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The role of ThPOK and T cell receptor signaling in $CD4^+$ versus $CD8^+$ T-cell lineage fate

par

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Résumé

Les lymphocytes T sont au cœur du système immunitaire adaptatif et leur dérégulation est à la base de pathologies. Les cellules T se développent dans le thymus et passent par de nombreuses étapes de maturations identifiables par l'expression des corécepteurs $CD4^+$/$CD8^+$ à la surface des cellules. À leur sortie du thymus, les cellules T sont divisées en deux sous-types principaux: les cellules T auxiliaires $CD4^+$ spécifique aux antigènes présentés sur complexe majeur d'histocompatibilité (CMH) de classe II et les cellules T cytotoxiques $CD8^+$ reconnaissant un antigène présenté sur un CMH-I. Toutes les cellules T proviennent d'un précurseur commun. Leur différenciation en cellule T $CD4^+$ et T $CD8^+$ est influencée par l'intensité et la durée de la signalisation du récepteur des cellules T (RCT) et des cytokines. Cette signalisation résulte en l'expression des facteurs de transcription ThPOK pour la différenciation de cellule T $CD4^+$ et Runx3 pour les cellules T $CD8^+$. Il a été démontré que ThPOK est à la fois nécessaire et suffisant pour le développement des lymphocytes T $CD4^+$, puisque le gain et la perte de la fonction de ThPOK favorise le développement de cellules lymphocytes T $CD4^+$ et $CD8^+$, respectivement. Ma thèse vise à approfondir notre compréhension du choix de la lignée $CD4^+$/$CD8^+$ en explorant les mécanismes moléculaires de la voix de signalisation de ThPOK et du RCT.

Dans cette étude, nous avons étudié l'impact d'un gain-de-fonction de ThPOK sur la différenciation des thymocytes, en utilisant trois lignées transgéniques exprimant des niveaux variables de ThPOK. Une analyse approfondie de ces transgènes chez des souris dont le RCT est restreint soit au CMH de classe I ou de classe II, a démontré que, comparés aux thymocytes restreints au CMH-II, les thymocytes restreints au CMH-I requéraient des niveaux plus importants de ThPOK pour se différencier en $CD4^+$. L'introduction d'un transgène exprimant un niveau moins élevé de ThPOK comparé aux deux autres transgènes, mais un niveau plus élevé de ThPOK par rapport au niveau endogène dans les cellules $CD4^+$ WT, n'induit qu'une réorientation partielle des cellules T $CD8^+$ en $CD4^+$, ce qui a mené à la génération, à la fois de lymphocytes T $CD4^+$, DN (doubles négatifs) et $CD8^+$ matures. L'analyse génotypique, plus précisément celle des cellules DN chez les souris porteuses du transgène ThPOK et dont le RCT est restreint au CMH-I, a révélé que l'inhibition des gènes spécifiques à la lignée $CD8^+$

nécessitait des niveaux d'expression différents de ThPOK comparés à ceux requis pour l'induction des gènes spécifiques à la lignée $CD4^+$. En effet, cette étude nous a permis de démontrer que l'intensité du signal dérivé du RCT ainsi que sa spécificité pour un CMH donné jouent un rôle essentiel dans le choix de différentiation $CD4^+/CD8^+$ induit par ThPOK. Ainsi, la réorientation $CD8^+/CD4^+$ chez les souris exprimant le transgène ThPOK-H est significativement augmentée par l'amplification de l'intensité du signal dérivé du RCT dans les cellules spécifiques aux CMH-I. De plus, la fréquence des cellules $CD4^+$ était plus élevée lorsqu'une quantité identique de ThPOK était exprimée dans des lymphocytes T spécifiques au CMH-II, suggérant qu'il existe un aspect qualitatif quant à la régulation de la différenciation des lymphocytes T $CD4^+$ par la signalisation induite par le RCT.

Nous avons également tenté d'étudier la voie de différenciation $CD4^+$ en l'absence de ThPOK, à la suite de la perturbation physiologique de la voie de signalisation induite par le RCT, par rapport à la perte de fonction de ThPOK. Bien que nous ayons observé une réorientation des thymocytes spécifiques au CMH-II vers la lignée $CD8^+$, aussi bien à la suite d'une délétion de *Thpok*, qu'à la perturbation de la signalisation RCT les deux modes de redirections semblent toutefois être différents. En effet, notre investigation a démontré qu'en l'absence de ThPOK, la signalisation induite par le RCT dans les cellules restreintes au CHM-II induit l'activation de certains gènes, suggérant ainsi leur implication dans la voie de différenciation $CD4^+$. Ces résultats suggèrent également que la contribution de la signalisation du RTC dans la différenciation des thymocytes restreints au CMH-II ne se limitait pas à l'induction de ThPOK. Étonnamment, seul un effet synergique limité a été observé sur la différenciation des thymocytes restreints au CMH-I, lorsque Gata3, un autre facteur de transcription également induit dans les thymocytes restreints au CMH-II, et ThPOK étaient surexprimés en même temps dans ces cellules, suggérant peu de chevauchement fonctionnel entre ces deux facteurs de transcription. L'ensemble de ces résultats indique que ThPOK et la signalisation induite par le RCT fonctionnent en synergie durant le développement des lymphocytes T $CD4^+$.

Mots-clés : ThPOK, lymphocyte T, RCT, choix de la lignée $CD4^+/CD8^+$, CMH, thymus, développement, Runx3, Gata3

Abstract

T lymphocytes are at the core of the adaptive immune system and their dysfunction is associated with several disorders and pathologies, which are at times fatal. The two main types of T-cells in mice and man are: the major histocompatibility complex (MHC) class-II-restricted $CD4^+$ helper T-cells, and the MHC-I-restricted $CD8^+$ cytotoxic T-cells. Developmental stages of the two types of T-cells occurs in the thymus in multiple sequential maturation stages that are identified by cell-surface $CD4^+/CD8^+$ co-receptor expression. Differentiation of the two types of T-cells in the thymus from a common precursor is influenced by the intensity and duration of signals derived from the T-cell receptor (TCR) and cytokines secreted by the thymic stromal cells. These signals lead to the activation of ThPOK or Runx/CBF transcription factors, which control the transcriptional network regulating $CD4^+$ and $CD8^+$ lineage fate, respectively. Studies have demonstrated that ThPOK is both necessary and sufficient for $CD4^+$ T-cell development as gain- and loss-of-ThPOK function redirects positively selected MHC-I- and MHC-II-restricted thymocytes into $CD4^+$ and $CD8^+$ T-cell lineage fate, respectively. However, the role of TCR signaling and the extent to which ThPOK expression influences $CD4^+$ lineage choice remains to be investigated. My thesis aims to elucidate the fundamental basis the $CD4^+/CD8^+$ lineage choice by exploring the molecular mechanism of action of ThPOK and TCR signaling in $CD4^+$ lineage fate of MHC-I- and MHC-II-specific thymocytes.

In this study, we have characterized gain-of-function of ThPOK in three independent transgenic mouse lines expressing varying amounts of ThPOK. Extensive analysis of the three ThPOK transgenic lines expressing MHC-I- and MHC-II-specific monoclonal TCR indicated that MHC-I-restricted, compared to MHC-II-restricted, thymocytes required significantly more ThPOK for efficient differentiation into the $CD4^+$ lineage. Interestingly, the founder line with the lowest transgene expression, despite expressing significantly higher amounts of ThPOK compared to the endogenous levels in WT $CD4^+$ T cells, induced a partial $CD8^+$ to $CD4^+$ redirection of MHC-I-restricted cells, leading to the generation of mature $CD4^+$, DN and $CD8^+$ T-cells in the same mouse. Lineage specific gene expression analysis, specifically in DN mature T cells from ThPOK transgenic mice expressing MHC-I-specific TCR, showed that, compared to induction of helper program, suppression of cytotoxic program required lower amount of ThPOK. Further investigation showed that TCR signal strength and MHC specificity of

developing thymocytes played a critical role in determining ThPOK-induced $CD4^+$ lineage fate. While increase in TCR signal strength augmented the efficiency of ThPOK-induced $CD4^+$ lineage choice of MHC-I-restricted thymocytes in part via endogenous ThPOK induction, it appeared to have ThPOK independent function as well as judged by significantly different $CD4^+$ T-cell frequencies in OTI mice expressing the same amount of ThPOK but transduced quantitatively different TCR signal. Importantly, the efficiency of $CD4^+$ lineage choice of MHC-I-specific thymocytes with augmented TCR signal strength was still significantly lower compared to the efficiency of $CD4^+$ lineage choice of MHC-II-restricted thymocytes expressing only the transgene-encoded ThPOK suggesting a qualitative role for TCR signaling as well in $CD4^+$ lineage choice.

We then evaluated $CD4^+$ lineage fate decision in the absence of ThPOK induction in physiologically relevant alteration in TCR signaling versus loss of ThPOK function. While we observed $CD4^+$ to $CD8^+$ lineage redirection of MHC-II-specific thymocytes due to *Thpok*-deficiency as well as lack of ThPOK induction due to disruption of TCR signaling, the two modes of lineage redirection appeared to be due to different mechanisms. Our investigation demonstrates that TCR signaling in MHC-II-restricted thymocytes induces the expression of select genes in loss-of-function of ThPOK model suggesting potential role for these genes in establishing the $CD4^+$ helper program. These results also suggest that the contribution of MHC-II-specific TCR signaling in driving $CD4^+$ lineage choice is not limited to *Thpok* induction. Interestingly, only a limited synergistic effect was observed when both Gata3, which is also induced in MHC-II-signaled thymocytes, and ThPOK were overexpressed in MHC-I-restricted thymocytes suggesting a limited functional overlap between the two transcription factors. Collectively, these data indicate that ThPOK and TCR signaling work synergistically to promote the development of $CD4^+$ T-cells with some ThPOK independent function for TCR signaling.

Keywords: ThPOK, TCR signaling, $CD4^+$/$CD8^+$ lineage choice, MHC, thymus, T lymphocytes, helper T-cells, development, kinetic signaling, Runx3, Gata3

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γc: Common γ chain

A: α-helix

Abs: Antibodies

ADAP: adhesion and degranulation promoting adaptor protein

Ade: Adenine

AGM: Aorta-gonad-mesonephros

AP-1: Activator protein-1

APC: Antigen presenting cell

Arg: Arginine

ATM: Ataxia telangiectasia mutated

B: β-strand

BAC: Bacterial artificial chromosome

BACH: Broad-complex, tramtrack and bric-à-brac domain and cap'n'collar homolog

BAF: Barrier-to-autointegration factor

Bcl: B cell chronic lymphoma

Bcl-XL: B cell lymphoma-2-like 1

BCoR: B cell lymphoma 6 corepressor

bHLH: Basic helix-loop-helix

BM: Bone marrow

BTB: Broad-complex, tramtrack and bric-à-brac

BTB-ZF: BTB/ Pox virus zinc finger

C terminus: Carboxyl terminus

C/EBPα: Cytosine cytosine adenine adenine thymine (CCAAT)-enhancer-binding proteins α

C2H2: Cysteine 2 histidine 2

CARMA1: CARD-containing MAGUK protein 1

Cbf: Core-binding factor

Cbfb: Core-binding factor subunit β

CD: Cluster of differentiation

$CD4^{+}8^{lo}$: $CD4^{+}CD8^{low}$

CDC42: Cell division control protein 42 homologue

cDNA: Complementary deoxyribonucleic acid

CDR1-3: Complementary determining regions (1-3)

CCR7: Chemokine cysteine-cysteine motif receptor 7

CCR9: Chemokine cysteine-cysteine motif receptor 9

ChIA-PET: Chromatin interaction analysis by paired-end tag sequencing

ChIP: Chromatin immunoprecipitation

CLP: Common lymphoid progenitor

CMP: Common myeloid progenitor

CRAC: calcium release-activated calcium channel

CSK: C-Terminal sarcoma (Src) Kinase

CTCF: Cytosine cytosine cytosine thymine cytosine (CCCTC)-binding factor

cTEC: Cortical thymic epithelial cells

CXCL12: Cytosine-x-cytosine motif chemokine ligand 12

CXCR4: C-X-C motif chemokine receptor type 4

DAG: Diacylglycerol

DHS: Deaxyribonuclease I hypersensitive sites

DLL4: Delta-like ligand 4

DC: Dendritic cell

DN: $CD4^{-}CD8^{-}$ double negative

DNA: Deoxyribonucleic acid

dNTP: Deoxynucleoside triphosphate

DP: $CD4^{+}CD8^{+}$ double positive

DRE: Distal regulatory element

DUSP: Dual Specificity Phosphatase

E2A: E-box E12/E47-α; also known as Transcription factor 3 (*Tcf3*)

E2F1: E2 transcription factor 1

$E8_{I-V}$: Enhancer elements (I – V)

ELP: Early lymphoid progenitors

ER: Endoplasmic reticulum

ERK: Extracellular signal regulated kinase

ETP: Early T-lineage progenitors

Ets: E-twenty-six

FACS: Fluorescence-activated cell sorting

FBS: Fetal bovine serum

Flt3: Fms-like tyrosine kinase 3

Flt3L: Flt3 ligand

FOXA: Forkhead box protein A

GADS: Growth factor receptor-bound protein 2-related adaptor downstream of Shc

Gata: [Adenine thymine] guanine adenine thymine adenine [adenine guanine] ([AT]GATA[AG]) binding protein

GFI1: Growth factor independent 1 transcriptional repressor

GFP: Green fluorescent protein

GLUT1: Glucose transporter 1

Gly: Glycine

GTE: General T enhancer

Grb2: Growth factor receptor-bound protein 2

Gua: Guanine

Gzmb: Granzyme b

HD: Helper deficient

HDAC: Histone deacetylase

Hes1: Hes Family bHLH Transcription Factor 1

HMG box: High mobility group box

HSC: Hematopoietic stem cells

IEL: Intraepithelial lymphocytes

IKZF3: IKAROS Family Zinc Finger 3

ID3: Inhibitor of DNA Binding 3

Ifn: Interferon

Ig: Immunoglobulin

IL: Interleukin

IL-7Rα: Interleukin-7 receptor α chain

ILC: Innate lymphocytes

InsP3: Inositol trisphosphate

IRF: Interferon regulatory factor

ITAM: Immunoreceptor tyrosine-based activation motif

ITK: IL-2-inducible T-cell kinase

JNK: Jun N-terminal kinase

Kb: Kilo base

KitL: Kit Ligand

KLF4: Krüppel like factor 4

Lin: Lineage marker

Lck: lymphocyte-specific protein tyrosine kinase

LSK: $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$

MALT1: Mucosa-associated lymphoid tissue lymphoma translocation protein 1

MAZR: Myelocytomatosis viral oncogene-associated zinc finger protein related factor

MAP: Mitogen-activated protein

MEKK: MAP/ERK kinase kinase

MFI: Mean fluorescence intensity

MHC: Major histocompatibility complex

miR: MicroRNA

MPP: Multipotent precursors

N terminus: Amino terminus

NCoR1: Nuclear receptor corepressor 1

NF-κB: Nuclear factor κ B

NFAT: Nuclear factor of activated Thymus-derived cell (T-cell)

NK: Natural killer

Nor-1: Neuron-derived orphan receptor 1

NR4A1: nuclear receptor subfamily 4 group A member 1

P-selectin: Platelet-selectin

p19Arf: Alternate reading frame protein 19

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PKCθ: Protein kinase Cθ

PLCγ1: Phosphoinositide-specific phospholipase C

PLZF: Promyelocytic leukaemia zinc finger

PI3K: Phosphatidylinositol 3-kinase

pMHC: Self-peptide/major histocompatibility complex

POZ: Pox virus zinc finger domain

PSGL1: P-selectin glycoprotein ligand 1

pTα: Pre-T cell receptor α (TCRα) chain

PtdIns(4,5)P2: Phosphatidylinositol 4,5-bisphosphate

PTPN22: Protein Tyrosine Phosphatase Non-Receptor Type 22

PTP-PEST: protein tyrosine phosphatase - rich in proline, glutamic acid, serine, and threonine

PRE: Proximal enhancer element

Prf: Perforin

PU.1: Purine-rich box1

QPCR: Quantitative polymerase chain reaction

R: Arginine

Rac1: Ras-related C3 botulinum toxin substrate 1

RAG: Recombination activating gene

RASGRP1: Ras guanyl-releasing protein 1

Rb: Retinoblastoma

RFI: Relative fluorescence intensity

rmIL: Recombinant murine IL

RORγt: Retinoic acid-related orphan receptor γ t

RT-PCR: Reverse transcription polymerase chain reaction

Runx: Runt-related transcription factor

Sca-1: Stem cell antigen

SCN4B: Sodium voltage-gated channel β subunit 4

SH: Src homology

SHP-2: Src homology region 2 domain-containing phosphatase-2

SLP76: SH2-domain-containing leukocyte protein of 76 kDa

Socs: Suppressor of cytokine signaling

Sos: Son of sevenless

Sos1: Son of sevenless homologue 1

SOX2: Sex determining region Y-box 2

SP: Single positive

SMRT: Nuclear receptor co-repressor 2

SWI/SNF: SWItch/Sucrose non-fermentable

STAT: Signal transducer and activator of transcription

T-cell: Thymus-derived cell

TCF: T-cell factor

TF: Transcription factor

Tg: Transgenic

THEMIS: Thymocyte-expressed molecule involved in selection

ThPOK: T helper Inducing POZ-krüppel like factor

Th: $CD4^+$ T helper cell

TM: Transmembrane

TNFα: Tumor necrosis factor α

TOX: Thymocyte selection-associated high mobility group box factor

Treg: Regulatory T lymphocyte

Tyr: Tyrosine

V(D)J: Variable, diversity, joining recombination

Vα: Variable α-chain

VGSC: Voltage-gated sodium channels

Vs: Versus

WT: Wild-type

Zap70: ζ chain of T-cell receptor associated protein kinase 70

ZBTB: Zinc finger and BTB

Zc: Zinc

ZF: Zinc finger

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Chapter 1: Introduction

1.1 Hematopoiesis

1.1.1 General Overview

Differentiation of progenitor stem cells into specialized cells endowed with unique biological functions is one of the most fundamental and critical biological processes for normal development of multicellular organisms including man. Amongst the various types of cells formed, blood lineage cells are essential for healthy living as they form the innate and adaptive arms of our immune system and play vital role in warding off myriads of pathogens that we encounter in our lifetime. Constant replenishment of cells of blood lineage is achieved by a process called hematopoiesis. In mammals, hematopoiesis is a structured process in which the hematopoietic stem cell (HSC) sits at the top of the hierarchy that self-renews and gives rise to all types of mature blood cells [1, 2].

The most reliable procedure that is routinely employed for identifying HSCs is to conduct an *in vivo* assay to evaluate the multi-lineage differentiation and self-renewal potential of these cells in primary and secondary irradiated hosts [3]. Despite considerable progress made in the purification and molecular characterisation of HSCs in recent years, no singular gene/molecular signature specific to HSCs has been identified so far. This is not surprising considering the extensive heterogeneity that exists within the HSC population.

HSC, which are of mesodermal origin, develop in localised niches that change over the course of a lifespan. As HSC progress through series of well-defined differentiation and proliferation stages, they gradually lose their self-renewal and multi-lineage potential [4, 5].

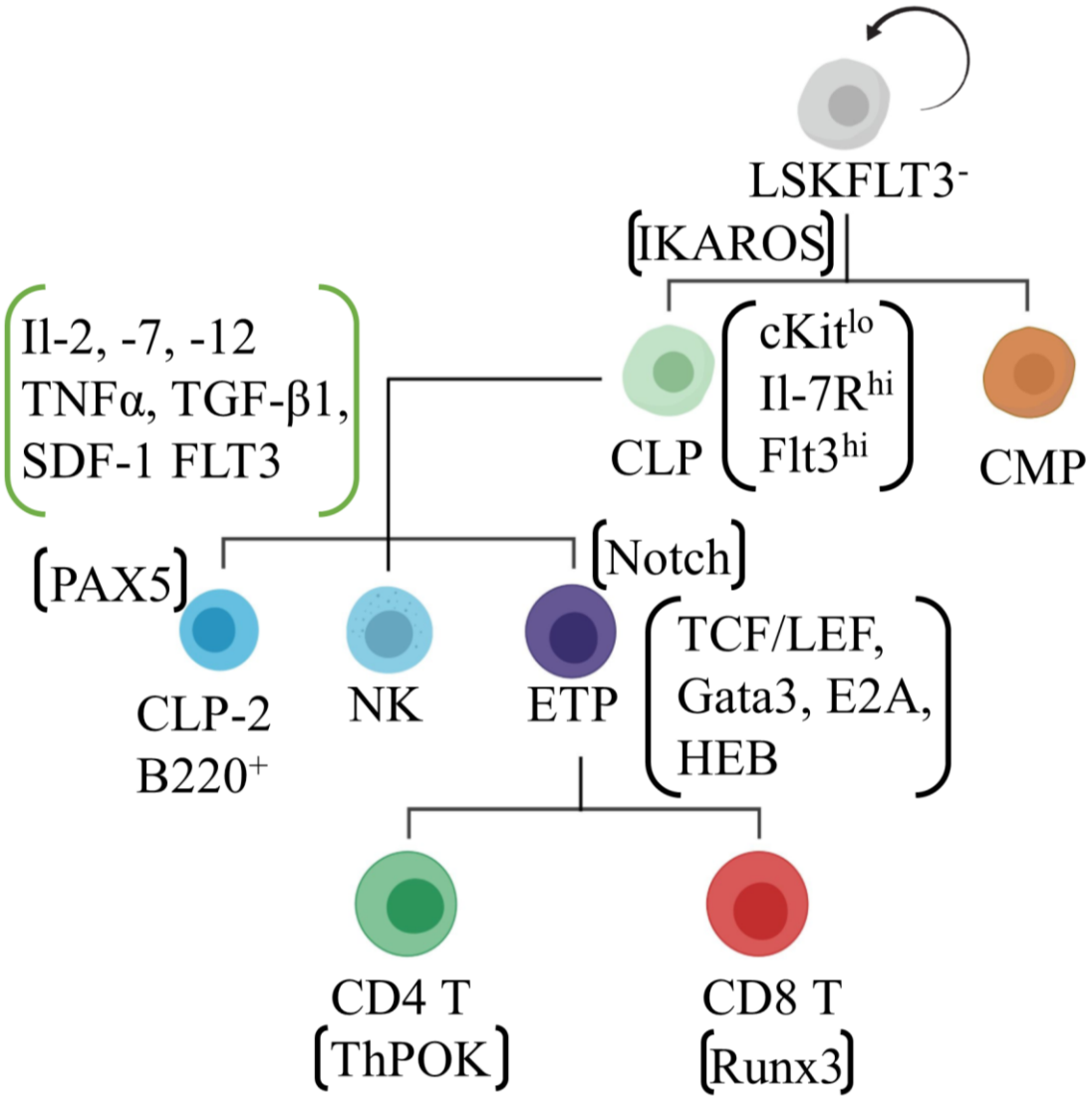

Figure 1. Schematic representation of hematopoietic lineage differentiation and specification

LSKFLT3^{-}, are self-renewable, multipotent HSC that reside in the bone marrow. CLP, which develop from LSK with the help of IKAROS, can give rise to all lymphoid subsets. Important cytokines for the development and lineage specification of CLP progenitors are depicted in green brackets, while black brackets represent transcription factors/cell intrinsic signaling molecules that regulate the differentiation of the various subsets. CLP, common lymphoid progenitor; CMP, common myeloid progenitor; ETP, early thymic progenitor; HSC, hematopoietic stem cell; IL, interleukin; LSK, Lin^{-}Sca1^{+}c-Kit^{+}; LSKFLT3^{-}, Lin^{-}Sca1^{+}c-Kit^{+}FLT3^{-}; NK, natural killer.

1.1.2 T-cell progenitors

The multi-lineage potential of HSCs, first described by James Till and Ernest McCulloch, proceeds in a hierarchical fashion through a stepwise loss of lineage potential [6-10]. The hematopoietic progenitors are defined as lineage markers negative (Lin^-, which refer to specific lineage defining cell surface antigens, such as TER119, Mac1, Gr1, B220, CD3, CD4, CD8) and expressing stem cell antigen (Sca-1) and stem cell growth factor called c-Kit (CD117 antigen). Thus, cells with $lin^-sca1^+c\text{-}Kit^{hi}$ (LSK) phenotype are the least differentiated hematopoietic progenitor cells in adult BM [11, 12]. LSK can be further differentiated based on surface expression of fms-related tyrosine kinase 3 (FLT3): $LSKFLT3^-$ are multipotent cells with self-renewing potential, whereas $LSKFLT3^+$ are multipotent progenitors (MPP) that lack self-renewing potential. [5, 13]. MPPs can differentiate into common myeloid progenitor (CMP) that produce cells of myeloid and erythroid lineages (monocytes, erythrocytes, thrombocytes, granulocytes) or common lymphoid progenitor (CLP) that gives rise to lymphoid cells (T and B cells and NK cells) [14-17] (Fig1).

Myeloid and lymphoid progenitors branch out early during hematopoiesis. CLPs arise from MPPs along a pathway that requires transcription factor IKAROS and FLT3 ligand [18, 19]. Surprisingly deletion of IKAROS, did not affect T-cell development, suggesting that CLP may not be the only progenitors with T lineage potential [18, 20]. Actually, a fraction of MPP have been shown to express *IL-7Rα* and the lymphoid specific recombination-activating gene 1 (*Rag1*), both needed for T-cell development [21-26].

Although mechanisms governing thymic migration of progenitors is not fully elucidated, it requires expression of different receptors such as glycoprotein ligand 1 (PSGL1), CD44, CCR7 and CCR9 and integrins such as platelet-selectin (P-selectin). [27, 28].

1.2 T-cell development

The thymus is a specialized primary lymphoid organ that provides a microenvironment suitable for the differentiation of T-cells from progenitor cells. Although the identity of the earliest thymus seeding progenitors is not clear, interactions of developing thymocytes with thymic epithelial cells drive the multistep differentiation process that result in gradual loss of

non-T cell lineage capacity of the progenitors and eventually establish the T-cell identity. This process begins during the early stages of T-cell development commitment of and persist through out many subsequent cycles [29, 30]. αβT-cells, which form the vast majority mature T-cell pool in man and mice, can be identified by the presence of a T-cell receptor (TCR) αβ heterodimer on their cell surface that can recognize foreign or self-protein-derived peptides presented by the major histocompatibility complex (MHC) class-I or -II. TCRα and TCRβ-chain encoded by *Tcra* and *Tcrb* genes, respectively, consists of several variable, joining and diversity (for TCRβ-chain) segments that rearrange somatically and join with constant region to produce diverse TCR repertoire [31]. As thymocytes lack self-renewing potential, continued thymopoiesis relies on the constant seeding of hematopoietic progenitor cells present in the BM.

Early thymocyte development is independent of antigen receptor engagement, whereas the specificity of the TCR for self-peptide/MHC (pMHC) ligand is a determining factor in the outcome of several steps during the later stages of T-cell development. While a vast majority of thymocytes fail to interact with pMHC on thymic epithelial cells and die by neglect, a fraction of them undergo thymic selection. Depending on the strength of the TCR with pMHC interactions, developing thymocytes survive and further differentiate in a process termed positive selection or eliminated by apoptosis in a process termed negative selection. Those thymocytes with strong affinity TCR for pMHC typically undergo negative selection although some differentiate into regulatory T-cells, while those expressing TCR with weak affinity for pMHC are positively selected and differentiate into the $CD4^{+}CD8^{low}$ ($CD4^{+}8^{lo}$) intermediate thymocytes before maturing into either CD4 helper or CD8 cytotoxic single positive T-cells [32]. This thymic selection checkpoint helps ensure that only thymocytes with “useful” TCR and devoid of potentially auto-reactive T-cells enter the circulation. The vast majority of MHC-I-restricted thymocytes differentiate into CD8 SP cytotoxic T-cells, whereas MHC-II-restricted thymocytes typically become CD4 SP helper T-cells. Below I describe various stages of thymocyte development and critical players regulating the process of generation of mature T-cells that surveillance our body for infection or transformed cells.

1.2.1 Early T-cell development

1.2.1.1 Characteristics of the early T-cell progenitors (ETPs)

The early T-cell progenitors (ETPs; $Lin^{lo}CD25^{-}Kit^{hi}$) constitute approximately 1:10,000 cells in the thymus and are capable of more than 10,000-fold expansion [18, 33]. Different subsets of ETPs have been identified based on CD24, C-C chemokine receptor 9 (CCR) and FLT3 expression, with $CCR9^{+}FLT3^{+}CD24^{-}$ ETP being the more efficient precursors [34-37]. They constitute ~10% of the ETP pool and have ten-fold greater expansion potential than $FTL3^{lo}$ ETPs [35, 38]. Following expansion and differentiation, ETP downregulate CCR9 and FLT3, and lose B-cell-lineage potential [39]. Surprisingly, cell-surface expression of CD4 can be detected on more than half of ETP that enter the thymus [40-43]. This suggests that CD4 expression is either rapidly downregulated following seeding the thymus or that a different developmental pathway is adopted by $CD4^{+}$ ETP.

1.2.1.2 Formation and maintenance of the T-cell identity

Thymus seeding progenitors lose their ability to adopt alternate lineage fate and initiate the T-cell differentiation program through a gradual process that is regulated by the thymic environment. The $CD4^{-}CD8^{-}$ (double negative-DN) thymocytes can be subdivided into four distinct sequential subsets based on the differential surface expression of CD25, CD44 and CD117 (DN1 to DN4). The most immature DN1 cells ($CD117^{hi}CD44^{hi}CD25^{-}CD24^{-/lo}$) consists of a heterogeneous population, which includes the ETP, and amounts to 0.01% of the total pool of thymocytes [18, 36]. Cells at this stage reside at the corticomedullary junction where they spend around 10 days and undergo massive proliferation [44]. DN1 to DN2 ($CD117^{hi}CD44^{hi}CD25^{hi}CD24^{+}$) differentiation is triggered by stimulatory signals from cortical thymic epithelial cells (cTECs) and fibroblasts in the subcapsular cortex region [45, 46]. The DN2 stage is characterized by the induction of lymphoid lineage specific genes, like *Rag1/2* (coding for Rag1/2) and T lineage specific tyrosine kinase *Lck* (coding for Lck), and the loss of potential to differentiate into dendritic cells (DC) [47]. As the cells progress from DN2 to DN3, the recombinase enzymes Rag1/2 mediate random recombination of V(D)J elements at the TCRβ locus, and thereby contribute to diverse TCR repertoire. Productively rearranged TCRβ associates with invariant pTα and CD3 chains to form preTCR complex whose surface

expression and oligomerization triggers cell autonomous signal transduction with critical role for Lck in this process [48-52]. DN3 ($CD117^{lo}CD44^{lo}CD25^{+}CD24^{+}$) thymocytes encounter the first important checkpoint during T-cell development, which ensures the functionality of the rearranged TCRβ chain [47, 53, 54]. This developmental checkpoint, referred to as β-selection, results in downregulation of *Rag1/2* and is influenced by at least two additional signals: C-X-C motif chemokine receptor type 4 (CXCR4) and Notch signaling [55-58]. Notch suppresses the activity of the E proteins factors (E-box E12/E47-α [E2A] and HEB, encoded by *T-cell factor 3* (*Tcf3)* and *Tcf12*, respectively), which impair thymocyte proliferation by regulating cell survival and metabolism. CXCR4, on the other hand, regulates β-selection and proliferation by associating with the preTCR and influencing localization of developing thymocytes in thymic sub-compartments [55-59]. DN4 thymocytes ($CD24^{+}CD25^{-}CD44^{-}CD117^{-}$) migrate away from the subcapsular region deeper into the cortex of thymus. At the DN4 stage, thymocytes experience a proliferation burst followed by the re-expression of *Rag* genes to initiate recombination at the *Tcra* locus [44].

The thymus is a separate and specialized organ with a unique microenvironment that largely supports T-cell development. Signals from the thymus environment trigger multiple rounds of proliferation of progenitors, while simultaneously initiating the T-cell specification program [45, 60-66]. Early events of T-cell development are marked by the consecutive and stepwise loss of potential for alternative cell lineage fates and concomitant acquisition of T-cell identity. The ubiquitous presentation of Notch ligands, mainly delta-like ligand 4 (DLL4), is a key feature that characterizes the thymic environment and that drives all direct and indirect events critical for establishing the T-cell identity [64, 66, 67].

1.2.1.3 Transcriptional control of early T-cell development

Transcriptional regulation of hematopoiesis serves as a model system for studying developmental biology. Understanding the basis of immune cell development from multi-lineage progenitors by transcriptional regulators has shed significant light on the molecular mechanisms governing developmental biology [61, 68]. T-cell development, from early progenitor settling events in the thymus to $CD4^{+}/CD8^{+}$ lineage commitment, occurs in a progressive stepwise fashion.

T-cell development, like many other developmental processes, needs to be tightly regulated by a network of transcription factors to avoid detrimental physiological consequences. The transcriptional regulation of temporal and spatial gene expression helps ensure that the right gene is expressed at the right time and in right amount. It is important to note that transcription factors recruited to *cis*-regulatory motifs in eukaryotes can control promoter activity over large genomic distances. Chromatin looping is a common mechanism for long-range regulation of gene expression that in some cases is mediated by the CCCTC-binding transcription factor (CTCF). By binding to multiple sequences throughout the genome, homodimerized CTCF can regulate long-range DNA looping and gene expression [69].

The access of the transcription factors to their target sites is regulated by the acetylation and methylation status of histones (at specific lysine residues). Thus, the activating or repressing multi-step process initiated by a transcription factor, requires chromatin structure remodeling in advance, which mostly implicates nucleosome unpacking mediated by the chromatin remodeling complex SWItch/Sucrose non-fermentable (SWI/SNF) and by epigenetic modifications [70]. Recent studies have found that several transcription factors, referred to as "pioneer factors", can open up chromatin without the help of chromatin remodeling or histone modifying complexes. Some of these pioneer factors include the forkhead box protein A (FOXA) factors, purine-rich box1 (PU.1), [AT]GATA[AG] (Gata) binding factors, krüppel like factor 4 (KLF4) and sex determining region Y-box 2 (SOX2) [71, 72]. These pioneer factors influence gene expression in two ways: 1) by regulating chromatin accessibility and 2) by regulating the actual transcription of a given gene [71, 72]. Although it is speculated that pioneer factors are recruited to DNA sites that are marked with epigenetic features such as H3K4me1, H3K4me2 and H3K9me3, their mechanism of actions remains poorly elucidated [71].

Based on the expression of different surface molecules and transcription factors, early T-cell development can be divided into three phases **(Fig. 2)**. While the first two, phase 1 and phase 2 depend on signals from the Notch receptor, the phase 3 differentiation requires pre-TCR signaling. While phase 1 is characterized by the expansion of multipotent progenitors, and in phase 2 differentiation to the T-cell lineage occurs by establishing the cells' ability to respond to the pre-TCR signaling in the differentiation of DN3 to DN4 during the phase 3. The three phases and their corresponding transcriptional networks are described below.

1.2.1.3.1 Phase 1

In the first phase, Notch signaling triggers the differentiation of IL-7Rα^{lo} ETP progenitors into IL-7Rα^{hi} DN2a cells.

Notch signaling (Notch1-4) is an evolutionarily conserved signaling pathway, with Notch1 being the functionally predominant receptor in T-cell development [73-76]. While T-cell differentiation in *Notch1*-deficient ETP is impaired at the DN1 stage, experimental studies showed that Notch signaling is also needed for DN2 to DN3 transition and in the β-selection [75-77]. Notch1-signaling is needed to antagonize the development of ETP into alternative non-T-lineages and to induce transcription factors such as *Hes1* (codes for Hes1), *B cell chronic lymphoma 11b* (*Bcl11b* codes for Bcl11b), *Gata3* (codes for Gata3) and *Tcf7* (codes for Tcf1) that regulate the T-cell identity [78-80]. Notch1-mediated inhibitory effect on non-T-cell lineage development of ETP occurs in a stepwise fashion. First, Notch signaling shuts off B-cell development by inducing the expression of Gata3 in ETP [81]. Following this, the potential of DN2a to develop into DC, macrophages and innate lymphocytes (ILC), is antagonized by sustained Notch signaling via Hes1 expression so that DN2b thymocytes are T-committed [82-90].

TCF-1-induced Gata3 plays a critical role in the establishment of the T-cell-regulatory network of transcription factors [91-95]. Gata3's role is not restricted to the early steps of T-cell development, but functions throughout T-cell development and mature T-cell function in the periphery [95-99]. However, in ETP, Gata3 supports T-cell specification mainly by blocking B-cell lineage potential in ETP thymocytes and inducing the expression *Bcl11b* [81, 85, 95, 99-102]. Later during T-cell development, Gata3 is needed for activating the TCRβ locus and for the production of CD4$^+$ SP T-cells [96]. Interestingly, Gata3 has a very limited range of dose-response, in DN cells at least. While increased levels of Gata3 is tolerated in periphery in CD4$^+$ Th2 cells, overexpression of Gata3 in ETPs is just as toxic to cells as Gata3 deficiency [85]. Several posttranslational modifications have been found to regulate Gata3 function [103-106]. IL-7 drives the proliferation of DN2a thymocytes and their differentiation into DN2b during phase 1 to phase 2 transition [22, 23, 107-111]. IL-7 regulates T-cell proliferation at several stages of development and function by activating both phosphoinositide 3-kinase (PI3K) and

signal transducer and activator of transcription 5 (STAT5) signaling pathways [22, 23, 107-111].

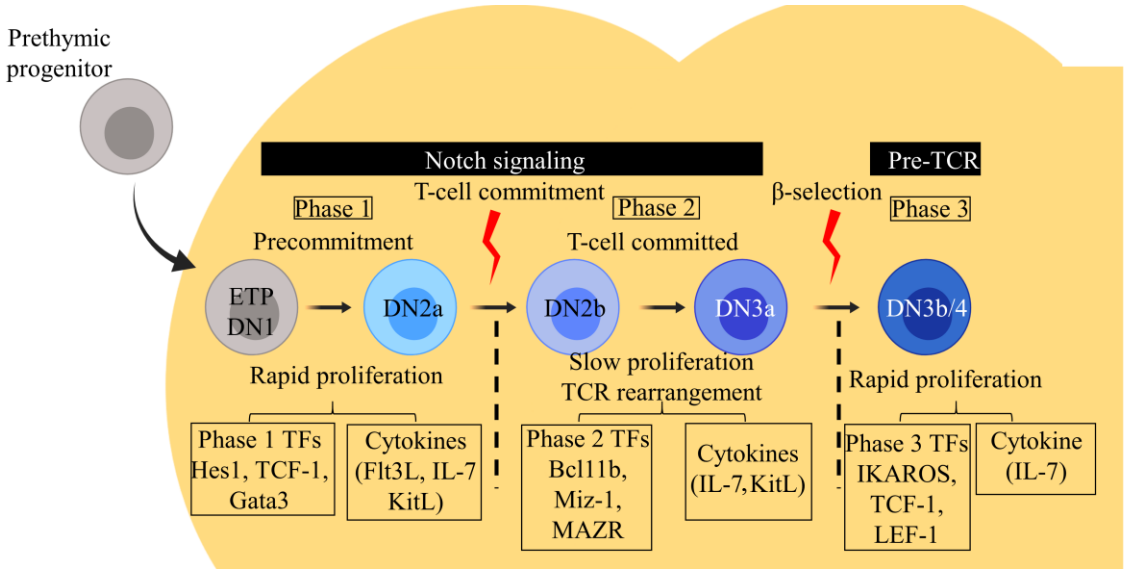

Figure 2. The three different phases of early T-cell development and role of cytokines and transcription factors

Notch signaling activates the T-cell differentiation program in T-cell progenitors. The early events of T-cell development consist of the sequential progression of early thymic progenitors (ETP or DN1) through the consecutive DN2a, DN2b, DN3a, DN3b/4 stages before they develop into double positive (DP) thymocytes. Based on Notch or pre-TCR signaling, early T-cell development can be divided into phases with distinct cytokine signaling and transcription factors (TF) that regulate proliferation rate and T-lineage commitment program. DN, Double negative; IL, interleukin; TCR, T-cell receptor. Figure adapted from Hosokawa and Rothenberg 2018 [79].

1.2.1.3.2 Phase 2

The transition from phase 1 to phase 2 is marked by a dynamic shift in the expression of several family of transcription factor [101]. The activation of the zinc-finger transcriptional repressor *Bcl11b* is one of the crucial transcriptional changes that marks the phase 2 [112-114]. By downregulating Kit expression and repressing all phase 1 specific transcription factors, Bcl11b prevents deviation to alternative cell lineage fates and dedifferentiation of developing thymocytes [112-114]. Bcl11b-mediated exclusion of myeloid/NK lineage fate and activation of Notch-signaling propels the DN2b cells into the T-committed DN3a stage in which T-cell restricted genes like *Rag1*, *Rag2*, *Ptcra*, and *Cd3e* are expressed by a process that is largely

mediated by Notch and E proteins (E2A and HEB, encoded by *Tcf3* and *Tcf12*, respectively) [115-119]. Alongside their role in the expression of T-cell specific genes, E proteins also regulate Rag-mediated recombination of the *Tcrb* locus by inducing the suppressor of cytokine signaling (*Socs*) genes, which uncouple growth factor receptors, such as IL-7R, from their signaling pathway and induce cell-cycle arrest needed for Rag-mediated recombination at the *Tcrb* locus [117, 120].

Myelocytomatosis viral oncogene-associated zinc finger protein related factor (MAZR) is a transcription factor that regulates gene expression in a context-dependent manner [121-125]. MAZR plays an important role in silencing *Cd8* gene expression by recruiting the nuclear coreceptor nuclear receptor corepressor 1 **(**NCoR1), during the DN3a to double positive (DP) transition [124]. MAZR was first described as a corepressor that functioned by recruiting the B-cell and T-cell regulatory factor, broad-complex, tramtrack and bric-à-brac domain and cap'n'collar homolog (Bach2) [121, 126-128]. Moreover, MAZR deficient mice are smaller in size and show increased risk of developing Bcl6-dependent lymphomas [129].

1.2.1.3.3 Phase 3

During the third phase, Notch-signaling, is quickly turned off by the newly formed pre-TCR signaling complex in an Ikaros-dependent manner [57, 130-132]. Thymocytes that have undergone a productive β-selection, experience a massive expansion and upregulate *IKAROS family zinc finger 3* (*Ikzf3*) and *retinoic acid-related orphan receptor γ t* (*Rorγt*), needed for the development of DP thymocytes [79, 133-135].

Repressive epigenetic marks accumulate at the promoters of Notch target genes and other phase 1-related regulatory loci as DN4 cells proliferate/differentiate into DP thymocytes [101, 136]. At the same time, multiple DP thymocyte-specific genes are epigenetically activated, making the T-cell differentiation process irreversible [101, 136, 137]. The newly formed regulatory network of transcription factors that includes mainly, TCF-1, HEB/E2A and RORγt supports survival of DP cells by inducing the anti-apoptotic molecule B cell lymphoma-2-like 1 (BCL-XL) [138-141].

1.2.2 Positive/negative thymic selection

Immature DP cells make up around 90% of developing thymocytes and can be separated into several stages. After a successful β-selection checkpoint, thymocytes differentiate into the highly proliferative pre-TCR$^+$ DP blasts. This is followed by a more quiescent phase during which DP thymocytes contract in size and downregulate pre-TCR complex [142]. Rearrangement at the *Tcra* locus is initiated at the small DP pre-TCR$^-$ stage before a fraction of cells audition for thymic selection. As mentioned earlier, the strength of the TCR interaction with pMHC complex will determine the fate of the developing thymocytes[142, 143][142, 143][142, 143][142, 143]. While strong interactions result in negative selection, positive selection is promoted by weak interactions. In some cases, strong affinity and/or avidity promotes the development of T_{Reg}. Ordinarily, deviation into the T_{Reg} cell lineage occurs when the strength of the interaction is not strong enough to cause negative selection [144]. When the TCR on developing thymocytes fails to engage in a productive interaction with the pMHC on the stromal cells, cells undergo a type of programmed cell death, called death by neglect.

Recently, genome wide analyses have uncovered a dynamic gene expression pattern that is unique to each DP subsets (pre-TCR$^+$, pre-TCR$^-$, αβTCR$^+$) [143]. The mapping of the transcriptional landscape of the DP subpopulations revealed, among other things, that cells destined for positive selection versus apoptotic deletion display unique gene signatures. Equally important, results showed a large-scale transcriptional shutdown of several genes that accompanies the differentiation of the proliferative pre-TCR$^+$ DP blasts to the resting pre-TCR$^-$ DP cells of smaller size. Then, the relatively transcriptionally quiescent stage is followed by another major transcriptional modification that is initiated by the TCR-mediated positive selection signaling. Some of these modifications include the reactivation of several important signaling pathways, such as the canonical TCR pathway, the metabolic pathway and distinct positive and negative selection-related genes, like *Id2* and *Id3* [145-147]. The transition of the small resting DP thymocytes into the transcriptionally dynamic CD69$^+$ DP thymocytes is associated with increased glycolytic and oxidative phosphorylation activity [143].

Signaling downstream of the newly formed TCR complex leads to the regulation of several factors involved in positive and negative selection. Some of these factors are involved in both positive and negative selections, like ID3 (encoded by *Id3)*, ifn-regulatory factor 1

(IRF1, encoded by *Irf1*), and nuclear factor κ-B (NF-κB), while others, described below, function specifically in one or the other selection process.

While this section covers the details of the complex regulatory network of transcription factors regulated by TCR-mediated signaling at the DP stage, the structural components of the TCR complex and the signaling pathways downstream of the TCR signaling will be discussed later.

1.2.2.1 Transcription factors regulating positive selection

1.2.2.1.1 Bcl11b

At the DP stage, Bcl11b expression has also been shown to control positive selection efficiency [148]. *Bcl11b*-deficient mice displayed defective proximal TCR signaling events, leading to dysregulated expression of genes involved in positive selection as well as $CD4^+/CD8^+$ lineage commitment [148]. Interestingly, introduction of the antiapoptotic Bcl2 transgene, but not a TCR transgene, rescued the phenotype in *Bcl11b*-deficient mice [148].

1.2.2.1.2 Tox

Thymocyte selection-associated high mobility group box protein (TOX, encoded by *Tox*) is induced following TCR-stimulation. Its deficiency severely impairs positive selection of developing thymocytes with a predominant impact on the development of MHC-II-restricted $CD4^+$ thymocytes [149-151].

1.2.2.1.3 Gata3

Although Gata-3 deficiency does not impair positive selection (as identified by CD69 upregulation on DP thymocytes), it is upregulated by the TCR-mediated positive selection signals and required for survival of selected MHC-II-restricted thymocyte. Gata-3 is induced in positively selected thymocytes immediately after TOX expression and increases gradually as cells undergo positive selection before peaking at the $CD4^+8^{lo}$ stage, which is when $CD4^+/CD8^+$ lineage choice occurs [152]. Subsequently, while Gata-3 expression is maintained in MHC-II restricted $CD4^+$ T-cells, it is downregulated in MHC-I-signaled CD8 committed thymocytes [153].

1.2.2.1.4 Notch signaling

The role of Notch-signaling in positive selection remains controversial [154-156]. A study showing an inhibitory effect of Notch on TCR signaling during thymic selection was later challenged by a study indicating that Notch-signaling potentiated TCR signaling by regulating late (or delayed) responding genes during positive selection [157, 158]. It was later elucidated by high-throughput screening that Notch- and positive-selection-induced genes (which interestingly could not be activated by *in vitro* TCR activation) overlapped significantly [158].

1.2.2.1.5 Nuclear factor of activated T-cells (NFAT)

Similar to Notch signaling, the role of nuclear factor of activated T-cells (NFAT) in positive selection has been controversial. While three of the four members of the NFAT family of transcription factors are expressed by the immune cells (NFATc1, NFATc2, and NFATc3), only NFATc3 was shown to be potentially involved in positive selection [159]. Surprisingly, the detrimental effect of *Nfatc3*-deficiency on thymic selection was not aggravated by the additional loss-of-function of *Nfatc2*. As for NFATc1, experimental results so far are inconclusive [159-162]. Nevertheless, NFAT reporter mice are commonly used to monitor TCR signaling [163].

1.2.2.2 Transcription factors regulating negative selection

DP and SP expressing a TCR with high affinity for self-peptide/MHC complexes are eliminated by negative selection to ensure that self-reactive T-cells are prevented from entering circulation [164, 165]. Important regulators of negative selection include transcription factors such as Nur77 (encoded by *nuclear receptor subfamily 4 group A member 1* [*Nr4a1*]) and Bim (encoded by *Bcl2l11*).

1.2.2.2.1 Nur77

The transcription factor Nur77 belongs to the steroid nuclear hormone receptors superfamily of transcription factors that includes two other members, Nurr1 (encoded by *Nr4a2*) and neuron-derived orphan receptor 1 (Nor-1 – encoded by *Nr4a3*) [166, 167]. Nur77 orphan receptor is a dynamic transcription factor that is induced in response to TCR stimulation and exhibits context dependent pro- or anti-apoptotic properties [168]. In thymus, expression of a

dominant negative version of Nur77, or its downregulation, correlated with reduced pro-apoptotic activity [169, 170]. The Nur77 transcription factor has been shown to mediate apoptosis by two main mechanisms: 1) by transcriptionally regulating its downstream gene *Ndg1*, which codes for a protein that can trigger apoptosis through caspase-8, and 2) by a transcriptional independent mechanism involving depolarisation of the mitochondria, through the transformation of Bcl2 into a toxic protein [166, 171-177]. While overexpression of Nur77 results in overt apoptosis of DP thymocytes, *Nur77*-deficient mice show no perturbation of clonal deletion, probably due to redundancy with Nurr1 or Nor-1 [170, 178].

1.2.2.2.3 Bim

The proapoptotic factor Bim, which functions by inhibiting *Bcl2*, induces apoptosis by regulating mitochondrial permeability [179, 180]. Surprisingly, loss-of-function of *Bim,* does not result in autoimmune diseases, suggesting that other redundant mechanisms maintain peripheral tolerance [179-181].

1.2.2.3 Transcription factors regulating positive and negative selection

1.2.2.3.1 ID3, IRF1 and NF-κB

ID3 is a Helix-Loop-Helix inhibitor protein that generally regulates E protein function by antagonising the DNA binding potential of E2A (encoded by *TCF3*) and HEB (encoded by *TCF12*) [182]. Although more rigorous research is needed, expression profile and genetic manipulations show that, unlike ID3, IRF1 and NF-κB control mainly positive and negative selection of MHC-I-restricted $CD8^+$ cytotoxic T-cells [183-187].

1.2.2.3.2 HDAC7

A high-throughput screen identified histone deacetylase 7 (HDAC7) as a potential regulator of thymic selection. The introduction of various dominant negative mutant forms of HDAC7 not only impaired thymic selection, but failed to rescue thymic selection in *Hdac7*-deficient mice as well [188].

The induction of the regulatory nuclear factors discussed above form a part of a broad transcriptional modifications induced by TCR-mediated positive selection signals. Thorough

investigations are needed to better elucidate the complete transcriptional landscape governing positive and negative selection.

Following positive selection, MHC-I- and MHC-II-restricted thymocytes terminate *Cd8* transcription to become $CD4^+8^{lo}$ intermediates, and differentiate into $CD4^+$ and $CD8^+$ single positive thymocytes by a process that is influenced by co-receptor, TCR and cytokine signaling [189]. Surprisingly, the differentiation of TCR-signaled thymocytes into $CD4^+$ helper or $CD8^+$ cytotoxic lineage is accompanied by differences in the expression of only a few lineage specific genes. These genes include *Runx3* and *Eomes* for $CD8^+$ cytotoxic lineage fate, and *zinc-finger-and-broad-complex, tramtrack and bric-à-brac-domain containing 7* (*Zbtb7b* also called "*Thpok*" here, encoding ThPOK protein) and *Gata3* for $CD4^+$ helper lineage [143].

