship and protective immunity of memory CD8 T cell subsets.* Nat Immunol, 2003. **4**(3): p. 225-34.
231. Klebanoff, C.A., et al., *Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells.* Proc Natl Acad Sci U S A, 2005. **102**(27): p. 9571-6.
232. School, E.V.M., *A Safety, Tolerability and Bioavailability Study of Lisofylline After Continuous Subcutaneous (12 mg/kg) and Intravenous (9 mg/kg) Administration in Subjects With Type 1 Diabetes Mellitus*. 2012-2016, National Library of Medicine (US): Clinicaltrials.gov.
233. Caprioli, A., et al., *Wnt4 is essential to normal mammalian lung development.* Dev Biol, 2015. **406**(2): p. 222-34.

234. Wang, Q., et al., *Wnt6 is essential for stromal cell proliferation during decidualization in mice.* Biol Reprod, 2013. **88**(1): p. 5.
235. Fu, J., et al., *Reciprocal regulation of Wnt and Gpr177/mouse Wntless is required for embryonic axis formation.* Proc Natl Acad Sci U S A, 2009. **106**(44): p. 18598-603.
236. Li, L., et al., *Prostaglandin E2 promotes survival of naive UCB T cells via the Wnt/beta-catenin pathway and alters immune reconstitution after UCBT.* Blood Cancer J, 2014. **4**: p. e178.
237. Apte, U., et al., *Activation of Wnt/beta-catenin pathway during hepatocyte growth factor-induced hepatomegaly in mice.* Hepatology, 2006. **44**(4): p. 992-1002.
238. Pircher, H., et al., *Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen.* Nature, 1989. **342**(6249): p. 559-61.
239. Kaplan, M.H., et al., *Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice.* Nature, 1996. **382**(6587): p. 174-7.
240. Chai, Q., et al., *Maturation of lymph node fibroblastic reticular cells from myofibroblastic precursors is critical for antiviral immunity.* Immunity, 2013. **38**(5): p. 1013-24.
241. Kuhn, R., et al., *Inducible gene targeting in mice.* Science, 1995. **269**(5229): p. 1427-9.
242. Hogquist, K.A., et al., *T cell receptor antagonist peptides induce positive selection.* Cell, 1994. **76**(1): p. 17-27.
243. Wu, C.y., et al., *IL-12 Receptor 2 (IL-12R 2)-Deficient Mice Are Defective in IL-12-Mediated Signaling Despite the Presence of High Affinity IL-12 Binding Sites.* The Journal of Immunology, 2000. **165**(11): p. 6221-6228.
244. Battegay, M., et al., *Quantification of lymphocytic choriomeningitis virus with an immunological focus assay in 24- or 96-well plates.* J Virol Methods, 1991. **33**(1-2): p. 191-8.
245. Oberle, S.G., et al., *A Minimum Epitope Overlap between Infections Strongly Narrows the Emerging T Cell Repertoire.* Cell Rep, 2016. **17**(3): p. 627-635.
246. Hamilton, S.E., et al., *MHC class Ia-restricted memory T cells inhibit expansion of a nonprotective MHC class Ib (H2-M3)-restricted memory response.* Nat Immunol, 2004. **5**(2): p. 159-68.

Publication list

Boudousquie, C., Danilo, M., et al., *Differences in the transduction of canonical Wnt signals demarcate effector and memory CD8 T cells with distinct recall proliferation capacity.* J Immunol, 2014. 193(6): p. 2784-91.

Utzschneider, D.T., et al., *T Cell Factor 1-Expressing Memory-like CD8(+) T Cells Sustain the Immune Response to Chronic Viral Infections.* Immunity, 2016. 45(2): p. 415-27.