1.2.3 $CD4^+$/$CD8^+$ Lineage fate of positively selected thymocytes

Following positive selection, developing thymocytes undergo a crucial lineage fate decision to differentiate into either $CD8^+$ cytotoxic or $CD4^+$ helper T-cells. DP thymocytes are unique among the developing T-cell subsets in that they express both CD4 and CD8 co-receptors and are unresponsive, due to high expression of SOCS proteins, to the pro-survival cytokine IL-7 [189-191]. The intracellular domains of both co-receptors are bound by the lymphocyte specific tyrosine kinase Lck. Engagement of TCR/co-receptor with MHC brings the co-receptor associated Lck in close proximity to the TCR complex leading to a cascade of phosphorylation events [192]. The extracellular domains of CD4 and CD8 co-receptors, on the other hand, bind to the MHC-II and MHC-I molecules, respectively [193, 194]. TCR specificity for MHC then determines thymocytes developmental fate; thymocytes expressing MHC-I- and MHC-II-specific TCR invariably differentiate into $CD8^+$ and $CD4^+$ SP mature T-cells, respectively. The development of DP thymocytes into either of the two lineages is a classical example of bipotential lineage-fate development of precursor cells. The last two decades have witnessed a significant progress in our understanding of the cellular and molecular mechanisms underlying the $CD4^+$/$CD8^+$ lineage choice, leading to development of various models as described below.

It was previously thought that uncommitted positively selected DP thymocytes downregulated one or the other of the co-receptors to ultimately give rise to either $CD4^+$ or $CD8^+$ SP T-cells. The classical models of $CD4^+$/$CD8^+$ lineage choice typically considered that

the same TCR-signals in DP thymocytes regulated, simultaneously, positive selection and lineage commitment, resulting in the irreversible termination of either one of the *Cd4* or *Cd8* co-receptor genes [189]. Opinions, however, were divided over whether co-receptor termination was "stochastic" or 'instructive' **(Fig. 3)**. The kinetic signaling model, on the other hand, is a non-classical model and is currently the most widely accepted model of $CD4^+/CD8^+$ lineage choice. It proposes that positively selected DP thymocytes develop into $CD4^+8^{lo}$ uncommitted intermediates before differentiating into either MHC-II-restricted $CD4^+$ or MHC-I-restricted $CD8^+$ SP mature thymocytes **(Fig. 4)** [195].

1.2.3.1 Stochastic model of $CD4^+/CD8^+$ lineage choice

The stochastic model of lineage choice proposes that TCR induces random termination of one of the co-receptors during positive selection, which generates a pool of SP thymocytes with matching and mismatching TCRs and co-receptors. Only selected thymocytes with matching co-receptors and TCRs, capable of transmitting a productive TCR signal, would proceed to differentiate into mature T-cells. Thymocytes with mismatching co-receptors and TCRs, which in theory should be observed in 50% of selected cells, are destined to die by apoptosis **(Fig. 3)**. Actually, the presence of a significant number of MHC class-II-specific $CD4^+CD8^+$ (DP) mature T cells in CD4 co-receptor transgenic mice supported this model [196-199]. This prompted the authors to argue that forced CD4 expression rescued MHC class-II-restricted SP thymocytes that had incorrectly terminated *Cd4* co-receptor expression and, hence, had died by apoptosis. However, the number of DP T-cells in periphery was fewer than 15%, much lower than the 50% frequency predicted by the stochastic model [196, 198, 200]. Studies of thymic selection using TCR transgenic mice have helped shed light on this matter. Positive selection efficiency in several TCR transgenic mice can reach up to 90%, which would not be feasible if co-receptor termination was a random event [201]. Another observation arguing against the stochastic lineage commitment model is the fact that long-lived and functionally mature TCR/co-receptor mismatched T-cells can be generated even in normal mouse [202, 203]. So, why does forced CD4 expression lead to the development of DP mature T-cells? It is possible that early transgenic co-receptor expression interfered in thymic selection leading to generation of mismatched thymocytes [196, 197, 204]. When investigators addressed this issue by placing a CD4 co-receptor transgene under the control of the CD8 enhancer I ($E8_I$), which is

active only in positively selected $CD8^+$-committed T-cells, mature T-cells expressing both the co-receptors were not produced contradicting the original study [205]. Taken together, these experimental observations have challenged the core principles of the stochastic model and demonstrate that lineage fate is neither error-prone nor stochastic [204, 206, 207].

1.2.3.2 Instructive $CD4^+$/$CD8^+$ lineage choice models

An alternate model explaining $CD4^+$/$CD8^+$ lineage choice proposes that TCR specificity for pMHC instructs positively selected thymocytes to develop into $CD4^+$ or $CD8^+$ lineage. Thus, MHC-II- and MHC-I-specific thymocytes almost always develop into $CD4^+$ helper and $CD8^+$ cytotoxic T-cells, respectively. Subsequent studies led to refinement of the instructive models as described below.

1.2.3.2.1 Strength-of-signal

The strength-of-signal model proposes that in the positively selected DP thymocytes a strong and weak TCR-signal terminates *Cd8* and *Cd4* transcription, respectively. As tyrosine kinase Lck binds the cytosolic tail of CD4 with more affinity than CD8 co-receptor [192, 208], MHC-II-restricted thymocytes would be predicted to receive quantitatively stronger signal compared to MHC-I-restricted thymocytes. Thus, according to this model, the relative strength of the TCR signaling instructs co-receptor transcription termination [201]. Redirection of MHC-I-specific thymocytes to the $CD4^+$ lineage upon introduction of a transgenic form of CD8α co-receptor engineered to express the cytosolic domain of CD4 (CD8.4) provided the first evidence that TCR-signal strength influenced the lineage fate of selected thymocytes [209]. A similar pattern was observed when components of the TCR complex, such as Lck, ζ-chain-associated protein kinase 70 (Zap70), C-terminal SRC kinase (CSK) and extracellular signal–regulated kinase (ERK), were manipulated to affect strength-of-signal in developing thymocytes in mice [210-218]. Basically, when a component downstream of TCR signaling was modulated to augment TCR-signal strength more $CD4^+$ T-cells were generated, while modifications leading to lower TCR-signal strength generated more $CD8^+$ T-cells. Interestingly, when TCR signal strength was modified by altering the number of immunoreceptor tyrosine-based activation

motifs (ITAM) of CD3ζ chain, a critical component of TCR/CD3 signaling complex, the efficiency of positive selection but not $CD4^+/CD8^+$-lineage choice was affected [219]. More specifically, increasing and decreasing TCR signaling, enhanced and hindered selection efficiency, respectively. These results prompted a careful re-examination of the original study showing $CD8^+$ to $CD4^+$ lineage redirection of MHC-I-restricted thymocytes following forced expression of the CD8.4 co-receptor. The concern was that a constitutively active *Cd8* transgene would complicate the analysis because, unlike endogenous *Cd8*, it is not downregulated after positive selection, which could lead to a constitutively active downstream signaling pathway. Indeed, developing thymocytes expressing CD8.4 transgene knocked into the *Cd8* locus failed to alter $CD4^+/CD8^+$ lineage choice arguing against a simple signal strength model of lineage choice [205, 220]. Collectively, these experimental observations demonstrated that strength-of-signal model cannot explain $CD4^+/CD8^+$ lineage fate decision of signaled thymocytes.

1.2.3.2.2 Duration-of-signal

Nonetheless, results described above strongly suggested that TCR specificity plays a critical role in the regulation of $CD4^+/CD8^+$ lineage choice. The duration-of-signal model is an extension of the strength-of-signal model, which suggests that, in addition to the signal strength, $CD4^+/CD8^+$ lineage fate is influence by TCR signal duration. The evidence in support of this model comes mostly from an important study showing that MHC-I- and MHC-II-restricted thymocytes exhibit different duration of TCR signaling [156]. This report showed that in ex vivo system longer incubation of pre-selection DP thymocytes and APCs (DCs or thymic stromal cells) resulted in CD4 development, while shorter incubation generated CD8 SP cells. This temporal signal duration model proposes that TCR signal of longer duration promotes $CD4^+$ lineage development by terminating *Cd8* expression, while shorter duration signal favors $CD8^+$ lineage development by terminating *Cd4* transcription [156]. Although, the evidence in support of this model is compelling, it was unclear why and how duration of MHC-I- and MHC-II-restricted TCR signals influences the $CD4^+/CD8^+$ lineage choice. Experiments addressing this question have led to the development of the kinetic signaling model (described in the following section): reports have shown that all positively selected DP thymocytes, irrespective of their MHC specificity, terminate *Cd8* expression to become $CD4^+8^{lo}$ intermediates with bipotential

lineage fate capacities, i.e. $CD4^+8^{lo}$ intermediates are uncommitted cells capable of giving rise to both $CD4^+$ and $CD8^+$ mature T-cells. Accordingly, CD8 co-receptor downregulation would be expected to disrupt TCR signal in MHC-I- but not MHC-II-specific thymocytes, leading to a transient or intermittent TCR-signal in MHC-I- compared to MHC-II-signaled thymocytes [221-223]. However, the development of an intermediate subset with an asymmetric phenotype that is precursor of both $CD4^+$ and $CD8^+$ T-cells contradicts one of the fundamental requirements of the all classical models, including the duration-of-signal model: lineage choice must occur in thymocytes that are transcriptionally $Cd4^+Cd8^+$ [189]. However, all experimental evidences obtained so far suggest that $CD4^+8^{lo}$ precursor cells are transcriptionally $Cd4^+Cd8^-$ [224]. Taken together, these findings, are in sharp contradiction with several critical principles of all classical models, to which the "stochastic" and the "instructive" models belong.

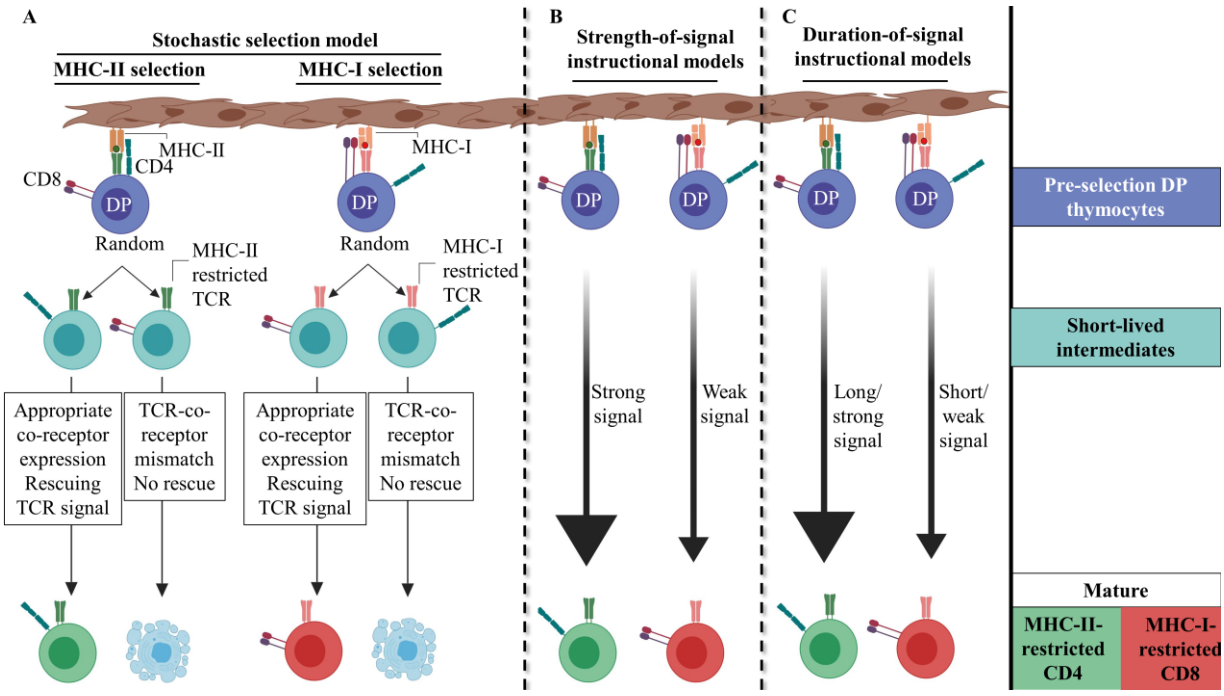

Figure 3. Previous models of CD4/CD8 lineage fate

(A) The stochastic model proposes that positively selected double positive (DP) thymocytes downregulate one co-receptor randomly to differentiate into short lived intermediate thymocytes expressing mismatched T-cell receptor (TCR) and co-receptor. Unless they are rescued by signal induced by matched TCR/co-receptor, short lived intermediates die by programmed cell death. **(B)** and **(C)** The strength-of-signal and duration-of-signal instructional models propose that a strong and long positively selecting TCR signal in major histocompatibility (MHC)-II-restricted thymocytes terminate *Cd8* transcription, whereas a weak and short signal terminates *Cd4* transcription, respectively. Figure adapted from Singer et. al. 2008 [189].

1.2.3.3 Kinetic signaling model

For the remaining part of this thesis, the "duration" of the TCR signaling, unless otherwise stated, refers to the signal duration in $CD4^{+}8^{lo}$ thymocytes, in which lineage choice is made based on the selective downregulation of CD8 expression. Accordingly, at $CD4^{+}8^{lo}$ stage continued CD4 expression results in persistent/continuous signaling in MHC class-II-specific thymocytes and $CD4^{+}$ lineage choice, while CD8 down regulation causes signal disruption in MHC class-I-specific thymocytes and $CD8^{+}$ lineage choice [189]. This process is expected to last several days and involve multiple individual encounters with pMHC bearing stromal cells [156, 163, 225].

The kinetic signaling model is based on a different set of principles than those proposed by the classical models. In the kinetic signalling model, positive selection and lineage choice are two independent and sequential events triggered by TCR-signals, and that reversible termination of *Cd8* co-receptor transcription is required to assess duration of TCR-signals. Thus, in MHC-II-signaled thymocytes continued transcription and surface expression of CD4 is permissive for longer duration of continuous TCR signaling thereby committing these cells to the $CD4^+$ lineage. In contrast, in MHC-I-signaled thymocytes ablation of *Cd8* transcription results in surface CD8 down regulation leading to disrupted or shorter duration of TCR signaling causing these cells to respond to cytokine signaling essential for $CD8^+$ lineage choice.

1.2.3.3.1 Cytokine signaling

On the basis of the evidence acquired thus far, signal emanating from TCR and cytokine receptors of the common γ-chain (γc) family play reciprocal antagonistic role in CD8 lineage choice **(Fig. 4, 6)** [221, 226, 227]. For MHC-I-signaled thymocytes to differentiate into $CD8^+$ T-cells, the $CD4^+8^{lo}$ intermediate cells must terminate *Cd4* gene expression and re-express *Cd8* gene. This event is referred to as the 'co-receptor reversal' and is considered a central feature of the kinetic signaling model [189]. Co-receptor reversal is mainly driven by IL-7, and potentially other γ_c cytokines, that transmits survival signals to $CD4^+8^{lo}$ thymocytes with disrupted TCR signals [221, 228]. Disruption of TCR signaling is critical for upregulation of IL7-Ra and thus rendering $CD4^+8^{lo}$ thymocytes permissive to cytokine signaling as *in vitro* and *in vivo* studies have shown that IL-7-dependent signal transduction and co-receptor reversal is inhibited by persistent TCR signaling [221, 228-230]. Although the underlying mechanism remains to be elucidated, IL-7 has been shown to regulate *Cd4* silencing and *Cd8* re-expression [228].

Several observations support the concept that IL-7 signaling promotes $CD8^+$ but not $CD4^+$ lineage choice as indicated by high cell-surface expression of the IL-7-regulated glucose transporter 1 (GLUT1) on $CD8^+$ but not $CD4^+$ T-cells [228, 231, 232]. The significance of IL-7-receptor signaling in $CD4^+/CD8^+$ lineage commitment is further exemplified by studies showing impaired $CD8^+$ T-cell development following inhibition of γ_c cytokine signal transduction, and conversely, deficiency of *Socs1* (cytokine-signaling inhibitor) or growth-

factor independent 1 (*Gfi1*, a negative regulator of IL-7-signaling) favored $CD8^+$ T-cell development and [190, 191, 221, 228, 233-236].

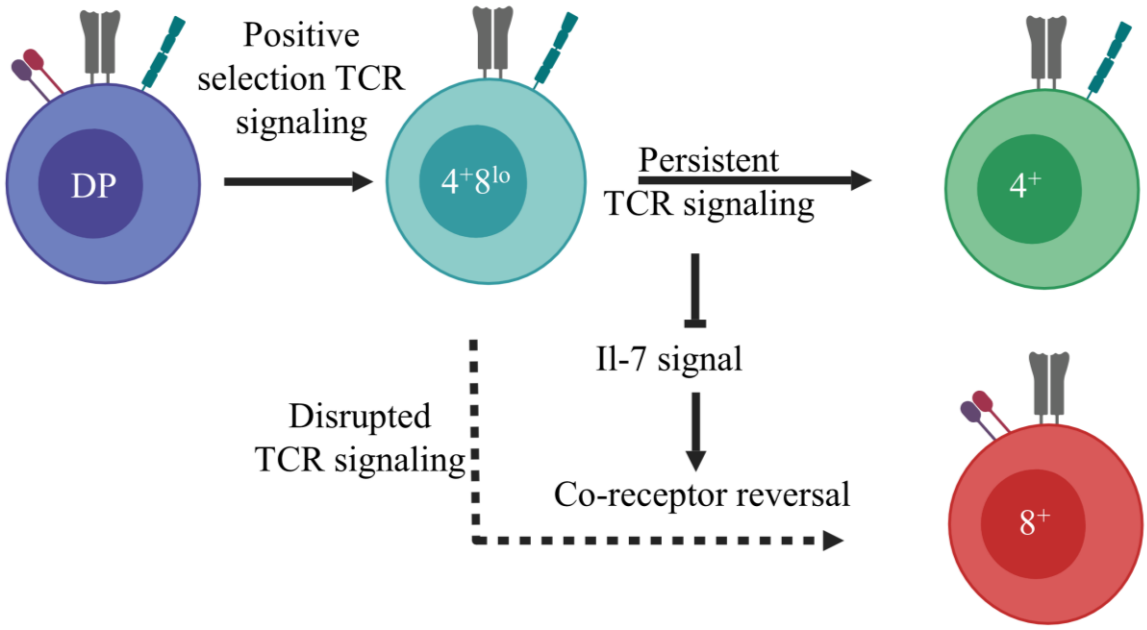

Figure 4. The kinetic signaling model

The kinetic signaling model of $CD4^+/CD8^+$ lineage fate proposes that T-cell receptor (TCR)-signaled double positive (DP) thymocytes terminate Cd8 transcription irrespective of major histocompatibility complex (MHC) specificity to differentiate into $CD4^+8^{lo}$ uncommitted intermediates. Thymocytes at the $CD4^+8^{lo}$ stage assess the effect of the loss of the CD8 co-receptor on TCR signaling. In MHC-I-restricted thymocytes, termination of the CD8 co-receptor disrupts TCR signaling, allowing for interleukin (IL)-7-mediated signaling, which promotes co-receptor reversal and differentiation into $CD8^+$ T-cell. In MHC-II-restricted thymocytes, a persistent TCR signaling promotes the development of $CD4^+$ by blocking IL-7-mediated signaling. Adapted from Singer et. al. 2008 [189].

1.2.3.3.2 Regulation of Cd4 and Cd8 expression

Exhaustive biochemical and genetic studies were conducted to identify and characterize transcriptional regulators of CD4 and CD8 co-receptor expression. These studies demonstrated that *Cd4* and *Cd8* gene expression is principally controlled by a combination of enhancers and/or silencers whose activities are regulated by lineage specific transcription factors **(Fig. 5)** [237, 238].

1.2.3.3.2.1 *Cd4* gene regulation

The studies revealed that a *Cd4* regulatory element containing a 339-bp proximal enhancer located 13-kb upstream of the transcription start site initiated *Cd4* transcription in MHC-II-specific thymocytes and included binding sites for several nuclear proteins such as E2A, HEB and TCF-1 that are active during the early steps of T-cell development. Results from the *in vivo* genetic manipulations revealed that these factors were critical for the proper expression of the *Cd4* gene, particularly at the DP stage [145, 237, 239-241]. More recent genetic studies have implicated chromatin remodeling factors, such as Mi-2β, in the positive regulation of *Cd4* expression in TCR-signalled DP thymocytes by acting on the *Cd4* proximal enhancer [242]. The observation that the proximal enhancer lacks lineage specificity led to the identification of an intronic *Cd4* silencer element that selectively inhibits *Cd4* gene expression in $CD8^+$ T-cells [238, 243]. The study of the CD4 silencer has resulted in the identification of a 300-bp and 190-bp core sequence in mice and human *Cd4* gene, respectively, and contains Runx-binding sites, which are indispensable for *Cd4*-silencing [244-246]. Surprisingly, conditional deletion of the CD4 silencer in mature $CD8^+$ T-cells did not reverse the inhibition of the *Cd4* gene expression in SP CD8 T-cells suggesting that the Runx-mediated epigenetic silencing of *Cd4* expression was irreversible in mature $CD8^+$ T-cells [247]. It is now believed that the chromatin remodelling complex barrier-to-autointegration factor (BAF) may be involved in the irreversible *Cd4* silencing in $CD8^+$ T-cells by relocating the *Cd4* locus near transcriptionally inert heterochromatin [248-251].

The CD4 protein consists of four extracellular immunoglobubin (Ig)-like domains linked to a transmembrane (TM) and a cytoplasmic domain by a short stalk [252]. While the cytoplasmic domain of the CD4 co-receptor binds Lck with more affinity than the cytoplasmic domain of the CD8 co-receptor, the binding affinity of CD4 to MHC class II, is much weaker than that of CD8 to MHC class I molecule [253].

1.2.3.3.2.2 *Cd8* gene regulation

A complex array of stage-specific enhancers ($E8_{I\text{-}V}$) control lineage-specific *Cd8* expression. The enhancer $E8_I$, which is active selectively in mature CD8 SP T-cells and $CD8\alpha\alpha^+$ intraepithelial cells, was the first to be characterized. Interestingly, CD8α and CD8β expressions

were not affected by deletion of $E8_I$ in thymocytes, suggesting redundancy between the different enhancer elements. Results from reporter assays revealed that while $E8_{II}$, $E8_{IV}$ and $E8_V$ regulated *Cd8* expression in DP and mature CD8 thymocytes, $E8_{III}$ regulated *Cd8* expression only in DP thymocytes.

The two main protein isoforms of CD8, CD8αα and CD8αβ, are expressed by different cell types with CD8αβ being the most common isoform [253]. The two α and β chains are connected by a disulfide bond and comprise each of one Ig-like domain linked to the TM and cytoplasmic domains by a long stalk [254, 255]. While glycosylation of both CD8α and CD8β stalks has been shown to regulate CD8 affinity to MHC class-I, only the CD8α cytoplasmic domain contains the binding motif for the tyrosine kinase Lck [253, 256, 257].

The primary function of the co-receptors is to recruit the tyrosine kinase Lck to the TCR complex in order to initiate signal transduction. However, affinity measurement experiments suggest that CD8 co-receptor may also function to reinforce the binding TCR-pMHC complex [258].

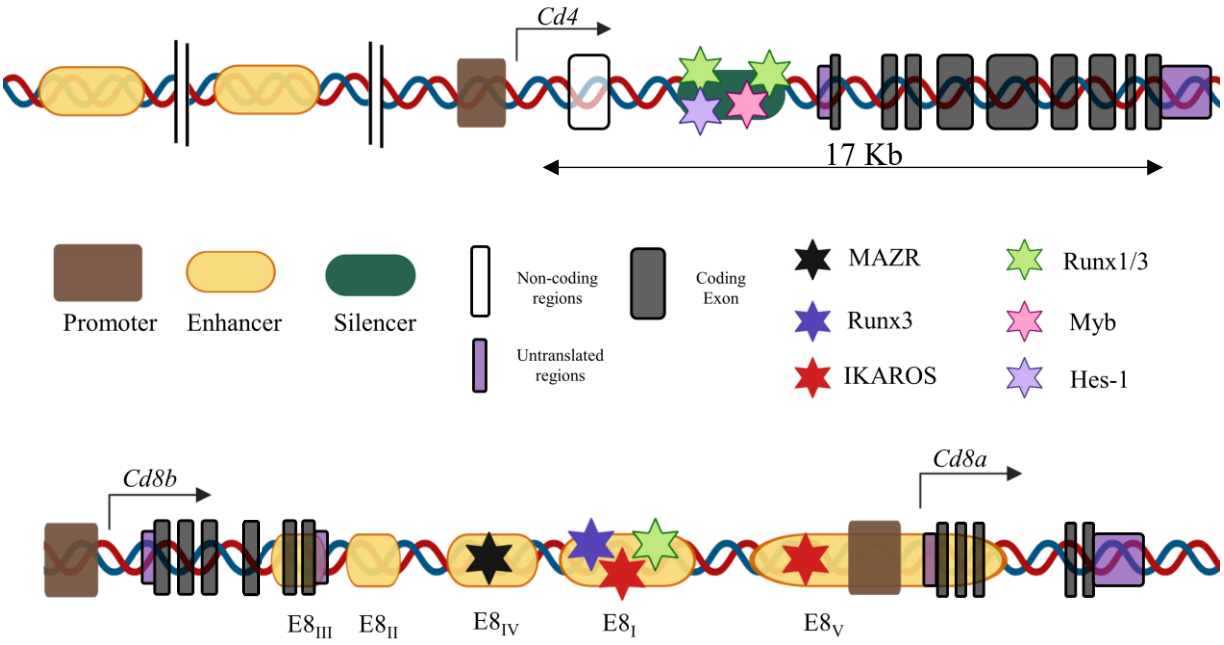

Figure 5. *Cd4* and *Cd8* gene structure and regulation

The stage-specific enhancers of *Cd8* ($E8_{I-V}$) can be bound and regulated by transcription factors, such as Runx proteins, MAZR and IKAROS. *Cd4* transcription is regulated by the activity a silencer motif, which can be bound by nuclear factors such as Hes-1, Runx proteins, and Myb. MAZR, myelocytomatosis viral oncogene-associated zinc finger protein related factor.

1.2.3.3.3 Co-receptor reversal

The elucidation of the molecular mechanism underlying the transcriptional regulation of *Cd4*/*Cd8* co-receptor expression helped reinforced important aspects of the kinetic signalling model, such as co-receptor reversal. Contrary to *Cd4* gene expression, which is primarily governed by the activity of one silencer, transcriptional regulation of the *Cd8* gene is controlled by five stage-specific enhancers ($E8_I$ – $E8_V$) [238, 243, 259, 260]. Two of these enhancers are particularly important for $CD4^+/CD8^+$ lineage commitment, $E8_{III}$ and $E8_I$. It has been shown that TCR-signals in DP thymocytes disrupt *Cd8* expression by inhibiting $E8_{III}$ enhancer activity, and that IL-7R-mediated co-receptor reversal, reinitiated *Cd8* gene expression by regulating $E8_I$ enhancer activity [203, 230].

1.2.3.3.4 TCR signaling

The importance of the activity of the TCR-mediated signaling in $CD4^+/CD8^+$ lineage choice has been assessed by several *in vivo* studies. Mainly, genetic manipulations of the TCR complex, intended to disrupt the duration of the TCR-signalling in MHC-II-restricted thymocytes, were found to affect thymocytes lineage choice. For instance, disrupting Zap70 activity, critical for TCR signaling, at the $CD4^+8^{lo}$ intermediate stage redirected MHC-II-restricted thymocytes to the CD8 lineage [261-264]. A similar $CD4^+$ to $CD8^+$ lineage redirection of TCR-signalled thymocytes was observed when MHC-II-specific TCR signaling was disrupted due to ablation of CD4 expression in positively selected thymocytes [203].

1.2.3.4 The network of transcription factors in $CD4^+/CD8^+$ lineage fate decision

The identification of critical transcription factors involved in regulating the lineage choice has greatly improved our understanding of the underlying molecular mechanism of lineage choice. Some of these factors include ThPOK, Gata3, TOX, Bcl11b, Runx3, and MAZR, and are discussed in greater details in the following section **(Fig. 6)** [149-151, 153, 265-273].

1.2.3.4.1 Gata3

The enhancer-binding zinc finger transcription factor Gata3 functions in many steps of T-cell development [153, 272]. *Gata3* is preferentially expressed in MHC-II-signaled thymocytes suggesting that it may be required for their development. Indeed, *Gata3* disruption selectively affected $CD4^+$ T-cell development without lineage redirection, while its constitutive expression hindered $CD8^+$ T-cell development confirming indispensable role for Gata3 in the $CD4^+$ helper program in MHC-II-restricted thymocytes [153, 273]. Interestingly, while Gata3 is recruited to the *Thpok* locus suggesting its possible role in transcriptional regulation of ThPOK expression, forced expression of Gata3 in MHC-I-restricted thymocytes, unlike ThPOK, did not influence the lineage fate of signaled thymocytes [274]. Collectively, these results indicated that Gata3 may require the induction of a binding partner for ThPOK induction in MHC-II-signaled thymocytes which is absent from MHC-I-restricted thymocytes. Alternately, Gata3 may activate ThPOK expression indirectly by inducing expression of an undefined factor in MHC-II-signaled thymocytes. It is important to note that Gata3 expression levels are directly regulated by the strength of TCR signaling and its role upstream of ThPOK

makes it a strong candidate protein in the TCR regulatory pathway governing $CD4^+/CD8^+$ lineage fate [189] Nonetheless, its specific role as well as its downstream effector molecules remain to be elucidated.

1.2.3.4.2 Tox

The HMG box protein TOX was implicated in positive selection and/or lineage commitment as it was found to be upregulated in TCR-signaled DP thymocytes [150]. However, results from genetic manipulations designed to elucidate the role of TOX in TCR-signalled DP thymocytes were inconclusive. In *Tox*-deficient mice, all lineages of MHC-II-restricted thymocytes failed to develop past a poorly characterized $CD4^{lo}CD8^{lo}$ intermediate stage, that appeared to precede *Thpok* and succeed *Gata3* induction [149]. However, in contrast to the loss-of-function experiments, phenotypes from gain-of-function mutants were hard to interpret, making it difficult to characterize TOX strictly as a $CD4^+$ lineage promoting transcription factor [150].

1.2.3.4.3 Bcl11b

Apart from its role in thymic selection, Bcl11b plays a role in $CD4^+/CD8^+$ lineage choice as well. During T-cell development, MHC-I- and MHC-II-restricted thymocyte differentiation into $CD8^+$ or $CD4^+$ steps is mainly regulated by ThPOK and Runx3, respectively. However, very little is known about the mechanisms by which TCR regulates expression of lineage-specifying genes. Bcl11b is a transcription factor expressed in TCR-signalled DP thymocytes that has recently been found to regulate expression of lineage-specifying *Thpok* and *Runx3* [275]. Conditional deletion of *Bcl11b* at the DP stage caused, irrespective of MHC specificity of the signaled thymocytes, random expression of ThPOK and Runx3 in the signaled thymocytes leading to the production of lineage confused DP mature T-cells. Surprisingly, while the Bcl11b-mediated repression of *Thpok* in preselection DP thymocytes was shown to be silencer-independent, Bcl11b was found to regulate Runx3 in MHC-II specific thymocytes by binding to and controlling the activity of two enhancers located −39 kb and −21 kb upstream of the distal promoter [275, 276].

1.2.3.4.4 Runx proteins

Runx family of proteins consists of three members, Runx1-3, which share several properties, like structural motifs and DNA-binding sites [277]. While Runx proteins show some functional redundancy, their spatiotemporal expression in developing thymocytes differ with Runx1 mainly active in DN and DP cells and Runx3 in the CD8-commited thymocytes [245]. Nonetheless, regardless of the distinct expression pattern, Runx1 and Runx3 function redundantly in $CD4^+/CD8^+$ lineage fate decision. Loss- and gain-of-function studies show critical role for the two Runx proteins in the silencer-mediated repression of *Cd4* expression in the thymus [245, 278]. While deletion of *Runx3* relieved the *Cd4* gene silencing in positively selected thymocytes leading to the development of MHC-I-specific DP mature T-cells, compound *Runx1* and *Runx3* deficiency repressed the *Thpok* silencer activity leading to redirection of some MHC-I-restricted thymocytes to the $CD4^+$ lineage [279]. Conversely, overexpression of either Runx protein disrupted *Cd4* upregulation in MHC-II-restricted thymocytes [245, 246, 271, 280]. *Runx1*, induced by Notch1, is ubiquitously expressed throughout T-cell development and functions differentially depending on its binding partner [281]. When complexed with cofactors like p300/core binding factor (Cbf) or DNA binding proteins like E-twenty-six-1 (Ets-1), Runx1 mainly acts as a transcriptional activator [282]. However, when bound to cofactors like HDAC, mSin3a and Groucho, via its VWRPY motif, Runx1 functions mainly as a repressor [283]. In contrast, Runx3 is specifically upregulated in MHC-I-signaled thymocytes as they transition from $CD4^+8^{lo}$ intermediates to CD8 SP cells [217, 270]. At molecular level, Runx3 promotes CD8-lineage choice in MHC-I-restricted thymocytes in several ways: 1) by directly interacting and activating the *Cd4* silencer to inhibit *Cd4* gene expression, 2) initiating *Cd8* expression via the $E8_I$ enhancer activation, and 3) heterodimerizing with MAZR to direct epigenetic silencing of *Thpok* in the CD8-committed thymocytes [245, 267, 270, 278]. Current hypothesis proposes that IL-7R signaling upregulates Runx3 and thus links cytokine signaling to CD8-commitment although the precise mechanism of cytokine action in Runx3 induction remains unclear.

1.2.3.4.5 MAZR

Similar to Bcl11b, the role of MAZR in T-cell development is not restricted to the early stages of T-cell development as it was shown to influence $CD4^+/CD8^+$ lineage choice as well. MAZR was shown to activate, in association with Runx proteins, *Thpok* silencer during thymocyte development [278]. While in preselection DP thymocytes MAZR represses *Thpok* induction by recruiting Runx1 to the *Thpok* silencer, in mature CD8 SP thymocytes MAZR/Runx3 complex binds to the *Thpok* silencer thereby preventing MHC-I-signaled thymocytes from adopting $CD4^+$ lineage fate [125, 278]. Interestingly, the stage-specific deletion of *Mazr* resulted in only a partial depression of *Thpok*, suggesting that other factors may regulate the *Thpok* silencer activity, particularly in DP thymocytes [125, 278].

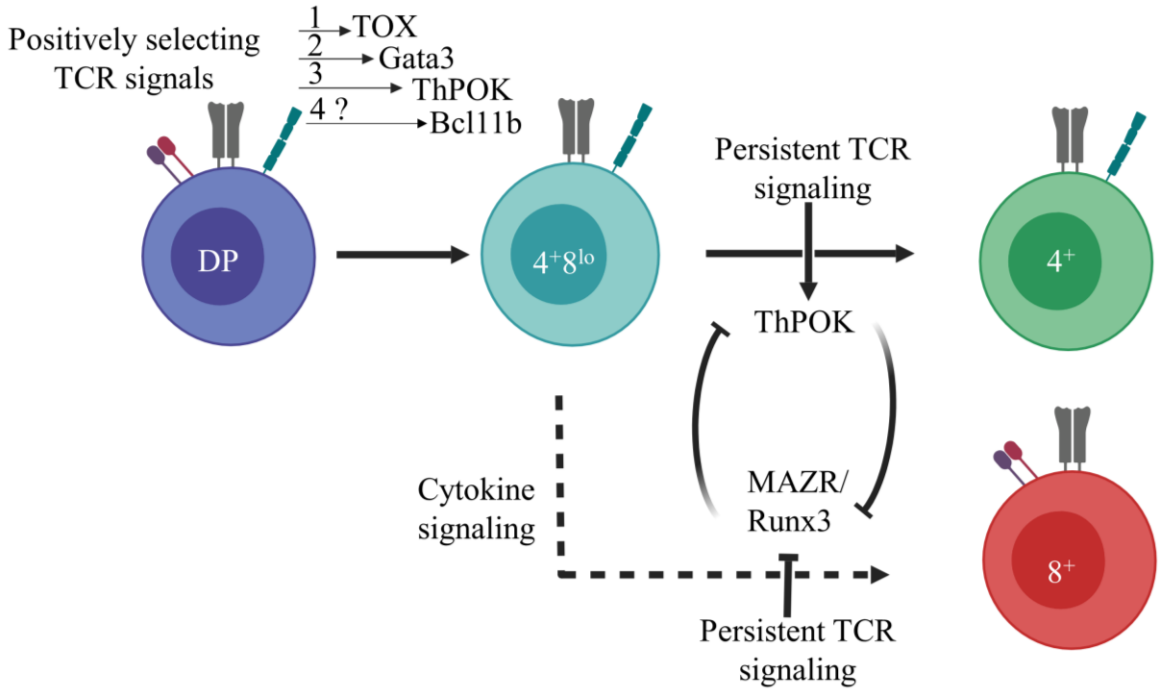

Figure 6. Nuclear proteins and environmental factors that regulate CD4/CD8 lineage choice

Lineage defining transcription factors translate the environmental cues into molecular events by regulating co-receptor gene expression and lineage specific genes. T-cell receptor (TCR)-mediated positive selection signals results in the sequential induction of TOX (thymus high-mobility group box protein), Gata3 (GATA-binding protein 3) and ThPOK (T-helper-inducing pox virus zinc finger /krüppel-like factor). TOX and Gata3 are upregulated at the DP stage and ThPOK at the $CD4^+8^{lo}$ stage. While TOX and Gata3 are required for the development and survival of $CD4^+8^{lo}$ uncommitted intermediates, ThPOK commits thymocytes into the $CD4^+$ lineage choice. Although the environmental signal that induces *Runx3* remains to be determined, it is thought to be dependent on IL-7-signaling. *Runx3* is upregulated in MHC-I-restricted thymocytes at the $CD4^+8^{lo}$ intermediate stage and is required for CD8-lineage commitment. Runx3 functions by silencing expression of *Cd4* and *Thpok* by binding to their respective silencers, and by reinitiating *Cd8* gene expression by being recruited to the $E8_I$ enhancer element. While Bcl11b affects lineage fate by priming *Thpok* and *Runx3*, promotes $CD8^+$ T-cell development by dimerizing with Runx3 and inhibiting *Thpok* expression in a silencer dependent manner. Bcl11b, B cell chronic lymphoma 11b; IL-7, interleukin-7; MAZR, myelocytomatosis viral oncogene-associated zinc finger protein related factor.

1.2.4 ThPOK, the master regulator of $CD4^+/CD8^+$ lineage choice

The transcription factor ThPOK is a member of the BTB/POZ zinc finger (BTB-ZF) family of transcription factors. The BTB-ZF family is comprised of close to 50 proteins in

humans and the majority of them function as transcription factors [284]. Although members of the BTB-ZF transcription factors differ in the number and type of ZFs, and the length of the DNA sequence spacing between ZFs, they have multiple general organization of motifs with BTB domain and ZF domain located at the amino (N) and (C) terminal regions, respectively **(Fig. 7)** [129, 285].

1.2.4.1 Study of the helper deficient (HD) mutation and the discovery of ThPOK

The identification of the HD mouse that does not produce $CD4^+$ T-cells, despite normal positive selection of MHC-II-restricted thymocytes, has provided a major breakthrough in our understanding of $CD4^+/CD8^+$ lineage choice. Although the HD mouse originated from complex intercrosses between knockout and transgenic mice, genetic backcrossing demonstrated that the mutation was not specific to any mouse strain suggesting spontaneous nature of the mutation. The almost complete absence of peripheral $CD4^+$ T-cells in HD mice was compensated by a proportional increase in $CD8^+$ T-cells, such that the total number of peripheral T-cells was unchanged compared to WT [202, 286]. More detailed analysis of the $CD8^+$ compartment showed that the HD mutation redirected MHC-II-restricted thymocytes into $CD8^+$ rather than $CD4^+$ lineage [287, 288]. The analysis of competitive mixed bone-marrow chimeras using WT and HD donors, revealed that the HD phenotype was due to T-cell intrinsic defect [265]. Genotype/phenotype experiments showed that the HD phenotype was not caused by a defect in *Cd4* gene expression or TCR signal transduction [202, 265]. It is important to note that HD mice manifested features such as impaired female fertility and augmented embryonic lethality displayed by other immune compromised mouse models (e.g., $Rag^{-/-}$) [287, 288].

Impaired $CD4^+$ T-cell development in homozygous HD ($HD^{-/-}$), but not $HD^{+/-}$ mice suggested the HD mutation was recessive. PCR-based linkage mapping using wild mice subspecies (*Mus musculus castaneus* and *M. m. molossinus*), enabled localisation of the HD mutation on chromosome 3, between markers D3Mit49 and D3Mit341. Transgenic mice expressing bacterial artificial chromosome (BAC) encompassing the *Hd* locus located the mutation within a 30-kb long region that comprised the gene coding for the zinc finger transcription factor T helper-inducing POZ/Krüppel-like factor (ThPOK, also known as Zbtb7b, Zfp67 or cKrox) [265, 266, 268, 287, 288]. Sequencing of the mutant allele revealed a single

transversion adenine to guanine (Ade>Gua) mutation in the coding sequence resulting in arginine to glycine (Arg>Gly) mutation at position 389 in the second zinc finger of ThPOK [265]. That the Arg>Gly point mutation in the evolutionary conserved motif of ThPOK was responsible for the HD defect was attested in transgenic rescue experiments as WT ThPOK but not ThPOK$^{389R>G}$ mutant rescued the HD phenotype [265, 287].

ThPOK, similar to other BTB-ZF factors, primarily acts as a transcriptional repressor [289]. In fact, ThPOK was first cloned as a repressor of the collagen promoter [290-292]. Although, the precise mode of action of ThPOK in CD4$^+$/CD8$^+$ lineage fate remains to be determined, recent reports suggest that ThPOK may function as a transcriptional activator as well in the thymus [276, 293]. This is consistent with findings that, depending on their binding partners, BTB-POZ transcription factors can act as activators or repressors, however, at present, ThPOK binding partners remain unknown [121, 294].

In thymocytes, ThPOK is expressed in stage- and lineage-specific fashion, despite the fact that ThPOK is also expressed abundantly in other tissues [287, 288]. Transcriptional profile of ThPOK show that it is first induced in CD4$^+$8^{lo} intermediates, irrespective of MHC-restriction. However, compared to MHC-II-, ThPOK induction levels in MHC-I-restricted thymocytes were much lower and quickly decreased to background levels. In contrast, in MHC-II-restricted thymocytes, ThPOK expression increased persistently and peaked in CD4$^+$ SP thymocytes [265, 268]. Importantly, gain- and loss-of-function experiments demonstrated that ThPOK is both necessary and sufficient for the differentiation of positively selected thymocytes into the CD4$^+$ helper lineage irrespective of their MHC specificity [202, 265, 266, 287].

The temporal pattern of *Thpok* expression and TCR signaling suggested that the two processes may be connected. Above described studies suggest that strong TCR signaling may be necessary for sustained ThPOK induction in signaled thymocytes. To better elucidate the link between TCR signals and *Thpok* induction, anti-TCRβ antibody was administered intraperitoneally into *MHC-II*$^{-/-}$ mice to mimic strong TCR signaling in DP thymocytes *in vivo* [266]. Surprisingly, at the DP stage before the lineage commitment steps, the *Thpok* locus was found to be insensitive to TCR signaling and not susceptible to induction, regardless of the strength of the TCR signaling [266]. Nonetheless, antibody-mediated stimulation induced higher levels of *Thpok* than normal in intermediate CD4$^+$8^{lo} and CD4$^+$ thymocytes, thus,

establishing a strong link between TCR activation and ThPOK induction [266]. TCR signaling, however, is dispensable for maintenance of ThPOK expression in mature T-cells [274, 287].

Interestingly, the characterization of the HD mutant mouse validated several core principles of the kinetics signaling model, primarily, that positive selection and $CD4^+/CD8^+$ lineage choice are mechanistically distinct events, involving two separate transcriptional networks [202]. This was mainly evidenced by the observation that efficiency of positive selection in *Thpok*$^{-/-}$ mice, which lack $CD4^+$ T-cells, was comparable to WT. Moreover, the results revealing that MHC-II-restricted T-cells in $HD^{-/-}$ mice go through a $CD4^+8^{lo}$ stage where CD8 co-receptor is downregulated before they are redirected to $CD8^+$ lineage fate, confirmed that $CD4^+8^{lo}$ cells are uncommitted intermediate precursors [189, 265, 287, 288].

1.2.4.2 General Structure of BTB-POZ domain of ThPOK

1.2.4.1.1 BTB domain

The eukaryotic BTB or POZ domain is found in approximatively 200 genes in human with wide ranging biological functions like transcription regulation, cancer development, protein ubiquitination, and these domains can vary in length from 90 – 120 amino acids [295-299]. Although its role remains largely unknown, the BTB/POZ protein-protein interacting motif is typically involved in homo- and hetero-oligomerization. Recent publications, however, have highlighted novel functions for the BTB domain in gene regulation. When bound to nuclear co-repressors like NCoR1, B cell lymphoma 6 corepressor (BCoR) and SMRT, BTB-domains can influence gene expression by recruiting chromatin-remodelling factors such as histone deacetylases (HDACs), members of the BAF family, and methylated-DNA binding proteins [124, 289, 300-306]. Moreover, BTB-domains have also been shown to act as adaptors for substrate binding. The BTB-ZF transcription factors Bcl-6 and promyelocytic leukaemia zinc finger **(**PLZF), for example, were found to mediate effector function differentiation in T-cells partly by interacting with the ubiquitin ligase Cullin 3 and regulating chromatin-modifying complexes associated with Bcl-6 and PLZF [307]. Although BTB-domains have evolutionary been associated with gene suppression, it is likely that the nature of the complex recruited by a specific BTB-ZF factor determines their function.

The structure of the core BTB domain consists of five α-helices (A) and three β-strands (B) [308]. Computer generated models from crystallographic analyses revealed a ribbon-like structure with A4/A5 region connected to B1/B2/A1/A2/B3 region by A3 and a linker [309-311].

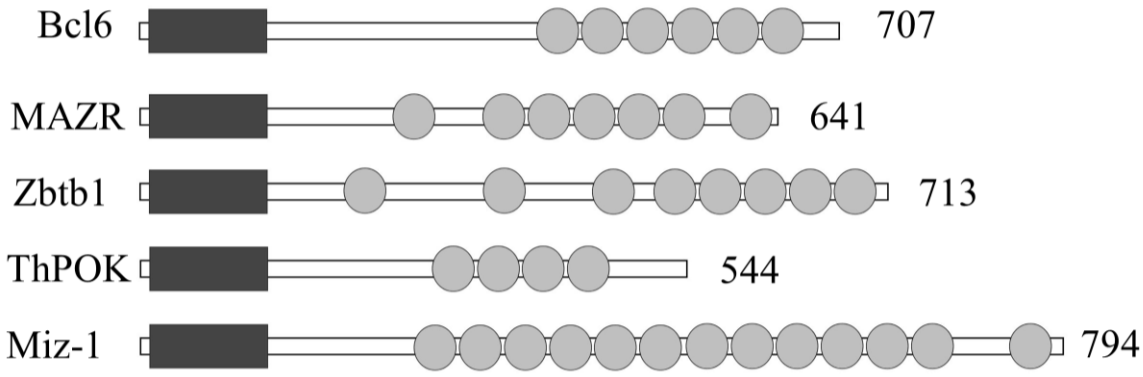

Figure 7. Protein structure of BTB-ZF transcription factors

The diagram shows the domain structures of the different BTB-ZF proteins that are involved in T-cell development. BTB (dark box) is always located at the N-terminal domain, whereas ZF (gray circles) are located at the C-terminal domain. The amino acid lengths are indicated at the right, and the protein names at the left. BTB-ZF, broad-complex, tramtrack and bric-à-brac/pox virus zinc finger; ZF, zinc finger. Figure adapted from [129].

1.2.4.1.2 Zinc finger domain

One of the most common DNA-binding motifs present in eukaryotic transcription factors is the zinc finger (ZF) motif. The classical ZF domain, as present in ThPOK, is made up of two cysteines separated from two histidine by 20-30 amino acids. The finger like structure, which is formed by two cysteines on one chain and two histidines on another chain (C2H2) is stabilized by a zinc (Zc) ion in the center plane [312]. Electron crystallographic structure revealed that a classical ZF consists of two β-sheets and one α-helix [313]. The DNA sequence specificity of the ZF is determined by the stretch of amino-acids in its α-helix chain that comes in contact with DNA [314]. All ZF-containing proteins are made up of multiple ZF motifs. Adjacent fingers are connected through a highly conserved region referred to as the linker. The canonical linker Threonine-Glycine-Aspartate-Lysine-Phenylalanine (TGEKP) is present in more than half of

C2H2-containing transcription factors [315-318]. Each additional ZF connected by a linker increases binding affinity of the transcription factor for DNA by 1000-fold [319]. Although the role of the α-helix amino-acids in DNA binding is well-known, whether the linker plays an active role in DNA binding remains an open question.

1.2.4.3 Transcriptional regulation of ThPOK induction in positively selected thymocytes

Thpok expression is primarily regulated at the transcriptional level, as evidenced from the tight coordination between *Thpok* transcription and translation [268, 320]. Lineage and stage specific ThPOK expression suggested a silencer/enhancer-driven regulation of expression. Indeed, a bottom up approach led to the identification of several cis-regulatory elements governing ThPOK expression. Studies using BAC reporter transgenes revealed that a fragment extending 17-kb upstream of the transcription start site was sufficient for recapitulating stage and lineage specific ThPOK expression in developing thymocytes and mature T-cells [266]. Initial mapping of the cis-regulatory regions revealed that the *Thpok* locus was made up of a proximal and a distal promoter that are 6-kb apart. Because the first start codon is situated within a common downstream exon, both distal and proximal promoters encode the same proteins, although the 5' untranslated region differ between the two transcripts [266]. The promoters display distinct activation patterns during thymocyte development with the distal promoter preferentially active in $CD4^+8^{lo}$ intermediates, while the proximal promoter is active in mature $CD4^+$ SP thymocytes [266, 287]. Mapping studies by He et. al. involving sequence homology and DNaseI hypersensitivity site (DHS) analysis identified six important highly conserved regulatory sites [266, 287]. Two of these DHS sites mapped to the two promoter elements, whereas the other DHS sites suggested novel *cis* regulatory elements. Mice expressing transgenic constructs containing different *Thpok* cis elements and regulating a reporter gene expression determined the nature of these regulatory sites [266]. This approach identified a 500-bp lineage-specific silencer (named the distal regulatory element – DRE) as deletion of this motif led to GFP expression in $CD4^+$ as well as $CD8^+$ SP thymocytes but not in DP thymocytes. Further investigation showed that the DRE could act as an enhancer as well. The DRE-element could augment the expression of a reporter gene under the control of heterologous promoter such as human CD2 promoter, which by itself lacked any significant transcriptional activity. Together, these findings demonstrated that the DRE governed lineage- and stage-specific

expression of *Thpok* [266]. Several transcription factor consensus binding sites were identified in the 500-bp long DRE element suggesting complex regulation of ThPOK during thymocyte development. However, their physiological importance needs further elucidation due to experimental inconsistencies **(Fig. 8)**. For instance, when the two Runx-binding sites were mutated to study their role in the regulation of the DRE silencer activity discrepancies were observed. While their mutations in one study resulted in the derepression of *Thpok* expression, in another study *Thpok* expression was not affected [266, 267, 274].

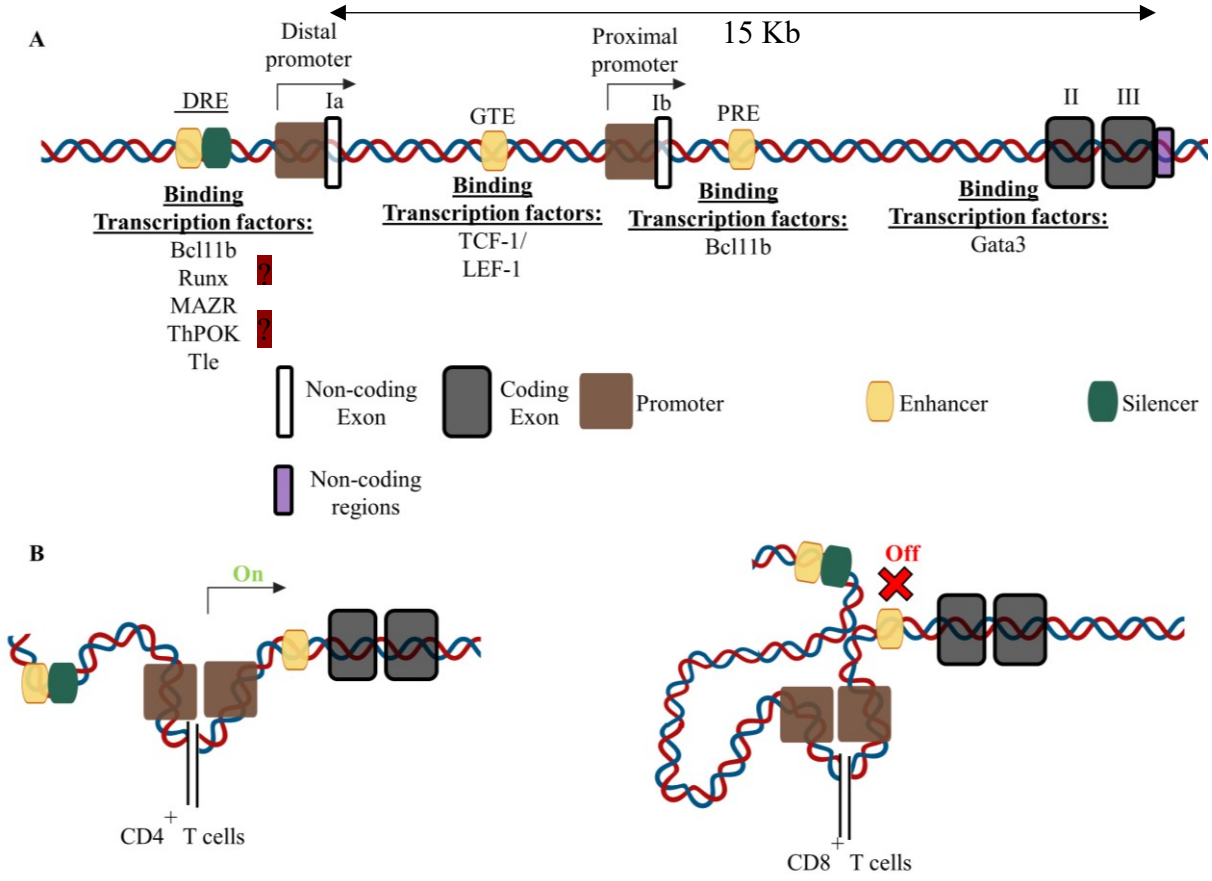

Figure 8. Mouse *Thpok* gene structure and regulating transcription factors

(A) The ThPOK regulatory region consists of the two promoters, the distal and proximal promoter, which is active selectively in T-cells. *Thpok* gene contains three *cis*-regulatory domains: a proximal enhancer (*PRE*), a general T lymphoid element (*GTE*) and the distal regulatory element (*DRE*), which consists of silencer and an enhancer. While the *PRE* is activated mostly in mature $CD4^+$ T-cells, the *DRE* enhancer element initiates *Thpok* induction at the $CD4^+8^{lo}$ stage. Gata3 and TCF-1/LEF-1 regulate *Thpok* expression by binding to a region upstream of exon II and the *GTE*, respectively. While Bcl11b is involved regulation of enhancer element of the *DRE,* ThPOK antagonizes the silencer activity. Runx proteins, on the other hand, silence *Thpok* expression by acting on the silencer. **(B)** Possible DNA configuration in $CD4^+$ and $CD8^+$ T-cells [276]. Gata3, Gata binding protein 3; Bcl11b, B cell lymphoma 11b; ThPOK, T helper Inducing pox virus zinc finger -krüppel like factor.

Apart from the DRE motif with dual silence and enhancer activities, two other enhancers have been mapped to the *Thpok* locus: a general T enhancer (GTE) located downstream of the distal promoter that is active early during $CD4^+$ T-cell development and a proximal regulatory

enhancer (PRE) located near the proximal promoter that regulates *Thpok* expression mainly in mature $CD4^+$ T-cells; PRE deletion resulted in 20- and 5-fold lower *Thpok* expression in peripheral and thymic $CD4^+$ SP T-cells, respectively. The DRE element is proposed to regulate the lineage- and stage-specific function of GTE, but not PRE [266, 321]. Taken together, these results suggest that persistent/strong TCR signaling may favor $CD4^+$ T-cell development mainly by regulating the DRE and enhancer activities in MHC-II-restricted thymocytes. Interestingly, *Thpok* silencing in DP thymocytes, prior to its TCR-mediated induction at the $CD4^+8^{lo}$ stage, is only partially dependent on the DRE element as its deletion leads to mild derepression of *Thpok* in only a fraction of DP thymocytes [322].

1.2.4.4 Upstream regulatory pathway involved in regulating ThPOK expression

The upstream regulatory network of transcription factors that controls ThPOK expression in MHC-II-restricted thymocytes is unclear. Most of the transcription factors that have been reported to induce ThPOK expression, also seem to be involved in the regulation of the $CD8^+$ lineage fate [275, 323, 324]. The group of Hai-Hui Xue reported that the transcription factors TCF-1 and LEF-1 governed $CD4^+$ T-cell development by directly regulating *Thpok* induction **(Fig. 8)** [323]. However, this was quickly followed by another publication by the same group, revealing that the TCF/LEF transcription factors were also needed for the development of the $CD8^+$ lineage fate [324]. Similarly, the zinc finger transcription factor Bcl11b was found to be equally important for the $CD4^+$ and $CD8^+$ T-cells development by "priming" *Thpok* and *Runx3* in post-selection thymocytes **(Fig. 8, 9)** [275]. It is possible that the function of TCF/LEF and Bcl11b in MHC-I and MHC-II-restricted thymocytes is regulated by lineage-specific binding partners. Finally, the transcription factors Tox and Gata3, which are part of the network of proteins that control $CD4^+$ T-cell development, have been proposed to regulate ThPOK expression. Impaired $CD4^+$ T-cell development as a result of *Gata3* or *Tox* deficiency could not be rescued by overexpression of ThPOK. One possibility for these observations is that Gata3 and Tox act upstream of ThPOK. Indeed, Gata3 was shown to associate with the proximal promoter region and positively regulate *Thpok* transcription **(Fig. 8)** [149, 274]. Nonetheless, results from Gata3 and Tox gain-of-function experiments indicated that their regulatory roles on $CD4^+$ lineage fate are more complex than previously expected [149, 274, 325]. Transgenic overexpression of Gata3 in MHC-I-restricted thymocytes, had no effect on lineage fate and

failed to induce *Thpok* expression. Together, these studies suggest that potential role of Gata3 and, to a much lesser extent Tox in positively regulating *Thpok* transcription is likely dependent on the presence of yet undefined factors. In contrast, the regulatory mechanisms underlying *Thpok* inhibition in MHC-I-restricted thymocytes is far less complex. Ample evidence exists to show that recruitment of Runx proteins, with the help of MAZR and/or Tle corepressors, to the *Thpok* silencer is necessary for its inhibitory function and epigenetic silencing of *Thpok* in MHC-I-restricted thymocytes [125, 278, 326].

1.2.4.5 ThPOK-regulated pathways

The regulation of $CD4^+$ lineage choice by ThPOK can be accomplished by two mechanisms, 1) by activating or suppressing $CD4^+$ or $CD8^+$ lineage specific genes, respectively, or 2) by controlling the expression of genes implicated in TCR signaling. Analysis of $HD^{-/-}$ mice strongly argues against the latter possibility as functional and molecular readouts of TCR signaling show no change in thymic selection or phospho status of molecules critical for TCR signaling (CD3ζ, Zap70 and Lck) in the mutant thymocytes [202, 265]. Additionally, the results showing that constitutive expression of CD4, which mechanistically restores TCR signaling in MHC-II-restricted *Thpok*$^{-/-}$ thymocytes, did not rescue the HD phenotype providing a strong evidence that ThPOK does not regulate lineage commitment by modulating TCR signaling [202, 287, 288].

Although evidence for guanine-rich (GGGAGGG) ThPOK recognition sequence is poor, which complicates the identification of potential target genes, several studies support the idea that ThPOK regulates expression of lineage-specific genes [327]. For instance, transduction of mature $CD8^+$ T-cells with ThPOK downregulated, albeit partially, $CD8^+$ lineage-specific genes including *Cd8* expression itself, however, it failed to upregulate *Cd4* expression. [287, 328]. The effect on lineage fate was more striking when ThPOK was introduced in developing thymocytes, which revealed a negative reciprocal transcriptional regulation between ThPOK and Runx3 **(Fig. 8, 9)** [287, 328]. ThPOK and Runx3 are the two major transcription factors regulating $CD4^+$/$CD8^+$ lineage choice, with ThPOK exerting a dominant role over Runx3 [329]. Their antagonistic interplay has been proposed to play a central role in $CD4^+$/$CD8^+$ lineage commitment of signaled thymocytes. By counteracting Runx3-mediated epigenetic

silencing of its own locus, ThPOK establishes a positive feed forward loop that is required to establish and maintain the helper program in $CD4^+$ committed thymocytes [321]. Direct and indirect reciprocal inhibitory functions were reported for ThPOK and Runx3. While the direct transcriptional inhibitory effect of ThPOK on the *Runx3* locus is poorly characterised, ThPOK is proposed to bind to the *Cd4* and *Thpok* silencer and, thereby, indirectly antagonize the Runx3-induced *Cd4* and *Thpok* silencer activity [321]. Recently, it was found that ThPOK suppressed cytokine signaling required for *Runx3* induction by inducing *suppressor of cytokine signaling* (*Socs)* genes [293]. Forced expression of one of the members of the SOCS family of proteins, SOCS1, was sufficient to restore $CD4^+$ T-cell development in ~50% of thymocytes in $ThPOK^{-/-}$ mice [293]. Conversely, ThPOK-mediated $CD8^+$ to $CD4^+$ lineage redirection was impaired if *Socs1* was ablated [293]. Despite the significant amount of knowledge gained so far about the mechanism by which ThPOK promotes $CD4^+$ commitment, important questions remain unanswered as outlined below in section 1.3.

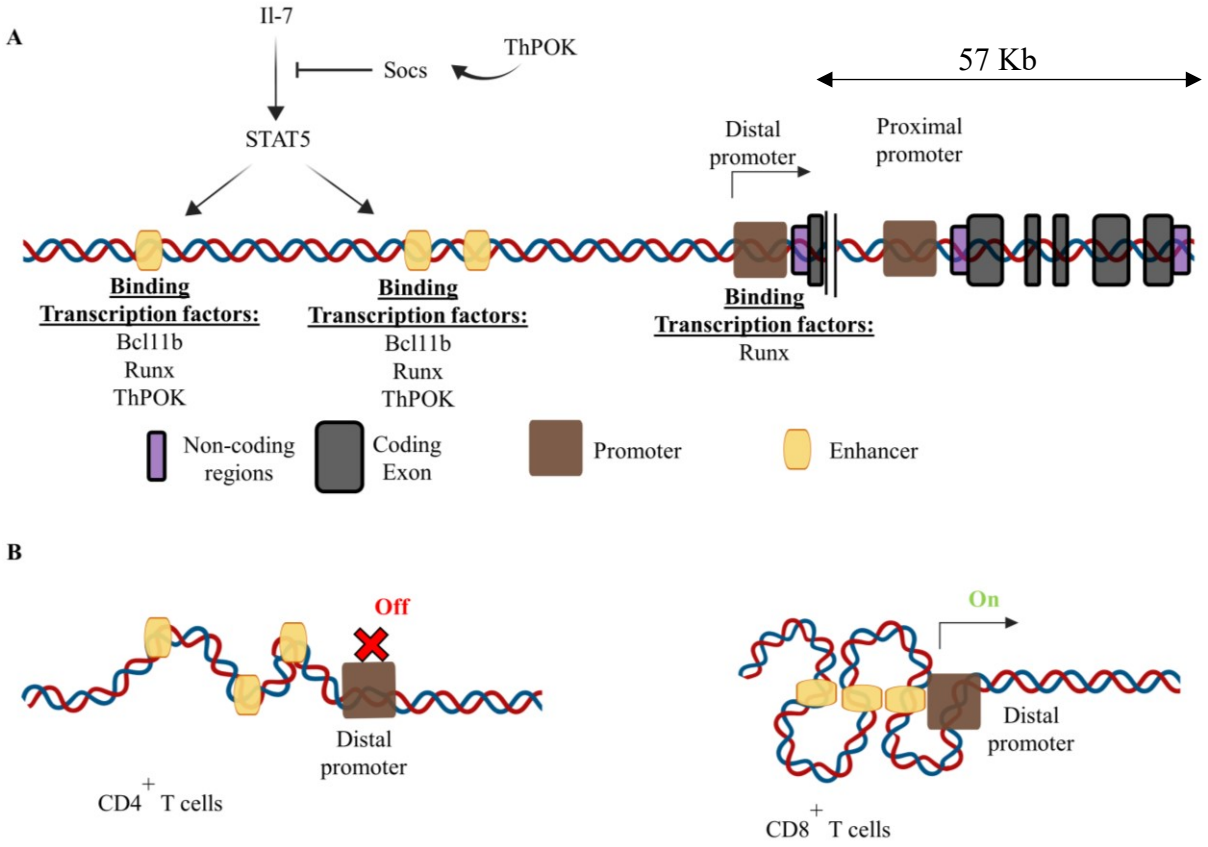

Figure 9. Mouse *Runx3* gene structure

(A) *Runx3* can be transcribed from two promoters, a distal and a proximal promoter, similar to *Thpok*. Only the distal-driven transcript is sufficient for protein synthesis. Genetic manipulations have helped determine that the three regulatory regions of *Runx3* function as enhancers [276]. Although the two most upstream regulatory regions contain ThPOK consensus sequences, the evidence of direct binding is lacking [276]. The exact mechanism by which IL-7-signaling induces *Runx3* remains to be determined. However, ThPOK-induces Socs proteins, which terminate cytokine signaling and inhibits *Runx3* upregulation. **(B)** Proposed DNA structure of *Runx3* gene in $CD4^+$ and $CD8^+$ T-cells [276]. IL-7, interleukin-7; ThPOK, T helper Inducing pox virus zinc finger -krüppel like factor.

1.2.4.6 Evidence for a dose-dependent effect of ThPOK on $CD4^+/CD8^+$ lineage fate

Multiple lines of evidence suggest that ThPOK acts in a dose dependent manner in $CD4^+$ lineage choice of MHC-II-signaled thymocytes. In mice carrying a hypomorphic allele of *Thpok*, inefficient induction of ThPOK resulted in a lineage redirection of a fraction of MHC-II-restricted thymocytes into $CD8^+$ lineage. In these mice the cells that remained as $CD4^+$ SP T-

cells expressed significantly lower ThPOK levels than WT $CD4^+$ T-cells and displayed cytotoxic functions. This suggested that lower amounts of ThPOK, while enough for $CD4^+$ T-cell development, failed to establish/maintain the helper program, demonstrating that higher physiological ThPOK expression levels are required in MHC-II-restricted thymocytes for proper $CD4^+$ helper T-cell development [320, 321]. In the same way, ablating *Thpok* in the peripheral $CD4^+$ T-cells led to expression of cytotoxic effector molecules indicating that even after the establishment of the $CD4^+$ lineage fate in the thymus ThPOK is essential for the maintenance of helper lineage [330-332]. Collectively, these observations clearly indicate that a threshold of ThPOK expression is needed to activate and maintain the expression of helper function genes in MHC-II-signaled thymocytes.

1.2.5 Structural biology of the TCR complex

The role of TCR signal strength in lineage commitment is well described in a previous section. In this section, I will describe the different components of the TCR complex, regulators of TCR signaling, the downstream effector molecules, and how they come together to influence T-cell development and lineage fate.

1.2.5.1 The αβTCR-CD3 complex

TCR complex consists of a covalently linked heterodimer of αβ TCR chains and three dimeric signaling modules, CD3δε, CD3γε, and ζζ, that are noncovalently associated with TCR chains [333]. While ζζ homodimer is covalently linked, two heterodimers of the CD3ε chain are formed by non-covalent pairing with CD3δ and CD3γ [334-338]. The formation of the heterodimers CD3δε, CD3γε and TCR is mediated through the interaction of their extracellular Ig domain with cysteine residues in the constant regions of TCR chains forming disulfide bond [339-342]. By contrast, because the ζ-chain has a very short ectodomain, it is through the TM domain that the disulfide-linked ζζ homodimer is formed [334, 343, 344]. TCR and CD3 chains assemble primarily via their TM domains that results in charged residues neutralized leading to stable assembly, transport and surface expression of the complex. While CD3 ε, δ, and γ contain one ITAM, ζ-chains have three ITAMs in their cytoplasmic tail. A close examination of the TM regions of the TCR proteins reveal that TCRα protein has two basic amino acids, while TCRβ chain has a single basic residue in their TM domains. Likewise, each subunit of the signaling

CD3 modules contains one TM acidic residue that can interact with the TCR basic residues [345-348]. Detailed analyses have revealed the following architecture of the αβTCR-CD3 complex: each TCRα associates with one of each CD3δε and ζζ modules, while the TCRβ associates with the CD3γε module [334, 338, 349]. This suggests that there are 10 ITAMs per TCR complex, which serves to amplify TCR signal transduction following receptor-ligand engagement during thymocyte development or mature T-cell response to an antigen.

Phosphorylation of ITAMs in the CD3 and ζ chains is the first detectable biochemical readout following TCR stimulation [350]. The efforts made to understand the early events of TCR signal transduction has unquestionably helped us elucidate more precisely the role of TCR signaling in T-cell development. The cytoplasmic domains of the TCR signaling modules are portrayed, by most textbook as flexible chains in the cytoplasm, with full access to the downstream effector molecules that could result in constant signal transduction. However, closer proximity of the negatively charged phosphatidylserine present in the inner leaflet of membrane lipid bilayer to the positively charged cytoplasmic domains of CD3ε and ζ raised the possibility of potential interactions between the cytosolic tails of the TCR complex and the phospholipid bilayer [349, 351-354]. Indeed, using synthetic lipid bilayer it was established that in resting T-cells ITAM domains of the CD3ε and ζ proteins are buried inside the lipid bilayer. The initial triggering events leading to the release of the cytoplasmic tail of CD3ε and ζ from the membrane remain elusive. Several hypotheses have been put forward:1) T-cell – antigen presenting cell (APC) interactions generating mechanical force could drive the dissociation of the ITAM from the membrane, 2) microclusters of TCR-CD3 complexes and their could cause spontaneous dissociations of CD3 cytoplasmic tails as a consequence of competitive binding between the cytosolic molecules of the different TCR complexes to the phospholipid bilayer, 3) microcluster formation could initiate spontaneous release of the cytoplasmic domains by modulating distribution of phospholipids in the inner leaflet within the vicinity of the ligand engaged TCR/CD3 complex [355, 356] [349, 354]. However, these hypotheses have yet to be tested in a reliable T-cell activation model [357, 358].

1.2.5.2 Lck and regulation of TCR signaling

The tyrosine kinase Lck, a member of the Src family of kinases, is the most proximal signal transducer and, like all Src family kinases, consists of a single src homology 2 (SH2) and SH3 domains and a catalytic domain (SH1). Phosphorylation status of two tyrosine residues, Tyr394 present in the catalytic and Tyr505 present in the non-catalytic C-terminal domains, regulate the kinase function [49]. Tyr394, when phosphorylated, positively regulates kinase activity by stabilizing the active form of Lck. Tyr394 can be trans-phosphorylated by the other Src kinase such as Fyn or other Lck molecule or autophosphorylated. On the other hand, phosphorylation of Tyr505, a highly conserved regulatory tyrosine present in all Src kinases, acts as a negative regulator of the kinase activity by forming an autoinhibitory conformational loop via association with SH2 domain. Accordingly, mutation of these regulatory tyrosine residues alters Lck activity and TCR signal strength [210, 211, 359, 360]. These mutant variants of Lck provided an important tool for the study of TCR signaling in T-cell development and $CD4^+/CD8^+$ lineage choice.

The kinase Csk and the phosphatase CD45 have been reported to phosphorylate and dephosphorylate Tyr505 residue in Lck, respectively [361, 362]. Other negative regulators include tyrosine-protein phosphatase non-receptor type 22 (PTPN22) and the protein tyrosine phosphatase - rich in proline, glutamic acid, serine, and threonine (PTP-PEST) tyrosine phosphatases, which act primarily by dephosphorylating Tyr394 [363, 364] In thymocytes, CD45 deficiency impairs ITAM phosphorylation and downstream activation events by increasing the pool of inhibitory Tyr505 phosphorylated form of Lck [50, 365-368]. These results strongly suggest that CD45 is involved in the early events of T-cell activation. Interestingly, several reports suggest that CD45 may negatively regulate Lck function by dephosphorylating Tyr394 as well, although studies employing physiologically relevant models have failed to confirm this [366, 368-371]. The counteracting forces of Csk and CD45 on Lck activity provide a tonic signal that maintain T-cells in a steady state **(Fig. 10)**. When a Csk mutant with reduced activity was produced, investigators observed spontaneous TCR activation in the absence any stimulus due to decreased phosphorylation of inhibitory Tyr505 of Lck [372]. Interestingly, these TCR activation events were subsequently found to require the action CD45 [373]. The evidence showing that titration of Csk and CD45 levels correlated with increased

and decreased phosphorylation at Tyr394 or Tyr505, respectively, indicated that basal level of phospho-turnover at these regulatory domains is highly dynamic [366, 368].

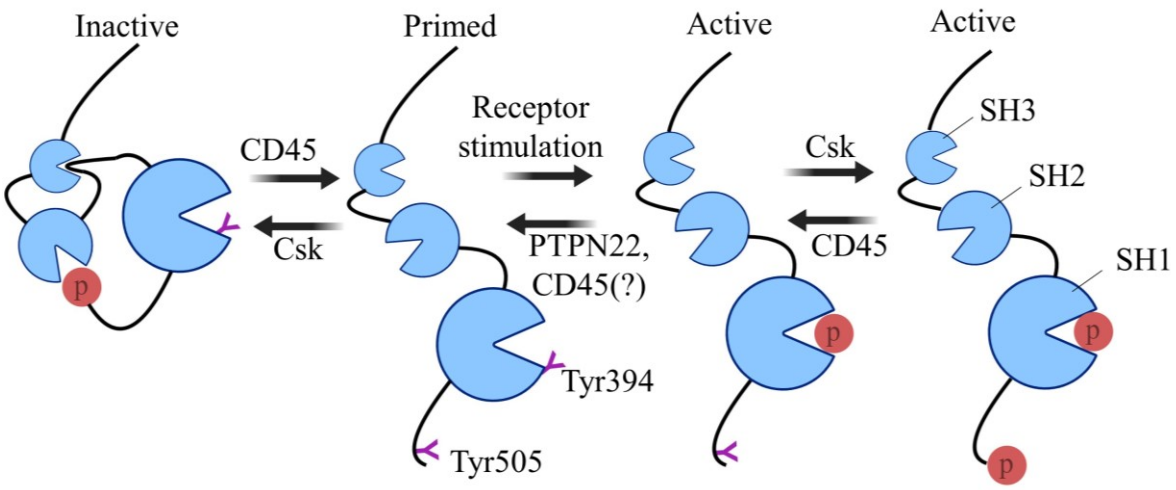

Figure 10. Regulation of Lck function

This diagram shows the four different phosphorylation states of Lck on the basis of the phosphorylation status of Tyr394, and Tyr505. Phosphorylation of Tyr394, which is located in the catalytic domain, and Tyr505 are associated with increased and decreased enzymatic activity, respectively. This drawing also shows the kinase (Csk) and phosphatases (CD45 and PTPN22) that are thought to control the alteration between the different phosphorylation states. While SH2 and SH3 are structural domains that contribute to the overall protein conformation, the catalytic domain corresponds to SH1. PTPN22, protein tyrosine phosphatase, non-receptor type 22; SH, Src homology. Figure adapted from Chakraborty and Weiss [49].

It is important to note that most reports looking into the initial TCR triggering events have primarily focused on assessing ITAM phosphorylation levels. This may be misleading, as there are several T-cell subsets that have constitutively phosphorylated ζ-chain, even in the resting state [374-377]. In fact, Csk inhibition, which induces spontaneous T-cell activation, had minimal effects on the phosphorylation status of ζ-chain when measured in *ex vivo* thymocytes [372]. Hence, assessing phosphorylation/activation status of downstream effector molecules, such as Zap70, may be more accurate. In stimulated, but not resting, thymocytes Zap70 is bound to the doubly phosphorylated ζ-chain ITAM via its tandem SH2 domain [378]. This step is required to release Zap70 from its inhibitory conformation and facilitate Lck-mediated

transphosphorylation, which are critical events for TCR signal amplification. Interestingly, transphosphorylation of Zap70 by Lck does not seem to occur in steady state T-cells and thymocytes, suggesting that Zap70 phosphorylation status may be a more reliable predictor of functional activation.

1.2.5.3 TCR signaling pathway

Upon TCR-ligand engagement a signal amplifying effect, initiated by Lck-mediated activation of Zap70, can trigger productive downstream TCR signaling [49]. Co-localization of the co-receptor-linked Lck to the ITAM-docked Zap70 occurs upon TCR stimulation by selecting ligand. Once in close proximity, Lck phosphorylates Zap70 at the Tyr319, thereby relieving the autoinhibitory constraint [379]. This phosphorylation event activates Zap70 and enables trans-autophosphorylation of Zap70 at Tyr493, which serves as a docking site for Lck. This is thought to stabilize the active conformation of Lck and help antagonize the negative feedback loop mediated by Csk and PTPN22 phosphatases [49, 379]. Subsequently, Zap70-bound active Lck can generate a positive feedback loop by promoting the phosphorylation of other molecules of Lck Tyr394 and Zap70 Tyr319. Ultimately, the phosphorylation build-up helps ensure T-cells reach the TCR signaling threshold required for signal transduction.

Zap70 phosphorylation primarily regulates phospholipase C γ1 (PLCγ1) activation, cytosolic Ca^{2+} mobilization, and activation of distal signaling pathways like NFAT, Activator protein-1 (AP-1), and NF-κB [380, 381]. Intense research helped unveil several components that constitute the proximal signalosome for TCR signal transduction. Two key adaptor proteins phosphorylated by Zap70 are the transmembrane linker for the activation of T-cells (LAT), and the cytosol-associated SH2 containing leukocyte phosphoprotein of 76 kDa (SLP76) [382, 383]. These two adaptors form the proximal signaling complex. The function of this complex is to orchestrate the recruitment of several effector molecules such as PLCγ1, PI3K, growth factor receptor-bound protein 2 (Grb2) and growth factor receptor-bound protein 2-related adaptor downstream of Shc (Gads) [384-387]. While LAT recruits Grb2-Sos complex for Ras activation, SLP76 recruits Vav1, IL-2-inducible T-cell kinase (Itk), and other adaptor proteins to the complex [388-391]. The synchronized loading of the different components to the complex is

important for stability of TCR signalosome leading to optimal activation. SLP76-recruited Vav1 is important to activate Ras-related C3 botulinum toxin substrate 1 (Rac1)-dependent actin reorganization, which is critical to sustain TCR-induced proliferation and migration [380, 391]. Activation of PLCγ1 represents a key event in connecting the proximal and distal signaling branches of the TCR signaling pathway. Activated PLCγ1 catalyses the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP2) into two secondary effector molecules – inositol triphosphate (InsP3), and diacylglycerol (DAG) [380, 392]. InsP3 triggers the influx of the secondary messenger Calcium (Ca^{2+}), which in turn triggers the activation of the calcineurin-NFAT signaling pathway. DAG, on the other hand, can activate multiple pathways, like protein kinase Cθ (PKCθ) and PDK1-mediated pathways [392, 393]. These events culminate in the activation and translocation of NFAT, AP-1 and NF-κB transcription factors to nucleus leading activation/suppression of genes that regulate various aspects of T-cell development, such as survival and differentiation. However, at present it is not known which of these TCR-induced signaling pathway lead to the activation of *Thpok* in the signaled thymocytes **(Fig. 11)**.

1.2.5.4 TCR signaling threshold in immature and mature T-cells

Detailed transcriptional analysis revealed, as expected, that gene-expression profile of DP and SP thymocytes differ greatly (22). Some of these genes that are distinctively regulated, encode proteins involved in TCR proximal signaling events, include *thymocyte-expressed molecule involved in selection* (*Themis*), *Tespa* and *Scn4b* [394-396]. Ca^{2+} mobilization is a very important signaling event triggered by TCR stimulation [397]. While *Tespa*'s specific function remains to be fully elucidated, *Tespa* and *Scn4b* encoded proteins regulate Ca^{2+} mobilisation [395, 396]. *Scn4b* encodes a subunit (SCN4B) of the voltage-gated sodium channel (VGSC) that is critical for the regulation of Ca^{2+} influx, as its deletion was shown to block Ca^{2+}-dependent positive selection [396]. Conversely, VGSC overexpression in mature T-cells enhanced sensitivity to TCR signals. *Themis*, on the other hand, facilitates positive selection by attenuating TCR signaling threshold with the help of the zinc-finger protein Schnurri-2 [394, 398].

Apart from these TCR signaling modulating molecules, microRNAs, a group of small non-coding RNAs, have recently been found to play an essential role in T-cell development. The microRNA, miR-181 in particular, shows preferential expression in DP thymocytes compared to SP thymocytes. Results from genetic manipulations in rodents have demonstrated that miR-181 can regulate responsiveness to TCR stimulation by repressing negative regulators of early TCR signaling events, such as Src homology region 2 domain-containing phosphatase-2 (SHP-2), PTPN22, Dual Specificity Phosphatase 5 (DUSP5) and DUSP6 [399-401]. Together these results demonstrate that DP thymocytes have a lower minimal threshold for TCR signaling compared to SP thymocytes [402].

1.2.5.5 MHC class-I- vs MHC class-II-specific TCR signaling

According to the kinetic signaling model for $CD4^+/CD8^+$ lineage fate, the persistence of TCR activity during the $CD4^+8^{lo}$ stage is the main lineage determining factor. Hence, if $CD4^+/CD8^+$ lineage fate is quantitatively proportional to the activity of TCR signalling, then the constitutive expression of the CD8 co-receptor in MHC class-I-restricted thymocytes is expected to induce a $CD8^+$ to $CD4^+$ lineage redirection. However this was only partially observed, which is suggestive of the presence of a complementary qualitative signal in MHC class-II-restricted thymocytes [220, 287].

For the rest of this section, the "duration" of the TCR signaling does not refer to the same temporal duration-of-signal mentioned earlier in the "kinetic signaling" section, but to the individual TCR-pMHC encounters at the cellular level.

It has recently been shown that a voltage-gated Na^+ channel (VGSC) increases the duration of activation of Ca^{2+} signaling in MHC class-II- vs class-I-restricted thymocytes by stimulating Ca^{2+} influx [163, 396, 403]. This in turn could potentially affect the transcription outcome in MHC class-II- vs class-I-restricted thymocytes as different levels of Ca^{2+} regulate distinct target genes [160, 404]. Similarly, *in situ* analyses have observed nuclear translocation of cytosolic NFAT during positive selection of MHC class-II- but not class-I-restricted thymocytes [163, 405-407]. While this would certainly affect the transcriptional outcome of positively selected MHC class-II- compared to class-I-restricted thymocytes, differences in

NFAT localization could be the result of a different pattern of Ca^{2+} signaling in MHC class-II-restricted thymocytes [160, 404, 408].

It is important to note that prolonged activation of Ca^{2+} signaling could also regulate the quantitative properties of TCR signaling by increasing the strength of the TCR-pMHC interaction [163, 396, 405]. Indeed, individual interactions with pMHC bearing stromal appeared to last longer for MHC class-II-restricted thymocytes (15 – 30 min) compared to MHC class-I-restricted thymocytes (~4 min) [163, 403].

Hence, although there is considerable evidence in support of qualitative differences between positively selecting class-II- and class-I-restricted signaling, like Ca^{2+}- and NFAT-dependent gene expression, further work needs to be done for better characterization. [160, 163, 396, 403, 404].

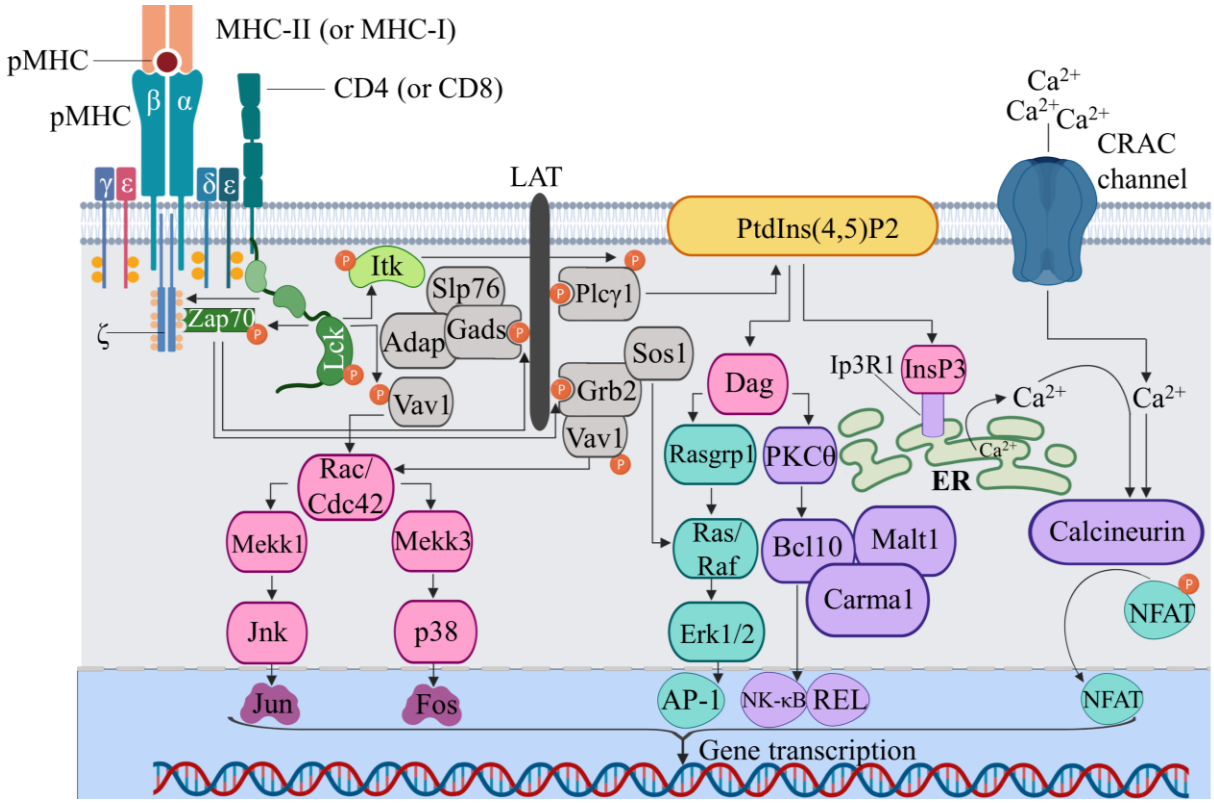

Figure 11. Overview of the most important TCR signaling pathways

Activation of the T-cell receptor (TCR) is mediated by binding to the peptide-MHC-complex expressed on the antigen presenting cells. The tyrosine kinase Lck binds to the cytosolic tail of the co-receptor, which is recruited to the TCR-pMHC complex upon stimulation, leading to a cascading phosphorylation events that triggers the activation of the linker for activation of T-cells (LAT)-associated effector molecules. Signal, subsequently, is propagated through three major pathways: the Ca^{2+}-calcineurin signaling pathway, which results in the translocation of the nuclear factor of activated (NFAT) into the nucleus, the mitogen-activated protein kinase (MAPK) pathway, which results in the activation of Fos, Jun and activator protein-1 (AP-1), and nuclear factor-κB (NF-κB) signalling pathway, which results in the transport of the REL and NF-κB transcription factors into the nucleus. Together, these events lead to T-cell proliferation and effector functions. ADAP, adhesion and degranulation promoting adaptor protein; BCL-10, B-cell lymphoma 10; CARMA1, CARD-containing MAGUK protein 1; CDC42, cell division control protein 42 homologue; CRAC, calcium release-activated calcium channel; DAG, diacylglycerol; ER, endoplasmic reticulum; ERK, extracellular signal regulated kinase; GADS, growth factor receptor-bound protein 2 (GRB2)-related adaptor protein 2; InsP3, inositol trisphosphate; ITK, IL-2-inducible T-cell kinase; JNK, Jun N-terminal kinase; MALT1, mucosa-associated lymphoid tissue lymphoma translocation protein 1; MEKK, Mitogen activated protein (MAP)/ERK kinase kinase; MHC, Major histocompatibility complex; PKCθ, protein kinase Cθ; PLCγ1, phospholipase Cγ1; pMHC, peptide-MHC; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; RASGRP1, Ras guanyl-releasing protein 1; SLP76, SH2-domain-containing leukocyte protein of 76 kDa; SOS1, son of sevenless homologue 1. Figure adapted from Hosokawa and Rothenberg 2018 [79]

1.3 Rationale

Following successful positive selection, αβ T-cells migrate to the medulla where they differentiate into either $CD4^+$ helper or $CD8^+$ cytotoxic T-cells from a common DP precursor. This important decision is known as $CD4^+/CD8^+$ lineage choice with T-cell receptor (TCR)-mediated signaling playing a central role in this process. While sustained TCR signaling promotes $CD4^+$ lineage choice, disrupted TCR signaling results in $CD8^+$ lineage choice. Although, the expression pattern of various components of the TCR complex, and its downstream signal-transducing factors, is modulated as thymocytes transition from the signaled DP stage to the $CD4^+$ or $CD8^+$ SP stage, their expression and regulation in mature $CD4^+$ SP thymocytes compared to CD8 thymocytes remain to be fully elucidated. The BTB-ZF transcription factor ThPOK has been identified as a critical factor for commitment to and maintenance of the $CD4^+$ T helper lineage. Reduced *Thpok* expression from hypomorphic allele results in the upregulation of $CD8^+$-specific cytotoxic genes in MHC-II-restricted T-cells. The transcription factors directly controlling *Thpok* expression are not well characterized. Nonetheless, there is a strong evidence linking TCR signaling to *Thpok* induction. In fact, it has been suggested that the "dose-dependent" effect of TCR signaling on lineage fate and cell function is mediated molecularly by ThPOK, implying that the quantitative MHC-II-restricted TCR signaling instructs thymocytes to become $CD4^+$ T-cells by inducing higher ThPOK expression levels. This fits nicely with the kinetic signaling model for lineage fate commitment, where ThPOK is proposed to act as a molecular sensor for the duration of TCR signaling. If this concept is correct, then, sustained TCR signaling in MHC-I-restricted thymocytes would induce similar levels of ThPOK detected in MHC-II-restricted thymocytes and make them become $CD4^+$ T-cells. Unexpectedly, this was not observed. Knock-in expression of the CD8.4 co-receptor construct, which increases TCR signaling in MHC-I-restricted T-cells, did not result in the redirection of MHC-I-specific thymocytes into $CD4^+$ lineage. Moreover, assuming that TCR signaling influences lineage fate by inducing higher levels of ThPOK expression, it implies that ThPOK is the only $CD4^+$ lineage commitment factor downstream of the TCR signaling pathway. Yet, in mice deficient for ThPOK and Runx proteins, more than 40% of selected cells were $CD4^+$ SP T-cells. These results are in contradiction with the previously proposed notion that

ThPOK is the only $CD4^+$ lineage fate determining factor. Moreover, it is not clear how TCR signaling influences ThPOK-mediated $CD4^+$ lineage choice.

1.4 Hypothesis and aims

The work presented here is aimed at better understanding the mechanism that governs $CD4^+/CD8^+$ lineage commitment in the thymus. In the positively selected thymocytes, sustained/stronger TCR signaling is proposed to "open" the spatio-temporal lineage commitment window for a longer time for *Thpok* induction and to exert its impact. If so, we hypothesize that the higher amount of ThPOK may be necessary for redirecting MHC-I, compared to MHC-II, -specific thymocytes into $CD4^+$ lineage. Further, as $CD4^+$ lineage fate can occur in the absence of ThPOK/Runx proteins and that quantitatively different MHC-I- and MHC-II-specific TCR signaling appear to differ qualitatively as well, we hypothesized that TCR-intrinsic properties in MHC-II-restricted thymocytes regulate $CD4^+/CD8^+$ lineage choice via a mechanism other than inducing *Thpok* expression. However, the elucidation of the effect of TCR signaling on lineage fate has been hindered by the lack of appropriate mouse models. In order to test our hypothesis, first, we have aimed to study independent effect of enforced ThPOK and TCR signaling on lineage commitment of MHC-I-specific thymocytes, and subsequently assess their combined impact on lineage rescue or redirection. We have then evaluated the impact of MHC-I- and MHC-II-specific TCR signaling on $CD4^+$ lineage choice in the presence of the same amount of ThPOK allowing us to evaluate quantitative and qualitative aspect of TCR signaling on ThPOK-mediated $CD4^+$ lineage choice.

Chapter 2: Critical role for TCR signal strength and MHC specificity in ThPOK-induced CD4+ helper lineage choice (Manuscript #1)

Contributions of authors:

Nabil Zeidan and Dr. Vibhuti P. Dave designed the experiments. Hassan Damen performed the experiments presented in Fig. 17D, Fig. 18H, Fig. 23E, H. Dr. Vibhuti P. Dave performed and analysed the experiments presented in Fig. S2A and S3D, E, as well as producing the ThPOK-H transgenic line. Nabil Zeidan performed and analyzed all the remaining experiments. Nabil Zeidan and Dr. Vibhuti P. Dave and wrote the manuscript. Dr. Vibhuti P. Dave conceptualized and supervised the study. Dr. Denis Claude Roy provided reagents and resources.

Critical role for TCR signal strength and MHC specificity in ThPOK-induced CD4$^+$ helper lineage choice

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2.1 Résumé

Une signalisation continue par le RCT est essentielle pour l'induction du facteur de transcription ThPOK dans les thymocytes restreints au CMH de classe II. ThPOK, dont

l'expression est indispensable pour le choix de la lignée $CD4^+$, inhibe les gènes requis pour le développement de la lignée $CD8^+$. La perte et le gain de fonction de ThPOK redirigent les thymocytes restreints au CMH de classe II et de classe I dans les lignées $CD8^+$ et $CD4^+$, respectivement. Cependant, l'impact d'un même taux d'expression de Thpok sur la différentiation en $CD4^+$ des thymocytes restreints au CMH de classe I ou de classe II et le rôle de la signalisation du RCT dans ce processus restent à élucider. Par ailleurs, il n'est pas sûr que la suppression du programme cytotoxique par ThPOK soit suffisante pour rediriger les thymocytes restreints du CMH de classe I en lignée $CD4^+$. Dans ce travail, nous avons étudié la différenciation des thymocytes restreints au CMH de classe I dans la voie $CD4^+$ dans trois lignées de souris transgéniques surexprimant le facteur de transcription ThPOK. Nos analyses montrent que dans l'une des lignées transgéniques, malgré la surexpression de ThPOK bloquant le programme cytotoxique par rapport aux $CD4^+$ WT contrôle, la redirection des thymocytes restreints au CMH de classe I en $CD4^+$ n'est que partielle. Cela nous a conduit à retrouver un grand nombre de lymphocytes T matures $CD8^+$ et DN en périphérie. Cependant, ce même transgène peut restaurer complètement la fonction endogène de ThPOK dans des thymocytes $Thpok^{-/-}$ restreints au CMH de classe II. De plus, nous avons observé que pour un même taux d'expression de ThPOK, l'augmentation de la puissance du signal du RCT dans les thymocytes restreints au CMH de classe I génère moins de cellules T $CD4^+$ par rapport à ceux restreints au CMH de classe II. Ces résultats suggèrent que la fonction de ThPOK dans le développement des cellules T $CD4^+$ est fortement influencé par la force du signal du TCR et par la spécificité du CMH.

2.1 Abstract

Sustained TCR signaling is critical for ThPOK induction in MHC-II-signaled thymocytes leading to the $CD4^+$ helper lineage commitment. ThPOK suppresses cytotoxic program in the signaled thymocytes and is shown to be necessary and sufficient for the $CD4^+$ lineage choice. Accordingly, loss and gain of ThPOK function redirects MHC-II- and MHC-I-signaled thymocytes into the $CD8^+$ and $CD4^+$ lineage, respectively. However, the impact of a defined ThPOK level on the $CD4^+$ helper lineage choice of MHC-II- and MHC-I-specific thymocytes and the role of TCR signaling in this process is not evaluated. Equally, it is not clear if suppression of the cytotoxic program by ThPOK is sufficient in redirecting MHC-I-restricted thymocytes into the $CD4^+$ lineage. Here, we have investigated $CD8^+$ to $CD4^+$ lineage redirection in three independent ThPOK overexpressing transgenic mouse lines. Our analysis show that one of the transgenic lines, despite overexpressing ThPOK compared to $CD4^+$ WT control and compromising cytotoxic program, failed to redirect all MHC-I-signaled thymocytes into the $CD4^+$ lineage resulting in the continued presence of $CD8^+$ mature T-cells and the generation of a large number of DN mature T-cells. Critically, the same ThPOK transgene completely restored the $CD4^+$ lineage commitment of MHC-II-specific *Thpok*$^{-/-}$ thymocytes. Importantly, augmenting TCR signaling significantly enhanced the ThPOK-mediated $CD4^+$ lineage choice of MHC-I-specific thymocytes but was still substantially less efficient than that of MHC-II-specific thymocytes expressing the same amount of ThPOK. Together, these data suggest that the ThPOK-induced $CD4^+$ lineage commitment is strongly influenced by TCR signal strength and MHC specificity of developing thymocytes.

2.2 Introduction

Functionally competent mature αβ T-cells play a central role in the cell-mediated immune responses [79, 409-411]. Development of these cells in the thymus is an ordered process consisting of distinct differentiation stages defined by the expression of CD4 and CD8 co-receptors. Precursor thymocytes are $CD4^{-}CD8^{-}$ double negative (DN), which following pre-T-cell receptor (pre-TCR) transduced signaling differentiate into $CD4^{+}CD8^{+}$ double positive (DP) thymocytes. The DP thymocytes expressing low level of TCRαβ receptor and the associated CD3 chains undergo thymic selection such that those expressing high affinity TCR for self-peptide/self-MHC (pMHC) are negatively selected, while those expressing low affinity TCR for pMHC are positively selected [64, 68, 412]. Positively selected thymocytes further differentiate into MHC-II-specific $CD4^{+}$ helper and MHC-I-specific $CD8^{+}$ cytotoxic mature thymocytes that populate the peripheral lymphoid organs [189, 222, 223]. How pMHC specificity of TCR/co-receptor translates into MHC-II-specific $CD4^{+}$ helper and MHC-I-specific $CD8^{+}$ cytotoxic lineage is not completely understood.

The $CD4^{+}/CD8^{+}$ binary lineage fate decision is strongly influenced by the duration and intensity of TCR signaling. A widely accepted kinetic signal strength model posits that positively selected DP thymocytes, irrespective of their MHC specificity, transcriptionally terminate *Cd8* expression and become lineage uncommitted $CD4^{+}CD8^{lo}$ thymocytes [189, 269, 413]. Continued *Cd4* transcription at this stage induces sustained/stronger signal in MHC-II-specific thymocytes leading to an error-free CD4 lineage choice [414], whereas down-regulation of CD8 results in disrupted/weaker signal in MHC-I-specific thymocytes leading to $CD8^{+}$ lineage choice. Lck, a Src family tyrosine kinase essential for T-cell development, is strongly

associated with the cytoplasmic tail of CD4 than that of CD8 [192, 210, 415]. Thus, increased Lck activity due to continued CD4 expression then results in stronger TCR signaling in MHC-II- than in MHC-I-specific thymocytes [210, 211]. Indeed, altered Lck activity is shown to direct positively selected thymocytes into alternate lineages [211, 416].

Induction of ThPOK (encoded by *ZBTB7B*, hereafter referred to as *Thpok*) in MHC-II-signaled thymocytes is both necessary and sufficient for the $CD4^+$ helper lineage commitment [276]. Similarly, Runx3 induction in MHC-I-signaled thymocytes establishes cytotoxic program in the $CD8^+$ committed thymocytes [413]. ThPOK is proposed to suppress Runx3 expression and thereby impair initiation of cytotoxic program in MHC-II-signaled thymocytes leading to the $CD4^+$ helper lineage choice [276, 293, 320, 417]. Accordingly, loss and gain of ThPOK function results in the production of MHC-II-specific $CD8^+$ cytotoxic and MHC-I-specific $CD4^+$ helper T-cells, respectively [265, 268]. The *Thpok* silencer-mediated heritable epigenetic modifications control ThPOK expression in the signaled thymocytes and is suggested to play an important role in the $CD4^+/CD8^+$ lineage choice [322]. These studies suggest that ThPOK induction during a temporal developmental window is critical for the $CD4^+$ lineage choice [322]. Persistent TCR signaling in MHC-II-specific thymocytes is proposed to reverse silencer-induced epigenetic modifications at the *Thpok* locus leading to stable ThPOK expression, which then suppresses cytotoxic program and thereby commits these cells into the $CD4^+$ helper lineage [293, 329]. Based on these and other studies, it is proposed that persistent TCR signaling leading to ThPOK induction and extent of this induction during a temporal lineage commitment window affects the $CD4^+/CD8^+$ lineage fate of positively selected thymocytes [320-322, 418]. Although published data suggest that developmental constrain on the $CD4^+$ lineage commitment of MHC-

I-signaled thymocytes can be overcome by enforced ThPOK expression [265, 268], several questions remain to be addressed. For example, it is not clear why ThPOK induction in MHC-I-signaled thymocytes lacking Tle proteins, which disrupt Runx3 function, or Runx1 and Runx3 or MAZR and Runx3 results in an incomplete $CD8^+$ to $CD4^+$ lineage redirection or generation of "confused" DP mature T-cells [267, 278, 326]. Further, role of TCR signaling in ThPOK-induced $CD4^+$ lineage choice of MHC-II- and MHC-I-signaled thymocytes is not evaluated. Is suppression of the cytotoxic program in itself sufficient for establishing the $CD4^+$ helper lineage? Thus, it remains to be investigated if the CD4+ lineage choice, irrespective of MHC specificity, requires the same level of ThPOK or it is also influenced by TCR signaling in MHC-I- vs MHC-II-specific thymocytes.

In the present investigation, we show that the efficiency of $CD4^+$ lineage commitment of MHC-I-signaled thymocytes is proportional to ThPOK dose. Further, a ThPOK dose that induced the partial $CD8^+$ to $CD4^+$ lineage redirection of MHC-I-signaled thymocytes expressing monoclonal or polyclonal TCRs completely restored the $CD4^+$ lineage commitment of MHC-II-signaled thymocytes expressing monoclonal or polyclonal TCRs in ThPOK-deficient mice. Importantly, this differential ThPOK-induced $CD4^+$ lineage commitment correlated, at least in part, with TCR signal strength as augmenting TCR signaling significantly enhanced the $CD4^+$ lineage choice of MHC-I-signaled thymocytes; however, still it was significantly less efficient than the $CD4^+$ lineage choice of MHC-II-signaled cells. Together, our results provide crucial insights into the mechanism of ThPOK-induced $CD4^+$ helper lineage choice of thymocytes specific for disparate MHC and critical role for TCR signaling in this process.

2.3 Materials and methods

2.3.1 Mice

MHC-I-restricted OTI^{+}*Rag*$^{-/-}$ (chicken ovalbumin antigen specific) and P14^{+}*TCRα*$^{-/-}$ (LCMV GP33 peptide specific) transgenic mice were obtained from Taconic Farm or Nathalie Labrecque (CRHMR). MHC-II-specific OTII^{+}*Rag*$^{-/-}$ mice were from Jackson Lab. *MHC-II*$^{-/-}$ and *Nur77-GFP* mice [419] were obtained from The Jackson Laboratory. All TCR transgenic mice were in Rag-deficient background unless mentioned otherwise. ThPOK transgenic mice were generated by cloning the genomic DNA encompassing the two coding exons flanking an intron into human CD2 expression vector. Following primers were used for cloning the ThPOK transgene; forward primer 5`-GGCGGAATTCCCAGGGAAGCAGAAG**ATG**GGGAGCCCCGAGGA-3` and reverse primer 5`-GCCCTTCCCCGGGCTTTTAAGAGGACTCCATGGCACC-3` (ThPOK sequence is underlined and ThPOK start codon in the forward primer is in bold letters). PCR product was digested with EcoRI and XmaI restriction enzymes, agarose gel purified and cloned into the EcoRI and XmaI cut hCD2 expression vector. The cloned DNA insert was sequenced to ensure fidelity of the ThPOK coding sequence. DNA was digested to release the insert from the vector backbone and agarose gel purified DNA devoid of the vector backbone was injected into the fertilized mouse eggs. Three independent founder lines were established and all of them showed increased frequency of CD4^{+} T-cells and severely reduced number of CD8^{+} T-cells in the lymphoid organs. ThPOK deficient mice were generated in the lab or acquired from Dan Littman (NYU). Constitutively active Lck transgenic mouse line (dLGF) is described elsewhere [211] and was obtained from Paul Jolicoeur [420]. Mice were genotyped by peripheral blood analysis and/or PCR of genomic DNA isolated from tail snippets. Lymphoid organs harvested

from five to seven-week-old mice were analyzed. Any mice that showed signs of ThPOK-induced thymic leukemia [421], usually observed in more than 12 week old mice, were excluded from the analysis. All mice were housed under specific pathogen free conditions at the Research Center Hopital Maisonneuve-Rosemont (CRHMR). Animal care was approved by the institutional Animal Care Committee in accordance with the Canadian Committee on Animal Care.

2.3.2 Flow cytometry

One x 10^6 thymocytes or red blood cell-depleted spleen cells or stimulated T-cells were incubated with a combination of fluorescently labeled antibodies to CD4 (GK1.5), CD8 (53-6.7), TCRβ (H57-957), CD5 (53-7.3), CD69 (H1.2F3), CD24 (M1/69), CD44 (IM7), CD62L (MEL-14), NK1.1 (PK136), CD154 (MR1), IFNγ (XMG1.2), IL-4 (11B11), Vα2 (B20.1), Vβ5 (MR9-4), ThPOK (D9V5T) or donkey anti-rabbit secondary antibody (Poly4064), phospho-Src (pY418; clone K98-37), phosphor-CD3z (pY142, clone 3ZBR4S) and analyzed by flow cytometry using LSRFortessa X-20 (BD Bioscience) or LSRII (BD bioscience). Antibodies were obtained from Ebioscience, Biolegend or Cell Signaling Technology. For ThPOK staining, the human Foxp3 staining kit (eBioscience) was used for cell fixation and permeabilization using the manufacturer's protocol. Data were analyzed using FlowJo software (Tree Star, Inc.). Gating strategy involving TCR transgenic mice is shown in Figure 1. Unless mentioned otherwise, this flow cytometry gating strategy was used for the analysis of all the thymic and splenic T-cells described in the manuscript.

2.3.3 Quantitative RT-PCR (QPCR)

Various thymic or splenic T-cell subsets were FACS purified and total RNA was isolated using Trizol (Invitrogen Inc.). Complementary DNAs were synthesized using a commercial kit (Bio-Rad). QPCR for *Thpok*, *Runx3d*, *Socs1*, *Nur77*, *Perforin*, *St8sia6*, *St3gal2*, *Cxxc5*, and endogenous *Thpok* was performed in triplicate using SyBR green dye (Bio-Rad) or EvaGreen (Abcam). Amplification of housekeeping gene *Hprt* served as an internal control. QPCR data were analyzed by Applied Biosystem software ABI 7500 v2.0.5. Data were normalized to *Hprt* expression in each population. Relative expression values were calculated using ΔΔCt method. Ratio of gene specific values to housekeeping gene for wild type or OTI reference subset was treated as one. Data are presented as an average of triplicate values and standard deviation. Following QPCR primers were obtained from the Integrated DNA Technologies or designed in our lab;

total *Thpok*, TGTCACAAGATAATCCACGGG and GGTCGTAGCTATGCAGGAAG;

Runx3d, CGACATGGCTTCCAACAG and CGGCGGAGTAGTTCTCATC;

Socs1, CAGAAAAATGAAGCCAGAGACC and ATTCCACTCCTACCTCTCCAT;

Nur77, CCATGTGCTCCTTCAGACAG and GCTCTGGTCCTCATCACTG;

Perforin, GTACAACTTTAATAGCGACACAGTA and AGTCAAGGTGGAGTGGAGGT;

Endogenous *Thpok* CCTCAGCGTTCAGGAGAAGAT and GCTGCTGTGGTCTGGGAAT (sequence unique for endogenous *Thpok* is underlined);

St8sia6, CCACCTCGTAGCTCATGTTAG and CGGCAAGCAGAAGAATATGAC;

St3gal2, GGTGTTGTGTGACTTGAATTGG and GTTTGACAGCCACTTTGACG;

Cxxc5, ATCACTGAAACCACCGGAAG and TTGTAGGAACCGAAAGACTGG;

Hprt, CCTCATGGACTGATTATGGACAG and TCAGCAAAGAACTTATAGCCCC;

Thpok transgene copy number, TTGAGGCTGTGGTGGTGGCAGT and GGTGAGGAAGAAGAGGAGGA.

2.3.4 Functional assays

Mature T-cell subsets from spleen of OTI ($CD8^+$) and OTI mice expressing specific ThPOK transgene ($CD4^+$, $CD8^+$, and DN) mice were purified and cultured in the presence of irradiated (2500 rads) BL/6 splenocytes pulsed with cognate OVA peptide (SIINFEKL) for 5 to 7 days. Purified mature T-cell subsets from WT ($CD4^+$ and $CD8^+$) and ThPOK-H^+*MHC-II*$^{-/-}$ ($CD4^+$, $CD8^+$, and DN) mice were stimulated with irradiated splenocytes obtained from Balb/c mice. In some cases, purified T-cells were stimulated with plate-bound anti-CD3 and anti-CD28 (in suspension). The stimulated cells were stained with a combination of CD69, CD154, CD4, CD8, and TCRβ specific antibodies and analyzed by flow cytometry. For cytokine staining, the activated T-cells were re-stimulated with PMA plus ionomycin in the presence of brefeldin for 4 hours, surface stained, fixed in 2% paraformaldehyde, permeabilized, washed and stained with anti-IL-4 and anti-IFNγ antibodies and analyzed by flow cytometry.

***In vitro* differentiation culture**: FACS sorted thymic subsets were cultured in 96-well flat bottom plates at a concentration of 1 x 10^6 cells/ml. Cultures were maintained in RPMI 1640 medium (Gibco BRL) supplemented with 10% (vol/vol) FBS, L-glutamine (2mM), 2-ME (50μM), streptomycin (100 mg/ml), penicillin (10U/ml) and IL-7 (1ng/ml). After two days of culture, cells were collected and analyzed by flow cytometry.

2.3.5 Luciferase reporter Assay

For luciferase reporter assay promoter sequence of *Actin* and *Nur77* was cloned into the EcoRV and HindIII cut pGL4.17 vector (Promega). Promoter sequence was amplified using genomic DNA and following primers.

Nur77 promoter, TCGCCGGTCGACTCGATATCAGGAGATGGAGTTCGATGGCCC and GTCGCCTCTAGATCAAGCTTACCAAGCACCTTGCAGACCCTTC;

Actin promoter, GGGGTGGCCGGTACCAGAGACACTAGCTAACGGCCC AND GGGCCCGGGAAGCTTCTGGTGGCGGGTGTGGACCGG.

The promoter-reporter DNA was co-transfected with either a ThPOK-YFP or control YFP plasmid (pMSCV) using the lipofectamine 2000 (Invitrogen) at a ratio of 3:1 (promoter-reporter to YFP) in 293T HEK cells. Twenty four hours after transfection, an equal number of YFP expressing cells were seeded in a 96 flat-bottom plate. *Socs1* promoter driven luciferase plasmid was used as a positive control (kind gift of Hyun Park, NIH). Luciferase activity was measured 48 h after transfection using the Luciferase Assay System (Promega).

Statistical analyses: was performed using Graphpad Software or Microsoft Excel software. Data are displayed as a mean with standard deviation error bar. Unpaired two-tailed Student *t*-test was used for determining the statistical significance when thymic and splenic T-cell subsets from different mice were compared. For experiments involving comparison of T-cell subsets isolated from the same mouse, paired Student *t*-test was used for evaluating the statistical significance. $P \leq 0.05$ was considered statistically significant. * $P \leq 0.05$, ** $P \leq 0.005$, and *** $P \leq 0.0005$.

2.4 Results

2.4.1 Characterization of ThPOK transgenic mice

To investigate if ThPOK-mediated suppression of the cytotoxic program in MHC-I-signaled thymocytes is in itself sufficient for inducing the $CD4^+$ T-cell helper program and, role of TCR signaling and MHC specificity in this process we generated three independent ThPOK founder lines (ThPOK-H, ThPOK-163 and ThPOK-611) in which ThPOK expression is driven by human CD2 promoter/enhancer cassette [422]. All the progenies of three ThPOK founders showed, in agreement with the previously published reports [265, 268], increased and decreased frequencies of $CD4^+$ and $CD8^+$ mature T-cells, respectively, in the lymphoid organs (**Fig. S1**). While $CD8^+$ mature T-cells in the spleen (TCR^+) and thymus ($CD24^-CD69^-TCR^+$) of ThPOK-611^+ and ThPOK-163^+ mice were almost completely absent, we consistently detected a small number $CD8^+$ mature T-cells in the lymphoid organs of ThPOK-H^+ mice (**Fig. S1A, S1B)**. Accordingly, compared to WT control, $CD4^+/CD8^+$ ratio of mature T-cells increased by ~20-fold in ThPOK-H^+ mice and >100-fold in ThPOK-163^+ and ThPOK-611^+ mice (**Fig. S1C**).

In order to investigate the basis of differential $CD4^+/CD8^+$ phenotype of the three transgenic mice, we analyzed ThPOK protein expression by intracellular staining. In WT mice, the basal ThPOK staining observed in preselection DP thymocytes increased as signaled thymocytes matured into $CD4^+CD8^{lo}$ and $CD4^+$ thymocytes (**Fig. S1D**). This ThPOK specific staining pattern in WT thymic subsets is in agreement with ThPOK induction in MHC-II-, but not MHC-I-, signaled thymocytes and its continued expression in $CD4^+$ mature T-cells [265]. Importantly, compared to WT control, significantly higher ThPOK expression was observed in all the thymic subsets including the preselection DP thymocytes from the three ThPOK transgenic mice (**Fig. S1E, S1G**), which correlated with the observed $CD4^+/CD8^+$ phenotype in

the thymus of these mice. Interestingly, ThPOK levels in DP thymocytes showed hierarchical pattern with that in ThPOK-611 > ThPOK-163 > ThPOK-H; DP thymocytes from ThPOK-611^{+} mice showed significantly higher ThPOK level compared to ThPOK-H^{+} DP thymocytes (**Fig. S1G**). Similar to the thymic subsets, significantly higher ThPOK expressed was observed in the splenic CD4^{+} mature T-cells from the three transgenic mice compared to WT CD4^{+} mature T-cells (**Fig. S1F, S1G**). The differential ThPOK staining in thymocytes from the three transgenic mice was not correlated with transgene copy number (**Fig. S1H**). Interestingly, variegated ThPOK expression observed in the preselection DP thymocytes was lost as the signaled thymocytes matured as judged by largely uniform ThPOK staining in CD4^{+}CD8^{lo} and CD4^{+} thymocytes and CD4^{+} splenic T-cells (**Fig. S1E, S1F**) from the three ThPOK transgenic mice; a small number of CD4^{+}CD8^{lo} and CD4^{+} thymocytes and mature T-cells, particularly from ThPOK-H^{+} and ThPOK-163^{+} mice, showed a slightly lower ThPOK staining. At present reason for this change in ThPOK expression pattern in DP thymocytes vs mature T-cells from these mice is not clear. Irrespective, we consistently observed about 1.5- to 2-fold more ThPOK expression in CD4^{+} mature T-cells from the spleen of three transgenic mice compared to that in CD4^{+} mature T-cells from the spleen of WT mice.

2.4.2 Impact of ThPOK dose on the CD4^{+} lineage choice of MHC-I-signaled thymocytes

To evaluate the impact of differential ThPOK levels on the CD8 to CD4 lineage redirection, we bred the three ThPOK transgenic lines to mice expressing MHC-I-restricted OTI-TCR (Vβ5^{+}Vα2^{+}; all mice *Rag*$^{-/-}$). In these mice intra-thymic signaling in MHC-I-specific thymocytes does not induce endogenous ThPOK expression and thus, allow us to study the role of transgenic ThPOK expression in the CD8^{+} to CD4^{+} lineage redirection. Indeed, we observed

a ThPOK dose-dependent impact on the $CD8^+$ to $CD4^+$ lineage redirection in OTI mice expressing each ThPOK transgene as judged by the hierarchical pattern of the $CD4^+$ mature T-cell frequency in the thymus and spleen of these mice (**Fig. 1A, 1B**) with that in OTI^+ThPOK-611^+ > OTI^+ThPOK-163^+ > OTI^+ThPOK-H^+ mice. Thus, there were only 16% $V\alpha2^+CD4^+$ mature T-cells in the spleen of OTI^+ThPOK-H^+ mice, whereas it was 44% in OTI^+ThPOK-163^+ and 68% in OTI^+ThPOK-611^+ mice compared to <1% in OTI control mice (**Fig. 1A**). Increase in the $CD4^+$ frequency observed in the spleen was reflected in the thymus of these mice as well indicating an efficient lineage redirection in OTI^+ThPOK-611^+ mice compared to partial lineage redirection in OTI^+ThPOK-163^+ and OTI^+ThPOK-H^+ mice (**Fig. 1B**). As expected, the frequency of $CD8^+$ mature T-cells in the thymus and spleen showed opposing pattern (**Fig. 1A, 1B**) resulting in the $CD4^+/CD8^+$ ratio in OTI^+ThPOK-611^+ mice significantly higher than that in OTI^+ThPOK-163^+ or OTI^+ThPOK-H^+ mice (**Fig. 1C**). We also noticed a significant number of DN mature T-cells in OTI^+ThPOK-H^+ and OTI^+ThPOK-163^+ mice; as many as 25 to 40% of total splenic T-cells were DN in these mice (**Fig. 1A**). The DN mature T-cells in OTI^+ThPOK-H^+ mice did not express NK1.1 and were $CD62L^{hi}CD44^{lo}$ indicating that they were not innate or memory T-cells (**Fig. S2A**) [423, 424].

Similar to non-TCR transgenic background, ThPOK specific staining in DP thymocytes was hierarchical with that in OTI^+ThPOK-611^+ > OTI^+ThPOK163^+ > OTI^+ThPOK-H^+ cells, and was substantially higher compared to similar subsets from OTI control (**Fig. 1D, 1E**). Similar to the analysis of ThPOK transgenic mice with WT background, variegated ThPOK expression observed in DP thymocytes was lost in a majority to the redirected $CD4^+$ mature T-cells from the three ThPOK transgenic mice expressing OTI TCR, and was about 1.5 to 2-fold higher compared to $CD4^+$ mature T-cells from WT mice (**Fig. 1D, 1E**). Interestingly, DN and

$CD8^+$ mature T-cells from the spleen of $OTI^+ThPOK\text{-}H^+$ or $OTI^+ThPOK\text{-}163^+$ mice continued to express a significant amount of ThPOK compared to ThPOK levels in $CD4^+$ mature T-cells from WT mice (**Fig. 1D** bottom panels, **1E**). While total thymocytes in OTI mice expressing or not individual ThPOK transgene were comparable, the frequency and number of selected thymocytes were reduced in ThPOK expressing mice likely due to impaired Runx3 expression (**Fig. 1F**; [245, 246, 269]; see below)). As expected, the frequency and number of $CD4^+$ single positive thymocytes was significantly increased, while that of $CD8^+$ single positive thymocytes decreased in all three ThPOK transgenic OTI lines compared to control. Similarly, frequency and number of TCR^+ splenic cells were reduced in ThPOK expressing OTI mice compared to control likely reflecting reduced thymic maturation and survival/expansion of the redirected T-cells in the periphery (**Fig. 1F**; ref 23, 24). Nevertheless, the frequency and cell number compilation data show significant increase in $CD4^+$ and/or DN mature T-cells and decrease in $CD8^+$ mature T-cells in all OTI mice expressing individual ThPOK transgene compared to control (**Fig. 1F**). Additionally, the DN mature T-cells appeared to be mostly derived from $CD4^+$ thymocytes in $OTI^+ThPOK\text{-}H^+$ mice (**Fig. S2B**).

To rule out the possibility that the ThPOK-H-mediated partial $CD8^+$ to $CD4^+$ lineage redirection was not specific to OTI model, we introduced ThPOK-H transgene into mice expressing MHC-I-specific monoclonal TCR (P14-TCR) or polyclonal TCRs (*MHC-II*$^{-/-}$). Similar to $OTI^+ThPOK\text{-}H^+$ mice, $P14^+ThPOK\text{-}H^+$ mice also showed partial $CD8^+$ to $CD4^+$ lineage redirection as judged by the presence of $CD4^+$, DN and $CD8^+$ mature T-cells in the spleen of these mice (**Fig. S3A**). Importantly, introduction of ThPOK-H transgene in *MHC-II*$^{-/-}$ mice also resulted in the partial $CD8^+$ to $CD4^+$ lineage redirection (**Fig. 2A, 2B**) and significant increase in the $CD4^+/CD8^+$ ratio in the thymus and spleen of $ThPOK\text{-}H^+$*MHC-II*$^{-/-}$ mice

compared to *MHC-II*$^{-/-}$ mice (**Fig. 2C**). Similar to OTI model, we noted decrease in thymic selection and mature splenic T-cell frequency and number in ThPOK-H^{+}*MHC-II*$^{-/-}$ mice (**Fig. 2D**). In the thymus of ThPOK-H^{+}*MHC-II*$^{-/-}$ mice the frequency and number of CD4^{+} thymocytes was significantly increased, while that of CD8^{+} thymocytes was significantly decreased compared to control mice (**Fig. 2D**). In the spleen, we observed a similar pattern except that number of CD4^{+} mature T-cells were only slightly higher in ThPOK-H^{+}*MHC-II*$^{-/-}$ mice compared to control mice likely due to their differentiation into DN mature T-cells in ThPOK-H^{+}*MHC-II*$^{-/-}$ mice (**Fig. 2B, 2D**). Together, the generation of a small number of CD4^{+} mature T-cells and the presence of a substantial number of DN mature T-cells with cytotoxic function but lack of activation of helper function suggest that ThPOK-H induces partial CD8^{+} to CD4^{+} lineage redirection of thymocytes expressing MHC-I-specific monoclonal or polyclonal TCRs.

To determine the basis for the presence of CD4^{+}, CD8^{+} and DN mature T-cells in OTI^{+}ThPOK-H^{+} mice, we assessed *Thpok*, *Runx3d*, *Socs1*, and *Nur77* levels in each T-cell subset purified from the same mouse. *Socs1* is positively regulated by ThPOK [293], and *Nur77* influences CD8^{+} T-cell development via modulating *Runx3* expression [425] and is suggested to be preferentially expressed in CD4^{+} mature T-cells [143]. QPCR and flow cytometric analysis of mature T-cells from OTI^{+}ThPOK-H^{+} mice showed graded ThPOK expression levels with that in CD4^{+} > DN > CD8^{+} mature T-cells (**Fig. 3A**), which is in agreement with staining data (**Fig. 1D**, **1E**). In agreement with ThPOK expression analysis, expression of *Runx3* from distal promoter (*Runx3d*) was completely abolished in CD4^{+} and DN mature T-cells, and reduced in CD8^{+} mature T-cells from OTI^{+}ThPOK-H^{+} mice compared to CD8^{+} mature T-cells from OTI mice (**Fig. 3B**). Similarly, *Socs1* was mostly expressed in CD4^{+} mature T-cells (**Fig. 3C**), while *Nur77* expression was directly proportional to ThPOK levels in the three mature T-cell subsets

from OTI$^+$ThPOK-H$^+$ mice (**Fig. 3D**). Note that Nur77 expression was significantly higher in CD4$^+$ mature T-cells (*P* 0.0032) but not in DN or CD8$^+$ mature T-cells from OTI$^+$ThPOK-H$^+$ mice compared to CD8$^+$ mature T-cells from OTI mice. Comparable CD5 levels (a surrogate marker for TCR signal strength [426]) in CD4$^+$ and CD8$^+$ mature T-cells (**Fig. S3B**) from OTI$^+$ThPOK-H$^+$ and OTI control suggest that the differential *Nur77* expression observed mature T-cells may be due to differential ThPOK expression [419]. We then evaluated Nur77-GFP reporter expression in DP thymocytes (to exclude influence of intra-thymic signaling on Nur77 expression) from OTI$^+$ThPOK-H$^+$ and control mice; comparable CD5 levels but higher GFP expression was detected in DP thymocytes from OTI$^+$ThPOK-H$^+$ mice compared to OTI control expressing Nur77-GFP reporter (**Fig. 3E**); however, increase in Nur77-GFP expression in the presence of transgenic ThPOK did not appeared to be significant. In cell transfection studies we did not observe any increase in *Nur77* promoter driven luciferase expression in the presence of ThPOK (**Fig. S3C**). These data suggest that ThPOK may not be involved in regulating *Nur77* expression.

2.4.3 Functionality of mature T-cell subsets in OTI$^+$ThPOK-H$^+$ mice

As ThPOK is proposed to suppress the cytotoxic program in mature T-cells [418], we wondered about the functionality of the three T-cell subsets, particularly CD8$^+$ and DN mature T-cells that expressed a significant amount of ThPOK and reduced levels of Runx3. To this end, we evaluated expression of genes involved in cytotoxic and helper function in these T-cell subsets. In agreement with Runx3 and ThPOK expression analysis, we observed, compared to CD8$^+$ mature T-cells from OTI mice, almost complete ablation of perforin and severely reduced IFNγ expression in CD4$^+$ and DN mature T-cells (**Fig. 3F**, **3G**). Interestingly, perforin and IFN□ expression was significantly reduced in CD8$^+$ mature T-cells isolated from OTI$^+$ThPOK-H$^+$

mice as well (**Fig. 3F, 3G**). Upon activation $CD4^+$, but not DN or $CD8^+$, mature T-cells from $OTI^+ThPOK\text{-}H^+$ mice upregulated CD154, a $CD4^+$ lineage marker (**Fig. 3H**). This was also observed in mature T-cells subsets isolated from $ThPOK\text{-}H^+$*MHC-II*$^{-/-}$ mice; activated $CD4^+$ mature T-cells from $ThPOK\text{-}H^+$*MHC-II*$^{-/-}$ mice expressed CD154 and IL-4 (**Fig. S3D, S3E**), while DN and $CD8^+$ mature T-cells from $ThPOK\text{-}H^+$*MHC-II*$^{-/-}$ mice continued to express IFNγ with DN mature T-cells expressing lower amounts. Together, these data suggest that a ThPOK level sufficient for suppressing the cytotoxic program does not activate the helper program (phenotype of DN mature T-cells) and that a higher amount of ThPOK is required for redirecting MHC-I-signaled thymocytes into the $CD4^+$ lineage.

2.4.4 Role of endogenous ThPOK in the $CD8^+$ to $CD4^+$ lineage redirection in $OTI^+ThPOK\text{-}H^+$ mice

ThPOK is proposed to form a positive auto-regulatory loop [321]. Hence, we wondered if the transgenic ThPOK induced the expression of endogenous ThPOK in the signaled thymocytes, and whether this contributed to the $CD8^+$ to $CD4^+$ lineage redirection in $OTI^+ThPOK\text{-}H^+$ mice. To address this, we first evaluated, using specific QPCR primers, endogenous ThPOK expression in various T-cell subsets from $OTI^+ThPOK\text{-}H^+$ mice. Indeed, we observed a significant increase in endogenous ThPOK expression in the splenic $CD4^+$ mature T-cells of $OTI^+ThPOK\text{-}H^+$ mice (**Fig. 4A**). To assess if this endogenous ThPOK induction played any role in the $CD8^+$ to $CD4^+$ lineage redirection in $OTI^+ThPOK\text{-}H^+$ mice, we analyzed $CD4^+/CD8^+$ phenotype of $OTI^+ThPOK\text{-}H^+$ mice expressing endogenous ThPOK or not. Surprisingly, we did not observe any significant changes in the frequency of $CD4^+$ and $CD8^+$ thymocytes in $OTI^+ThPOK\text{-}H^+$*Thpok*$^{-/-}$ mice compared to $OTI^+ThPOK\text{-}H^+$*Thpok*$^{+/+}$ control (**Fig. 4B**). A slight decrease in $CD4^+$ and increase in $CD8^+$ mature T-cell frequency in the spleen

of OTI$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice compared to OTI$^+$ThPOK-H$^+$*Thpok*$^{+/+}$ mice was noticed, however, it did not result in any significant change in the CD4$^+$/CD8$^+$ ratio in these mice (0.41±0.09 in OTI$^+$ThPOK-H$^+$*Thpok*$^{+/+}$ versus 0.28±0.035 in OTI$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice; (**Fig. 4C**; relative to OTI control)). The frequency/number of various thymic and splenic T-cell subsets in OTI$^+$ThPOK-H$^+$ mice were comparable irrespective of the presence or absence of endogenous ThPOK (**Fig. 4D**; some mice were in *Rag*$^{+/-}$ background, which did not affect the CD4$^+$/CD8$^+$ phenotype). These data suggest that endogenous ThPOK played an insignificant role in the CD8$^+$ to CD4$^+$ lineage redirection in OTI$^+$ThPOK-H$^+$ mice.

2.4.5 Evaluating role of ThPOK-H in the CD4$^+$ lineage choice of MHC-II-specific thymocytes

The presence of a large number of DN and CD8$^+$ mature T-cells in ThPOK-H$^+$ mice expressing MHC-I-specific TCR (OTI, P14 or *MHC-II*$^{-/-}$), despite expressing about 1.5 to 2-fold more transgenic ThPOK compared to endogenous ThPOK levels in CD4$^+$ mature T-cells from WT mice, suggest that differential amount of ThPOK may be required for the CD4 lineage choice of MHC-I- and MHC-II-specific thymocytes. Alternately, the observed phenotype of OTI$^+$ThPOK-H$^+$ mice could be due to ThPOK-H transgene specific effect. To address these questions, we evaluated the impact of ThPOK-H on the rescue of CD4$^+$ choice of MHC-II-specific thymocytes in ThPOK deficient mice. To this end, we generated OTII$^+$*Thpok*$^{-/-}$ mice expressing or not ThPOK-H transgene. In OTII$^+$*Thpok*$^{+/+}$ mice >95% of the Vα2$^+$ mature T-cells are CD4$^+$, which are directed into CD8$^+$ lineage in the absence of ThPOK (**Fig. 5A**). Indeed, introduction of ThPOK-H transgene into OTII$^+$*Thpok*$^{-/-}$ mice completely rescued CD4 lineage commitment; more than 95% of Vα2$^+$ mature T-cells were CD4$^+$ in the thymus and spleen of OTII$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice, which was similar to that in OTII$^+$*Thpok*$^{+/+}$ mice (**Fig. 5A, 5B**).

ThPOK specific staining of the mature $CD4^+$ thymocytes and splenic T-cells showed ~2-fold higher expression compared to endogenous ThPOK expression in control $CD4^+$ mature T-cells from $OTII^+$*Thpok*$^{+/+}$ mice (**Fig. 5C**). The frequency and absolute cell numbers in the thymus and spleen of $OTII^+$ThPOK-H^+*Thpok*$^{-/-}$ mice were comparable to that in littermate control $OTII^+$ThPOK-H^+*Thpok*$^{+/+}$ mice (**Fig. 5D**).

To further support the observation that the same level of ThPOK differentially influences $CD4^+$ lineage choice of MHC-I- and MHC-II-specific thymocytes, we introduced each of the three ThPOK transgene into *Thpok*$^{-/-}$ mice expressing polyclonal TCR repertoire. In *Thpok*$^{-/-}$ mice, positively selected MHC-II-specific thymocytes are redirected into the $CD8^+$ lineage and thus, the peripheral $CD8^+$ mature T-cell population consists of MHC-I- and MHC-II-specific T-cells. Indeed, each of the ThPOK transgene rescued $CD4^+$ development and impaired $CD8^+$ development in *Thpok*$^{-/-}$ mice (**Figure S4A-C**). Together, these data strongly suggest that, compared to $CD4^+$ lineage choice of MHC-II-specific thymocytes, an efficient $CD8^+$ to $CD4^+$ lineage redirection of MHC-I-specific thymocytes requires a higher amount of ThPOK, and the partial $CD8^+$ to $CD4^+$ lineage redirection in OTI^+ThPOK-H^+ mice is unlikely due to the ThPOK-H transgene specific effect.

2.4.6 Impact of augmented TCR signal strength on the ThPOK-induced $CD4^+$ lineage choice of MHC-I-signaled thymocytes

The partial $CD8^+$ to $CD4^+$ lineage redirection of MHC-I-signaled thymocytes but complete rescue of $CD4^+$ lineage choice of MHC-II-signaled thymocytes prompted us to ask if differential TCR signaling played a role in the ThPOK-H-mediated $CD4^+$ lineage choice of MHC-I- and MHC-II-specific thymocytes. We considered the possibility that weak TCR signaling in MHC-I-specific thymocytes, compared to that in MHC-II-specific thymocytes, may

be responsible for an inefficient $CD4^+$ lineage choice of MHC-I-specific thymocytes expressing ThPOK-H transgene. If so, we reasoned that increasing TCR signal strength may enhance the efficiency of ThPOK-H-mediated $CD4^+$ lineage choice of MHC-I-specific thymocytes. To test this notion, we introduced constitutively active Lck transgene (dLGF [210, 211, 420]) into OTI^+ThPOK-H^+ mice. We bred mice to obtain OTI^+dLGF$^+$ThPOK-H^+ triple transgenic mice with *Rag*$^{-/-}$ background and analyzed $CD4^+/CD8^+$ development in these mice. As reported previously [210], increased TCR signaling due to constitutively active Lck led to an increase in the frequency of $V\alpha2^+CD4^+$ and a decrease in the frequency of $V\alpha2^+CD8^+$ mature T-cells in the thymus and spleen of OTI^+dLGF$^+$ mice compared to control mice (**Fig. 6A**). Importantly, analysis of mature T-cells in the thymus and spleen of OTI^+dLGF$^+$ThPOK-H^+ mice showed a significant increase in the frequency and number of $V\alpha2^+CD4^+$ T-cells, while that of $V\alpha2^+CD8^+$ mature T-cells was significantly reduced compared to OTI^+ThPOK-H^+ mice (**Fig. 6A, 6B**). About 70% of mature T-cells were $CD4^+$ in the spleen and thymus of triple transgenic mice resulting in a significantly higher $CD4^+/CD8^+$ ratio compared to OTI^+ThPOK-H^+ or OTI^+dLGF$^+$ mice (**Fig. 6C**). Of note, the frequency and absolute number of DN mature T-cells were also reduced in the spleen of triple transgenic mice compared to OTI^+ThPOK-H^+ mice but was still higher compared to OTI^+dLGF$^+$ mice (**Fig. 6A, 6B**). To ascertain that the efficient $CD8^+$ to $CD4^+$ lineage redirection was not specific to the introduction of dLGF transgene into OTI^+ThPOK-H^+ mice, we analyzed the $CD4^+/CD8^+$ phenotype of OTI^+dLGF$^+$ThPOK-163^+ mice as well. Indeed, an increase in the $CD4^+$ and a decrease in the $CD8^+$ mature T-cell frequency was observed in the thymus and spleen of OTI^+dLGF$^+$ThPOK-163^+ mice compared to OTI^+ThPOK-163^+ mice (**Fig. S4D**). We then evaluated expression of *St8sia6* and *St3gal2*, the helper lineage associated genes [143, 326], and *Cxxc5*, a ThPOK target gene that negatively

regulates CD154 and is highly expressed in $CD8^+$ mature T-cells [427]. Indeed, QPCR analysis showed significantly elevated expression of *St8sia6* and *St3gal2*, and decreased expression of *Cxxc5* in $CD4^+$ mature T-cells from the triple transgenic mice, which was similar to expression of these genes in $CD4^+$ mature T-cells but opposite to their expression in $CD8^+$ mature T-cells from WT mice (**Fig. 6D**). Note that the DN mature T-cells from the triple transgenic mice showed significantly lower *St8sia6* and *St3gal2* expression compared to $CD4^+$ mature T-cells from the same mice. Interestingly, DN mature T-cells, which upon activation failed to upregulate CD154, expressed very little *Cxxc5* suggesting possible complex regulation of CD154 expression in the activated $CD4^+$ mature T-cells [427]. Upregulation of *St8sia6*, *St3gal2* and suppression of *Cxxc5* was also observed in the redirected $CD4^+$ mature T-cells isolated from OTI^+ThPOK-163^+ and OTI^+ThPOK-611^+ mice as well (**Fig. 4SE**). Together, these data strongly suggest that elevated TCR signal strength and transgenic ThPOK act synergistically in redirecting MHC-I-signaled thymocytes into the $CD4^+$ helper T-cell lineage.

2.4.7 Evaluating contribution of transgenic and endogenous ThPOK in the $CD8^+$ to $CD4^+$ lineage redirection in the presence of augmented TCR signaling

An efficient $CD8^+$ to $CD4^+$ lineage redirection of MHC-I-specific thymocytes in the presence of augmented TCR signaling and ~ two-fold more transgenic ThPOK protein (compared to endogenously expressed ThPOK in WT $CD4^+$ mature T-cells) in OTI^+dLGF$^+$ThPOK-H^+ and OTI^+dLGF$^+$ThPOK-163^+ mice could be due to two overlapping possibilities; augmented TCR signaling (a) induces endogenous ThPOK that contributes to this lineage redirection or (b) plays a role in the $CD4^+$ lineage choice of MHC-I-specific thymocytes that is independent of ThPOK. To investigate these possibilities, first we measured endogenous

ThPOK levels in the positively selected thymocytes from OTI^{+}dLGF^{+} mice. As expected, we detected a significant ThPOK induction in the CD4^{+}CD8^{lo} thymocytes from OTI^{+}dLGF^{+} mice compared to OTI control (**Fig. 7A**); ThPOK induction was essential for the generation of CD4^{+} mature T-cells in these mice as indicated by the absence of these cells in the thymus and spleen of OTI^{+}dLGF^{+}*Thpok*$^{-/-}$ mice (**Fig. 7B**). Thus, it was conceivable that the induction of endogenous ThPOK due to increased TCR signal strength substantially contributed to the increased frequency of CD4^{+} mature T-cells in OTI^{+}dLGF^{+}ThPOK-H^{+} mice. Therefore, to evaluate relative contribution of the two sources of ThPOK (transgenic and endogenous) in the CD4^{+} lineage choice in triple transgenic mice we ablated ThPOK expression in these mice (all mice *Rag*$^{-/-}$). We predicted that if endogenous ThPOK induced by augmented TCR signaling primarily contributed to the increased frequency of CD4^{+} mature T-cells in OTI^{+}dLGF^{+}ThPOK-H^{+} mice then ablating endogenous ThPOK in these mice would result in the CD4^{+} frequency that would be lower compared to the CD4^{+} frequency in OTI^{+}dLGF^{+}ThPOK-H^{+}ThPOK$^{+/+}$ mice but it would be comparable to the CD4^{+} frequency in OTI^{+}ThPOK-H^{+}ThPOK$^{-/-}$ mice (**Fig. 4B**). Indeed, ablating the endogenous ThPOK expression resulted in a small but significant decrease (P<0.02) in the splenic CD4^{+} mature T-cell frequency in OTI^{+}dLGF^{+}ThPOK-H^{+}*Thpok*$^{-/-}$ mice compared to ThPOK-sufficient control mice; more than 50% of mature T-cells were still CD4^{+} in the thymus and spleen of OTI^{+}dLGF^{+}ThPOK-H^{+}*Thpok*$^{-/-}$ mice compared to more than 60% in triple transgenic ThPOK sufficient mice (**Fig. 7C, 7D**). Importantly, despite expressing only the transgene encoded ThPOK the CD4^{+} mature T-cells frequency (52%) in the spleen of OTI^{+}dLGF^{+}ThPOK-H^{+}*Thpok*$^{-/-}$ mice was still significantly higher than the CD4^{+} mature T-cell frequency (20%) observed in the spleen of OTI^{+}ThPOK-H^{+}*Thpok*$^{-/-}$ mice (**Fig. 7C, 7D**; P < 0.0001). The frequency and number of DN and CD8^{+} splenic T-cell subsets were comparable in

triple transgenic mice expressing endogenous ThPOK or not (**Fig. 7D**). To ascertain the observed differential $CD4^+$ frequency in $OTI^+dLGF^+ThPOK\text{-}H^+$*Thpok*$^{-/-}$ mice, we analyzed CD4/CD8 phenotype of $OTI^+dLGF^+ThPOK\text{-}163^+$*Thpok*$^{-/-}$ mice as well. Indeed, we observed only a small decrease in the splenic $CD4^+$ mature T-cell frequency in $OTI^+dLGF^+ThPOK\text{-}163^+$*Thpok*$^{-/-}$ mice (48%) compared to $OTI^+dLGF^+ThPOK\text{-}163^+$*Thpok*$^{+/+}$ mice (54%) but it was higher compared to that in $OTI^+ThPOK\text{-}163^+$*Thpok*$^{-/-}$ mice (29%; **Fig. S4D**). Of note, ThPOK expression analysis showed slightly more frequency of $ThPOK^{lo}$ DP thymocytes from $OTI^+dLGF^+ThPOK\text{-}H^+$*Thpok*$^{-/-}$ mice compared $OTI^+ThPOK\text{-}H^+$*Thpok*$^{-/-}$ control suggesting a possible influence of augmented TCR signaling on the transgenic ThPOK levels in DP thymocytes (**Fig. 7E**). Significantly higher phospho-Src staining in DP and $CD4^+8^{lo}$ thymocytes confirmed augmented TCR signaling in OTI^+dLGF^+ mice expressing ThPOK-H or not compared to OTI or WT control (**Fig. 7F, 7G**). We also observed elevated, albeit insignificant, phospho-CD3ζ levels in DP and $CD4^+CD8^{lo}$ thymocytes from these mice compared to OTI control (it was significantly higher compared to similar subsets from WT mice (**Fig. 7F, 7G**)). Note that the increased pSrc and pCD3ζ staining observed in DP thymocytes in OTI mice expressing dLGF transgene became less pronounced in $CD4^+CD8^{lo}$ thymocytes reflecting possible impact of intra-thymic signaling and/or the limit of sensitivity of phospho specific antibody staining. We then evaluated expression of $CD4^+$ lineage genes in purified T-cells from the triple transgenic *Thpok*$^{-/-}$ mice. Indeed, expression pattern of *St8sia6*, *St3gal2* and *Cxxc5* in $CD4^+$ mature T-cells from $OTI^+dLGF^+ThPOK\text{-}H^+$*Thpok*$^{-/-}$ mice was similar to that in $CD4^+$ mature T-cells from WT mice, which is upregulation of *St8sia6*, *St3gal2* and down regulation of *Cxxc5* (**Fig. 7H**). Collectively, these data strongly suggest that augmenting TCR signal strength in MHC-I-specific thymocytes significantly promotes the ThPOK-induced $CD8^+$ to

$CD4^+$ lineage redirection. These data also suggest that TCR signaling plays a role in $CD4^+$ lineage choice that may be independent of ThPOK.

The $CD4^+$ mature T-cell frequency in OTI^+dLGF^+ThPOK-H^+*Thpok*$^{-/-}$ mice, while higher than the $CD4^+$ mature T-cell frequency in OTI^+ThPOK-H^+*Thpok*$^{-/-}$ mice, was still significantly lower than the $CD4^+$ mature T-cell frequency in $OTII^+$ThPOK-H^+*Thpok*$^{-/-}$ mice (**Fig. 8A**; $P < 0.0005$). $CD4^+$ mature T-cells in all these three mouse strains expressed the same amount of ThPOK but received differential intra-thymic signaling (MHC-I-induced signaling, MHC-I-induced signaling combined with augmented TCR signaling, and MHC-II-induced signaling). Thus, it was possible that TCR signaling in MHC-I-specific $CD4^+$ mature T-cells from OTI^+dLGF^+ThPOK-H^+ mice, while elevated compared to that in MHC-I-specific $CD4^+$ mature T-cells from OTI^+ThPOK-H^+ mature T-cells, may still be lower than that in MHC-II-specific $CD4^+$ mature T-cells from OTII mice. Therefore, we compared CD5 levels in the thymic and splenic T-cells from OTI mice expressing or not dLGF with that from OTII mice to assess their TCR signal strength. In each experiment, we calculated CD5 levels in the thymocytes and mature T-cell subsets from various mice relative to CD5 levels in the relevant thymic subsets or $CD8^+$ mature T-cells from OTI mice. As expected, DP and $CD4^+CD8^{lo}$ thymocytes from OTI mice expressing dLGF transgene showed significantly higher CD5 levels compared to OTII subset (**Fig. 8B**). Interestingly, CD5 levels in $CD4^+$ thymocytes from the two mice were quite comparable (**Fig. 8C**) likely reflecting stronger intra-thymic signaling transduced in $OTII^+$ thymocytes but became significantly higher in the $CD4^+$ splenic T-cells from OTI^+dLGF^+ mice compared to that from OTII mice (**Fig. 8D**). In OTI^+dLGF^+ThPOK-H^+ mice, we observed a similar trend in CD5 expression levels in DP, $CD4^+CD8^{lo}$ thymocytes (**Fig. 8B, 8C**) and $CD4^+$ splenic T-cell (**Fig. 8D**) subsets compared to similar subsets from OTII mice. Of note, CD5

levels were lower in $CD4^+$ mature thymocytes and splenic T-cells from $OTI^+dLGF^+ThPOK\text{-}H^+$ mice compared to CD5 levels in similar subsets from OTI^+dLGF^+ mice. As well, CD5 levels in DN and/or $CD8^+$ mature T-cells from OTI^+dLGF^+ or $OTI^+ThPOK\text{-}H^+$ mice were lower compared to that in $CD8^+$ mature T-cells from OTI mice. Analysis of CD5 levels in the splenic $CD4^+$ mature T-cells from $OTI^+dLGF^+ThPOK\text{-}163^+$ mice also showed similar trend. CD5 levels in $CD4^+$ mature T-cells from $OTI^+dLGF^+ThPOK\text{-}163^+$ mice was significantly higher compared to that in $CD4^+$ mature T-cells from OTII mice, and it was slightly lower compared to CD5 levels in $CD4^+$ mature T-cells from OTI^+dLGF^+ mice (**Fig. S4F**). Although unclear but differential intra-thymic signaling, which influences CD5 levels and correlates with mature T-cell function [375, 428], may be responsible for the altered CD5 levels in $CD4^+$ or $CD8^+$ mature T-cells in the presence of transgenic ThPOK in OTI^+dLGF^+ mice. Nevertheless, these data support the notion that TCR signaling in MHC-I-specific OTI^+ thymocytes expressing dLGF transgene is significantly higher than that in MHC-II-specific $OTII^+$ thymocytes. Collectively, our in-depth analysis of $CD4^+/CD8^+$ lineage choice of MHC-I-specific thymocytes with or without augmented TCR signaling and of MHC-II-specific thymocytes in the presence of same amount of ThPOK strongly suggest that ThPOK-induced $CD4^+$ lineage choice of developing thymocytes is critically influenced by quantitative as well as differential TCR signaling.

2.5 Discussion

In the present manuscript, we have investigated the impact of ThPOK levels on the $CD4^+$ lineage choice of MHC-I- and MHC-II-specific thymocytes, and role of TCR signaling in it. Specifically, we have evaluated the impact of ThPOK levels on the $CD4^+$ lineage choice of thymocytes with differential TCR signaling. Our data strongly suggest that MHC-I-restricted thymocytes require higher level of ThPOK in preselection thymocytes for an efficient $CD8^+$ to $CD4^+$ lineage redirection (ThPOK-611 mice), while relatively low/moderate levels (still higher than ThPOK levels in WT $CD4^+$ mature T-cells) result in the partial $CD8^+$ to $CD4^+$ lineage redirection (ThPOK-H and ThPOK-163 mice). The lower frequency of mature T-cells observed in the spleen of OTI^+ThPOK-163^+ and OTI^+ThPOK-611^+ mice likely reflects reduced thymic maturation and/or the effect of mismatched co-receptor expression ($CD4^+$ T-cells) or lack of co-receptor expression (DN T-cells) on survival and/or homeostatic expansion of the redirected T-cells (ref 23, 24). A consequence of partial lineage redirection is that a substantial number of $CD8^+$ and DN mature T-cells are detected in OTI^+ThPOK-H^+ or OTI^+ThPOK-163^+ mice. It is interesting that the DN mature T-cells in OTI^+ThPOK-H^+ mice fail to maintain CD4 expression despite almost complete suppression of Runx3 expression indicating complex regulation of *Cd4* expression requiring sustained TCR signaling in developing thymocytes [429]. *In vitro* differentiation culture data supports such a notion; disrupting intra-thymic TCR signaling in *in vitro* culture of purified $CD4^+$ thymic subsets from OTI^+ThPOK-H^+ mice results in the loss of CD4 expression in a significant number of cells leading to the generation of DN mature T-cells. Importantly, DN and $CD8^+$ mature T-cells, particularly the former, continued to express a substantial amount of ThPOK that compromised the cytotoxic function but still failed to activate the helper program in these cells. A simple explanation would be that activation of the helper

program in MHC-I-signaled thymocytes requires a higher amount of ThPOK than that required for suppression of the cytotoxic program (phenotype of DN mature T-cells). However, it was paradoxical that ThPOK level in DN mature T-cells from OTI^{+}ThPOK-H^{+} or OTI^{+}ThPOK-163^{+} mice was significantly higher compared to endogenous ThPOK levels in MHC-II-restricted CD4^{+} mature T-cells from WT mice, and yet failed to redirect them into the CD4^{+} lineage. The inability of ThPOK-H to induce efficient CD8^{+} to CD4^{+} lineage redirection is unlikely due to variegated expression as the same ThPOK-H transgene completely rescued CD4^{+} development in *Thpok*$^{-/-}$ mice expressing or not OTII-TCR.

We propose two mutually non-exclusive possibilities that may explain the ability of the same amount of transgenic ThPOK to completely rescue the CD4^{+} development in *Thpok*$^{-/-}$ or OTII^{+}*Thpok*$^{-/-}$ mice but induce an inefficient CD8^{+} to CD4^{+} lineage redirection of MHC-I-specific thymocytes (in OTI^{+}, P14^{+} or MHC-II$^{-/-}$ mice). It is possible that genes responsible for activating helper program in MHC-I-specific thymocytes, due to weak or shorter duration of TCR signaling, are epigenetically modified in such a way that they are inaccessible or accessible for a shorter time for ThPOK-mediated regulation, and in such a case significantly higher amount of ThPOK (than the one required for CD4^{+} lineage choice of MHC-II-specific thymocytes) would be required to override this constrain on CD4^{+} lineage choice of MHC-I-specific thymocytes. Significantly higher CD4^{+} mature T-cell frequency in OTI^{+}dGLF^{+}ThPOK-H^{+}*Thpok*$^{-/-}$ mice compared to OTI^{+}ThPOK-H^{+}*Thpok*$^{-/-}$ mice, both expressing the same amount of ThPOK but differing in their TCR signal strength, strongly suggest that TCR signal strength plays a critical role in establishing the ThPOK-mediated CD4^{+} lineage choice. We propose that augmented TCR signal strength, while critical for ThPOK induction, promotes the CD4^{+} lineage choice by extending the window of lineage choice during which the target gene loci are

accessible readily or for a longer time for ThPOK-mediated modulation. Any MHC-I-signaled thymocytes expressing ThPOK at levels comparable to that induced in MHC-II-signaled thymocytes but remain outside this temporal lineage commitment window will differentiate into $CD8^+$ mature T-cells with compromised cytotoxic function. Such a possibility is supported by the observation that ThPOK induction in MHC-I-signaled thymocytes, due to compound deficiency of Runx1 and Runx3 or Tle1/3/4 or MAZR and Runx3, while upregulates helper lineage genes including *Cd4*, fails to completely suppress expression of cytotoxic lineage genes including *Cd8* resulting in the generation of a large number of $CD4^+CD8^+$ mature T-cells of undefined functional potential [267, 278, 326]. Compromised cytotoxic function but failure to upregulate CD4 or secrete IL-4 following retroviral-mediated ThPOK expression in the peripheral mature $CD8^+$ mature T-cells also supports such a notion [328]. These data are in agreement with signal strength model of $CD4^+/CD8^+$ lineage commitment; irrespective of MHC specificity stronger TCR signaling may alter chromatin structure such that not only the $CD4^+$ lineage specifying genes such as *Gata3*, *Tox* or *c-Myb* are induced [149, 272, 430, 431] but accessibility of the target gene loci by ThPOK is enhanced as well leading to suppression of the $CD8^+$ cytotoxic lineage choice and imprinting of the $CD4^+$ helper lineage choice in these cells. These data also suggest that stronger TCR signaling may be sufficient for the $CD4^+$ lineage commitment even in the absence of ThPOK provided those critical for the $CD8^+$ lineage commitment are suppressed [320].

While strong TCR signaling is critical for the $CD4^+$ lineage choice, the MHC specificity of developing thymocytes appears to play an equally important role in the process. Comparing the $CD4^+$ development of MHC-I-specific thymocytes in OTI^+dLGF^+ThPOK-$H^+Thpok^{-/-}$ and MHC-II-specific thymocytes in $OTII^+$ThPOK-H^+*Thpok*$^{-/-}$ mice provides some insight into this

issue. Significantly higher frequency of $CD4^+$ mature T-cells in $OTII^+$ThPOK-H^+*Thpok*$^{-/-}$ mice (MHC-II-specific) compared to OTI^+dLGF$^+$ThPOK-H^+*Thpok*$^{-/-}$ mice (MHC-I-specific), both expressing the same amount of ThPOK, cannot simply be explained by TCR signal strength model as thymic subsets and $CD4^+$ mature T-cells from $OTII^+$ mice show significantly lower TCR signaling compared to that in similar subsets from OTI^+dLGF$^+$ mice expressing or not ThPOK transgene. Our data then suggest that TCR signaling in MHC-I- and MHC-II-specific thymocytes, while quantitatively different, are likely to be qualitatively different as well, and introducing constitutively active Lck in MHC-I-specific thymocytes mimics the quantitative aspect. We propose that continuous TCR signaling in the positively selected MHC-II-specific thymocytes not only results in stronger TCR signal that keeps the lineage commitment window "open" for longer time but also induces expression of $CD4^+$ lineage establishing genes whose continued expression likely requires ThPOK.

Based on these data we propose a model that links TCR signaling to $CD4^+$/$CD8^+$ lineage choice of MHC-I- and MHC-II-signaled thymocytes. We propose that qualitatively distinct and stronger TCR signaling opens the window of lineage commitment during which the $CD4^+$ lineage specifying genes are induced in MHC-II-signaled thymocytes. It is conceivable that during the $CD4^+$ lineage specification phase *Gata3* induced by TCR signaling functions, for instance, as a "pioneer" transcription factor that remodels the chromatin landscape, which then facilitates the ability of other transcription factors to access the target gene loci in association with or independently of the pioneering factor [71, 432, 433]. The persistent TCR signaling, along with Gata3 expression, would then initiate ThPOK induction [274, 322] in MHC-II-signaled thymocytes, which collectively play a role in the $CD4^+$ lineage commitment and maintenance. The induction of helper program in MHC-I-signaled thymocytes but inability to

sustain it [217] may be due to inadequate chromatin alterations leading to insufficient induction of ThPOK and/or its residency at the target gene loci. In such a case very high ThPOK expression would be necessary for efficient redirection of the MHC-I-signaled thymocytes into the $CD4^+$ lineage.

In conclusion, considerably different efficiency of $CD4^+$ lineage choice in three different mouse models expressing the same amount of ThPOK but different modes of TCR signaling (OTI^+ThPOK-H^+*Thpok*$^{-/-}$, OTI^+dLGF$^+$ThPOK-H^+*Thpok*$^{-/-}$ and $OTII^+$ThPOK-H^+*Thpok*$^{-/-}$) provides a critical in-sight into the mechanism of CD4 lineage choice of developing thymocytes. Our data link stronger TCR signaling to ThPOK induction and strongly suggest that the $CD4^+$ lineage choice by a defined amount of ThPOK is critically influenced by TCR signal strength and MHC specificity of developing thymocytes during a temporal window of lineage commitment.

2.6 Figures and figure legends

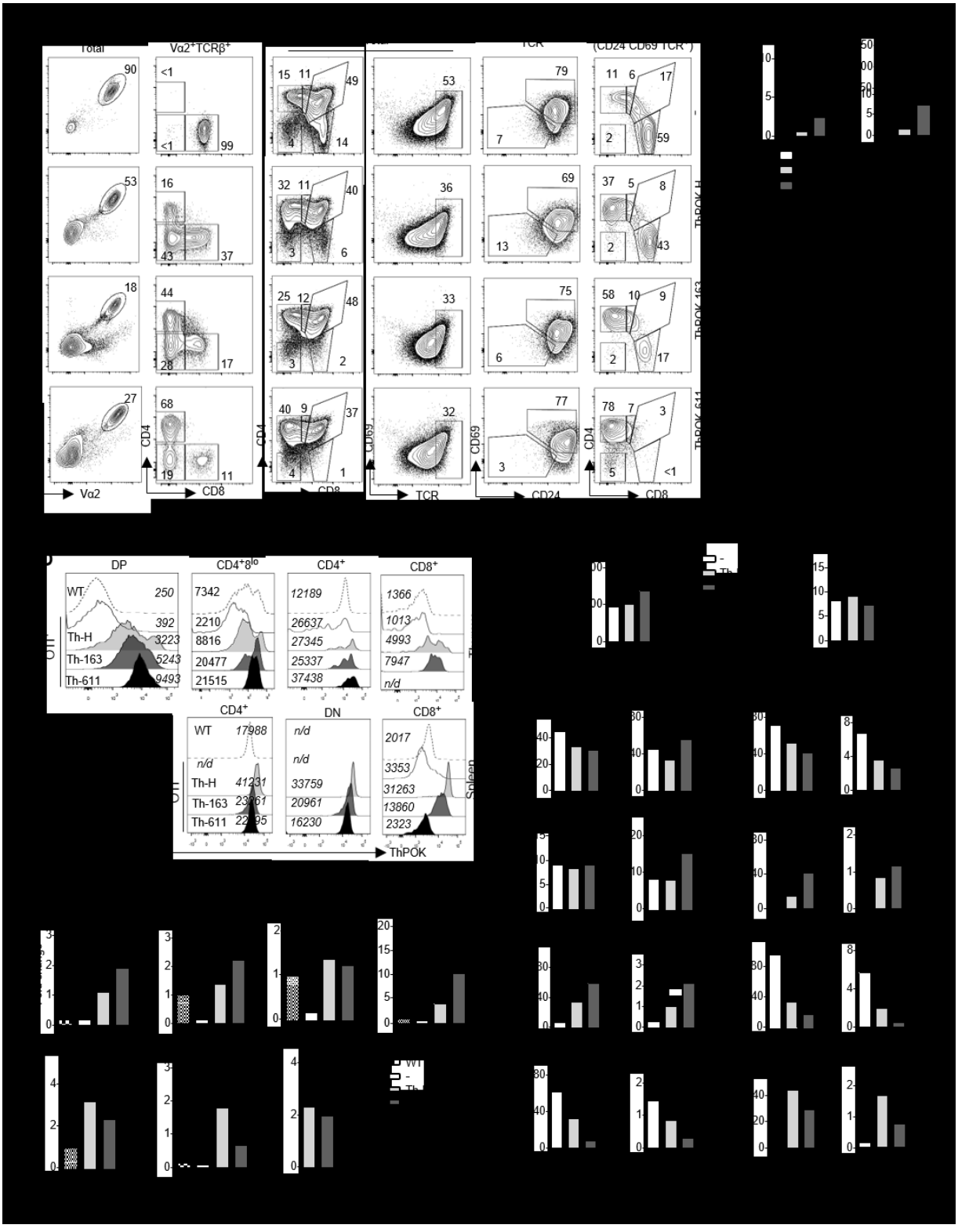
Total
Vα2+TCRβ+
(CD24+CD69+TCR+)
Vα2
CD4
CD8
TCR
CD24
CD69
ThPOK
DP
CD4+8lo
CD4+
CD8+
DN
WT
Th-H
Th-163
Th-611
CTH
Spleen

Figure 12. ThPOK dose impacts the $CD8^+$ to $CD4^+$ lineage redirection

To assess the impact of individual ThPOK transgene on the $CD8^+$ to $CD4^+$ lineage redirection, each ThPOK transgenic line was introduced into $OTI^+Rag^{-/-}$ mice and T-cells were analyzed by flow cytometry. **(A)** Frequency of mature T-cells ($TCR\beta^+V\alpha2^+$) and CD4/CD8 profiles of splenic T-cells in OTI mice expressing the indicated ThPOK transgene are shown. **(B)** CD4/CD8 and CD69/TCR profile of total thymocytes, CD69/CD24 profile of TCR^+ thymocytes and CD4/CD8 profile of mature thymocytes ($CD69^-CD24^-TCR^+$) from the indicated strain of mice are shown. **(C)** The $CD4^+/CD8^+$ ratio for $V\alpha2^+$ T-cells from the spleen **(left)** and mature thymocytes **(right)** for the indicated strain of mice is shown. **(D)** ThPOK protein levels in DP, mature $CD4^+$ and $CD8^+$ thymocytes ($CD69^-CD24^-TCR^+$; top histograms) and splenic T-cell subsets (bottom histograms) from the indicated strain of mice are shown. Numbers in histograms represent the Mean Fluorescence Intensity (MFI) values. **(E)** Compilations of ThPOK MFI for the indicated thymic and splenic T-cell subsets from the indicated mice are shown. For DP thymocytes ThPOK MFI is relative that in DP thymocytes from ThPOK-H. For all other thymic subsets, ThPOK MFI is relative to the corresponding WT thymic subset. ThPOK MFI for splenic subsets is relative to that in splenic $CD4^+$ mature T-cells from WT. Note that ThPOK MFI for $CD4^+$ mature T-cells from OTI mice and DN mature T-cells from OTI and WT mice are not determined due to lack of a substantial number of these cells. **(F)** The frequency and absolute number of TCR^+ and $CD4^+CD8^{lo}$ subsets in total thymocyte, and $CD4^+$ and $CD8^+$ mature thymocytes ($CD24^-CD69^-TCR^+$) in the indicated mice are shown. Also shown are total splenocytes and the frequency and number of splenic T-cells and T-cell subsets from OTI mice expressing or not the indicated ThPOK transgene ($n>12$). Data are representative examples of four or more independent experiments **(A, B and D)**. n/d not determined.

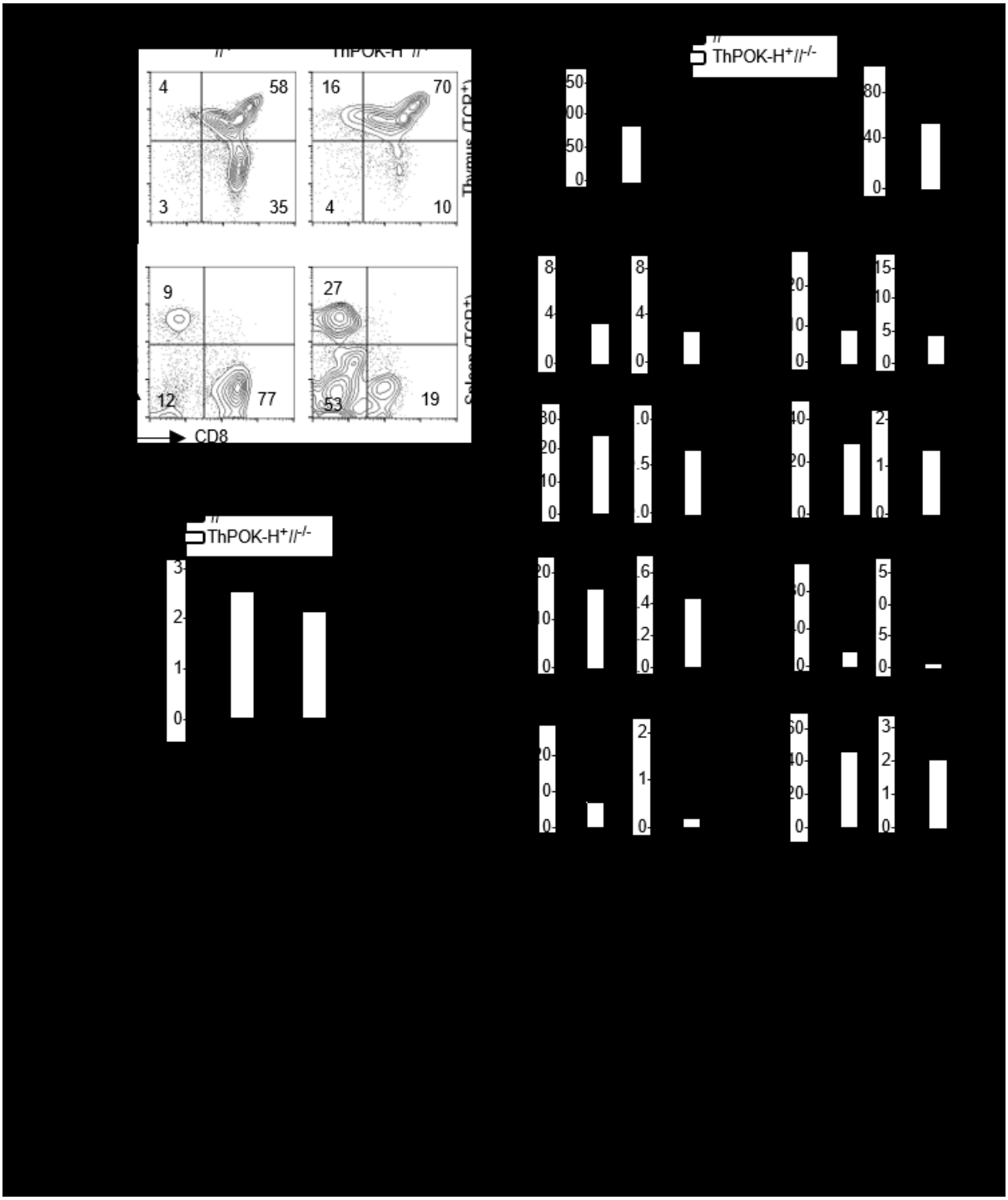
ThPOK-H+//-/-
4
58
3
35
16
70
4
10
9
12
77
27
53
19
CD8

Figure 13. ThPOK-induces partial $CD8^+$ to CD4+ lineage redirection in *MHC-II*$^{-/-}$ mice

The CD4/CD8 profiles of TCR^+ thymocytes **(A)** and splenocytes **(B)** from *MHC-II*$^{-/-}$ **(left)** and ThPOK-H^+*MHC-II*$^{-/-}$ **(right)** mice are shown. **(C)** $CD4^+/CD8^+$ ratio in the thymus and spleen of *MHC-II*$^{-/-}$ (black bars) and ThPOK-H^+*MHC-II*$^{-/-}$ (white bars) mice is shown. **(D)** The frequency and absolute number of TCR^+ and $CD4^+CD8^{lo}$ subsets in total thymocytes, and $CD4^+$ and $CD8^+$ mature thymocytes (TCR^+) in *MHC-II*$^{-/-}$ expressing or not ThPOK-H are shown. Also shown are total splenocytes and the frequency and number of splenic T-cells and T-cell subsets from *MHC-II*$^{-/-}$ mice expressing or not ThPOK-H transgene (n>8). Data are representative of six or more independent experiments **(A and B)**.

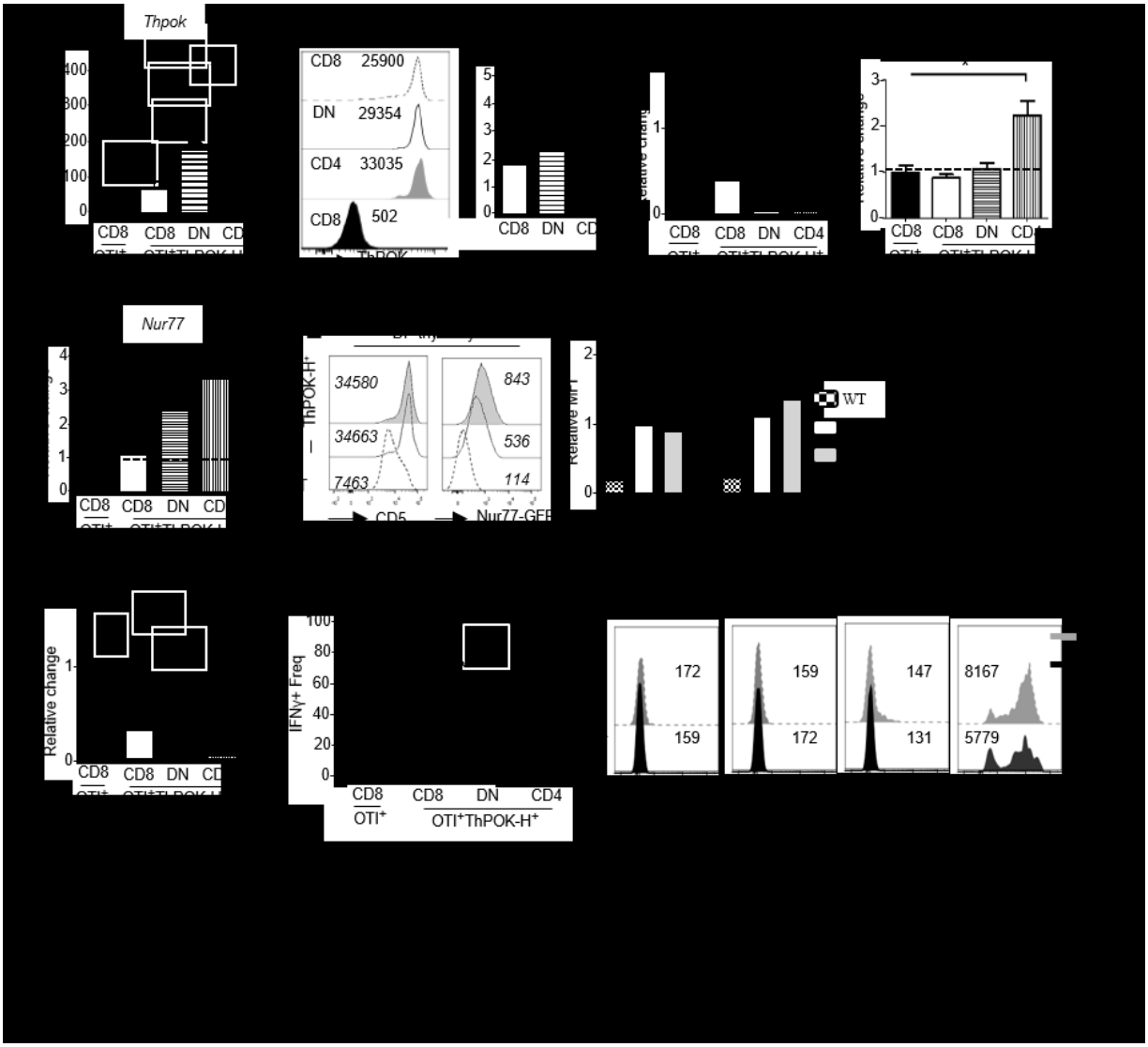
Thpok
CD8 25900
DN 29354
CD4 33035
CD8 502
CD8 CD8 DN CD4
Nur77
34580
34663
7463
843
536
114
WT
Relative change
IFNγ+ Freq
172
159
159
172
147
131
8167
5779
OTI+
OTI+ThPOK-H+

Figure 14. ThPOK modulates lineage specific gene expression in T-cell subsets

$CD4^+$, $CD8^+$, and DN mature T-cells from the spleen of OTI^+ThPOK-H^+ mice were isolated and expression levels of *Thpok* **(A, left)**, *Runx3d* **(B)**, *Socs1* **(C)**, and *Nur77* **(D)** were evaluated by QPCR, and were compared to that in $CD8^+$ mature T-cells from OTI mice (normalized to *Hprt* expression). Data depicts average of triplicate values with standard deviation and are expressed as fold increase over expression of individual genes in control $CD8^+$ mature T-cells from OTI mice. **(A, right)** ThPOK protein levels in the indicated splenic T-cell subsets from OTI^+ThPOK-H^+ or OTI^+ mice are shown. Also shown is ThPOK MFI compilation for the indicated T-cell subsets (relative to WT $CD4^+$ T-cells). **(E)** MFI of CD5 and Nur77-GFP expression in DP thymocytes from OTI^+ThPOK-H^+, OTI and WT mice are compared **(left)** and compiled (bottom; relative to OTI). **(F)** shows perforin levels by QPCR in $CD4^+$, DN and $CD8^+$ mature T-cells from the spleen of OTI^+ThPOK-H^+ mice compared to $CD8^+$ mature T-cells from OTI control mice (normalized to *Hprt* expression). **(G)** Purified T-cell subsets from OTI mice expressing or not ThPOK-H were activated for 5-7 days in the presence of irradiated splenocytes from BL/6 mice pulsed with OTI peptide (SIINFEKL) and then re-stimulated with PMA/ionomycin in the presence of brefeldin for analysis of IFN expression. **(H)** CD154 expression in the cognate-peptide activated indicated T-cell subsets from OTI^+ and OTI^+ThPOK-H^+ mice is shown. Data are representative of two to six independent experiments.

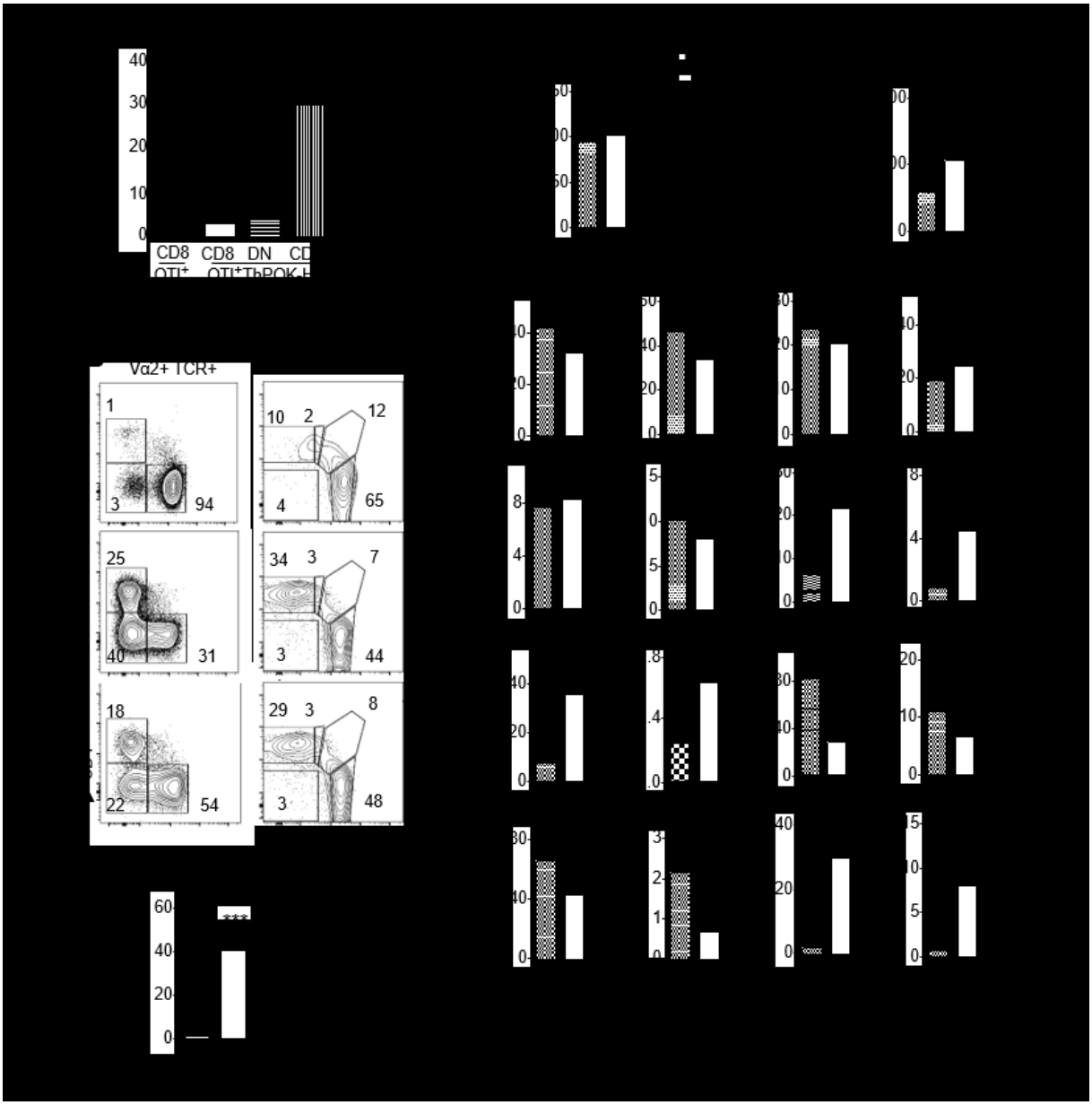
CD8
CD8
DN
Vα2+ TCR+
1
3
94
10
2
12
4
65
25
40
31
34
3
7
3
44
18
22
54
29
3
8
3
48
***

Figure 15. Insignificant contribution of endogenous ThPOK in the CD8$^+$ to CD4$^+$ lineage redirection

(A) To assess endogenous ThPOK levels various T-cell subset from OTI$^+$THPOK-H$^+$ mice were purified for QPCR analysis. Data shows endogenous ThPOK levels in the indicated splenic T-cell subsets from OTI$^+$ThPOK-H$^+$ mice compared to that in CD8$^+$ mature T-cells from OTI mice (normalized to *Hprt* expression). Data depicts average of triplicate values with standard deviation and are expressed as fold increase over endogenous ThPOK levels in control CD8$^+$ mature T-cells from OTI mice. To determine the impact of endogenous ThPOK expression on CD4 lineage choice OTI$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice. **(B)** shows flow cytometric analysis of the CD4/CD8 profiles of spleen and thymus from isolated from OTI$^+$, OTI$^+$ThPOK-H$^+$*Thpok*$^{+/+}$, and OTI$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice. **(C)** CD4$^+$/CD8$^+$ ratio in the spleen of indicated mice relative to OTI is shown. **(D)** The frequency and absolute number of TCR$^+$ and CD4$^+$CD8^{lo} subsets in total thymocytes, and CD4$^+$ and CD8$^+$ mature thymocytes (CD24$^-$CD69$^-$TCR$^+$) in the indicate mice are shown (n>6). Also shown are total splenocytes and the frequency and number of splenic T-cells and T-cell subsets in these mice. Data representative of three or more independent experiments **(A, B)**.

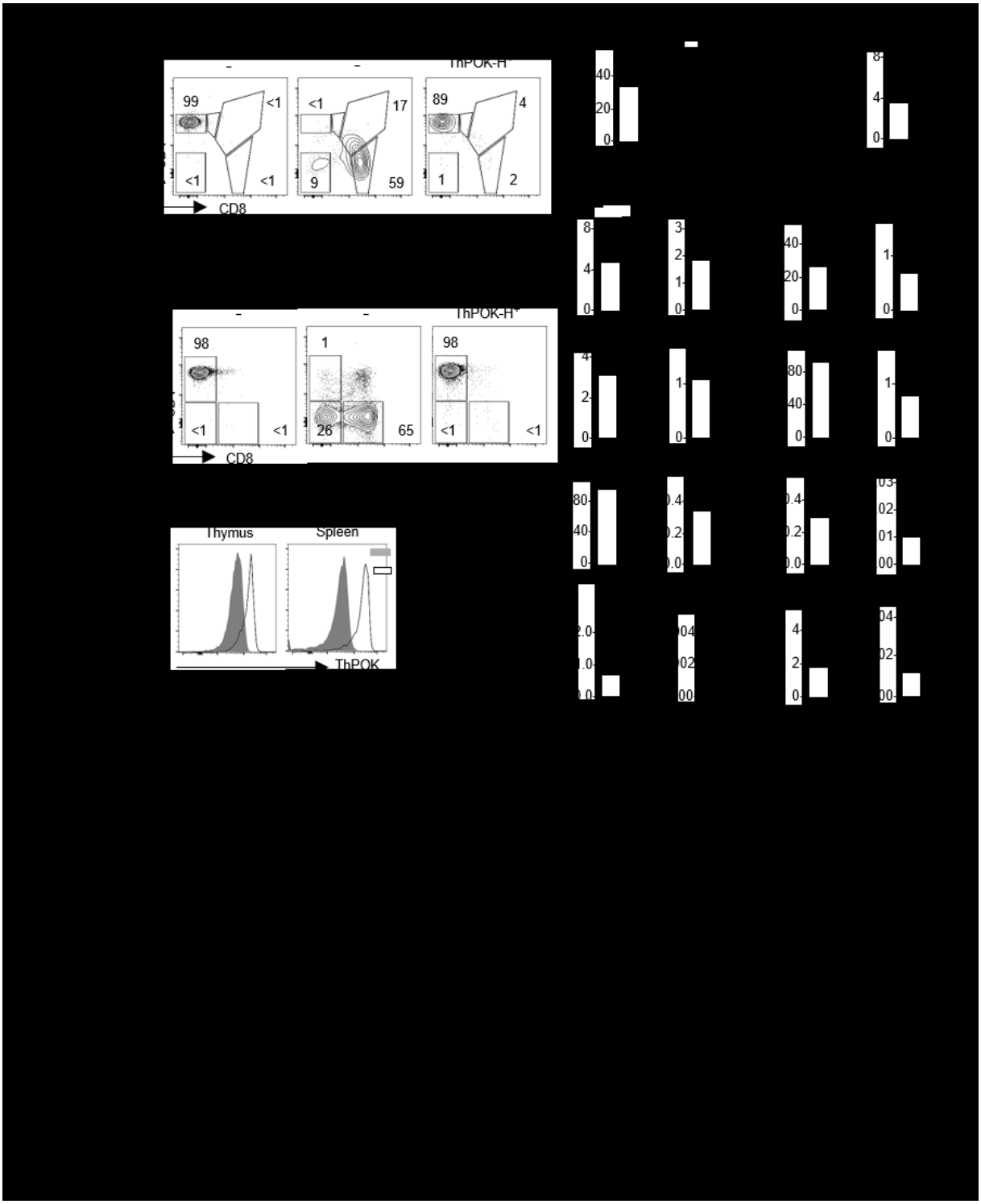
ThPOK-H+
99
<1
<1
<1
CD8
<1
17
9
59
89
4
1
2
98
<1
<1
CD8
1
26
65
98
<1
<1
Thymus
Spleen
ThPOK

Figure 16. ThPOK-H completely rescues $CD4^+$ development in $OTII^+$*Thpok*$^{-/-}$ mice.

To evaluate the ability of ThPOK-H transgene to rescue $CD4^+$ development of MHC-II-specific thymocytes, the transgene was introduced into $OTII^+$*Thpok*$^{-/-}$ mice. Panel **(A)** shows the CD4/CD8 profile of mature thymocytes ($CD24^-CD69^-TCR^+$) and panel **(B)** shows the CD4/CD8 phenotype of splenic T-cells from the indicated mice. **(C)** ThPOK protein levels in $CD4^+$ mature T-cells from the thymus and spleen of $OTII^+$*Thpok*$^{+/+}$ (shaded histogram) and $OTII^+$ThPOK-H^+*Thpok*$^{-/-}$ (open histogram) mice are shown. **(D)** The frequency and absolute number of TCR^+ and $CD4^+CD8^{lo}$ subsets in total thymocytes, and $CD4^+$ and $CD8^+$ mature thymocytes ($CD24^-$ $CD69^-TCR^+$) in $OTII^+$ThPOK-H^+*Thpok*$^{-/-}$ and littermate $OTII^+$ThPOK-H^+*Thpok*$^{+/+}$ (all *Rag*$^{-/-}$) control mice are shown. Also shown are total splenocytes and the frequency and number of splenic T-cells and T-cell subsets from the same mice (n>4). Data representative of three or more independent experiments **(A-C)**.

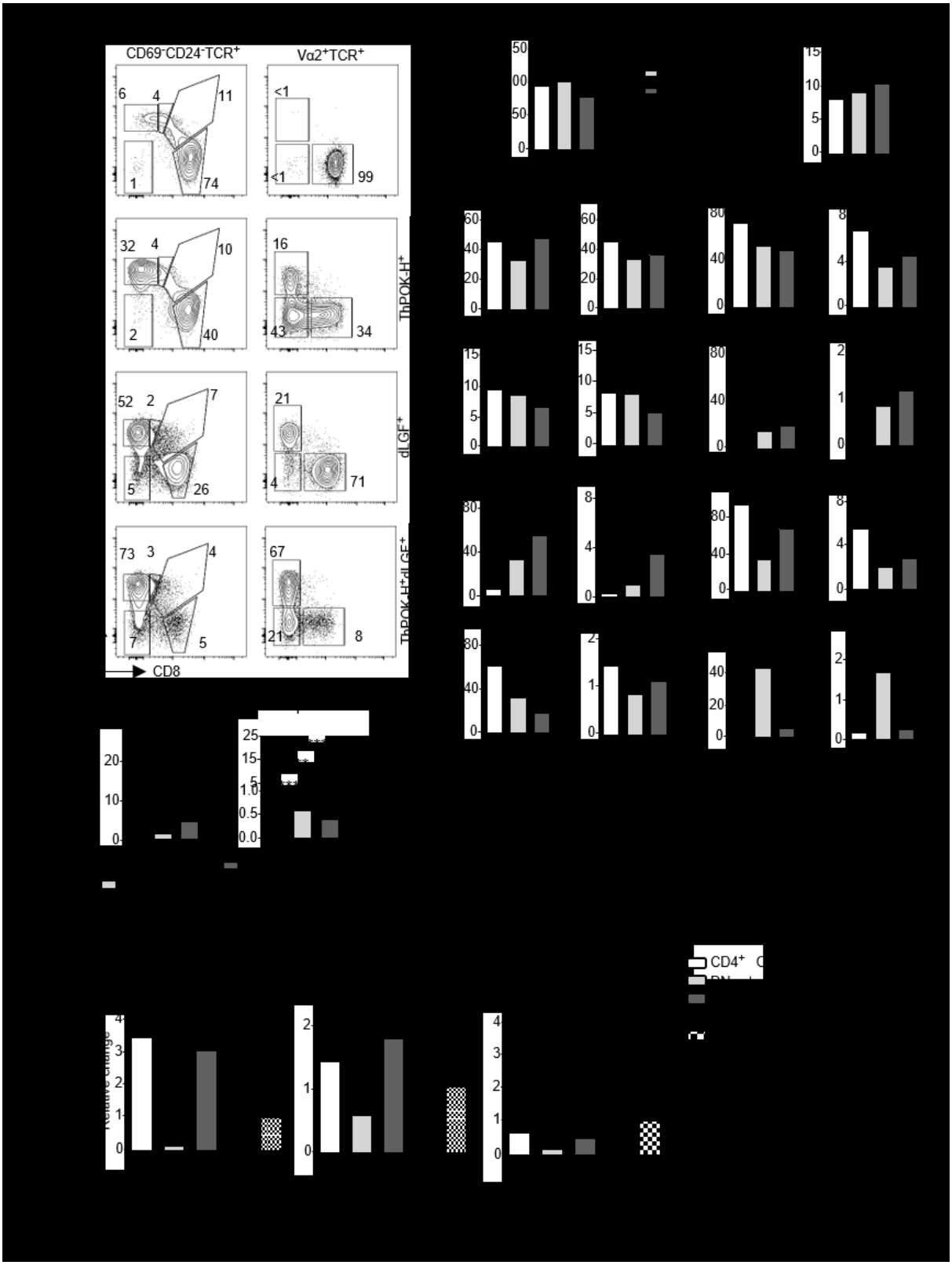
CD69-CD24-TCR+
Vα2+TCR+
6
4
11
1
74
<1
<1
99
32
4
10
2
40
16
43
34
ThPOK-H+
52
2
7
5
26
21
4
71
dLGF+
73
3
4
7
5
67
21
8
ThPOK-H+dLGF+
CD8
CD4+

Figure 17. Augmenting TCR signal strength enhances the $CD8^+$ to $CD4^+$ lineage redirection

Role of increased TCR signal strength in promoting $CD8^+$ to $CD4^+$ lineage redirection in $OTI^+ThPOK\text{-}H^+$ mice was investigated by introducing constitutively active Lck transgene (dLGF) into $OTI^+ThPOK\text{-}H^+$ mice. **(A)** shows a representative example of the CD4/CD8 profile of mature thymocytes **(left)** and splenic T-cells **(right)** from the indicated mice. **(B)** The frequency and absolute number of TCR^+ and $CD4^+CD8^{lo}$ subsets in total thymocytes, and $CD4^+$ and $CD8^+$ mature thymocytes ($CD24^-CD69^-TCR^+$) as well as splenic T-cells and subsets from the indicated mice are shown (n>6). **(C)** CD4/CD8 ratio of mature thymic and splenic T-cells in the indicated mice is shown. **(D)** Mature T-cells from the spleen of WT ($CD4^+$ and $CD8^+$), OTI^+dLGF^+ ($CD4^+$) and $OTI^+dLGF^+ThPOK\text{-}H^+$ ($CD4^+$ and DN) mice were isolated and expression of *St8sia6*, *St3gal2*, and *Cxxc5* was evaluated by QPCR. Data depicts average of triplicate values with standard deviation and are expressed as fold increase over expression of individual genes in control $CD4^+$ mature T-cells from WT mice (normalized to *Hprt* expression). Data are representative of more than six independent experiments **(A)** and two experiments **(D)**.

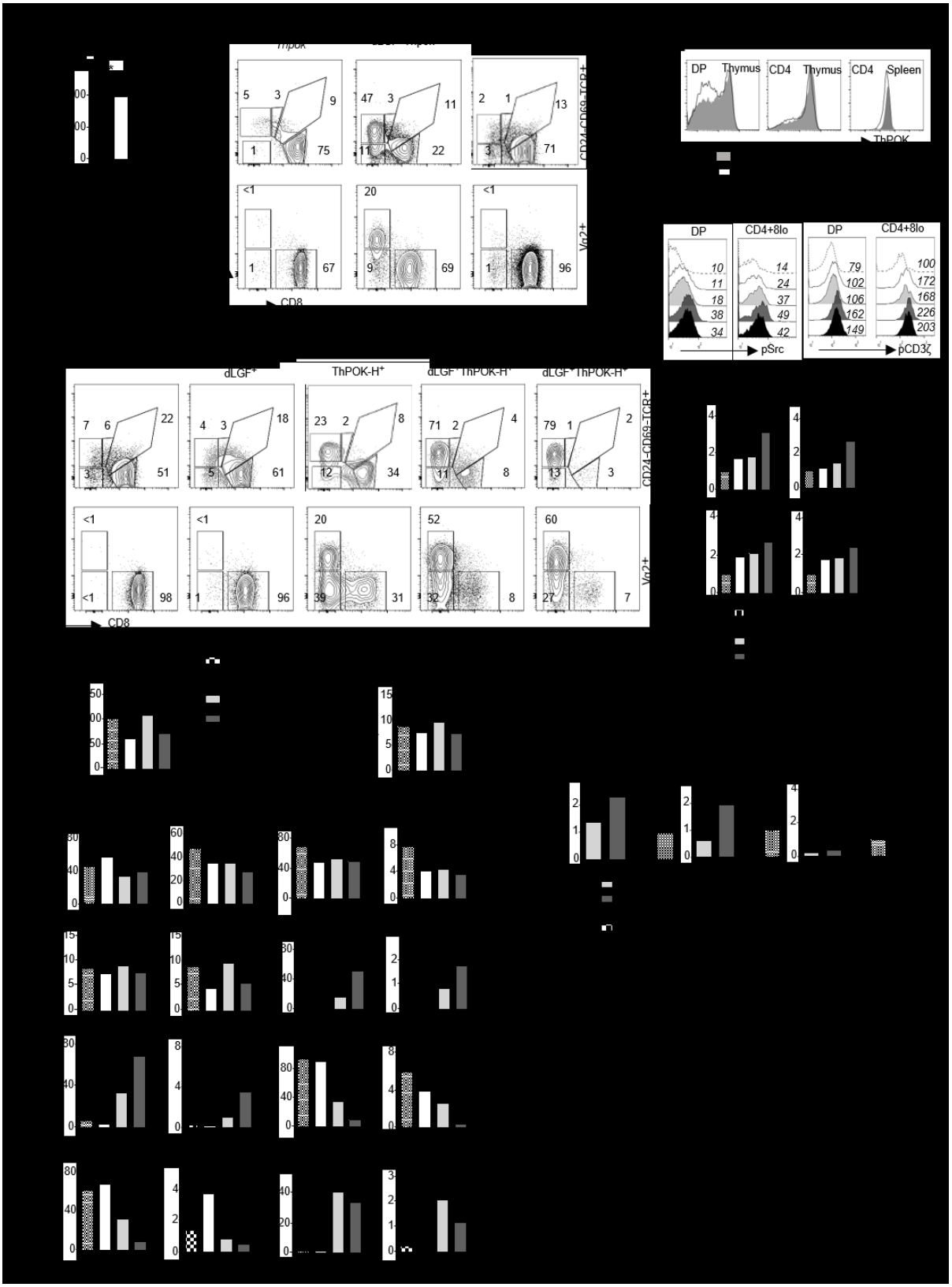
DP
Thymus
CD4
Thymus
CD4
Spleen
ThPOK
CD24+CD69+TCR+
Vα2+
CD8
pSrc
pCD3ζ
dLGF+
ThPOK-H+
dLGF+ThPOK-H+

Figure 18. Evaluating contribution of endogenous and transgenic ThPOK in $CD4^+$ lineage choice in $OTI^+dLGF^+ThPOK\text{-}H^+$ mice

(A) $CD4^+CD8^{lo}$ thymocytes were purified from OTI^+dLGF^+ and control OTI mice, and endogenous ThPOK expression was analyzed by QPCR. A representative example shows average of triplicate values $\pm$ SD and are expressed as relative fold increase over pre-selection DP thymocytes from OTI control mice. **(B)** shows the CD4/CD8 profiles of the mature thymocytes ($CD69^-CD24^-V\alpha2^+$) and splenic T-cells from OTI^+dLGF^+ mice expressing or not endogenous ThPOK. **(C)** Contribution of endogenous and transgenic ThPOK in the CD4 lineage choice of MHC-I-specific thymocytes with augmented TCR signaling was evaluated by assessing the CD4/CD8 phenotype of the mature thymocytes **(top)** and splenic T-cells (bottom) isolated from the indicated mice. **(D)** The frequency and absolute number of TCR^+ and $CD4^+CD8^{lo}$ subsets in total thymocytes, and $CD4^+$ and $CD8^+$ mature thymocytes ($CD24^-CD69^-TCR^+$) as well as splenic T-cells and subsets from the indicated mice are shown ($n>6$). **(E)** ThPOK specific staining in DP thymocytes and $CD4^+$ mature thymocytes and splenic T-cells from the indicated mice is shown. **(F)** Histograms show phosphor-Src (upper panels) and phosphor-CD3ζ (middle panels) expression levels in DP and $CD4^+CD8^{lo}$ thymocytes from the indicated mice. **(G)** Compilation of MFI data for pSrc and pCD3ζ for DP and $CD4^+CD8^{lo}$ subsets from the indicated mice are shown and are expressed relative to MFI values in WT subsets. **(H)** Expression of *St8sia6*, *St3gal2*, and *Cxxc5* was evaluated by QPCR in mature T-cell subsets purified from the spleen of WT ($CD4^+$ and $CD8^+$) and $OTI^+dLGF^+ThPOK\text{-}H^+$*Thpok*$^{-/-}$ ($CD4^+$ and DN) mice. Data depicts average of triplicate values with standard deviation and are expressed as fold increase over expression of individual genes in control $CD4^+$ mature T-cells from WT mice (normalized to *Hprt* expression). Data are representative of two to six independent experiments **(A-C, E, F and H).**

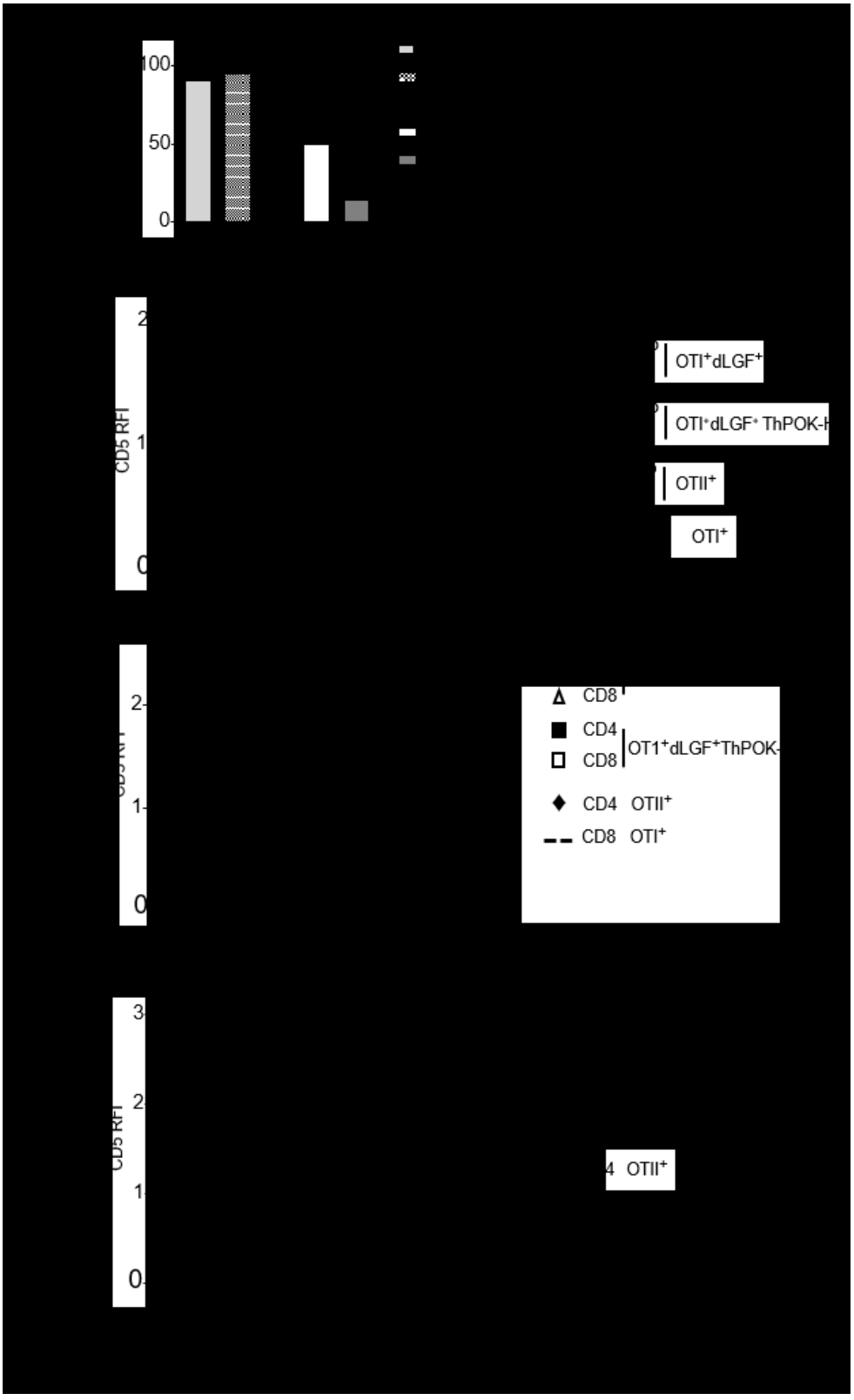
100
50
0
CD5 RFI
2
1
0
OTI+dLGF+
OTI+dLGF+ ThPOK-
OTII+
OTI+
CD8
CD4
CD8
OT1+dLGF+ThPOK-
CD4 OTII+
CD8 OTI+
3
2
1
0
4 OTII+

Figure 19. Comparison of $CD4^+$ mature T-cell frequency and TCR signal strength in MHC-I- and MHC-II-specific thymic and splenic T-cells from control mice and ThPOK-H mice expressing or not dLGF transgene

(A) Efficiency of ThPOK-H-mediated $CD4^+$ lineage choice of thymocytes with differential TCR signaling was evaluated by comparing the $CD4^+$ splenic T-cell frequencies in the indicated mice. To assess relative TCR signal strength, CD5 levels in various thymic subsets and mature T-cells from OTI^+, OTI^+dLGF^+, $OTI^+dLGF^+ThPOK\text{-}H^+$ and $OTII^+$ mice were compared. **(B)** CD5 levels in DP and $CD4^+CD8^{lo}$ thymocytes from the indicated mice were normalized to CD5 levels in the relevant thymic subsets from OTI mice. CD5 levels in the mature $CD4^+$ and $CD8^+$ thymocytes **(C)** and splenocytes **(D)** were normalized to CD5 levels in mature $CD8^+$ thymocytes and splenocytes from OTI mice. Each symbol represents one mouse.

Supplementary materials

Critical role for TCR signal strength and MHC specificity in ThPOK-induced $CD4^+$ helper lineage choice

Nabil Zeidan, Hassan Damen, Denis-Claude Roy, and Vibhuti P. Dave

Inventory

Supplemental Figures:

Figure S1 to S4

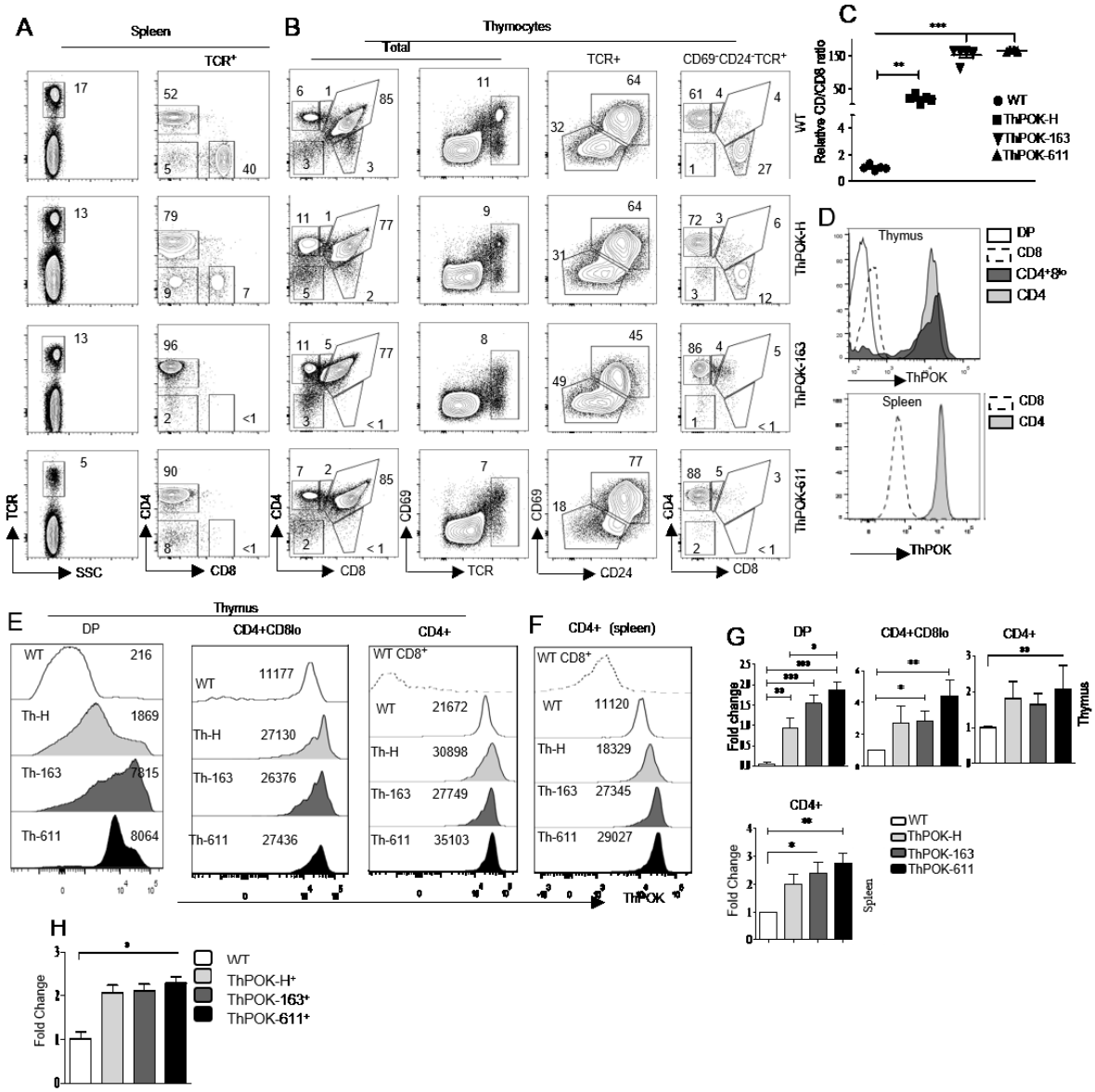
A
Spleen
TCR+
B
Thymocytes
Total
TCR+
CD69-CD24-TCR+
WT
ThPOK-H
ThPOK-163
ThPOK-611
TCR
SSC
CD4
CD8
CD69
TCR
CD24
C
Relative CD/CD8 ratio
WT
ThPOK-H
ThPOK-163
ThPOK-611
D
Thymus
DP
CD8
CD4+8lo
CD4
ThPOK
Spleen
CD8
CD4
ThPOK
E
Thymus
DP
CD4+CD8lo
CD4+
WT
Th-H
Th-163
Th-611
216
1869
7815
8064
11177
27130
26376
27436
WT CD8+
21672
30898
27749
35103
ThPOK
F
CD4+ (spleen)
WT CD8+
11120
18329
27345
29027
ThPOK
G
DP
CD4+CD8lo
CD4+
Fold change
Thymus
CD4+
Fold Change
Spleen
WT
ThPOK-H
ThPOK-163
ThPOK-611
H
Fold Change
WT
ThPOK-H+
ThPOK-163+
ThPOK-611+

Figure 20. Characterization of ThPOK transgenic mice

Three independent transgenic ThPOK founder lines (ThPOK-H, ThPOK-611, and ThPOK-163) were generated and $CD4^+/CD8^+$ T-cell lineage choice was determined by flow cytometry analysis of thymocytes and splenic T-cells isolated from the 5 to 7 week old indicated strain of mice. The CD4/CD8 profiles of TCR^+ cells in the spleen **(A)** and mature ($CD69^-CD24^-TCR^+$) thymocytes **(B)** are shown. Numbers in each FACS plot represent the frequency of the specific population. **(C)** shows the $CD4^+/CD8^+$ ratio in the blood of WT and ThPOK transgenic lines. Each symbol represents an individual mouse. **(D)** Thymocytes from WT mice were stained for CD4, CD8α, TCRβ, CD24 and CD69 followed by intranuclear staining for ThPOK. ThPOK staining histograms for the indicated thymic subsets **(top)** and the splenic $CD4^+$ and $CD8^+$ T-cells (bottom) from WT mice are shown, which concords with ThPOK expression profile in MHC-II-signaled cells. ThPOK expression in the indicated thymic subsets **(E)** and $CD4^+$ splenic T-cells **(F)** from WT and the indicated ThPOK transgenic mice was assessed by flow cytometry. $CD4^+$ and $CD8^+$ T-cells from WT thymus and spleen serve as controls. Numbers in the histogram represent Mean Fluorescent Intensity (MFI) values. **(G)** shows comparison of MFI for ThPOK staining for thymic subsets **(top)** and $CD4^+$ splenic T-cells **(bottom)** from the indicated mice. ThPOK staining in DP thymocytes is relative to that in ThPOK-H mice, while for all other subsets it is relative corresponding WT subset. **(H)** Transgene copy number was determined by QPCR of genomic DNA and expressed as fold increase over WT mice. Data shown are representative examples of four or more experiments **(A, B, D-F)** or an average of at least three independent experiments **(G, H)**.

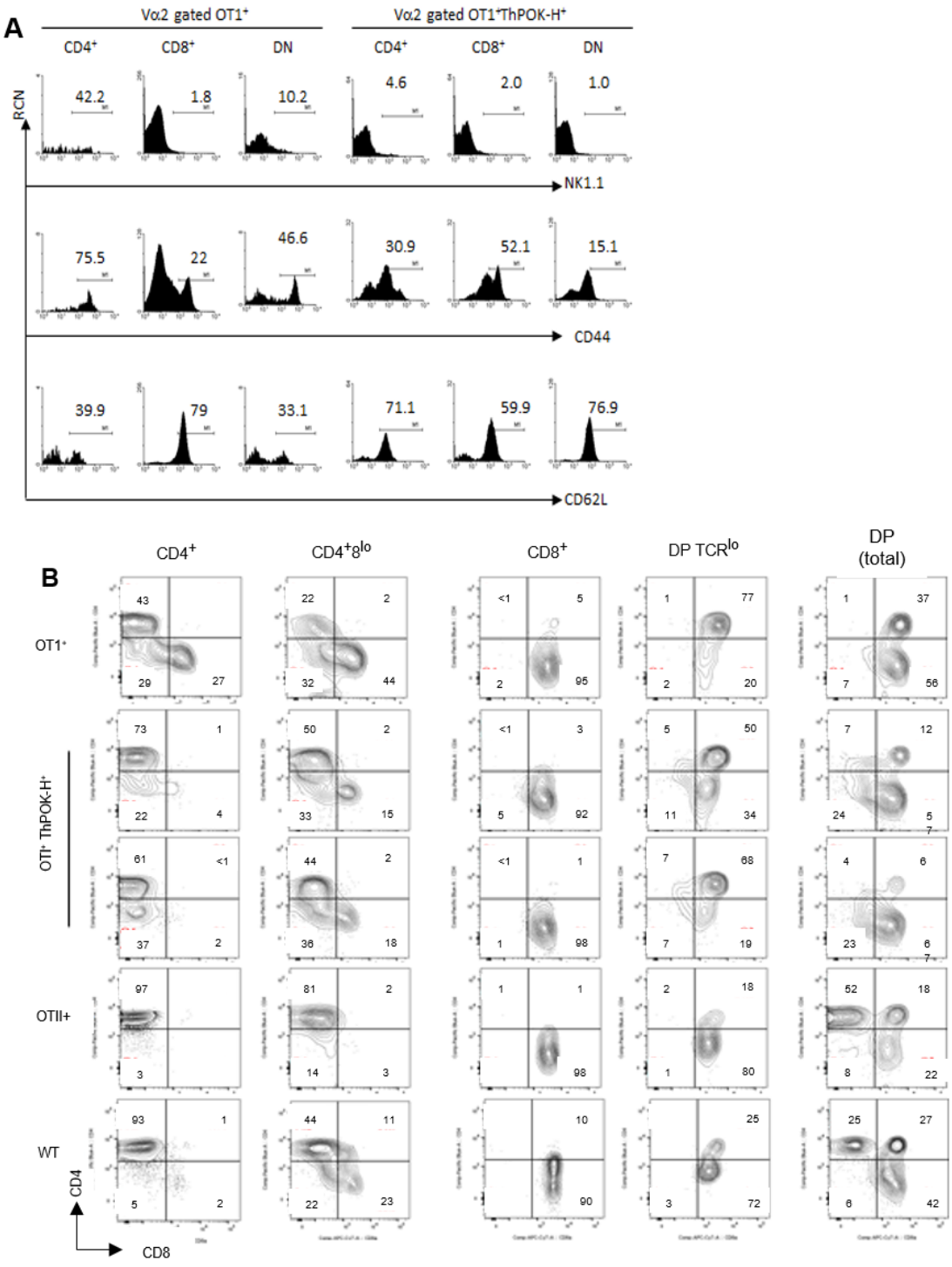
A
Vα2 gated OT1+
Vα2 gated OT1+ThPOK-H+
CD4+
CD8+
DN
CD4+
CD8+
DN
RCN
42.2
1.8
10.2
4.6
2.0
1.0
NK1.1
75.5
22
46.6
30.9
52.1
15.1
CD44
39.9
79
33.1
71.1
59.9
76.9
CD62L
B
CD4+
CD4+8lo
CD8+
DP TCRlo
DP (total)
OT1+
OTI+ ThPOK-H+
OTII+
WT
CD4
CD8

Figure 21. Mature DN T-cells in OTI$^+$ThPOK-H$^+$ mice are not innate like T-cells and transdifferentiate mostly from CD4$^+$ thymocytes

(A) Innate like phenotype of various splenic T-cell subsets from OTI and OTI$^+$ThPOK-H$^+$ mice was assessed by evaluating expression of NK1.1, CD44, and CD62L. Single color histograms for NK1.1, CD44, and CD62L for the indicated T-cell subsets from the two strains of mice are shown. Data representative of two or more experiments. **(B)** The indicated thymocyte populations were purified from WT, OTII$^+$, OTI$^+$ or OTI$^+$ThPOK-H$^+$ mice, and cultured in the presence of IL-7 for two days and then analyzed for the CD4 and CD8 surface expression. Data are a representative example of two independent experiments.

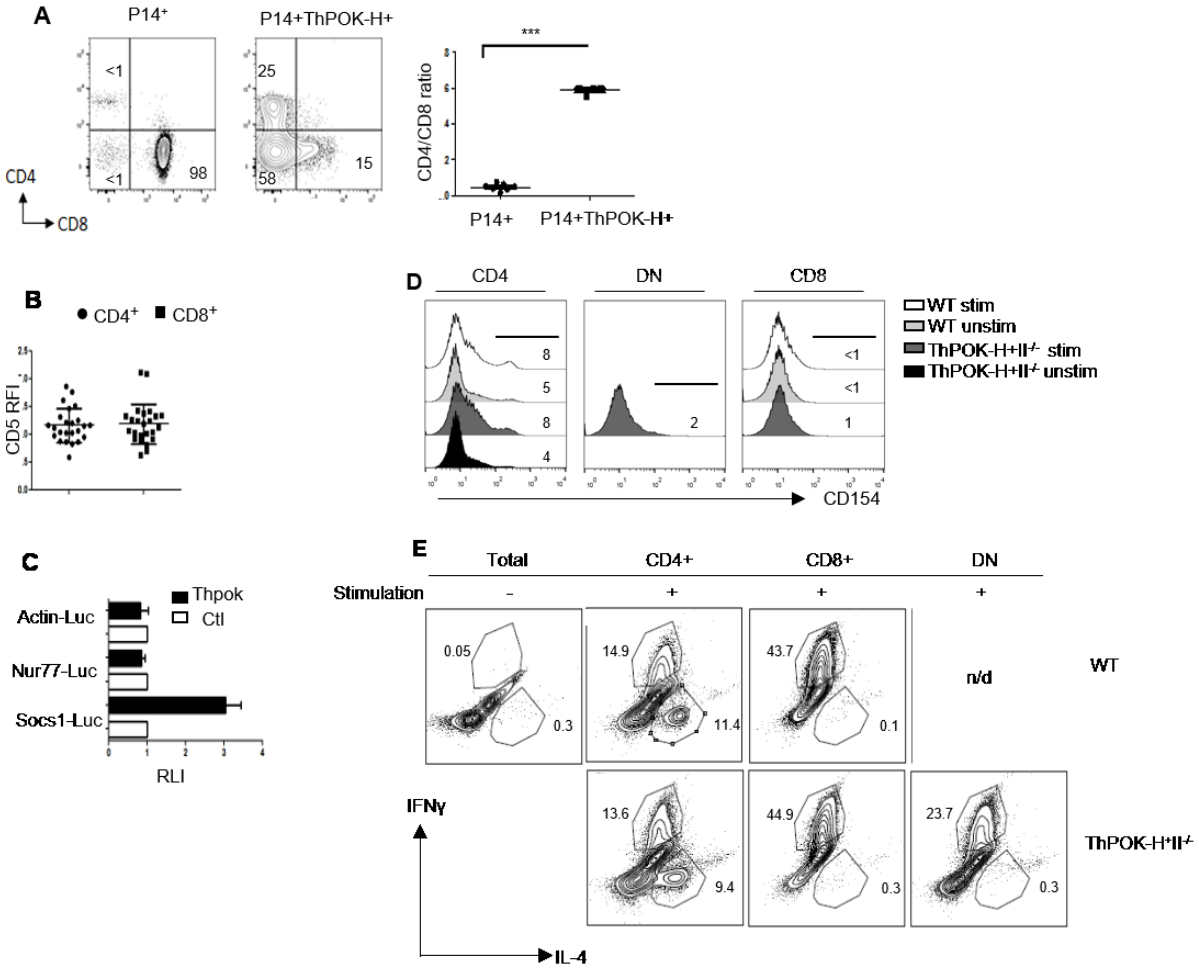
A
P14+
P14+ThPOK-H+
<1
<1
98
25
58
15
CD4
CD8
***
CD4/CD8 ratio
P14+
P14+ThPOK-H+
B
CD4+
CD8+
CD5 RFI
C
Thpok
Ctl
Actin-Luc
Nur77-Luc
Socs1-Luc
RLI
D
CD4
DN
CD8
8
5
8
4
2
<1
<1
1
CD154
WT stim
WT unstim
ThPOK-H+Il4-/- stim
ThPOK-H+Il4-/- unstim
E
Total
CD4+
CD8+
DN
Stimulation
-
+
+
+
0.05
0.3
14.9
11.4
43.7
0.1
n/d
WT
IFNγ
13.6
9.4
44.9
0.3
23.7
0.3
ThPOK-H+Il4-/-
IL-4

Figure 22. Impact of ThPOK-H on lineage choice in P14 mice, Nur77 reporter and function of mature T-cells

(A) Flow cytometric analyses of $V\alpha2^+$ splenic T-cells and the $CD4^+/CD8^+$ ratio of splenic T-cells for $P14^+$ThPOK-H^+ and P14 control mice are shown. Panel **(B)** compares CD5 levels on the $CD4^+$ and $CD8^+$ mature T-cells from OTI^+ThPOK-H^+ mice. Each symbol represents an individual mouse. **(C)** To evaluate if Nur77 was directly regulated by ThPOK, Nur77 promoter sequence was cloned into luciferase reporter plasmid pGL4.19 (Nur77-Luc). Reporter plasmid was transfected in 293T cells in the presence or absence of ThPOK expressing plasmid and luciferase activity was measured at 48 hours post-transfection. Luciferase expression under the control of Actin (Actin-Luc) and Socs1 (Socs1-Luc) promoters serve as negative and positive controls, respectively. RLI, Relative Luminescence Intensity. Purified mature T-cell subsets from WT ($CD4^+$ and $CD8^+$) and ThPOK-$H^+II^{-/-}$ ($CD4^+$, $CD8^+$ and DN) were stimulated in the presence of irradiated Balb/c splenocytes. Five to seven days later cells were stained for CD154 **(D)** or restimulated with PMA/Ionomycin and stained for intracellular IFNγ and IL-4 **(E)**. Unstimulated T-cells from WT mice served as a control. Data are representative of two or more independent experiments **(A, C-E)**. n/d not determined.

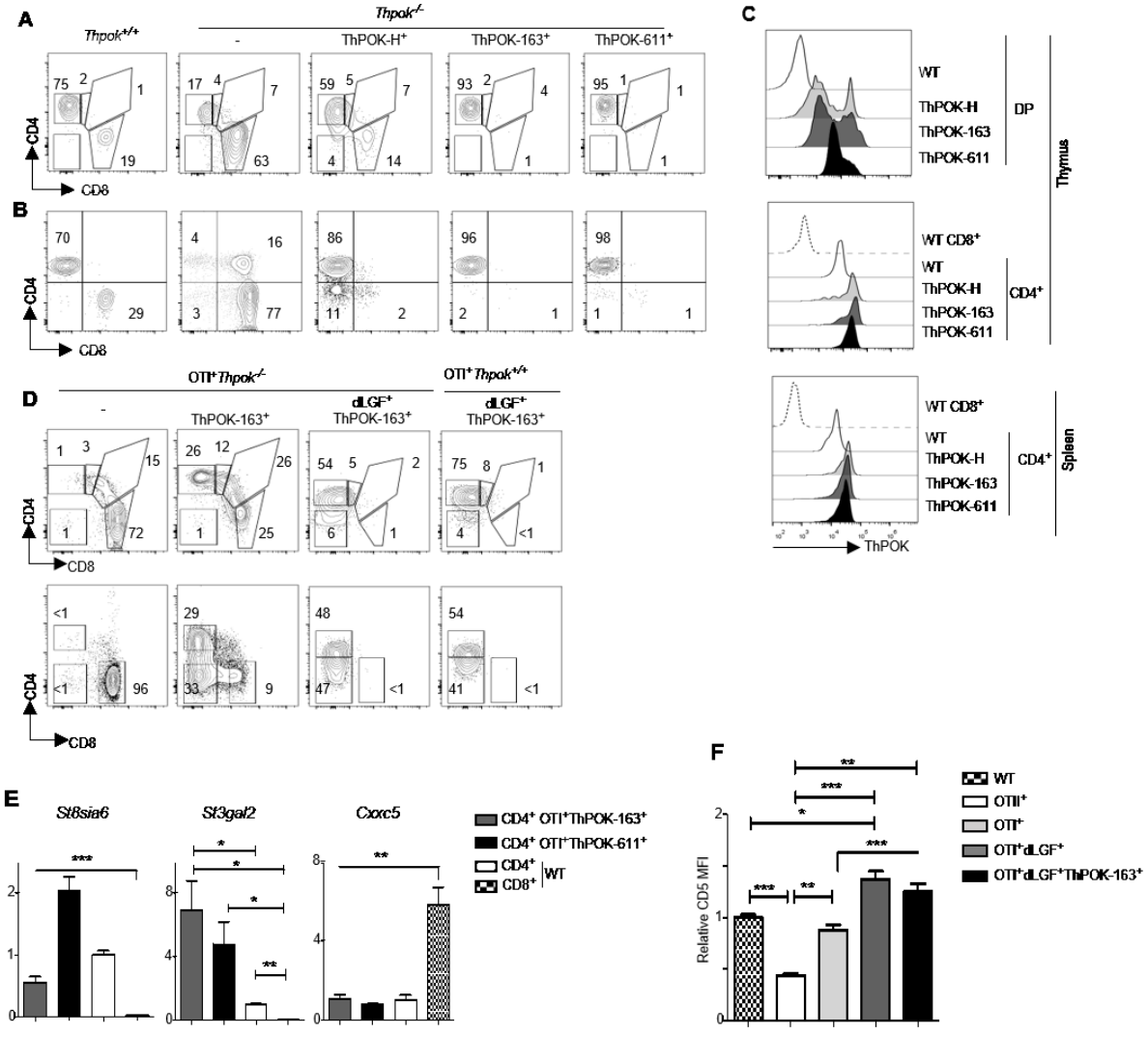
A
Thpok+/+
Thpok-/-
-
ThPOK-H+
ThPOK-163+
ThPOK-611+
CD4
CD8
B
C
WT
ThPOK-H
ThPOK-163
ThPOK-611
DP
Thymus
WT CD8+
CD4+
Spleen
ThPOK
D
OTI+Thpok-/-
OTI+Thpok+/+
dLGF+ ThPOK-163+
E
St8sia6
St3gal2
Cxxc5
CD4+ OTI+ThPOK-163+
CD4+ OTI+ThPOK-611+
CD4+ | WT
CD8+ | WT
F
Relative CD5 MFI
WT
OTII+
OTI+
OTI+dLGF+
OTI+dLGF+ThPOK-163+

Figure 23. The impact of individual ThPOK transgene on $CD4^+$ development in $ThPOK^{-/-}$ mice, the impact of augmented TCR signal strength on the $CD8^+$ to $CD4^+$ lineage redirection in $OTI^+ThPOK\text{-}163^+$ and $CD4^+$ lineage gene expression analysis

Each of the three ThPOK transgene was introduced into *ThPOK*$^{-/-}$ mice and thymocytes and splenic T-cells were analyzed by flow cytometry. Panel (A) shows the CD4/CD8 phenotype of the mature thymocytes ($CD24^-CD69^-TCR\beta^+$) from the indicated mice. (B) shows CD4/CD8 profile of splenic T-cells from the indicated strain of mice. Numbers represent the frequency of relevant thymocytes and splenic T-cells in the specified gates in each FACS plot. (C) shows ThPOK staining of the indicated thymocytes and splenic T-cells. $CD8^+$/DP and $CD4^+$ thymocytes and splenic T-cells from WT mice serve as ThPOK staining controls. Note the continued presence of a small number of $CD8^+$ thymocytes and splenic T-cells in ThPOK-H^+*Thpok*$^{-/-}$ mice compared to ThPOK-163^+*Thpok*$^{-/-}$ or ThPOK-611^+*Thpok*$^{-/-}$ mice. Also note higher ThPOK staining in $CD4^+$ thymocytes and splenic T-cells from the three transgenic mice compared to WT control. (D) Thymocytes (top panels) and splenic (bottom panels) T-cells from OTI^+ThPOK-163^+*ThPOK*$^{-/-}$ expressing or not dLGF transgene were analyzed by flow cytometry and compared to OTI^+dLGF^+ThPOK-163^+*Thpok*$^{+/+}$ mice. The CD4/CD8 phenotype of mature thymocytes and the $V\alpha2^+$ splenic T-cells from the indicated mice is shown. At least three mice of each genotype were analyzed. (E) St8sia6, St3gal2 and Cxxc5 expression in the redirected $CD4^+$ T-cells from OTI^+ThPOK-163^+ and OTI^+ThPOK-611^+ mice was analyzed by QPCR and was compared to expression in $CD4^+$ (St8sia6 and St3gal2) or $CD8^+$ (Cxxc5) T-cells from WT mice. Data are average of triplicate (normalized to Hprt expression; n=2). (F) CD5 MFI in $CD4^+$ splenic T-cells from the indicated mice relative to CD5 MFI in $CD4^+$ T-cells from WT mice is shown

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Chapter 3: Sustained TCR signaling protects the helper fate and suppresses the cytotoxic developmental program independently of ThPOK (manuscript #2)

Contributions of authors:

Nabil Zeidan and Dr. Vibhuti P. Dave designed the experiments. Dr. Vibhuti P. Dave performed the experiments presented in Fig. 28 and part of 29A, B, C and D. Nabil Zeidan performed and analyzed all the remaining experiments. Nabil Zeidan, Dr. Denis Claude Roy and Dr. Vibhuti P. Dave wrote the manuscript. Dr. Vibhuti P. Dave conceptualized and supervised the study. Dr. Denis Claude Roy provided reagents and resources.

Sustained TCR signaling protects the helper fate and suppresses the cytotoxic developmental program independently of ThPOK

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Keywords: $CD4^+/CD8^+$ lineage choice, ThPOK, Gata3, TCR signaling, MHC specificity

3.1 Résumé

La signalisation du RCT dans les thymocytes restreints au CMH de classe II, par l'induction du facteur de transcription ThPOK inhibant le développement du programme cytotoxique, joue un rôle essentiel dans l'engagement dans la lignée $CD4^+$. En l'absence de ThPOK, les thymocytes sélectionnés par le CMH de classe II sont redirigés dans la voie $CD8^+$. Hormis la fonction du signal RCT dans l'induction de ThPOK, le rôle que joue cette voie de signalisation dans l'orientation des thymocytes vers les voies $CD4^+$ ou $CD8^+$ reste à déterminer. De même, l'existence d'un chevauchement fonctionnel entre ThPOK et les autres facteurs de transcriptions induits par le RCT, tels que Gata3 est incertaine. Nous avons étudié ici l'effet de la modulation de la voie de signalisation du RCT sur le programme d'engagement vers la lignée $CD4^+$. Notre analyse démontre que les thymocytes restreints au CMH de classe II qui se différencient dans la voie $CD8^+$ en l'absence de ThPOK continuent à exprimer des facteurs spécifiques nécessaires au programme de développement de la lignée $CD4^+$. Cependant, nos études de surexpression transgénique n'ont montré aucune synergie entre Gata3 et ThPOK dans l'orientation des thymocytes vers la voie $CD4^+$ ou $CD8^+$. L'ensemble de ces résultats suggèrent que la signalisation du TCR spécifique au CMH de classe II joue un rôle dans l'activation d'un réseau de facteurs de transcription avec peu de chevauchement fonctionnel ne se limitant pas à ThPOK.

3.2 Abstract

The MHC class-II TCR signaling is essential for the development of $CD4^+$ helper T-cells, by inducing the transcription factor ThPOK, which suppresses the cytotoxic program. In the absence of ThPOK, MHC class-II-restricted thymocytes are redirected to the $CD8^+$ lineage fate. Apart from inducing ThPOK, the role of TCR signaling, and its downstream effector nuclear factors, on $CD4^+$/$CD8^+$ lineage fate remains elusive. Equally, it is not clear if there is any functional overlap between ThPOK and other TCR-induced transcription factors, such as Gata3, in lineage choice. Here, we have investigated the effect of modulating the TCR signaling pathway on the $CD4^+$ helper program. Our analysis showed that $CD8^+$-redirected MHC-II-restricted T-cells in *Thpok*$^{-/-}$ mice continued to express multiple genes specific to the helper lineage fate. Importantly, however, transgenic overexpression of Gata3 failed to show any synergistic effect with ThPOK in redirecting MHC-I-signaled thymocytes into $CD4^+$ lineage. Together, these data suggest a role for MHC-II-specific TCR signaling in activating a network of transcription factors with limited functional overlap.

3.3 Introduction

The $CD4^+/CD8^+$ lineage fate decision marks a critical step in the life of a T-cell, and is primarily regulated by TCR- and cytokine-derived signals [326]. These signals specify lineage fate by inducing a network of transcription factors. At the centre of this network are the mutually antagonistic transcription factors ThPOK and Runx3, which mediate $CD4^+$ and $CD8^+$ lineage choice, respectively [276, 326]. It is thought that TCR- and cytokine-regulated expression of Gata3 and STAT5 control transcriptional expression of the lineage-determining factors ThPOK and Runx3, respectively [275, 276, 326, 434]. While loss- and gain-of-ThPOK function redirects positively selected thymocytes into $CD8^+$ and $CD4^+$ lineage, respectively, constitutive Runx3 expression fails to induce $CD4^+$ to $CD8^+$ lineage redirection suggesting that, by itself, Runx3 is not sufficient to antagonise ThPOK [245, 246, 265, 268, 269, 271, 280, 286, 413]. The network of transcription factors in committed thymocytes also helps maintain the lineage integrity in the periphery by epigenetic modifications of target genes [276, 322].

We aim to provide an in-depth analysis of the role that TCR signaling plays in lineage commitment. It is generally agreed that timing, duration and strength of TCR signaling influence lineage fate decision of positively selected $CD4^+8^{lo}$ uncommitted thymocytes [156, 326, 435, 436]. While the disruption of TCR signals at the $CD4^+8^{lo}$ uncommitted intermediate stage results in the transcriptional activation of *Runx3* and commitment to the $CD8^+$ lineage fate, sustained TCR signaling activates two $CD4^+$ signature genes, Gata3 and ThPOK [98, 266, 287, 320, 321, 437]. Recent models propose that TCR-induced Gata3 participates in the development of $CD4^+$ T-cells by binding to a regulatory region upstream of the second exon of *Thpok* and provoking an epigenetic priming of the locus by antagonizing *Thpok* silencer-mediated repression of ThPOK [322, 438]. However, transgenic overexpression of Gata3 or ThPOK suggest that Gata3's function is independent of its effect on *Thpok* expression. Constitutive expression of Gata3 fails to induce *Thpok* expression and instead induces apoptosis of $CD8^+$ thymocytes [96, 153, 272, 273, 431]. Additionally, transgenic ThPOK failed to rescue $CD4^+$ development in Gata3-deficient mice [274].

Although ThPOK is at the centre of the transcriptional network regulating the helper program, several reports suggest that the expression of multiple genes that are active in $CD4^+$ helper T-cells does not positively correlate with ThPOK expression [268, 321, 438]. For

example, redirected MHC-II-specific $CD8^+$ T-cells in *Thpok*$^{-/-}$ mice can be induced to express similar levels of IL-4 as MHC-II-specific $CD4^+$ T-cells [293]. This is intriguing because differentiation of MHC-II-restricted *Thpok*$^{-/-}$ and MHC-I-restricted conventional thymocytes into the $CD8^+$ cytotoxic lineage is governed by the same Runx3 transcriptional network of proteins [320, 321]. We propose that these results correlate with the MHC specificity in developing thymocytes. Indeed, we previously showed that the efficiency of $CD4^+$ lineage choice of MHC-I-specific thymocytes with augmented TCR signaling and MHC-II-specific thymocytes in the presence of same amount of ThPOK was significantly different. By modulating TCR signaling in MHC-II-specific TCR transgenic mice models, we show here that TCR specificity could potentially regulate $CD4^+$ lineage commitment independently of ThPOK expression. Interestingly, constitutive Gata3 failed to promote ThPOK-mediated $CD8^+$ to $CD4^+$ lineage redirection suggesting a lack of functional overlap between these two T-helper signature genes.

3.4 Materials and methods

3.4.1 Mice

MHC class-I-restricted OTI-TCR (chicken ovalbumin peptide specific) and P14-TCR (LCMV GP33 peptide specific) transgenic mice were obtained from Taconic Farm or Nathalie Labrecque (Hôpital Maisonneuve-Rosemont Research Centre). MHC class-II-restricted OTII-TCR (chicken ovalbumin peptide specific), 5cc7-TCR (pigeon cytochrome c peptide specific) were obtained from Taconic, whereas AND-TCR (moth and pigeon cytochrome c peptide specific) and MHC-II-deficient mice were obtained from Jackson Lab. Generation of ThPOK transgenic mice was described earlier [439]. ThPOK deficient mice (*ThPOK*$^{gfp/gfp}$) were acquired from Dan Littman (NYU). Constitutively active Lck transgenic mouse line (dLGF) is described elsewhere and was obtained from Paul Jolicoeur [211, 420]. Gata3 transgenic mice are described in detail elsewhere [273]. All the TCR transgenic mice used in this study were on a Rag-sufficient background. Mice were genotyped by peripheral blood analysis and/or PCR of

genomic DNA isolated from tail snippets. Six to nine-week-old mice were analyzed. All mice were housed under specific pathogen free conditions at the Hôpital Maisonneuve-Rosemont Research Centre. In accordance with the Canadian Committee on Animal Care, animal protocols were approved by the local Animal care Committee.

3.4.2 Flow cytometry

Single-cell suspensions were prepared from harvested thymi, spleens and/or lymph nodes of 4 – 8-week-old mice. Cells were stained with the following fluorescein isothiocyanate-, peridinin-chlorophyll-protein complex-, peridinin-chlorophyll-protein complex cyanine 5.5-phycoerythrin-, phycoerythrin-cyanine 7-, pacific blue-, BV510-, allophycocyanin-, or allophycocyanin cyanine 7-conjugated antibodies: CD4 (GK1.5), CD8 (53-5.8), TCRβ (H57-597), CD5 (53-7.3), CD69 (H1.2F3), CD24 (M1/69), CD103 (2E7), CD25 (3C7), CD127 (A7R34), H-2Kb (AF6-88.5), H-2Kk (36-7-5), H-2Kd (SF1-1.1), Vα2 (B20.1), Vβ5 (MR9-4), IFN-γ (XMG1.2) or Gzmb (GB11) and analyzed by flow cytometry using an LSRII or LSRFortessa (BD Bioscience). To differentiate between YFP and GFP, the following filters were used: 510/20, 545/35, 495LP and 525LP. Data were analyzed using FlowJo software (Treestar Inc.). Antibodies were obtained from Ebioscience or Biolegend.

3.4.3 Quantitative RT-PCR (Q-PCR)

Various thymocyte or splenic T-cell subsets were FACS purified and total RNA was prepared using Trizol (Invitrogen Inc.). Complementary DNAs were synthesized using commercial kit (Invitrogen). QPCR for *Thpok*, *Gata3*, *Nur77*, distal *Runx3* (*dRunx3*) and *Nkg7* was performed in triplicate using SyBR green dye (Qiagen Inc.) and data was analyzed with the Applied Biosystem software ABI 7500 v2.0.5 using the ΔΔCt method. The housekeeping genes

Rp16 or *Hprt* or *Gapdh* served as an internal control. All QPCR primers were obtained from IDT or designed in our lab (primer information available upon request).

3.4.4 Functional assays

Mature T-cells from spleen of $OTI^{+}dLGF^{+}$, and OTI^{+} mice were isolated and resuspended at a concentration of 1milion/ml and activated with 2μg/ml of plate-bound α-CD3ε (clone 145-2C11, BioLegend®, catalogue number: 100301), 1ug/ml of soluble α-CD28 (clone 37.51, BioLegend®, catalogue number: 102101) in the presence of 50U/ml of rmIl2 (R&D Systems, catalogue number: 402-ML-020) for 3 days. Stimulated cells were stained with anti-TCRβ, CD69, CD4, CD8, interferon-γ and granzyme B specific antibodies and analyzed by flow cytometry. For intracellular cytokine staining, the activated T-cells were incubated with brefeldin A (BioLegend®, catalogue number: 420601) for 4 hours, then fixed with eBioscience™, IC Fixation Buffer (catalogue number: 00-8222-49) and permeabilized with eBioscience™, Permeabilization Buffer (catalogue number: 00-8333).

3.4.5 Retroviral transduction of peripheral murine T cells

Mature peripheral T-cells from the spleen of *Thpok*$^{-/-}$ mice were purified using the EasySep™ (STEMCELL™ technologies, catalogue number: 19851) isolation kit and activated for 48 hours in vitro with plate-bound anti-CD3ε and soluble anti-CD28, as described above. Activated peripheral mouse T-cells were transduced with either a ThPOK-YFP or control YFP retrovirus (produced by transfecting ecotropic GP+E86 packaging cell line (obtained from ATCC©)), for two days in the presence of polybrene.

3.4.6 Statistical analyses

Prism (Graphpad Software©) or Microsoft Excel software were used for all statistical analyses. Data are displayed as a mean with standard error bar. Unpaired or paired Student *t* test was used for determining significance. $P \leq 0.05$ was considered statistically significant. **P*<0.05; ***P*<0.005; ****P*<0.0005.

3.5 Results

3.5.1 Disruption of TCR signaling affects lineage fate

To evaluate the role of TCR signaling in $CD4^+/CD8^+$ lineage choice, we employed several MHC-II-specific TCR transgenic mice. We modified the TCR signaling in these mice by altering the expression level of the selecting ligand. The OTII-TCR is positively selected by K^b, but not K^d or K^k, MHC-II haplotype. We generated OTII mice heterozygous for selecting MHC haplotype by breeding $OTII^+H\text{-}2K^b$ to K^k mice. Indeed, $OTII^+H\text{-}2K^{b/k}$ mice, compared to $OTII^+H\text{-}2K^{b/b}$, showed impaired positive selection of developing thymocytes; there was almost 3-fold decrease in TCR^+ thymocytes in $OTII^+$ $H\text{-}2K^{b/k}$ mice compared to $OTII^+H\text{-}2K^{b/b}$ mice **(Fig. 24A)**. To better examine the effect of introducing a non-selecting MHC-II allele on TCR signaling, we measured cell-surface expression of CD5, a negative regulator of TCR signaling that is proportional to TCR signaling [428]. Compared to $OTII^+H\text{-}2K^{b/b}$, we observed that CD5 on selected DP thymocytes were lower in $OTII^+H\text{-}2K^{b/k}$. Although this difference is not statistically significant, the results suggest that the presence of only one selecting allele may reduce TCR signaling in MHC-II-specific thymocytes **(Fig. 24B)**. Since TCR signaling is a key determining factor in CD4 lineage fate, we analysed the effect of impairing MHC-II-specific TCR signaling on lineage fate in our model [189]. Indeed, diminished TCR signaling resulted in almost 9-fold decrease in the frequency of $CD4^+$ SP thymocytes with a significant frequency of signaled thymocytes adopting the $CD8^+$ lineage fate with some becoming DN mature T-cells in $OTII^+H\text{-}2K^{b/k}$ mice compared to control **(Fig. 24C)**. In $OTII^+H\text{-}2K^{b/k}$, regulatory T-cells (T_{Reg}) constitute a small proportion of the $CD4^+$ compartment. However, this frequency was increased by more than 10-fold in $OTII^+H\text{-}2K^{b/k}$, demonstrating that development of these cells

mediated by agonist ligand, and therefore stronger TCR signaling, was largely unaffected **(Fig. 24D)**.

Sustained TCR signaling favors $CD4^+$ T-cell development primarily by inducing the transcription factor *Thpok* [276]. Mouse with GFP knocked into the *Thpok* locus have been reported and $Thpok^{+/gfp}$ mice accurately recapitulates the endogenous ThPOK expression pattern in $CD4^+$ T-cells [320, 321]. Unlike MHC-I-specific $CD8^+$ T-cells lacking GFP expression, MHC-II-specific $CD8^+$ T-cells continue to express GFP in $Thpok^{gfp/gfp}$ mice. As H-$2K^{b/k}$ MHC background led to the generation of a significant number of $CD8^+$ T-cells, we asked if this lineage redirection was due to a lack of transcriptional activation or post-transcriptional inactivation of ThPOK. To address this, we introduced $Thpok^{+/gfp}$ background in $OTII^+$ H-$2K^{b/b}$ and $OTII^+$ H-$2K^{b/k}$ mice. Analysis of T-cells showed that MHC-II-specific redirected DN and $CD8^+$ mature T-cells failed to express GFP indicating that disrupted TCR signaling in $OTII^+$H-$2K^{b/k}$ mice impair *Thpok* induction **(Fig. 24E)**. Further investigation showed CD103 expression, a target of Runx3 and thus an indicator of *Runx3* induction, on $CD8^+$, but not $CD4^+$, mature T-cells from $OTII^+$H-$2K^{b/k}$ mice [271]. Interestingly, DN mature T-cells from $OTII^+$H-$2K^{b/k}$ mice, while failed to express GFP, showed lower CD103 expression, and therefore potentially lower Runx3 induction, compared to $CD8^+$ T-cells from the same mouse. These data suggest that during lineage fate, disruption of TCR signaling in $OTII^+$H-$2K^{b/k}$ thymocytes could fail to induce *Thpok* expression leading to Runx3-dependent $CD8^+$ T-cell differentiation **(Fig. 24E)**.

3.5.2 MHC-II redirected $CD8^+$ T-cells following disruption in TCR-signals can be rescued by transgenic ThPOK expression

To support the observed $CD4^+$ to $CD8^+$ lineage redirection following the introduction of a non-selecting allele in $OTII^+$H-$2K^{b/k}$ is not limited to OTII, we repeated the experiment using two other mice expressing MHC-II-specific TCR of different affinity for ligand. A previous report has shown that 5cc7- and AND-TCR have similar and higher TCR-signal strength as OTII-TCR, respectively [428]. $5cc7^+$ thymocytes are selected by K^k haplotype and so to reduce TCR signaling we introduced the non-selecting allele K^b [428]. AND-TCR, on the other hand, is selected by K^b and K^k, but not K^d, haplotypes, and so K^d allele was introduced to evaluate the impact of altered TCR signaling on thymic selection and lineage choice [428]. Similar to OTII

mice, the introduction of a non-selecting MHC-II molecule impaired $CD4^+$ T-cell development in AND mice and were partially redirected to the $CD8^+$ lineage fate with the generation of a large number of DN mature T-cells **(Fig. 25A)**. In the same way, disruption of TCR signaling in 5cc7 impaired $CD4^+$ T-cell development. However, unlike OTII and AND model, reduced TCR-signaling in 5cc7 mice favored the development of DP mature T-cells in the periphery **(Fig. 25B)**. To demonstrate that the lineage redirection of MHC-II-restricted thymocytes occurred as a consequence of impaired *Thpok* induction, we introduced ThPOK transgene in $5cc7^+$H-$2K^{k/b}$ mice. Indeed, $CD4^+$ development was completely rescued in $5cc7^+$ H-$2K^{k/b}$ThPOK-Tg^+ mice as judged by the comparable frequency of $CD4^+$ T-cells in these mice to control animals **(Fig. 25C)**.

3.5.3 MHC-II-specific redirected $CD8^+$ T-cells in ThPOK knock out mice show some functional and transcriptional differences compared to genuine MHC-I-specific $CD8^+$ T-cells

So far, we have provided solid evidence that modulating TCR signaling in MHC-II-restricted thymocytes impaired induction of the helper program potentially by hindering *Thpok* induction leading to Runx3 expression. However, it is not clear if there is any similarity between the mechanism that underlies the generation of MHC-II-specific $CD8^+$ T-cells caused by weakened TCR signaling or genetic ablation of ThPOK, which does not affect TCR signaling [287]. We used ThPOK knock-out mice (*Thpok*$^{gfp/gfp}$) mouse to test this prediction. As reported in the literature, the redirected MHC-II-specific $CD8^+$ T-cells expressed GFP indicating that, despite Runx3 expression, the *Thpok* locus is active in the redirected $CD8^+$ T-cells unlike conventional MHC-I-specific $CD8^+$ T-cells (which do not express GFP) **(Fig. 26A, B)**. Interestingly, CD5 levels on $CD8^+GFP^+$ T-cells were higher compared to $CD8^+GFP^-$ T-cells suggesting a stronger TCR-signaling in $CD8^+GFP^+$ T-cells despite lack of expression of matching CD4 co-receptor **(Fig. 26C)**. The expression levels of CD103 on $CD8^+GFP^+$ and $CD8^+GFP^-$ populations were similar, suggesting that both populations could potentially be expressing similar levels of Runx3 **(Fig. 26B)**. Moreover, we detected similar expression levels of *Gata3* and *Nur77* (which is induced by a strong TCR signaling, and is more abundant in MHC-II-restricted $CD4^+$ T-cells compared to MHC-I-restricted $CD8^+$ T-cells [439]) in $CD8^+GFP^+$ compared to those expressed by MHC-II-specific $CD4^+GFP^+$ T-cells, but not MHC-

I-specific $CD8^+GFP^-$ T-cells **(Fig. 26D)**. These data propose that $CD8^+GFP^+$ and $CD8^+GFP^-$ received different TCR signals during thymic selection. In MHC-I-restricted $CD8^+$ T-cells, *Thpok* and other $CD4^+$ specifying genes are epigenetically silenced by Runx3 as part of the $CD8^+$ differentiation. This is probably not the case for $CD8^+GFP^+$ T-cells which displayed several active components of the helper program. Hence, we reasoned that introducing ThPOK in the redirected $CD8^+GFP^+$ T-cells may rescue $CD4^+$ phenotype. While retroviral-expression of *Thpok* in $CD8^+GFP^+$ T-cells downregulated $CD8^+$ expression (by around 30%), CD4 expression was not affected **(Fig. 26E)**. These data may suggest either that (1) peripheral T-cells are unresponsive to exogenous expression of ThPOK in terms of *Cd4* induction, (2) the amount of ectopically expressed ThPOK was not sufficient to induce *Cd4* expression in our model, or that (3) a binding partner necessary for ThPOK-induced *Cd4*-expression is absent from $CD8^+GFP^+$ T-cells in $Thpok^{-/-}$ mice.

3.5.4 Sustained TCR signaling activates helper program and supresses cytotoxic program independently of ThPOK

To further support our hypothesis that TCR signaling could trigger the expression of $CD4^+$ lineage specific genes even in the redirected $CD8^+GFP^+$ T-cells, we introduced, in MHC-I-specific TCR transgenic OTI mice, a constitutively active Lck transgene (dLGF) as increased Lck activity is known to redirect MHC-I-restricted thymocytes to the $CD4^+$ lineage fate [210]. Augmenting the catalytic activity of Lck in MCH-I-restricted thymocytes partially phenocopied the effect of an MHC-II-restricted TCR signaling on the lineage fate **(manuscript#1, Fig. 17A)** as judged by a small but significant increase in the frequency of $CD4^+$ T-cells in OTI^+dLGF^+ mice **(Fig. 27A)**. Lineage redirection in OTI^+dLGF^+ mice required ThPOK induction because $OTI^+dLGF^+Thpok^{gfp/gfp}$ mice lacked mature $CD4^+$ T-cells. Importantly, a fraction of $CD8^+$ T-cells in $OTI^+dLGF^+Thpok^{gfp/gfp}$ mice continued to express GFP with higher CD5 levels (compared to $CD8^+GFP^-$ cells) which is indicative that these cells received stronger TCR signaling during thymic selection leading to the activation of the *Thpok* locus **(Fig. 27B, C & D)**. Although more experiments need to be performed, but these data complement the above described data showing that disrupted MHC-II TCR signaling fails to induce ThPOK expression. However, these data may also argue against the proposed role of ThPOK in forming an autoregulatory feed forward loop [267, 275, 321, 322]. Additionally, we also observed a

slight decrease in CD103 expression, and by extension probably lower Runx3 expression, in $CD8^+GFP^-$ T-cells from OTI^+dLGF^+*Thpok*$^{gfp/gfp}$ mice compared to control $CD8^+$ T-cells from OTI mice **(Fig. 27E)**. Although the difference is minimal, it is tempting to speculate that stronger TCR signaling may result in suppression of cytotoxic program even in the absence of ThPOK expression **(Fig. 27E)**. A similar pattern was detected in $P14^+dLGF^+$ mice as well, indicating that the observations were not limited to OTI^+ mice **(data not shown)**. Moreover, *in vitro* activated $CD8^+$ splenocytes from OTI^+dLGF^+ mice showed a small decrease in IFNγ expression but not enough to draw any significant conclusion **(Fig. 27G)**.

3.5.5 Impact of constitutive Gata3 expression on the ThPOK-induced lineage redirection

Gata3 expression is critical for $CD4^+$ lineage development and its expression levels in MHC-II-signaled thymocytes correlates with TCR signal strength [266, 272, 431]. Gata3, in turn, promotes TCR signal transduction and acts upstream of ThPOK [195]. We have previously shown that TCR signaling in MHC-II-restricted thymocytes can influence $CD4^+$/$CD8^+$ lineage fate because they require lower amounts of ThPOK compared to MHC-I-signaled thymocytes for $CD4^+$ lineage commitment **(Fig. 16, 17)**. Therefore, we reasoned that the ThPOK-independent effect of MHC-II-specific TCR signaling on the activation of helper program may be mediated by Gata3. To test this hypothesis, we introduced Gata3 transgene into OTI mice [273]. We observed reduced frequency and absolute number of $CD8^+$ SP thymocytes and splenocytes in OTI^+Gata3^+ mice without any significant increase in $CD4^+$ T-cells suggesting that Gata3 expression in MHC-I-specific thymocytes does not induce lineage redirection **(Fig. 28A, B & C).** Since it is reported that Gata3 and ThPOK have distinct functions during lineage fate [273, 274], we sought to determine if the two molecules act in synergistic fashion in promoting $CD8^+$ to $CD4^+$ lineage redirection. To address this issue, we used our ThPOK-H transgene, which causes a partial $CD8^+$ to $CD4^+$ lineage redirection in OTI mice but expresses more ThPOK than the endogenous locus **(manuscript#1 Fig. 12, 18)**. Much like the introduction of the dLGF transgene in $OTI^+ThPOK\text{-}H^+$ mice **(manuscript#1 Fig. 17, 18)**, we observed a significant decrease and increase in the frequency of $CD8^+$ and $CD4^+$ T-cells, respectively, in $OTI^+ThPOK\text{-}H^+Gata3^+$ compared to $OTI^+ThPOK\text{-}H^+$ **(Fig. 28A & B)**. The presence of Gata3 did not, however, affect the frequency of DN mature T-cells in triple transgenic mice compared to $OTI^+ThPOK\text{-}H^+$ mice. However, the increased frequency of $CD4^+$

T-cells did not appear to be due to augmented $CD8^+$ to $CD4^+$ lineage redirection but rather due to loss of significant number of not only $CD8^+$ but $CD4^+$ and DN mature T-cells in OTI^+Gata3^+ mice expressing or not ThPOK-H.

In the thymus, we observed similar pattern for the $CD4^+/CD8^+$ ratio with the highest in the triple transgenic mice **(Fig. 28C & D)**. However, unlike splenic T-cells, the absolute number of total or selected thymocytes were comparable in all groups of mice. Importantly, the absolute number of $CD4^+$ SP thymocytes in $OTI^+ThPOK\text{-}H^+$ and $OTI^+ThPOK\text{-}H^+Gata3^+$ was comparable, albeit the frequency was more elevated in $OTI^+ThPOK\text{-}H^+Gata3^+$ mice suggesting that the presence of Gata3 *per se* does not affect the $CD4^+$ lineage commitment in $OTI^+ThPOK\text{-}H^+$ mice. These data suggest that the redirected $CD4^+$ T-cells in $OTI^+ThPOK\text{-}H^+$ mice behave like $CD8^+$ T-cells within the context of Gata3-induced apoptosis, and point to potential differences in TCR signaling in MHC-I- and MHC-II-specific thymocytes irrespective of their CD4/CD8 lineage choice.

Significantly different $CD4^+$ mature T-cell number in spleen of $OTI^+ThPOK\text{-}H^+$ and $OTI^+ThPOK\text{-}H^+Gata3^+$ could be due to apoptotic effect of constitutive Gata3 expression in a strong affinity TCR transgenic mice. To address this issue, we introduced ThPOK-H and/or Gata3 transgene into MHC II-deficient ($I\!I^{-/-}$) mice. As expected, introducing Gata3 or ThPOK-H transgene into $I\!I^{-/-}$ mice significantly decreased in the frequency and number of T-cells in the spleen compared to control $I\!I^{-/-}$ mice **(Fig. 29A & B)**. This was further reduced, in the presence of both the transgenes in $I\!I^{-/-}$ mice. Interestingly, constitutive Gata3 expression in $I\!I^{-/-}$ mice expressing or not ThPOK-H resulted in severe depletion of total T-cells and this resulted in 1) a significant increase in the frequency but not absolute number of $CD4^+$ mature T-cells and 2) almost complete depletion of $CD8^+$ mature T-cells without any impact on a small number of DN mature T-cells. As a result, we observed a significant increase in the $CD4^+/CD8^+$ ratio in the spleen as well as thymus compared to $ThPOK\text{-}H^+I\!I^{-/-}$ and $Gata3^+I\!I^{-/-}$ **(Fig. 29A & C)**. We thought that Gata3 may be selectively inhibiting MHC-I-specific $CD8^+$ T-cell development by interfering with cytokine signaling since MHC-I-signaled $CD8^+$ committed thymocytes rely more on cytokine- rather than TCR-induced signaling for their continued differentiation. Indeed, cell-surface expression of CD127 was down regulated in $CD4^+8^{lo}$ thymocytes in MHC-I-specific $CD8^+$ T-cells from Gata-3 transgenic mice compared to WT **(Fig. 29E)**. However, more

research needs to be done in order to better understand Gata3-mediated apoptosis of $CD8^+$ T-cells.

3.6 Discussion

In the present investigation, we have studied the impact of TCR signaling on $CD4^+/CD8^+$ lineage commitment and how altering individual components of the downstream signaling pathway results in distinct $CD4^+/CD8^+$ phenotypes. Our data corroborate published reports suggesting the requirement of a persistent TCR signaling for $CD4^+$ T-cell development via induction of *Thpok* and other factors like *Gata3*. While our recently published data show that augmenting TCR signal strength promotes ThPOK-induced CD4 lineage choice of MHC-I-specific thymocytes, here we have employed converse approach of decreasing TCR signaling, by altering selecting ligand levels, to study $CD4^+/CD8^+$ lineage choice of MHC-II-specific thymocytes [439]. Our data show that decrease in TCR signaling in MHC-II-restricted thymocytes results in significant $CD4^+$ to $CD8^+$ lineage redirection (or generation of DP mature T-cells). The higher frequency of T_{Reg} within the remaining $CD4^+$ T-cells in $OTII^+H\text{-}2K^{b/k}$ is likely due to intrinsic characteristics of T_{Reg} as they may be less susceptible to signal strength alterations. We found evidence suggesting that TCR signaling may inhibit the cytotoxic program, albeit partially, in developing thymocytes independently of ThPOK expression. One possible explanation could be that MHC-II-restricted TCR signaling could antagonize cytokine signaling by inducing SOCS proteins independently of ThPOK. Indeed, β-selected DP thymocytes are reported to express high levels of SOCS proteins, suggesting that Lck-derived signals in DN3b stage could be initiating their expression [189]. This could help explain our results showing dLGF partially downregulated the expression of CD103 from OTI and p14 mice. We found that ThPOK was the main $CD4^+$ lineage specifying transcription factor induced by MHC-II-restricted TCR signaling as forced expression of ThPOK rescued the lineage redirection caused by disruption of TCR signaling in $5cc7^+H\text{-}2K^{k/b}$ [189, 275]

Nonetheless, the molecular mechanism that leads to lineage redirection of MHC-II-signaled cells due to the loss of ThPOK function, or the disruption of TCR signaling does not seem to be similar [265, 286]. The fact that genuine MHC-I-restricted $CD8^+$ T-cells in $ThPOK^{gfp/gfp}$ had lower TCR signal strength than MHC-II-specific redirected $CD8^+$ T-cells

suggest that the two subsets may share distinct features. Our results suggest that MHC-II TCR signaling has a broader effect on $CD4^+$ lineage fate than ThPOK-mediated $CD4^+$ lineage choice of MHC-I-specific thymocytes. The inability of Runx3 to silence *Thpok* locus in $CD8^+GFP^+$ T-cells in $ThPOK^{gfp/gfp}$ mice demonstrate that ThPOK is dispensable for antagonizing Runx3-mediated silencing of its own locus. This also suggest that ThPOK, rather than promoting its own expression during $CD4^+$ lineage choice, could be more generally required for protecting cells from inducing the cytotoxic program. How do we explain continued activity of the *Thpok* locus in $CD8^+GFP^+$ T-cells in $Thpok^{gfp/gfp}$ mice? We suggest that MHC-II-specific TCR signaling may regulate the expression of a *Thpok*-inducing factor that functions to counteract the strong epigenetic *Thpok* silencing activity of Runx3 in these cells; in $CD8^+GFP^-$ T-cells such factor will not be induced and thus, *Thpok* locus will be inactive. Gata3 could fit this role as it is suggested to act as a pioneer transcription factor that can regulate gene activity by directly binding and recruiting histone modifying enzymes on condensed chromatin [71, 440, 441]. Gata3 acts early in $CD4^+$ T-cell development by promoting the development of the $CD4^+8^{lo}$ intermediate stage in MHC-II-restricted double positive (DP) thymocytes [274]. In agreement with this, we find continued Gata3 expression in $CD8^+GFP^-$ T-cells. However, constitutive Gata3 expression in OTI mice did not upregulate ThPOK or redirect thymocytes into $CD4^+$ lineage. Importantly, concomitant constitutive Gata3 and ThPOK expression in OTI mice not only failed to show synergistic effect on the $CD8^+$ to $CD4^+$ redirection but resulted in significant loss of mature $CD4^+$ T-cells compared to OTI mice expressing only ThPOK transgene. While reduction in the $CD8^+$ T-cell number in $OTI^+ThPOK\text{-}H^+Gata3^+$ mice is likely due to ThPOK-induced lineage redirection and/or Gata3-mediated impaired maturation, reduction in $CD4^+$ or DN mature T-cell number in these mice was unexpected [98, 273, 434, 437]. These data suggest that the impact of constitutive Gata3 on inducing apoptotic pathway in MHC-I-signaled thymocytes appears to be potentially preserved even in the redirected $CD4^+$ T-cells in ThPOK transgenic mice. Reduced CD127 (IL-7Ra chain) expression on residual $CD8^+$ T-cells from Gata3 transgenic mice may partly supports such a possibility as thymocytes rely on tonic TCR and cytokine signaling for homeostasis and survival [225, 230, 442, 443]. Finally, if and how Gata3, may contribute to suppression of cytotoxic program via a potential ThPOK-independent mechanism needs further study [195].

Acknowledgements

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3.7 Figures and figure legends

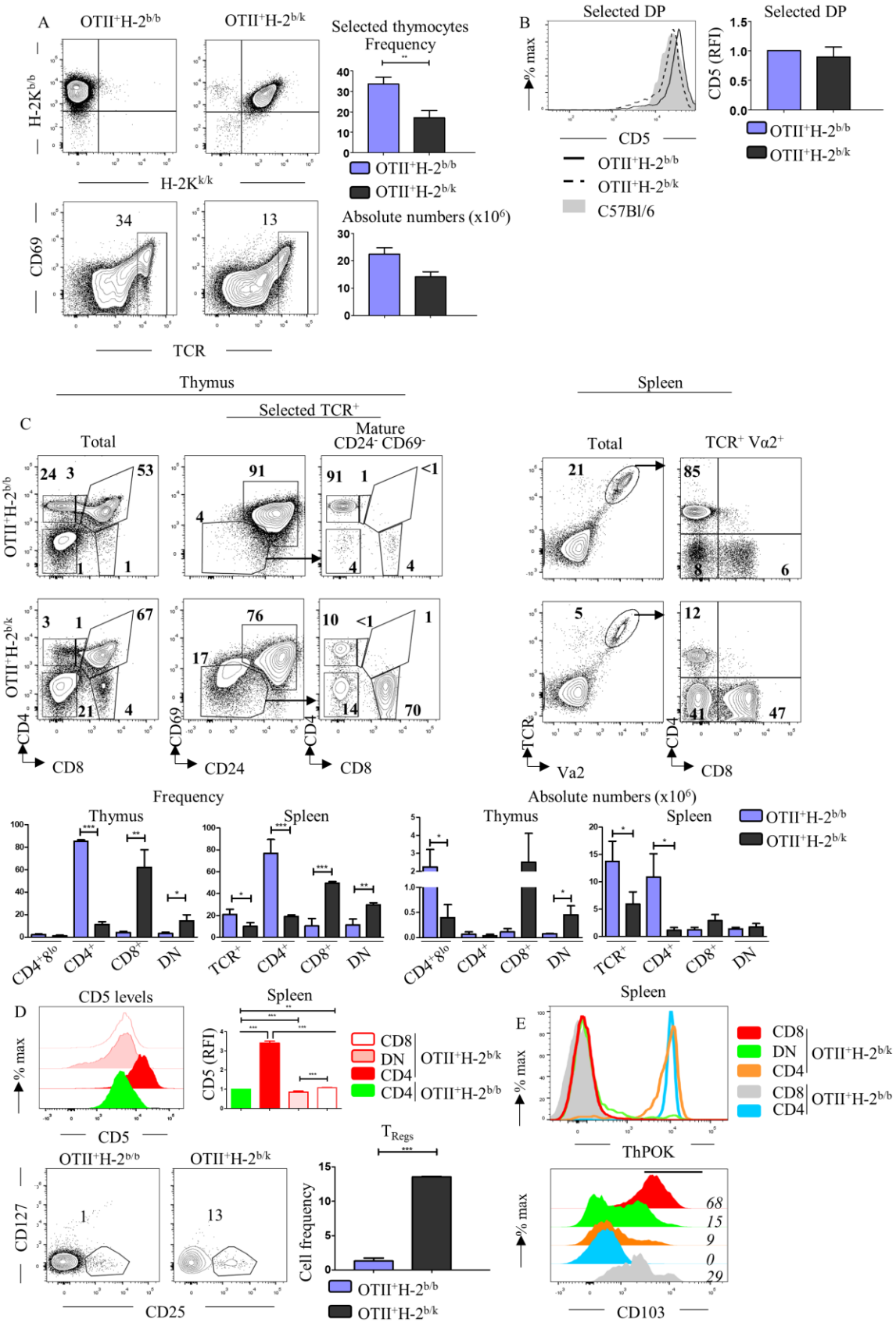
A
OTII+H-2b/b
OTII+H-2b/k
H-2Kb/b
H-2Kk/k
CD69
TCR
34
13
Selected thymocytes Frequency
OTII+H-2b/b
OTII+H-2b/k
Absolute numbers (x10^6)
B
Selected DP
% max
CD5
OTII+H-2b/b
OTII+H-2b/k
C57Bl/6
Selected DP
CD5 (RFI)
OTII+H-2b/b
OTII+H-2b/k
C
Thymus
Spleen
Selected TCR+
Total
Mature CD24- CD69-
TCR+ Vα2+
OTII+H-2b/b
OTII+H-2b/k
CD4
CD8
CD24
Va2
TCR
Frequency
Absolute numbers (x10^6)
Thymus
Spleen
CD4+8lo
CD4+
CD8+
DN
TCR+
OTII+H-2b/b
OTII+H-2b/k
D
CD5 levels
% max
CD5
Spleen
CD5 (RFI)
CD8
DN
CD4
CD4
OTII+H-2b/k
OTII+H-2b/b
CD127
CD25
1
13
TRegs
Cell frequency
E
Spleen
% max
ThPOK
CD103
68
15
9
0
29

Figure 24. Disruption of MHC-II TCR signaling during lineage commitment impairs CD4$^+$ lineage commitment

(A) FACS analysis showing expression of H-2K^k and H-2K^b alleles (upper panels) and positive selection (bottom panels) in OTII$^+$H-2K$^{b/b}$ and OTII$^+$H-2K$^{b/k}$ mice. Values indicate the percentage of cells in each gate. **(B)** Histogram showing cell-surface expression of CD5 on selected DP T-cell subsets of indicated genotypes. **(C)** Contour analysis showing CD4 and CD8 expression in total thymocytes, mature thymocytes (gated on TCR$^+$CD24$^-$CD69$^-$) and spleen TCRβ$^+$ Vα2$^+$ T-cells from mice of the indicated genotypes. Numbers indicate the percentage of cells in each gate. **(D)** FACS analysis showing the CD5 MFI (upper panel) and CD5 relative fluorescence intensity (RFI) (upper histogram) in the different subsets from OTII$^+$H-2K^{bk} as compared with CD5 levels on CD4$^+$ T-cells from OTII$^+$H-2K^b and frequency of T_{reg} (lower panel) in CD4$^+$ T-cells from mice of the indicated genotypes. **(E)** Histograms showing *ThPOK*^{GFP} reporter expression (top panel) and CD103 expression levels (bottom panel) in splenic T-cells of indicated genotypes. Values indicate the frequency of CD103$^+$ T-cells in the indicated gate. Data are representative of at least two independent experiments with n=3.

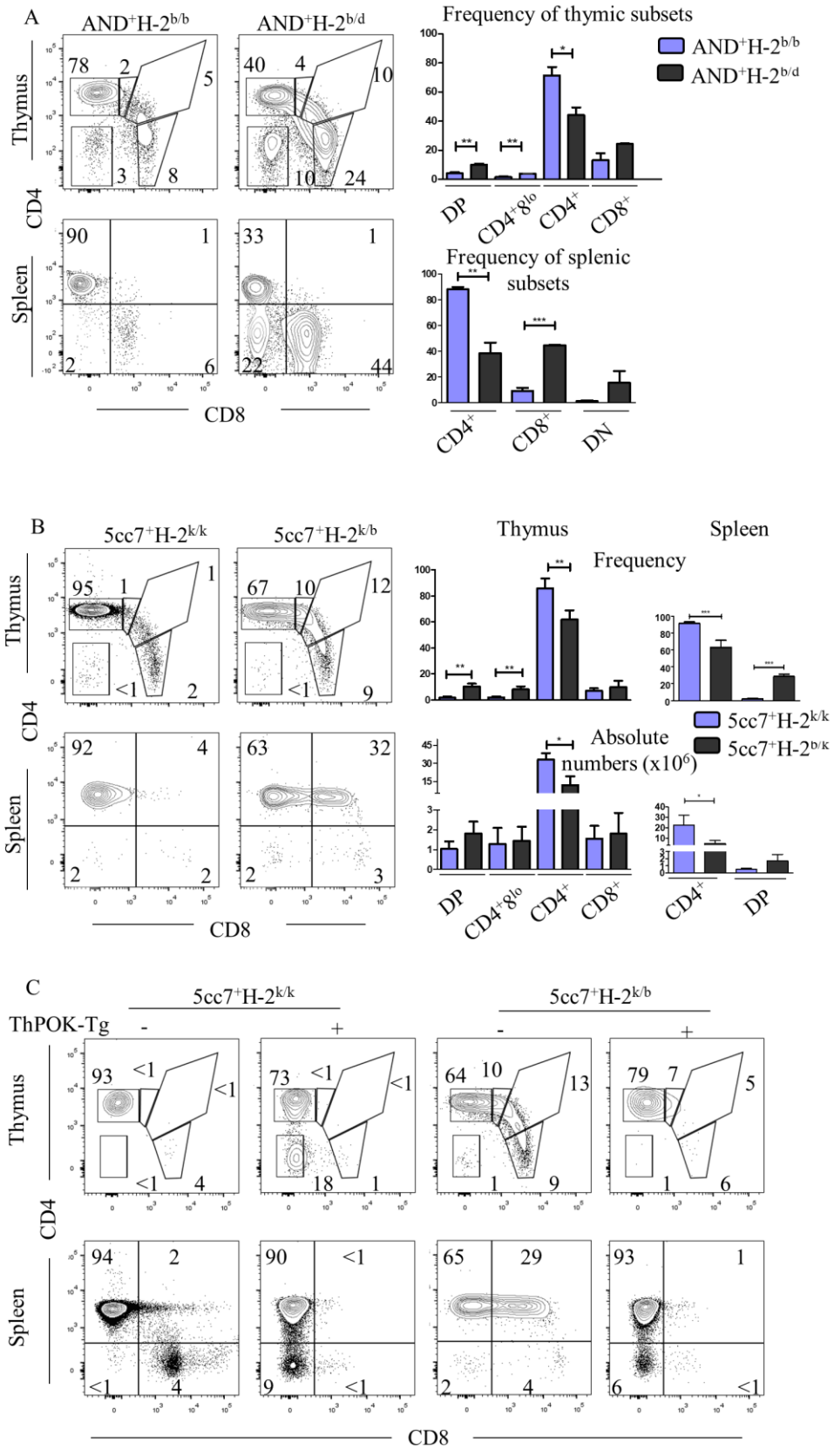
A
AND+H-2b/b
AND+H-2b/d
Thymus
Spleen
CD4
CD8
78
2
5
3
8
40
4
10
10
24
90
1
2
6
33
1
22
44
Frequency of thymic subsets
AND+H-2b/b
AND+H-2b/d
DP
CD4+8lo
CD4+
CD8+
Frequency of splenic subsets
CD4+
CD8+
DN
B
5cc7+H-2k/k
5cc7+H-2k/b
Thymus
Spleen
95
1
1
<1
2
67
10
12
<1
9
92
4
2
2
63
32
2
3
Frequency
Absolute numbers (x10^6)
5cc7+H-2k/k
5cc7+H-2b/k
DP
CD4+8lo
CD4+
CD8+
CD4+
DP
C
5cc7+H-2k/k
5cc7+H-2k/b
ThPOK-Tg
-
+
-
+
93
<1
<1
<1
4
73
<1
<1
18
1
64
10
13
1
9
79
7
5
1
6
94
2
<1
4
90
<1
9
<1
65
29
2
4
93
1
6
<1

Figure 25. Lineage redirection in MHC-II impaired TCR signaling can be rescued by constitutive expression of transgenic ThPOK

(A and B) FACS analysis showing CD4 and CD8 expression in mature thymocytes and spleen TCRβ^{+}Vα11^{+} T-cells from mice of indicated genotypes. **(C)** CD4 and CD8 cell-surface expression on mature thymocytes and TCRβ^{+}Vα11^{+} gated splenocytes were analysed from freshly isolated thymus and spleen organs from mice of indicated genotypes. Data are representative of at least two experiments with similar results with n=3. Numbers indicate the percentage of cells in each gate.

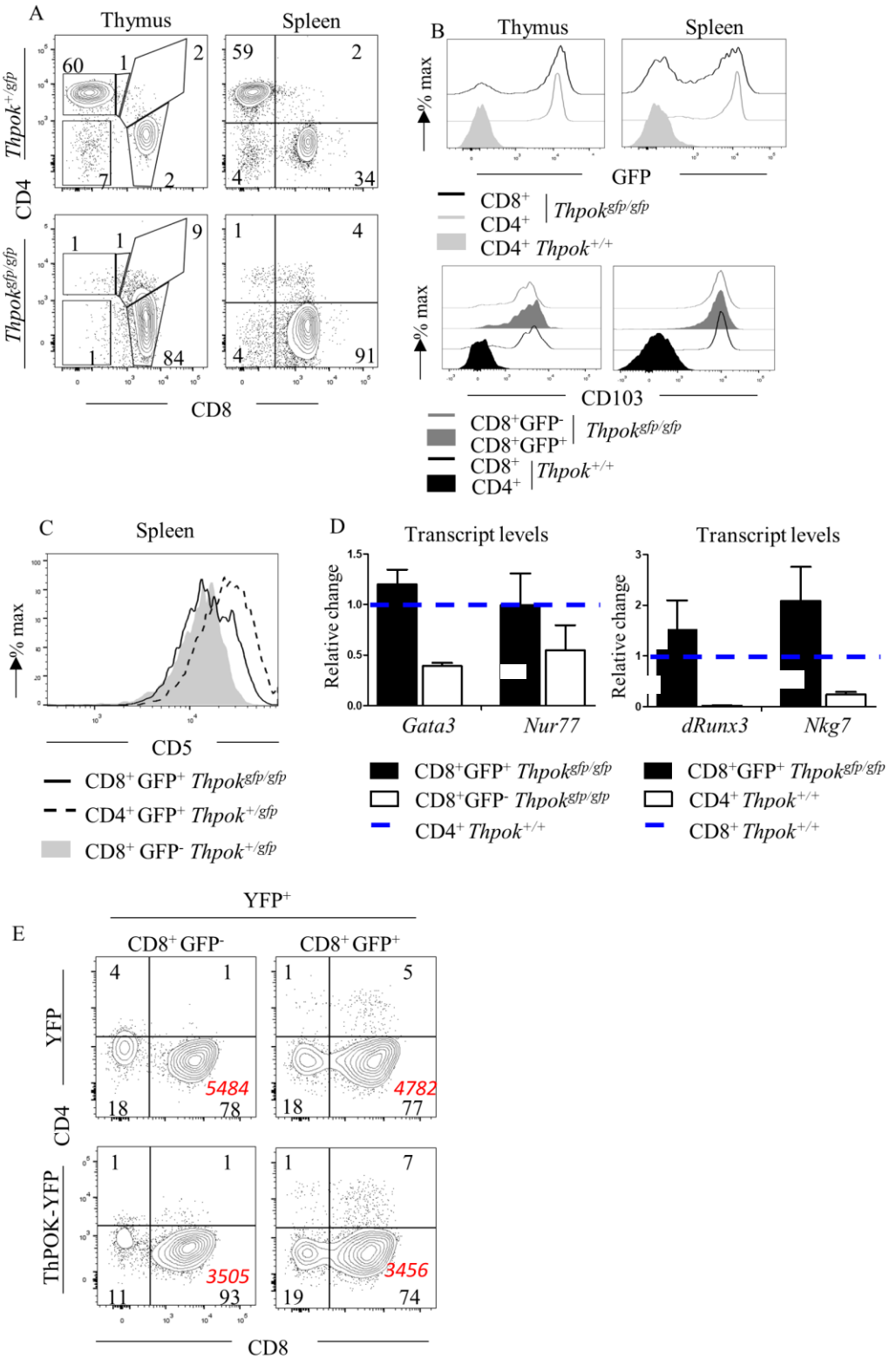
A
Thymus
Spleen
Thpok+/gfp
Thpokgfp/gfp
CD4
CD8
60
1
2
7
2
59
2
4
34
1
1
9
1
84
1
4
4
91
B
Thymus
Spleen
% max
GFP
CD8+
CD4+
Thpokgfp/gfp
CD4+ Thpok+/+
% max
CD103
CD8+GFP-
CD8+GFP+
Thpokgfp/gfp
CD8+
CD4+
Thpok+/+
C
Spleen
% max
CD5
CD8+ GFP+ Thpokgfp/gfp
CD4+ GFP+ Thpok+/gfp
CD8+ GFP- Thpok+/gfp
D
Transcript levels
Relative change
1.5
1.0
0.5
0.0
Gata3
Nur77
Transcript levels
Relative change
3
2
1
0
dRunx3
Nkg7
CD8+GFP+ Thpokgfp/gfp
CD8+GFP- Thpokgfp/gfp
CD4+ Thpok+/+
CD8+GFP+ Thpokgfp/gfp
CD4+ Thpok+/+
CD8+ Thpok+/+
E
YFP+
CD8+ GFP-
CD8+ GFP+
YFP
CD4
ThPOK-YFP
4
1
5484
18
78
1
5
4782
18
77
1
1
3505
11
93
1
7
3456
19
74
CD8

Figure 26. ThPOK deficiency does not affect class-II TCR signaling in redirected T-cells

(A) Contour plot showing CD4 and CD8 expression on mature thymocytes and TCRβ$^+$ splenocytes from mice of indicated genotypes. Numbers indicate the frequency of cells in designated gates. **(B)** (Upper panels) Histograms showing *Thpok*^{GFP} reporter expression in mature thymocytes and TCRβ$^+$ gated splenocytes, in the presence (*Thpok*$^{+/gfp}$) or absence (*Thpok*$^{gfp/gfp}$) of ThPOK, in T-cell subsets from mice of the indicated genotypes. Shaded histogram is GFP$^-$ control. (Lower panels) Histograms showing cell-surface expression of CD103 in CD4$^+$ and CD8$^+$ mature thymocytes and TCRβ$^+$ splenocytes from *Thpok*$^{+/gfp}$ and *Thpok*$^{gfp/gfp}$ mice. **(C)** Flow cytometry of expression of CD5 splenic T-cells subsets from mice of the indicated genotypes. **(D)** Quantitative RT-PCR analysis of MHC-II-specific gene, *Gata3* and *Nur77* and MHC-I-specific genes (*dRunx3* and *NKG7*) in CD8$^+$GFP$^+$ and CD8$^+$GFP$^-$ peripheral splenocytes from *Thpok*$^{gfp/gfp}$ mice and CD4$^+$ peripheral splenocytes sorted from and *Thpok*$^{+/gfp}$ mice. **(E)** Contour plots showing CD4 and CD8 expression on CD8$^+$GFP$^-$ or CD8$^+$GFP$^+$ peripheral T-cells infected with a ThPOK-expressing vector (ThPOK-YFP) or an empty vector (YFP). Values adjacent to outlined areas represent frequency cells in each. Values in red represent mean fluorescence intensity (MFI) of CD8. Data are representative of two analyses with similar results with n≥2.

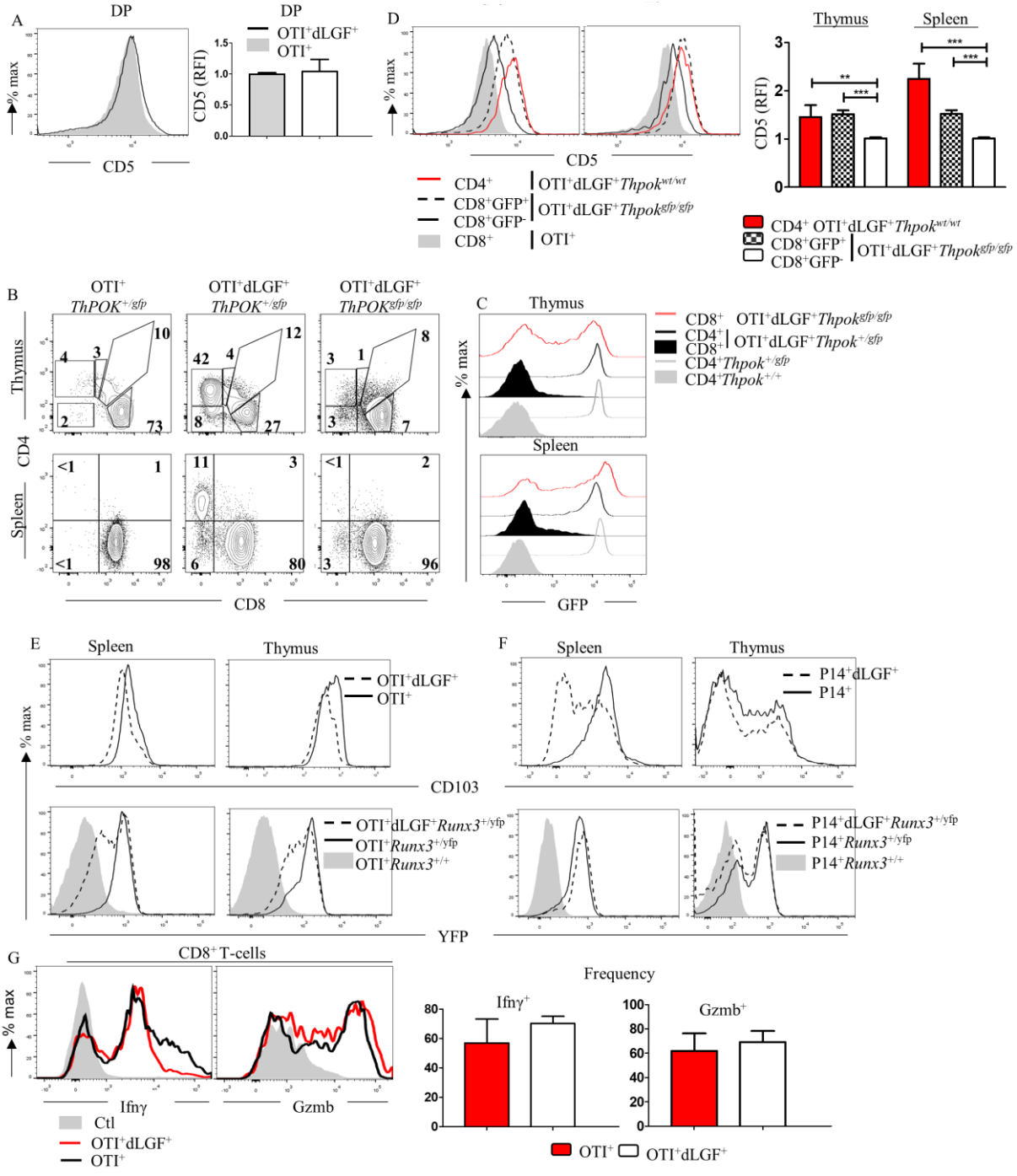
A
DP
% max
CD5
DP
OTI+dLGF+
OTI+
CD5 (RFI)
D
% max
CD5
CD4+
CD8+GFP+
CD8+GFP-
CD8+
OTI+dLGF+Thpokwt/wt
OTI+dLGF+Thpokgfp/gfp
OTI+
Thymus
Spleen
CD5 (RFI)
CD4+ OTI+dLGF+Thpokwt/wt
CD8+GFP+ OTI+dLGF+Thpokgfp/gfp
CD8+GFP-
B
OTI+
ThPOK+/gfp
OTI+dLGF+
ThPOK+/gfp
OTI+dLGF+
ThPOKgfp/gfp
Thymus
Spleen
CD4
CD8
C
Thymus
Spleen
% max
GFP
CD8+ OTI+dLGF+Thpokgfp/gfp
CD4+ CD8+ OTI+dLGF+Thpok+/gfp
CD4+Thpok+/gfp
CD4+Thpok+/+
E
Spleen
Thymus
% max
CD103
YFP
OTI+dLGF+
OTI+
OTI+dLGF+Runx3+/yfp
OTI+Runx3+/yfp
OTI+Runx3+/+
F
Spleen
Thymus
P14+dLGF+
P14+
P14+dLGF+Runx3+/yfp
P14+Runx3+/yfp
P14+Runx3+/+
G
CD8+ T-cells
% max
Ifnγ
Gzmb
Ctl
OTI+dLGF+
OTI+
Frequency
Ifnγ+
Gzmb+
OTI+
OTI+dLGF+

Figure 27. Strong TCR signaling can induce helper function and repress cytotoxic program independently of ThPOK

(A) CD5 expression on DP thymocytes from OTI and OTI^{+}dLGF^{+} mice. **(B)** Expression of CD4 and CD8 in mature thymocytes from mice of the indicated genotypes. Numbers next to the outlined areas represent percentage cells in each. **(C)** *Thpok*^{GFP} reporter expression in TCRβ^{+} splenocytes from mice of the indicated genotypes. **(D)** Histograms and bar graph compilation illustrating surface CD5 expression levels in indicated subsets from mice of the indicated genotypes. Bar graph shows CD5 relative fluorescence intensity (RFI) compilation in the different subsets in comparison with levels in CD8^{+}GFP^{+} from OTI^{+}dLGF^{+} mice **(E** and **F)** Surface CD103 expression and *Runx3*^{YFP} reporter expression in mature CD8 thymocytes and peripheral TCRβ^{+} CD8^{+} T-cells from mice of the indicated genotypes. **(G)** Purified CD8^{+} T-cells from mice of the indicated genotypes were activated with plate-bound α-CD3ε, soluble α-CD28 and rIL2 for three days and analysed for intracellular Ifnγ and Gzmb in the presence of brefeldin-A. The shaded histogram is unstimulated CD8^{+} cells from OTI^{+}dLGF^{+}. Bar graph shows compilation of Ifnγ and Gzmb. Representative example of two or more experiments with n≥2.

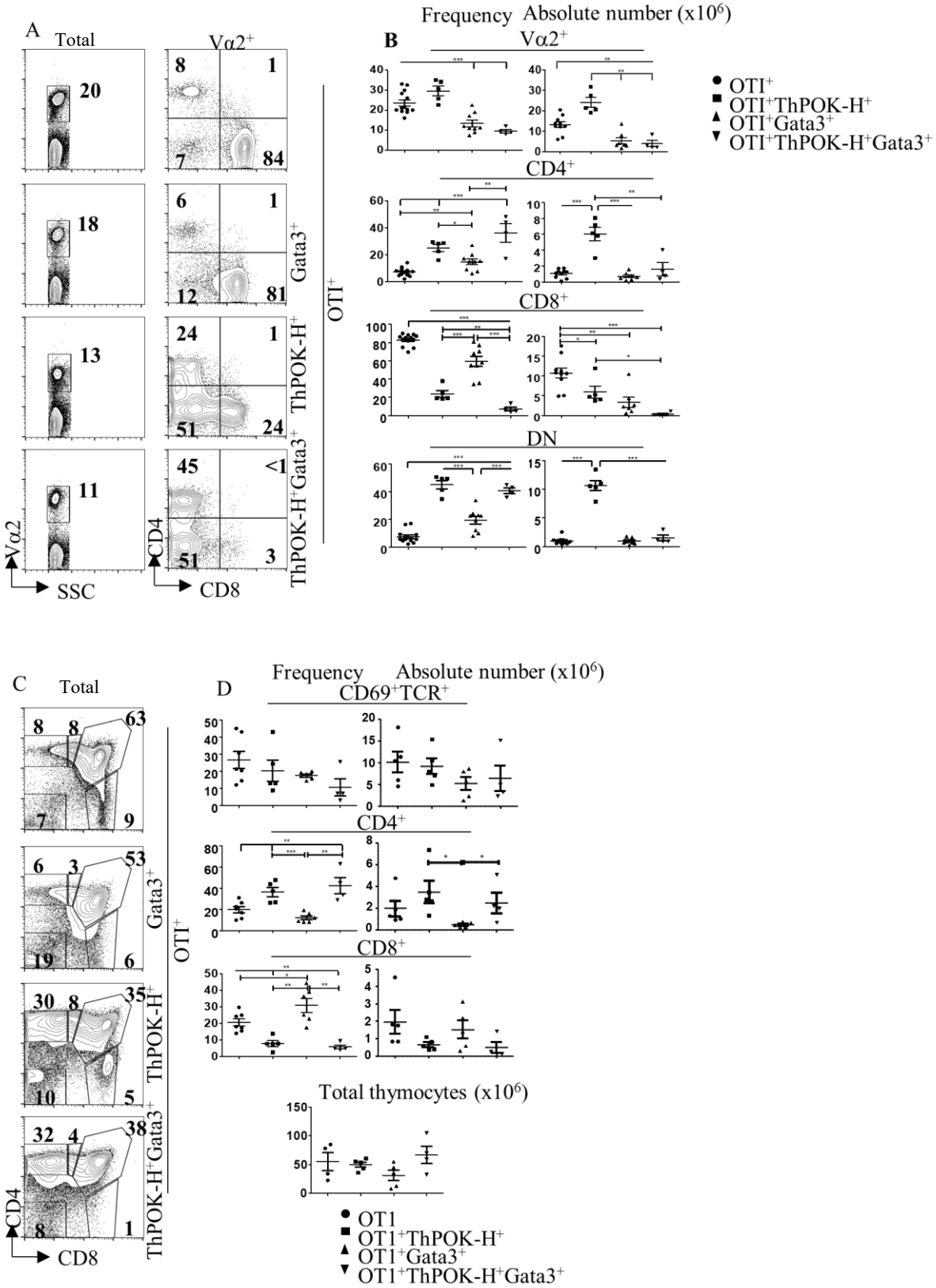
A
Total
Vα2+
20
8
1
7
84
18
6
1
12
81
13
24
1
51
24
11
45
<1
51
3
Vα2
SSC
CD4
CD8
Gata3+
ThPOK-H+
ThPOK-H+Gata3+
OTI+
B
Frequency
Absolute number (x10^6)
Vα2+
CD4+
CD8+
DN
OTI+
OTI+ThPOK-H+
OTI+Gata3+
OTI+ThPOK-H+Gata3+
C
Total
8
8
63
7
9
6
3
53
19
6
30
8
35
10
5
32
4
38
8
1
CD4
CD8
Gata3+
ThPOK-H+
ThPOK-H+Gata3+
OTI+
D
Frequency
Absolute number (x10^6)
CD69+TCR+
CD4+
CD8+
Total thymocytes (x10^6)
OT1
OT1+ThPOK-H+
OT1+Gata3+
OT1+ThPOK-H+Gata3+

Figure 28. Constitutive Gata3 differentially affects the frequency and number of redirected T-cells in OTI^{+}ThPOK-H^{+} mice

OTI^{+}ThPOK-H^{+}Gata3^{+} mice were generated to determine if constitutively expressed Gata3 enhanced CD8^{+} to CD4^{+} lineage redirection in OTI^{+}ThPOK-H^{+} mice. CD4 and CD8 expression of Vα2^{+} splenic T-cells **(A)** and thymocytes **(C)** of mice from the indicated strain was analyzed by flow cytometry. In panel **(B)**, the frequencies and absolute numbers of Vα2^{+} T-cells, CD4^{+}, CD8^{+} and DN mature T-cell population in the spleen of various mice are shown. In panel **(D)**, the frequencies and absolute numbers of positively selected thymocytes (CD69^{+}TCR^{+}), CD4 SP and CD8 SP thymocytes from mice of the indicated genotypes are shown. Total thymocytes number and CD4^{+}/CD8^{+} ratio for single positive thymocytes are also shown for these mice. Data in panels A and C are representative example of 3 to 5 independent experiments with n>5.

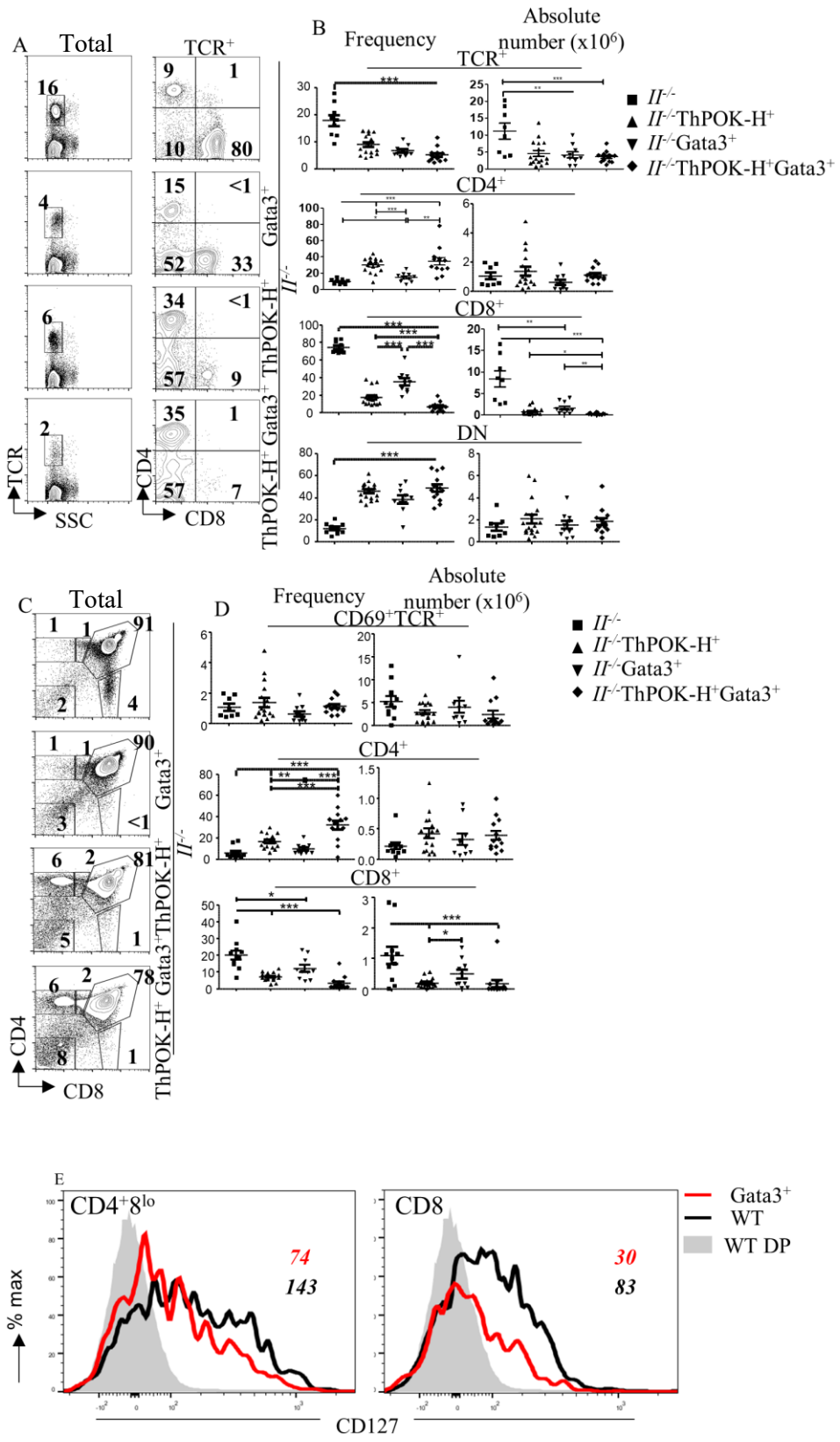
A
Total
TCR+
TCR
SSC
CD4
CD8
Gata3+
ThPOK-H+
Gata3+ ThPOK-H+
Il-/-
B
Frequency
Absolute number (x10^6)
TCR+
CD4+
CD8+
DN
Il-/-
Il-/-ThPOK-H+
Il-/-Gata3+
Il-/-ThPOK-H+Gata3+
C
Total
D
Frequency
Absolute number (x10^6)
CD69+TCR+
CD4+
CD8+
E
CD4+8lo
CD8
74
143
30
83
% max
CD127
Gata3+
WT
WT DP

Figure 29. Enforced GATA3 differentially affects the frequency and number of redirected T-cells in OTI^{+}ThPOK-H^{+} mice

ThPOK-H and GATA3 transgenes were expressed individually or together in MHC II-deficient mice to study the lineage redirection of polyclonal MHC class-I-restricted thymocytes. **(A)** Shows flow cytometric analysis of spleen of GATA3^{+}*II*$^{-/-}$, THPOK-H^{+}*II*$^{-/-}$, ThPOK-H^{+}GATA3^{+}*II*$^{-/-}$ and *II*$^{-/-}$ mice. Numbers in the quadrants indicate percent cells in each. **(B)** Graphs illustrating the frequencies and absolute number of T-cells and T-cell subsets in the spleen of the indicated strain of mice. **(B)** Also shows CD4^{+}/CD8^{+} ratio of splenic T-cells. **(C)** CD4^{+} and CD8^{+} profile of total thymocytes from the indicated strain of mice. The frequencies and absolute number of positively selected thymocytes, CD4^{+} SP, and CD8^{+} SP thymocytes are shown in **(D)**. Also shown are total thymocytes numbers and CD4^{+}/CD8^{+} ratio of SP thymocytes in GATA3^{+}*II*$^{-/-}$, THPOK-H^{+}*II*$^{-/-}$, ThPOK-H^{+}GATA3^{+}*II*$^{-/-}$ and *II*$^{-/-}$ mice. **(E)** Histograms showing CD127 expression in CD4^{+} and CD4^{+}8^{lo} thymocytes from mice of the indicated genotypes. Data are representative of at least two independent experiments with n>5.

Chapter 4: Discussion

T lymphocytes are at the heart of the adaptive immune system and play an important role in eliminating offending pathogens and transformed cells. T-cell progenitors originate in the BM and migrate to the thymus where the T-cell identity is established through discrete developmentally regulated steps comprising sequential commitment stages and lineage checkpoints [79, 189]. The $CD4^+/CD8^+$ cell-fate specification is an essential developmental event and is regulated by MHC specificity and duration of TCR signaling via expression of lineage-specific transcription factors [326]. The goal of this thesis was to investigate the role of ThPOK and TCR signaling and, thereby, further our understanding of the mechanisms of $CD4^+$ helper and $CD8^+$ cytotoxic lineage choice of developing thymocytes.

In the first part of this thesis (manuscript #1), our goal was to study, in the first place, the dose dependent effect of ThPOK on $CD4^+$ lineage fate and helper function and, secondly, to determine the role of TCR signaling and MHC specificity on ThPOK-mediated $CD4^+$ lineage fate. Studies with ThPOK hypomorphic mice showed that partial ThPOK loss-of-function redirected only a fraction of MHC-II-restricted T-cells into the $CD8^+$ lineage [276, 320, 321]. Importantly, the residual $CD4^+$ T-cells from ThPOK hypomorphic mice upregulated Runx3, which resulted in the loss of helper-lineage features and gain of cytotoxic characteristics [320, 321]. This suggested that higher ThPOK levels are required to prevent differentiation of mature $CD4^+$ T-cells towards the cytotoxic lineage, demonstrating that the ThPOK doses required for the acquisition of the $CD4^+$ SP surface phenotype and the helper-lineage functions are different. Results from our gain-of-function experiments showed a similar dose dependent role for ThPOK **(manuscript #1 Fig. 14)** as one of the transgenes, ThPOK-H, induced only a partial $CD8^+$ to $CD4^+$ lineage redirection. The ThPOK-H transgene expressed 1.5 to 2-fold more ThPOK levels compared to the endogenous locus in conventional $CD4^+$ T-cells. This suggests that the transgenic ThPOK should rescue conventional $CD4^+$ development in ThPOK deficient mice, which indeed was the case, as evident from the phenotype of $OTII^+$*Thpok*$^{-/-}$ mice expressing or not ThPOK-H. Partial lineage redirection of MHC-I-specific thymocytes, which transduce a different TCR signal compared to MHC-II-specific thymocytes, by ThPOK-H prompted us to define the role of TCR signaling in ThPOK-mediated $CD4^+$ helper fate decision. The ability of constitutively active Lck transgene (dLGF) to significantly augment $CD8^+$ to $CD4^+$ lineage redirection in OTI^+ThPOK-H^+ mice, supported a role for TCR signaling in $CD4^+$ lineage choice

in these mice. However, the $CD4^+$ SP frequency in $OTI^+dLGF^+ThPOK\text{-}H^+$*Thpok*$^{-/-}$ mice was still significantly lower than $OTII^+ThPOK\text{-}H^+$*Thpok*$^{-/-}$ **(manuscript #1, Fig. 16, 17, 18)** suggesting an important role for MHC specificity as well in ThPOK-mediated $CD4^+$ lineage choice.

In the second part of the thesis (manuscript #2), we further characterized the role of TCR signaling and downstream events in $CD4^+$ T-cell development. Our results showed that sustained TCR signaling may protect $CD4^+$ lineage integrity by potentially contributing to the inhibition of Runx3-dependent cytotoxic program and inducing $CD4^+$-specific genes, independently of ThPOK **(manuscript #2 Fig. 24, 26, 27)**. The fact that ThPOK and Gata3 are both induced by TCR signaling in MHC-II-restricted T-cells, motivated us to study their interplay in $CD4^+$ cell identity **(manuscript #2 Fig. 28, 29)** [276]. We have generated evidence possibly implicating Gata3 in the selective disruption of MHC-I-specific T-cells irrespective of their lineage fate **(manuscript #2 Fig. 28, 29)**. This study furthers our understanding of the role of TCR signaling in the of induction/suppression of the network of genes governing $CD4^+/CD8^+$ lineage fate of positively selected thymocytes.

4.1 Role of TCR signaling in ThPOK-mediated $CD4^+$ lineage commitment (manuscript#1)

4.1.1 The dose dependent effect of ThPOK

One purpose of this study was to investigate the dose-dependent effect of ThPOK on lineage commitment and helper function. In 2008, two independent research groups showed that the introduction of a ThPOK hypomorphic allele in MHC-II-restricted thymocytes redirected a fraction of them to the DN lineage fate, which expressed lower ThPOK levels compared to conventional $CD4^+$ T-cells [320, 321]. This indicated that lowering ThPOK levels below a certain threshold in MHC-II-restricted thymocytes does not necessarily result in $CD8^+$ lineage redirection. Instead, ThPOK exhibited a dose-dependent modulation of $CD4^+$ lineage fate. Importantly, compared to control mice, $CD4^+$ T-cells from the hypomorphic mice expressed lower amounts of ThPOK, which enabled Runx3 upregulation.

Our ThPOK transgenic founder lines were produced by cloning a DNA sequence that included the two coding exons of ThPOK downstream of the hCD2 promoter, resulting in an early constitutive expression of ThPOK that significantly increased the frequency of $CD4^+$ T-cells in the thymus and spleen **(manuscript #1 Fig. 12, 20)** [217, 276, 326, 331]. Further analysis demonstrated that the founder lines that we generated, expressed different levels of ThPOK **(manuscript #1 Fig. 12, 20)**. Previous reports describing ThPOK transgenic mice suggested a complete $CD8^+$ to CD4+ lineage redirection of MHC-I-specific transgenic TCR (HY-TCR, P14-TCR, OTI-TCR). However, both the studies were done in Rag-sufficient background thus making it difficult to assess if the complete $CD8^+$ to $CD4^+$ lineage redirection was due to ThPOK or that the endogenous TCR expression could have contributed to this process as well [265, 268]. Further, these studies failed to suggest if the ThPOK-mediated lineage redirection was dose dependent. In contrast, we have done extensive characterization of our three independent ThPOK transgenic mouse lines at RNA and, importantly, protein level. All three lines showed significant increase in $CD4^+$ and decrease in $CD8^+$ frequencies in the thymus and spleen, compared to WT control. Comparing the $CD4^+/CD8^+$ frequencies amongst the three lines showed that the line with the lowest ThPOK expression (ThPOK-H) showed significantly fewer $CD4^+$ T-cells compared with the line with the strongest expression (ThPOK-611). The third line, ThPOK-163, displayed an intermediate phenotype, thus highlighting the dose-dependent gain-of-function of ThPOK in $CD4^+$ lineage commitment. As the same ThPOK-expressing vector was used to generate all the founder lines, we propose that chromatin organisation at the transgene integration sites likely affected expression patterns. While ThPOK-611 transgene integration probably occurred in euchromatic regions of the genome, in ThPOK-H and ThPOK-163, integration likely occurred in both heterochromatic and euchromatic regions of the genome. The insertion of a transgene in heterochromatin DNA is often associated with a variegated pattern of expression [444, 445]. Indeed, analysis of ThPOK protein expression, by flow cytometry, in DP thymocytes from $OTI^+ThPOK\text{-}H^+$ mice show a wide peak, with some cells expressing high levels of ThPOK and others expressing lower or no ThPOK. In contrast, ThPOK protein levels in $OTI^+ThPOK\text{-}611^+$ mice were more uniform. This likely provides an explanation for the difference in mean fluorescence intensity between the two transgenic lines **(manuscript #1 Fig. 12, 20)**. It is tempting to speculate that in $OTI^+ThPOK\text{-}H^+$, mature $CD4^+$ T-cells originate from the $ThPOK^{hi}$ fraction, the DN from the $ThPOK^{med}$ cells, and $CD8^+$ T-

cells from the ThPOK^{lo} population **(Fig. 30)**. To determine the impact of variegated expression on lineage choice, would require transgenic mice expressing ThPOK and a reporter gene as a bicistronic message. In contrast to DP thymocytes, ThPOK staining in peripheral T-cells from ThPOK-H mice gives a relatively tight peak, further suggesting that thymic progenitors expressing similar ThPOK levels differentiate into a unique peripheral T-cell subset **(manuscript #1 Fig. 12)**. Regardless of the reasons behind the partial redirection of CD8$^+$ T-cells to the CD4$^+$ lineage, our model provides us with the unique possibility to study the dose-dependent effect of ThPOK on lineage fate and cell function.

During lineage commitment, MHC-II TCR signaling favors CD4$^+$ T-cell development by positively and negatively regulating the transcription of a set of genes involved in the helper and cytotoxic programs, respectively [446]. Indeed, our results show that the constitutively active Lck (dLGF) transgene redirected selected thymocytes to the CD4$^+$ lineage fate by inducing ThPOK expression and suppressing Runx3 **(manuscript #1 Fig. 17, 18; manuscript #2 Fig. 27)**. In the absence of ThPOK, selected MHC-II-restricted thymocytes are redirected to the CD8$^+$ lineage due to Runx3 induction [320]. However, the presence of CD4$^+$ T-cells in ThPOK-deficient mice that also lacked Runx complexes (*Core binding factor beta – Cbfb*-deficiency) suggest that the CD4$^+$ lineage commitment may be ThPOK independent [293, 320]. Assuming that CD4$^+$ T-cells in *Thpok*$^{-/-}$*Cbfb*$^{-/-}$ are MHC-II-specific, this study suggests that specific TCR signaling in itself may be sufficient for CD4$^+$ lineage choice. As a matter of fact, the observation that a significantly higher frequency of CD4$^+$ T-cells in OTI$^+$dLGF$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice compared to OTI$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ genotype strongly suggest that a constitutively active TCR signaling promotes CD4$^+$ lineage independent of ThPOK.

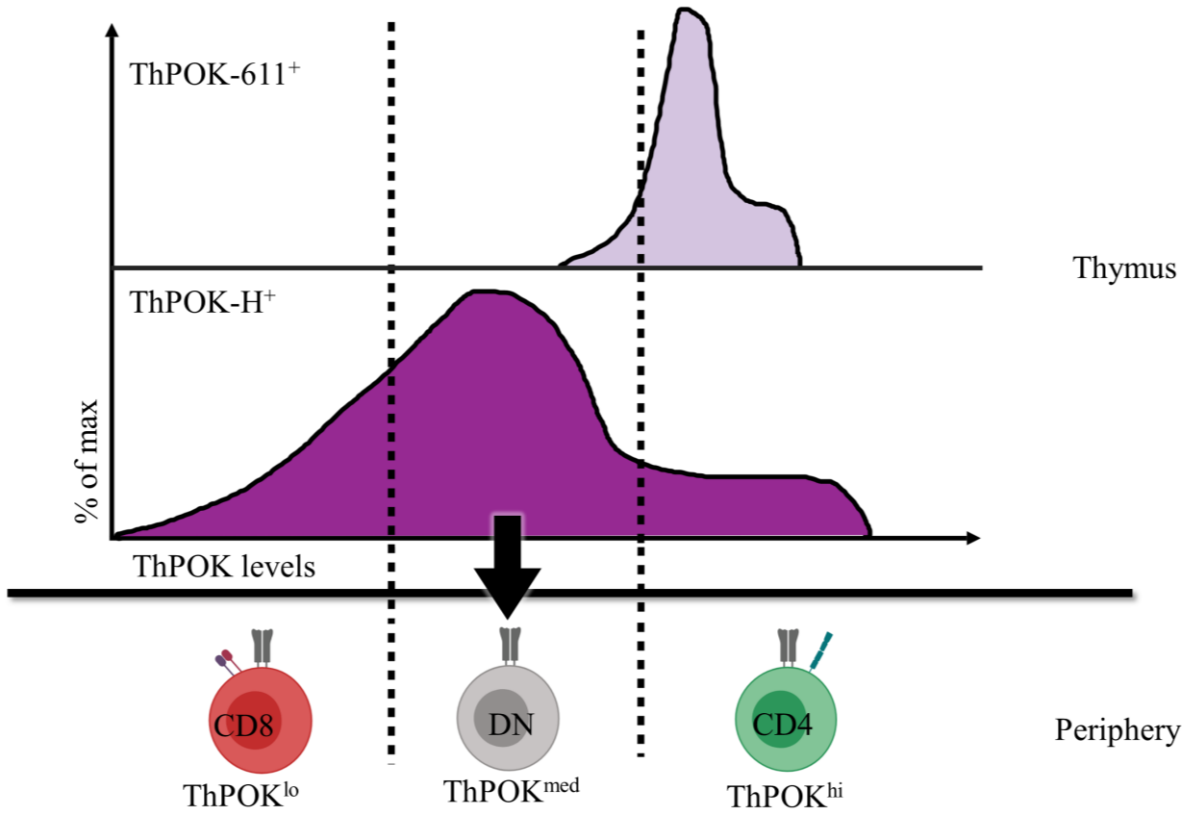

Figure 30. ThPOK expression profile in DP thymocytes determines lineage fate

This drawing shows ThPOK expression profile in double positive (DP) thymocytes from ThPOK-611$^+$ and ThPOK-H$^+$ transgenic mice. It is possible that different expression levels of ThPOK in ThPOK-H$^+$ results in the partial redirection of major histocompatibility complex (MHC)-I-restricted CD8$^+$ T-cells to the CD4$^+$ lineage fate.

ThPOK directs thymocytes to the helper fate by regulating CD4-specifying genes, like SOCS proteins, and by antagonising Runx3-mediated activation of the cytotoxic program [293, 322]. Our gene expression comparison of the three peripheral T-cell subsets in OTI$^+$ThPOK-H$^+$ has highlighted the mechanism of ThPOK dose-dependent regulation of CD4$^+$/CD8$^+$ lineage fate. While only high ThPOK expression levels could induce SOCS1, intermediate and higher amounts of ThPOK upregulated the anti-cytotoxic lineage factor, Nur77 [425]. However, it

remains to be determined if Nur77, which is reported to suppress Runx3, is involved in inducing the $CD4^+$ lineage fate [425]. Finally, we noted that the low levels of ThPOK in $CD8^+$ T-cells, which failed to induce any lineage-determining factors, could still disrupt the cytotoxic function following T-cell activation. Based on these data, we propose a model wherein low or no ThPOK leads to $CD8^+$ lineage choice, moderate ThPOK inducing *Nur77* expression promotes DN mature T-cell development and higher ThPOK resulting in *SOCS1* and *Nur77* induction mediates CD4 lineage choice **(Fig. 32)**.

It needs to be noted that our results provide the first experimental evidence to suggest that ThPOK may act through Nur77 to inhibit *Runx3* induction and cytotoxic program in MHC-I-specific thymocytes. The transcription factor Nur77, which serves primarily as an indicator of TCR signal strength, has been shown to regulate $CD8^+$ T-cell development by suppressing the expression of Runx3 [425]. We show here that Nur77 is part of the network of transcription factors that is regulated by ThPOK during lineage fate **(manuscript #1, Fig. 14)**. However, we were unable to validate in promoter-luciferase reporter assay that Nur77 is a direct target gene of ThPOK. Interestingly, we noticed some Nur77-reporter upregulation, albeit insignificant, in *in vivo* model **(manuscript #1, Fig. 14, 22)**. Several factors can explain the apparent discrepancy between our *in vivo* and *in vitro* studies. For instance, the cell line (HEK293 cells) we used in our experiment may be physiologically irrelevant for evaluating *Nur77* promoter activity in the presence of ThPOK possibly due to lack of a binding partner. Alternately, ThPOK may regulate Nur77 expression via some other *cis* acting motif that is not present in the DNA fragment used for reporter assay; the DNA fragment contained only the promoter sequence of *Nur77* (2kb DNA fragment upstream of the transcription start site and containing ThPOK binding sites). A recent chromatin IP and sequencing data strongly suggest that ThPOK may bind to *Nur77* sequence 3.5kb and 16kb upstream of the start codon [447]. To more precisely determine if ThPOK is recruited to the other regulatory regions of Nur77, reporter gene expression regulated by these motifs would be required.

Results from our transcriptional analysis, suggesting unique gene signature in $CD4^+$ and DN T-cells, coupled with our findings that the DN T-cell subset develop from the $CD4^+$ thymic compartment reflect a complex regulatory mechanism of gene expression in developing thymocytes **(manuscript #1 Fig. 14, 21)**. The fraction of $CD4^+$ thymocytes with intermediate

levels of ThPOK, most likely represents the population that is destined to give rise to DN T-cells **(manuscript #1 Fig. 19)**. They appear phenotypically as $CD4^+$ SP T-cells likely because of continued intra-thymic signaling leading to delayed CD4 downregulation possibly by Runx1 in association with Tle and Tcf/Lef proteins [326]. Although there is no evidence suggesting that the dynamics of Runx1-mediated inactivation of the *Cd4* locus is any different from that of Runx3, it is possible that in the presence of ThPOK, the Runx1-dependent activation of the *Cd4* silencer may take longer time thereby delaying the emergence of DN T-cells [276].

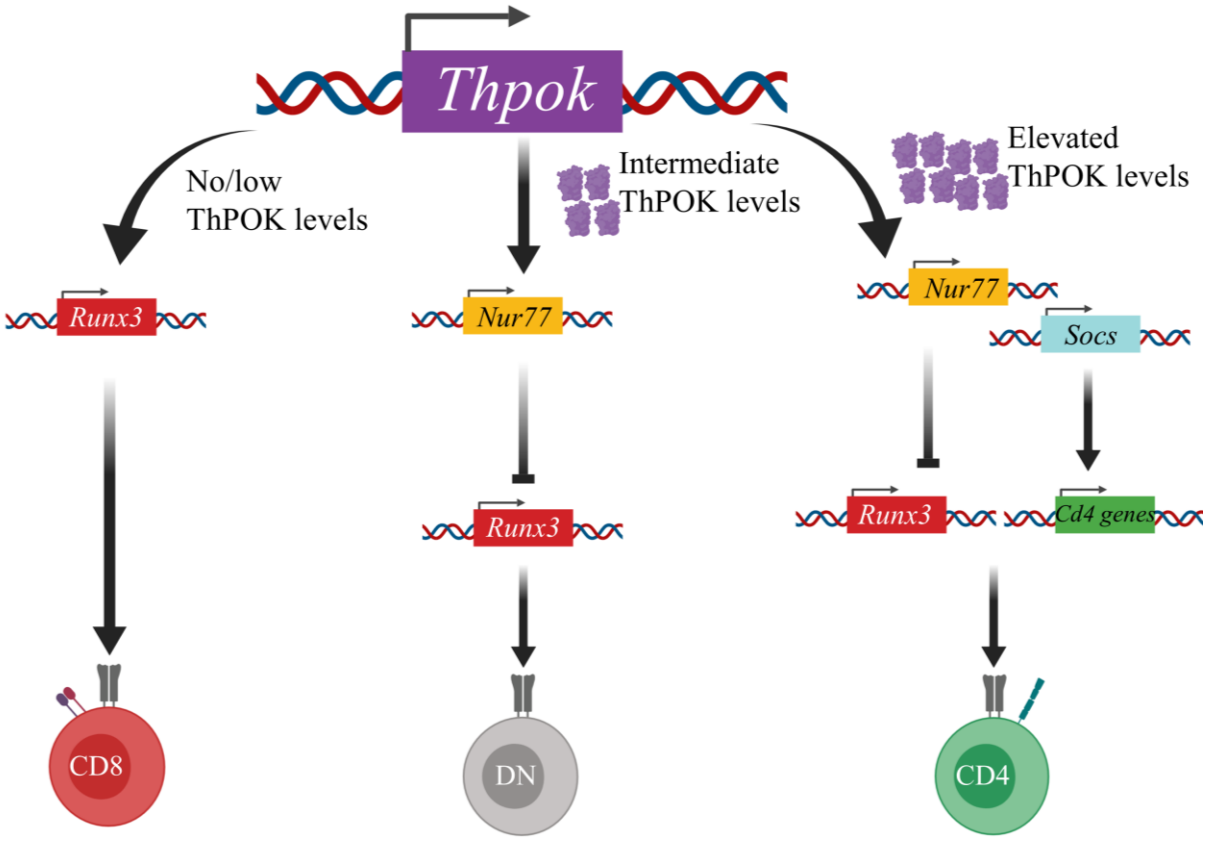

Figure 31. Dose-dependent gain of function of ThPOK in periphery

This drawing depicts the dose dependent gain of function of ThPOK model that we have established. When ThPOK expression levels are no/absent, thymocytes upregulate *Runx3*, which commits cells to the $CD8^+$ lineage. When ThPOK is expressed at intermediate levels, *Nur77* is induced, which blocks the $CD8^+$ program by inhibiting *Runx3* expression and cells adopt an intermediate phenotype. When high levels of ThPOK are present, on top of blocking the CD8 program, thymocytes induce the $CD4^+$ helper program by upregulating $CD4^+$ lineage specific genes like *Socs* genes. Socs, suppressor of cytokine signaling; ThPOK, T helper Inducing pox virus zinc finger -krüppel like factor.

We noted that constitutive expression of ThPOK in MHC-I-restricted T-cells reduced the absolute number of peripheral T-cells compared to OTI^+ control **(manuscript #1, Fig. 12)**. We argue that this decrease in mature T-cell number is probably due to a defect in the homeostasis of the redirected T-cells expressing MHC mismatched TCR and co-receptor (OTI-TCR and CD4 co-receptor), which may result in the inefficient recruitment of the co-receptor

bound Lck required for tonic signaling and T-cell survival [448]. While this could account for the lower number of T-cells in ThPOK-transgenic mice, many other factors can affect T-cell numbers in periphery. For instance, impaired positive selection can reduce peripheral T-cell numbers by hindering thymic output [449]. Actually, our analysis shows a drop in the frequency of positively selected T-cells in ThPOK-transgenic mice compared to OTI control. Such early transgenic ThPOK expression may affect positive selection of MHC-I-specific thymocytes, by a mechanism that remains to be elucidated.

4.1.2 Role of TCR specificity in ThPOK-mediated CD4$^+$ lineage redirection

OTI-TCR has a higher ligand affinity compared to P14-TCR and continue to signal with fewer remaining CD8 molecules (i.e., the duration of TCR signaling in OTI$^+$ thymocytes is higher than p14$^+$) [326, 446]. It is suggested that stronger affinity of OTI-TCR for its ligand, while renders thymocytes more susceptible to lineage choice errors, induces higher Runx3 expression. Given this, one would predict that more ThPOK would be needed to counteract higher *Runx3* induction in OTI$^+$ mice compared to P14 mice. On the other hand, as stronger TCR signaling favours CD4$^+$ lineage choice one would have predicted more efficient CD8$^+$ to CD4$^+$ lineage redirection in OTI$^+$ than in P14$^+$ mice expressing the same amount of ThPOK. However, comparable T-cell subset frequencies in P14$^+$ and OTI$^+$ mice **(manuscript #1, Fig. 22)** expressing ThPOK-H argues against TCR signal strength. It is quite likely that MHC-II TCR signaling influences ThPOK-induced CD4$^+$ lineage choice in a way that is less dependent on the strength of TCR signaling. The inability of constitutively expressed CD8 or knocked in CD8.4 (into *Cd8* locus) to generate significant lineage redirection supports such a notion [205, 287].

Nonetheless, this does not dismiss the hypothesis that TCR signaling cannot affect CD4$^+$/CD8$^+$ lineage fate as reported by several labs. Indeed, increase in the activity of the TCR signaling via constitutively active Lck expression dramatically improved CD4$^+$ lineage choice in OTI$^+$ThPOK-H$^+$ mice. To better elucidate the role of TCR signaling in CD4$^+$/CD8$^+$ lineage fate, we introduced our ThPOK-H transgene in OTII-TCR transgenic mice lacking endogenous ThPOK. Our objective was to confirm that stronger TCR signaling in OTII$^+$ thymocytes would result in similar efficiency of CD4$^+$ lineage choice by ThPOK-H transgene. However, we noted

a complete rescue of CD4 lineage in OTII^{+}ThPOK-H^{+}*ThPOK*$^{-/-}$ mice compared to OTI^{+}dLGF^{+}ThPOK-H^{+}*ThPOK*$^{-/-}$ mice **(manuscript #1 Fig. 17, 18)**. This is even more surprising given the fact that TCR signal strength, as measured by CD5 levels, was higher in OTI^{+} thymocytes compared to OTII^{+} thymocytes **(manuscript #1 Fig. 19)**. In fact, our three different mouse models (OTI^{+}ThPOK-H^{+}*Thpok*$^{-/-}$, OTI^{+}dLGF^{+}ThPOK-H^{+}*Thpok*$^{-/-}$ and OTII^{+}ThPOK-H^{+}*Thpok*$^{-/-}$) expressing the same amount of ThPOK but different levels of TCR signaling provide one of most unequivocal support for a potential role for a quantitative and qualitative aspect for TCR signaling in CD4^{+}/CD8^{+} lineage fate decision **(Fig. 33)**. We believe that MHC-II-restricted TCR signaling may help promote CD4^{+} T-cell development either, independently of ThPOK by inducing other lineage-specifying factors, or by improving ThPOK target gene accessibility [322]. The observation that high amounts of ThPOK (like in ThPOK-611^{+}) induced a full CD8^{+} to CD4^{+} lineage redirection supports the latter possibility. We think that there is a window of lineage commitment, regulated by the quantitative and qualitative aspects of TCR signaling, within which ThPOK must function. In MHC-II-restricted thymocytes both qualitative and quantitative TCR signaling may make target genes more accessible and/or for longer time for ThPOK-mediated activation and, thereby, enabling efficient lineage rescue in OTII^{+}ThPOK-H^{+}*Thpok*$^{-/-}$ mice. Such a situation may allow even ThPOK$^{lo/med}$ DP thymocytes to develop into CD4^{+} lineage. In contrast, in OTI^{+}dLGF^{+}ThPOK-H^{+}*Thpok*$^{-/-}$ mice only the quantitative aspect is operating, where both the aspects of TCR signaling are absent in OTI^{+}ThPOK-H^{+}*Thpok*$^{-/-}$ mice.

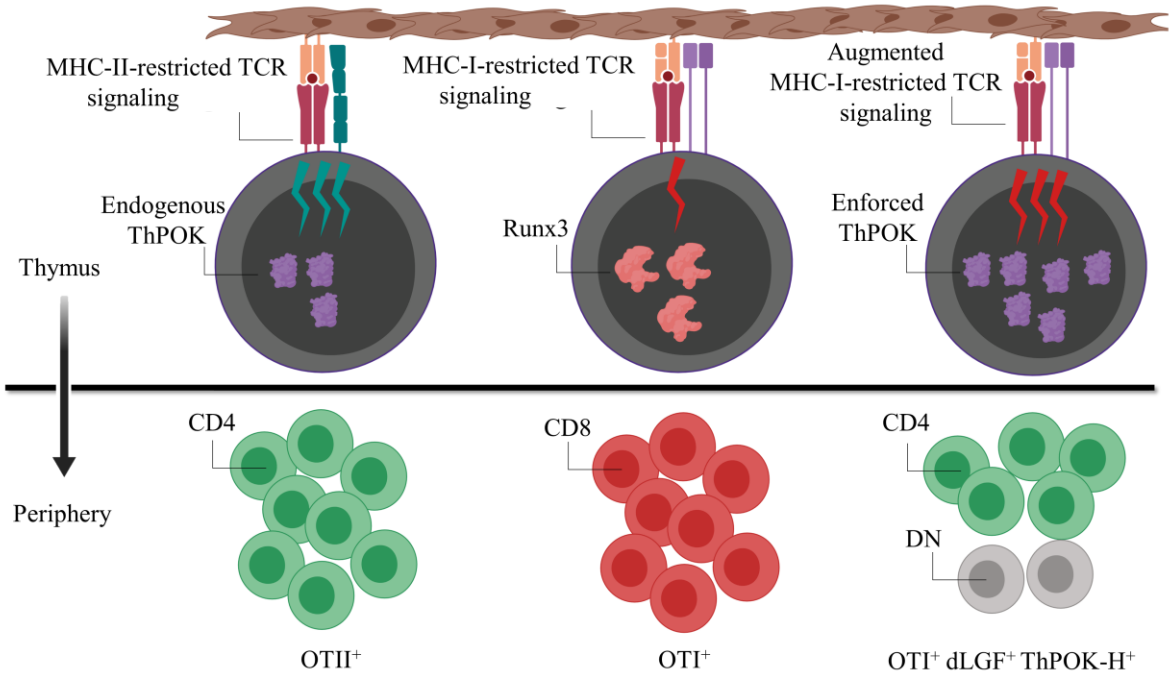

Figure 32. Representative model for the role of TCR specificity in ThPOK-mediated lineage redirection.

While T-cell receptor (TCR) signaling in major histocompatibility complex (MHC)-II-restricted thymocytes promotes $CD4^+$ lineage commitment by upregulating *Thpok* expression, MHC-I-restricted TCR signaling promotes $CD8^+$ lineage differentiation by inducing *Runx3*. Augmented MHC-I-restricted TCR signaling (quantitative aspect of TCR signaling), coupled with enforced expression of ThPOK, resulted in fewer $CD4^+$ T-cells than $OTII^+$ mice. This clearly demonstrated that the qualitative aspects of MHC-II-restricted TCR signaling are required for $CD4^+$ lineage commitment, possibly by potentiating the effect of ThPOK. ThPOK, T helper Inducing pox virus zinc finger -krüppel like factor.

4.2 TCR-activated downstream pathways in $CD4^+$ T-cell development (manuscript #2)

4.2.1 $CD4^+$/$CD8^+$ lineage fate is susceptible to changes in selecting ligand density

In the first manuscript presented in this thesis, we demonstrate that compared to MHC-I-restricted thymocytes, TCR signaling in MHC-II-restricted thymocytes is more potent in promoting ThPOK function.

The various developmental and maturation stages of positively signalled MHC-I- and MHC-II-restricted thymocytes are determined by TCR specificity [326]. MHC-I-restricted thymocytes also require STAT5/STAT6-mediated cytokine signaling for their development and survival [446]. The identification of signaling proteins and/or the genes that are differentially regulated by MHC-I- and MHC-II-specific TCR signaling during lineage commitment has proven to be difficult for several reasons: 1) it is difficult to separate positive selection from lineage commitment as they both are regulated by TCR signaling, making it hard to study one without affecting the other, and 2) that MHC-I- and MHC-II-restricted TCR signaling differ in several aspects that are challenging to elucidate, for instance TCR dwell time [49, 189]. In the second part of the thesis we have attempted to better elucidate the role TCR signaling and the different downstream nuclear factors, such as Gata3 and ThPOK, which exhibit differences in temporal regulation during $CD4^+$/$CD8^+$ lineage fate. We specifically asked if the ThPOK-independent function of TCR signaling on $CD4^+$/$CD8^+$ lineage fate requires Gata3.

In our TCR transgenic mice carrying a non-selecting allele, which is known to decrease positive selection efficiency, a significant fraction of MHC-II-restricted thymocytes failed to differentiate into $CD4^+$ T-cells and were redirected to alternative fates [450]. Hence, similar to previously published reports, we showed that altering TCR-mediated positive selection signals affected the lineage commitment [189, 210]. We noted reduced cell-surface expression levels of selecting MHC molecules in MHC-heterozygous mice, carrying one selecting and one non-selecting MHC-II allele, compared to the homozygous control, which is in line with previous experiments [450]. However, it is still unclear how the presence of a non-selecting MHC allele

disrupts TCR signals in thymocytes by selecting MHC ligand. It is possible that in mice heterozygous for selecting MHC-II, α- and β-chains of the selecting and non-selecting alleles form mismatched heterodimers. These could then result in MHC-II heterodimers with inefficient TCR binding properties leading to an altered duration/strength of TCR signaling. Indeed, mixed MHC haplotypes were first described more than 30 years ago, and although their specific function remains poorly understood developing thymocytes were shown to bind mixed MHC-II heterodimers with reduced affinity compared to the selecting MHC-II heterodimers [451, 452]. Although mixed haplotypes are less likely to assemble due to steric hindrance, those that are formed appear to have a structure comparable to that of parental haplotypes with some differences in the peptide-binding groove [451]. This is an important aspect to consider when evaluating the impact of mismatched MHC molecules on thymic selection and lineage choice. While the complementary-determining region (CDR) 1 and 2 of the TCR interact with the more constant segment of the MHC molecule, the more variable CDR3 is in direct contact with the selecting peptide residues facing the TCR [453]. Accordingly, alteration in peptide/MHC interaction could influence the activity of the TCR signaling in thymocytes. Alternatively, it could also be possible that mixed MHC heterodimers enabled the selection of non-conventional subset of thymocytes and committed them into $CD8^+$ lineage. Nonetheless, it remains to be determined if disruption of the TCR signaling in thymocytes was due to the formation of mixed MHC-II haplotypes, to reduced abundance of the selecting ligand, or a combination of both. We favour the latter possibility as it is suggested that thymocytes also require multiple TCR/peptide-MHC (pMHC) interactions for a successful positive selection and lineage choice [403]. Several other scenarios could be considered as well. For example, TCR-regulated immunological synapse formed between thymocytes and MHC-II-expressing cells is suggested to control signal transduction, and thus reduced number of these synapses may lower the signaling efficiency [454].

4.2.2 Role of TCR signaling on the integrity of the helper phenotype

Analysis of $OTII^+H\text{-}2K^{b/k}$ mice strongly suggest that disrupted TCR signaling redirects MHC-II-restricted thymocytes to the DN or $CD8^+$ lineage fates. Intriguingly, the MHC-II-specific redirected $CD8^+$ T-cells in $OTII^+H\text{-}2K^{b/k}$ mice were transcriptionally different from the MHC-II-specific redirected $CD8^+$ T-cells found in *Thpok*$^{-/-}$ mice. The indispensable role of

ThPOK in promoting the helper function and suppressing the cytotoxic program is well documented [276, 287]. Nonetheless, unlike disruption of TCR signaling, ThPOK deletion in MHC-II-restricted thymocytes did not result in a complete shutdown of the helper program [293]. There is mounting evidence that suggest that commitment to the $CD4^+$ lineage is orchestrated by a complex interplay between multiple TCR-induced effector molecules. In fact, the differentiation of DP thymocytes into $CD4^+8^{lo}$ intermediates requires the concerted action of Tox, Gata3 and Myb, and in their absence, ThPOK-fails to promote $CD4^+$ T-cell development [195, 274]. However, their precise role in $CD4^+$ lineage development remains to be fully elucidated. Although it is abundantly evident that Gata3 plays an important role during early $CD4^+$ T-cell development and lineage specification via ThPOK induction, constitutive expression of Gata3 failed to redirect MHC-I-specific thymocytes into $CD4^+$ lineage, and thus failed to replicate the $CD8^+$ to $CD4^+$ lineage redirection observed due to increased TCR signal strength in OTI^+dLGF^+ mice **(manuscript #1, Fig. 18; manuscript #2, Fig. 28, 29)** [195]. Importantly, constitutive Gata3 failed to act in synergy with ThPOK in promoting the $CD8^+$ to $CD4^+$ lineage redirection but continued to exert pro-apoptotic effect on MHC-I-specific cells, including $CD4^+$ T-cells, possibly via down regulation of Runx3-activation by cytokine signaling. This implies that MHC-I-specific $CD4^+$ T-cells in ThPOK transgenic mice likely continues to maintain some aspect of MHC-I-specific program, which likely underlies the apoptotic death of these cells in the presence of Gata3. Such a possibility complements similar results observed in the $CD4^+$ to $CD8^+$ redirected T-cells in *Thpok*$^{-/-}$ mice; MHC-II-specific $CD8^+$ T-cells in *Thpok*$^{-/-}$ mice continue to display active *Thpok* locus, continue to express Gata3 at levels comparable to conventional $CD4^+$ T-cells, and yet, do not die by apoptosis [274, 455].

We found lower levels of TCR signaling in DN T-cells compared to $CD8^+$ T-cells from $OTII^+H\text{-}2K^{b/k}$ mice, which challenges our assumption that TCR signaling affected lineage fate in a dose-dependent fashion. One explanation could be that in the absence of any co-receptor, Lck associates weakly to the TCR complex, resulting in poor signal transduction in DN T-cells compared to $CD4^+$ or $CD8^+$ T-cells [456]. Surprisingly, we noted that $CD4^+$ T-cells from $OTII^+H\text{-}2K^{b/k}$ mice showed increased TCR signaling compared to the same subset from $OTII^+H\text{-}2K^{b/b}$ mice, indicating that $CD5^{hi}$ T-cells, which may be less susceptible to changes in TCR signaling, accumulated within this particular subset. The observation that disruption of

TCR signaling led to the development of DN and $CD8^+$ T-cells in OTII and AND mice, and DP mature T-cells in 5cc7 mice suggests a dynamic molecular interaction between TCR signaling and co-receptor gene expression likely via stage-specific enhancers present in the *Cd4* and *Cd8* locus [189, 457].

How might we explain continued Thpok promoter-driven GFP expression in the redirected MHC-II-specific $CD8^+$ T-cells? We suggest a role for Gata3, albeit indirect one, in inducing and maintaining the *Thpok* locus activity only in MHC-II-, but not MHC-I-, signaled thymocytes. Gata3 is shown to antagonize Runx3 activity, and thus may impair Runx3-mediated silencing of *Thpok* in MHC-II-signaled thymocytes, an effect that is probably dependent on its chromatin modifying function [433, 440, 441]. These results suggest that TCR signaling plays a role in protecting the epigenetic integrity of MHC-II-restricted T-cells partly by inducing the pioneer transcription factor such as Gata3. It should be noted that the epigenetic silencing of helper-specific genes like *Cd4* in $CD8^+$ MHC-II-redirected T-cells from ThPOK-deficient mice, could not be rescued by ectopic expression of ThPOK in mature T-cells, which further reinforces the premise that the spatiotemporal regulation of *Thpok* expression is important to prevent Runx3-dependent adoption of the cytotoxic program [320].

The $CD8^+$ mature T-cells from thymus and spleen of OTI^+dLGF^+ mice, showed increased TCR signaling compared to $CD8^+$ T-cells from control OTI mice. This increase in TCR activity, seem to influence the expression of certain $CD8^+$ lineage-specific genes, implying that TCR signaling could affect the cytotoxic functional program without actually causing lineage redirection **(Fig. 27)**. It remains to be determined, however, if the TCR-mediated inhibition of the cytotoxic program was orchestrated by Gata3, as ThPOK is not expressed in these $CD8^+$ T-cells.

Collectively, these results strongly suggest that MHC specificity of developing thymocytes likely plays a critical role in modulating quantitative as well as qualitative TCR signaling.

Chapter 5: Conclusions

The $CD4^+/CD8^+$ lineage choice is one of the most intensively studied topics in developmental immunology as it is essential to our understanding of thymocyte development. The kinetic signaling model is a widely accepted model that explains how $CD4^+/CD8^+$ lineage fate matches with the MHC specificity of positively selected thymocytes [189, 326]. It argues that differentiation of uncommitted $CD4^+8^{lo}$ intermediates into helper or cytotoxic lineage is influenced by the strength and duration of TCR- and cytokines-derived signaling [189]. The transcriptions factors ThPOK and, its antagonist, Runx3 are at the center of the network of transcription factors establishing lineage specificity and functional program in the signaled thymocytes. While ThPOK is indispensable for the differentiation into the helper lineage, Runx3 promotes the development of the $CD8^+$ lineage. In this thesis, we first explored the dose-dependent gain-of-function of ThPOK and the dynamic interplay between TCR signaling and ThPOK-mediated $CD4^+$ lineage fate by using transgenic mice. High level of ectopic ThPOK expression (ThPOK-611) efficiently redirected MHC-I-restricted thymocytes to the $CD4^+$ lineage, while relatively lower amounts (ThPOK-H and ThPOK-163) induced incomplete lineage redirection resulting in the development of significant numbers of mature DN and $CD8^+$ T-cells in periphery. Interestingly, $CD8^+$ and DN splenocytes from $OTI^+ThPOK\text{-}H^+$ and $OTI^+ThPOK\text{-}163^+$ still expressed an amount of ThPOK that was higher than that expressed in conventional $CD4^+$ T-cells, making those mice great models to study the dose-dependent effect of ThPOK in $CD4^+/CD8^+$ lineage fate. Consistent with previously published results, our study demonstrated that ThPOK acted differently depending on its level of expression [195, 320, 321, 417]. Runx3, albeit reduced, was detected only in $CD8^+$ T-cells from $OTI^+ThPOK\text{-}H^+$ mice as DN and $CD4^+$ mature splenocytes from the same mice expressed higher ThPOK levels. We showed that high levels of ThPOK promoted the helper program by activating SOCS proteins, while intermediate levels disrupted the cytotoxic program, likely by upregulating Nur77 that is shown to suppress Runx3 [425]. Together, this confirms that the activation and suppression of the helper and cytotoxic programs, respectively, are two independently regulated pathways initiated by ThPOK when it is expressed at different levels. Our study highlighted the complexity of the network of transcription factors that need to be precisely regulated by ThPOK for efficient induction of the helper program. We have also provided new insight into the role of TCR signaling in $CD4^+$ T-cell development. Using various transgenic models, we have demonstrated that the establishment of the helper program requires a synergistic collaboration

between multiple independent but overlapping pathways downstream of the TCR signaling. The significantly higher numbers of $CD4^+$ T-cells in $OTII^+ThPOK\text{-}H^+$*Thpok*$^{-/-}$ compared to $OTI^+dLGF^+ThPOK\text{-}H^+$*Thpok*$^{-/-}$ indicated that differentiation into the helper program is influenced by quantitative as well as qualitative aspects of TCR signaling. We have showed that in $CD8^+$ T-cells, Gata3 may induce apoptosis potentially by regulating the expression of cytokine receptors. In MHC-II-restricted T-cells, Gata3 plays an essential role in maintaining the integrity of $CD4^+$ lineage-specific genes likely by regulating chromatin accessibility. Interestingly, we failed to observe any synergistic effect between ThPOK and Gata3 in promoting $CD8^+$ to $CD4^+$ lineage redirection as the number of $CD8^+$ SP and ThPOK-redirected $CD4^+$ SP T-cells were equally impaired by Gata3 overexpression. This study furthers our knowledge on $CD4^+/CD8^+$ lineage fate by demonstrating that, while transcription factors such as ThPOK, Runx3, Gata3, play an important role in $CD4^+/CD8^+$ lineage choice, functional integrity of these cells requires quantitatively and qualitatively different TCR signaling during their development in the thymus.

Chapter 6: Future Directions

In this thesis we have extensively characterized the dose-dependent effect of ThPOK and the role of TCR signaling in the development of helper and cytotoxic T-cells. Several issues, however, remain to be addressed as discussed below.

First, it remains to be demonstrated if, and how, the qualitative and quantitative aspects of the TCR signaling modulate $CD4^+/CD8^+$ lineage choice of developing thymocytes and whether this is also necessary for maintaining the functional integrity of mature T-cells in the periphery. Precisely, the pathways downstream of the TCR-mediated positive-selection signals in MHC-I- vs MHC-II-restricted thymocytes must be elucidated. In our study, we have found that increased TCR signaling in MHC-I-restricted thymocytes augmented ThPOK-mediated $CD4^+$ T-cell development less efficiently than genuine MHC-II-restricted TCR signaling expressing the same amounts of ThPOK (Fig. 21). Moreover, the results showing that TCR signaling in MHC-II-restricted cells helped ensure the integrity of the *Thpok* active locus despite the presence of Runx3, illustrates that TCR signaling could control $CD4^+$ T-cell development partly through epigenetic modifications. Therefore, a thorough investigation regarding the contribution of MHC-restricted TCR signaling in the development of $CD4^+/CD8^+$ lineage is crucial to shed some light on this unresolved issue. For comprehensive understanding of the effect that MHC-restricted TCR signaling on lineage fate, genome-wide epigenetic and transcriptomic analyses must be performed. The use of signaled but uncommitted ($CD4^+8^{lo}$) *Thpok*$^{-/-}$ MHC-I-specific thymocytes with or without augmented TCR signaling or MHC-II-specific thymocytes is essential for genome wide analysis to eliminate any ThPOK-mediated effect. While the analysis of $CD4^+8^{lo}$ from OTI^+ mice vs $CD4^+8^{lo}GFP^+$ (destined to become $CD4^+$ T-cells) from OTI^+dLGF^+*ThPOK*$^{+/gfp}$ mice, could help us better understand the how the quantitative aspect of TCR signaling could favor $CD4^+$ T-cell development, analysis of $CD4^+8^{lo}GFP^+$ from OTI^+dLGF^+*ThPOK*$^{+/gfp}$ mice vs $CD4^+8^{lo}$ from $OTII^+$ mice could help us better understand the role of qualitative aspect of TCR signaling in the development of $CD4^+$ T-cells. Our lab has, recently, done RNA sequencing using redirected mature T-cells from OTI^+ThPOK-H^+ mice and have identified several interesting target genes. Validation of these target genes would require gain- and loss-of-function experiments. Moreover, the contribution of the TCR signaling on chromatin structure could be tested by deleting chromatin remodeling

complexes in selected DP thymocytes by using the $E8_{III}$-Cre system that is active at the DP stage only.

Second, ever since its discovery in 2005, the efforts to elucidate the mechanism of ThPOK action have met with relatively little success [265]. In 2014, it was discovered that a crucial function of ThPOK in regulating the $CD4^+$ helper lineage involved the induction of *Socs* genes. However, transgenic expression of SOCS1 only partially restored $CD4^+$ T-cell development in *ThPOK*$^{-/-}$ mice, suggesting that the helper differentiating function likely requires the cooperation of several ThPOK-target genes [293]. Additionally, it remains to be elucidated how ThPOK induces *Socs1* as, similar to our results with *Nur77*, experimental evidence associating ThPOK to the *Socs1* locus is lacking [276, 293]. It is possible that, depending on the binding partner, ThPOK may induce different sets of genes. In fact, a recent publication revealed that the oligomerization of ThPOK and NF-κB, when bound to DNA elements referred to as NF-κB reception centres (NRCs), culminated in a stochastic inter-chromosomal interactions necessary for the induction of ThPOK target genes [458]. However, this model was established using human epithelial cell lines, and thus whether ThPOK/NF-κB plays any role in $CD4^+$ lineage choice remains to be investigated. Nevertheless, this inter-chromosomal model for ThPOK function and its dependence on the binding partner provides an attractive model to explore the mechanism of ThPOK action. If ThPOK/NF-κB interaction is required for the development of the helper lineage, then conditional NF-κB knock-down in the signaled thymocytes from OTI^+ ThPOK-611^+ should reduce or abrogate the lineage redirection. If so, a comprehensive map of inter-chromosomal interactions with target genes can be established by performing a Chromatin Interaction Analysis by Paired-End Tag Sequencing (ChIA-PET), which is a technique that incorporates chromatin immunoprecipitation and high-thoughput sequencing to map long range regulatory regions [459]. Enriching DNA sequences bound to the NF-κB-ThPOK complex, could help identify genome-wide *de novo* chromatin interactions.

Third, the evaluation of ThPOK's function in a therapeutic setting has been poorly investigated. Here we propose two studies to elucidate how regulation of *Thpok* induction could be used for therapeutic purposes. First, ThPOK expression, and that of its antagonist Runx3, are maintained in $CD4^+$ and $CD8^+$ peripheral T-cells where they continue to play an active role in maintaining functional integrity [332]. While ThPOK and Runx3 exhibit a mutually exclusive

expression pattern during the establishment of lineage fate, they can be simultaneously expressed in peripheral T-cells under certain physiological conditions. For instance, a fraction of $CD8^+$ T-cells upregulate ThPOK upon activation, which is stipulated to be necessary for their differentiation into long-lived memory cells [279]. Considering that ThPOK and its downstream effector molecules can inhibit the cytotoxic program in $CD4^+$ or $CD8^+$ peripheral T-cells along with the fact that tumor homing potentials of $CD8^+$ T-cells is dependent on Runx3 suggest that eliminating ThPOK in the engineered T-cells may enhance the therapeutic efficacy of cellular-based immunotherapies, such as CAR T-cell therapy [460]. Second, the helper function conferred by ThPOK is well documented. By suppressing *Runx3* expression in peripheral T-cells, ThPOK protects the integrity of the helper lineage. Previous studies have demonstrated that ThPOK safeguards the T_H2 response and prevents aberrant T_H17 differentiation of activated T-cells [332]. Psoriasis is a debilitating autoimmune disease that is primarily mediated by T_H17 polarized $CD4^+$ T-cells [461]. Several antibody-based immunotherapies, such as Etanercept, perform better than older conventional systemic anti-inflammatory medications by skewing the T_H17 response towards T_H2 [462]. In view of the role of ThPOK in plasticity of helper T-cells, it would be interesting to determine if reversal of the response exerted by immunotherapies like Etanercept is ThPOK-dependent, which can be readily tested by deleting or knocking down ThPOK in peripheral $CD4^+$ T-cells *in vitro* and *in vivo*. The transcription factor Nur77, which we have shown to be upregulated by ThPOK (manuscript #1), has recently been demonstrated to restrict and reverse the development of autoimmune diseases by regulating the metabolic response of activated T-cells [439, 463]. By deleting or knocking-down *Nur77*, and then assessing the effector function and metabolic program following treatment with the antibody it would be possible to investigate the potential role of *Nur77* in the reversal of polarization. The results of this study would help in better understanding the molecular mechanism behind the beneficial effect of antibody-based immunotherapies for autoimmune diseases and develop more targeted treatments with fewer side-effects.

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Annex

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Critical Role for TCR Signal Strength and MHC Specificity in ThPOK-Induced CD4 Helper Lineage Choice

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Critical Role for TCR Signal Strength and MHC Specificity in ThPOK-Induced CD4 Helper Lineage Choice

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Sustained TCR signaling is critical for ThPOK induction in MHC class II (MHCII)–signaled thymocytes leading to the CD4 helper lineage commitment. ThPOK suppresses the cytotoxic program in the signaled thymocytes and is shown to be necessary and sufficient for the CD4 helper lineage choice. Accordingly, loss and gain of ThPOK function redirects MHCII- and MHC class I (MHCI)–signaled thymocytes into the CD8 cytotoxic and CD4 helper lineage, respectively. However, the impact of a defined ThPOK level on the CD4 helper lineage choice of MHCII- and MHCI-specific thymocytes and the role of TCR signaling in this process is not evaluated. Equally, it is not clear if suppression of the cytotoxic program by ThPOK is sufficient in redirecting MHCI-restricted thymocytes into the CD4 helper lineage. In this study, we have investigated CD8 to CD4 helper lineage redirection in three independent ThPOK overexpressing transgenic mouse lines. Our analysis shows that one of the transgenic lines, despite overexpressing ThPOK compared with wild-type CD4 mature T cells and compromising cytotoxic program, failed to redirect all MHCI-signaled thymocytes into the CD4 helper lineage, resulting in the continued presence of $CD8^+$ mature T cells and the generation of a large number of double negative mature T cells. Critically, the same ThPOK transgene completely restored the CD4 helper lineage commitment of MHCII-specific *Thpok*$^{-/-}$ thymocytes. Importantly, augmenting TCR signaling significantly enhanced the ThPOK-mediated CD4 helper lineage choice of MHCI-specific thymocytes but was still substantially less efficient than that of MHCII-specific thymocytes expressing the same amount of ThPOK. Together, these data suggest that the ThPOK-induced CD4 helper lineage commitment is strongly influenced by TCR signal strength and MHC specificity of developing thymocytes. *The Journal of Immunology*, 2019, 202: 000–000.

Functionally competent mature αβ T cells play a central role in the cell-mediated immune responses (1–4). Development of these cells in the thymus is an ordered process consisting of distinct differentiation stages defined by the expression of CD4 and CD8 coreceptors. Precursor thymocytes are $CD4^-CD8^-$ double negative (DN), which following pre-TCR–transduced signaling, differentiate into $CD4^+CD8^+$ double positive (DP) thymocytes. The DP thymocytes expressing a low level of TCRαβ receptor and the associated CD3 chains undergo thymic selection such that those expressing high-affinity TCR for self-peptide/self-MHC (pMHC) are negatively selected, whereas those expressing low-affinity TCR for pMHC are positively selected (5–7). Positively selected thymocytes further differentiate into MHC class II (MHCII)–specific $CD4^+$ helper and MHC class I (MHCI)–specific $CD8^+$ cytotoxic mature thymocytes that populate the peripheral lymphoid organs (8–10). How pMHC specificity of TCR/coreceptor translates into MHCII-specific CD4 helper and MHCI-specific CD8 cytotoxic lineage choice is not completely understood.

The CD4/CD8 binary lineage fate decision is strongly influenced by the duration and intensity of TCR signaling. A widely accepted kinetic signal strength model posits that positively selected DP thymocytes, irrespective of their MHC specificity, transcriptionally terminate *Cd8* expression and become lineage uncommitted $CD4^+CD8^{lo}$ thymocytes (8, 11, 12). Continued *Cd4* transcription at this stage induces a sustained/stronger signal in MHCII-specific thymocytes, leading to an error-free CD4 helper lineage choice (13), whereas the downregulation of CD8 results in a disrupted/weaker signal in MHCI-specific thymocytes leading to CD8 cytotoxic lineage choice. Lck, a Src family tyrosine kinase essential for T cell development, is strongly associated with the cytoplasmic tail of CD4 than that of CD8 (14, 15). Thus, increased Lck activity due to continued CD4 expression then results in stronger TCR signaling in MHCII- than in MHCI-specific thymocytes (16, 17). Indeed, altered Lck activity is shown to direct positively selected thymocytes into alternate lineages (17, 18).

Induction of ThPOK (encoded by *ZBTB7B*, hereafter referred to as *Thpok*) in MHCII-signaled thymocytes is both necessary and sufficient for the CD4 helper lineage commitment (19). Similarly, Runx3 induction in MHCI-signaled thymocytes establishes a cytotoxic program in the CD8-committed thymocytes (12). ThPOK is proposed to suppress Runx3 expression and thereby impair the initiation of the cytotoxic program in MHCII-signaled thymocytes, leading to the CD4 helper lineage choice (19–22). Accordingly, the loss and gain of ThPOK function results in the production of MHCII-specific $CD8^+$ cytotoxic and MHCI-specific $CD4^+$ helper T cells, respectively (23, 24). The *Thpok* silencer-mediated

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The online version of this article contains supplemental material.

Abbreviations used in this article: DN, double negative; DP, double positive; MHCI, MHC class I; MHCII, MHC class II; pMHC, self-peptide/self-MHC; QPCR, quantitative PCR; WT, wild-type.

heritable epigenetic modifications control ThPOK expression in the signaled thymocytes and is suggested to play an important role in the CD4/CD8 lineage choice (25). These studies suggest that ThPOK induction during a temporal developmental window is critical for the CD4 helper lineage choice (25). Persistent TCR signaling in MHCII-specific thymocytes is proposed to reverse silencer-induced epigenetic modifications at the *Thpok* locus, leading to stable ThPOK expression, which then suppresses the cytotoxic program and thereby commits these cells into the CD4 helper lineage (21, 26). Based on these and other studies, it is proposed that persistent TCR signaling leading to ThPOK induction and the extent of this induction during a temporal lineage commitment window affects the CD4/CD8 lineage fate of positively selected thymocytes (22, 25, 27, 28). Although published data suggest that developmental constrain on the CD4 helper lineage commitment of MHCI-signaled thymocytes can be overcome by enforced ThPOK expression (23, 24), several questions remain to be addressed. For example, it is not clear why ThPOK induction in MHCI-signaled thymocytes lacking Tle proteins, which disrupt Runx3 function, or Runx1 and Runx3 or MAZR and Runx3 results in an incomplete CD8 to CD4 helper lineage redirection or the generation of "confused" DP mature T cells (29–31). Further, the role of TCR signaling in ThPOK-induced CD4 helper lineage choice of MHCII- and MHCI-signaled thymocytes is not evaluated. Is suppression of the cytotoxic program in itself sufficient for establishing the CD4 helper lineage? Thus, it remains to be investigated if the CD4 helper lineage choice, irrespective of MHC specificity, requires the same level of ThPOK or if it is also influenced by TCR signaling in MHCI- versus MHCII-specific thymocytes.

In the present investigation, we show that the efficiency of CD4 helper lineage commitment of MHCI-signaled thymocytes is proportional to ThPOK dose. Further, a ThPOK dose that induced the partial CD8 to CD4 helper lineage redirection of MHCI-signaled thymocytes expressing monoclonal or polyclonal TCRs completely restored the CD4 helper lineage commitment of MHCII-signaled thymocytes expressing monoclonal or polyclonal TCRs in ThPOK-deficient mice. Importantly, this differential ThPOK-induced CD4 helper lineage commitment correlated, at least in part, with TCR signal strength as augmenting TCR signaling significantly enhanced the CD4 helper lineage choice of MHCI-signaled thymocytes; however, it was still significantly less efficient than the CD4 helper lineage choice of MHCII-signaled cells. Together, our results provide crucial insights into the mechanism of ThPOK-induced CD4 helper lineage choice of thymocytes specific for disparate MHC and a critical role for TCR signaling in this process.

Materials and Methods

Mice

MHCI-restricted OTI^{+}*Rag*$^{-/-}$ (chicken OVA Ag-specific) and P14^{+}*TCR*$\alpha^{-/-}$ (lymphocytic choriomeningitis virus GP33–) transgenic mice were obtained from Taconic Biosciences or Nathalie Labrecque (Centre de Recherche Hopital Maisonneuve-Rosemont). MHCII-specific OTII^{+}*Rag*$^{-/-}$ mice were from The Jackson Laboratory. *MHCII*$^{-/-}$ and *Nur77-GFP* mice (32) were obtained from The Jackson Laboratory. All TCR transgenic mice were in Rag-deficient background unless mentioned otherwise. ThPOK transgenic mice were generated by cloning the genomic DNA encompassing the two coding exons flanking an intron into human CD2 expression vector. The following primers were used for cloning the ThPOK transgene: forward primer 5′-GGCGGAATTCCCAGGGAAGCAGAAGA**ATG**GGGAGCCCCGAGGA-3′ and reverse primer 5′-GCCCTTCCCCGGGCTTTTAAGAGGACTCCATGGCACC-3′ (ThPOK sequence is underlined, and ThPOK start codon in the forward primer is in bold letters). The PCR product was digested with EcoRI and XmaI restriction enzymes, and agarose gel purified and cloned into the EcoRI and XmaI cut hCD2 expression vector. The cloned DNA insert was sequenced to ensure fidelity of the ThPOK coding sequence. DNA was digested to release the insert from the vector backbone, and agarose gel purified DNA devoid of the vector backbone was injected into the fertilized mouse eggs. Three independent founder lines were established, and all of them showed an increased frequency of CD4^{+} mature T cells and severely reduced number of CD8^{+} mature T cells in the lymphoid organs. ThPOK-deficient mice were generated in the laboratory or acquired from Dan Littman (New York University). Constitutively active Lck transgenic mouse line, dLGF, is described elsewhere (17) and was obtained from Paul Jolicoeur (33). Mice were genotyped by peripheral blood analysis and/or PCR of genomic DNA isolated from tail snippets. Lymphoid organs harvested from 5–7-wk-old mice were analyzed. Any mice that showed signs of ThPOK-induced thymic leukemia (34), usually observed in >12-wk-old mice, were excluded from the analysis. All mice were housed under specific pathogen-free conditions at the Centre de Recherche Hopital Maisonneuve-Rosemont. Animal care was approved by the Institutional Animal Care Committee in accordance with the Canadian Committee on Animal Care.

Flow cytometry

A total of 1×10^{6} thymocytes or RBC-depleted spleen cells or stimulated T cells were incubated with a combination of fluorescently labeled Abs to CD4 (GK1.5), CD8 (53-6.7), TCRβ (H57-957), CD5 (53-7.3), CD69 (H1.2F3), CD24 (M1/69), CD44 (IM7), CD62L (MEL-14), NK1.1 (PK136), CD154 (MR1), IFN-γ (XMG1.2), IL-4 (11B11), Vα2 (B20.1), Vβ5 (MR9-4), ThPOK (D9V5T) or donkey anti-rabbit secondary Ab (Poly4064), phospho-Src (pY418; clone K98-37), and phospho-CD3z (pY142, clone 3ZBR4S) and analyzed by flow cytometry using LSRFortessa X-20 (BD Biosciences) or LSR II (BD Biosciences). Abs were obtained from eBioscience, BioLegend, or Cell Signaling Technology. For ThPOK staining, the human Foxp3 staining kit (eBioscience) was used for cell fixation and permeabilization using the manufacturer's protocol. Data were analyzed using FlowJo software (Tree Star). Gating strategy involving TCR transgenic mice is shown in Fig. 1. Unless mentioned otherwise, this flow cytometry gating strategy was used for the analysis of all the thymic and splenic T cells described in the manuscript.

Quantitative RT-PCR

Various thymic or splenic T cell subsets were FACS purified, and total RNA was isolated using TRIzol (Invitrogen). cDNA were synthesized using a commercial kit (Bio-Rad Laboratories). Quantitative PCR (QPCR) for *Thpok*, *Runx3d*, *Socs1*, *Nur77*, *Perforin*, *St8sia6*, *St3gal2*, *Cxxc5*, and endogenous *Thpok* was performed in triplicate using SYBER green dye (Bio-Rad Laboratories) or EvaGreen (Abcam). Amplification of housekeeping gene *Hprt* served as an internal control. QPCR data were analyzed by Applied Biosystems software ABI 7500 v2.0.5. Data were normalized to *Hprt* expression in each population. Relative expression values were calculated using ΔΔ cycle threshold method. Ratio of gene-specific values to housekeeping gene for wild-type (WT) or OTI reference subset was treated as one. Data are presented as an average of triplicate values and SD. The following QPCR primers were obtained from the Integrated DNA Technologies or designed in our laboratory: total *Thpok*, 5′-TGTCACAAGATAATCCACGGG-3′ and 5′-GGTCGTAGCTATGCAGGAAG-3′; *Runx3d*, 5′-CGACATGGCTTCCAACAG-3′ and 5′-CGGCGGAGTAGTTCTCATC-3′; *Socs1*, 5′-CAGAAAAATGAAGCCAGAGACC-3′ and 5′-ATTCCACTCCTACCTCTCCAT-3′; *Nur77*, 5′-CCATGTGCTCCTTCAGACAG-3′ and 5′-GCTCTGGTCCTCATCACTG-3′; *Perforin*, 5′-GTACAACTTTAATAGCGACACAGTA-3′ and 5′-AGTCAAGGTGGAGTGGAGGT-3′; endogenous *Thpok* 5′-CCTCAGCGTTCAGGAGAAGAT-3′ and 5′-GCTGCTGTGGTCTGGGAAT-3′ (sequence unique for endogenous *Thpok* is underlined); *St8sia6*, 5′-CCACCTCGTAGCTCATGTTAG-3′ and 5′-CGGCAAGCAGAAGAATATGAC-3′; *St3gal2*, 5′-GGTGTTGTGTGACTTGAATTGG-3′ and 5′-GTTTGACAGCCACTTTGACG-3′; *Cxxc5*, 5′-ATCACTGAAACCACCGGAAG-3′ and 5′-TTGTAGGAACCGAAAGACTGG-3′; *Hprt*, 5′-CCTCATGGACTGATTATGGACAG-3′ and 5′-TCAGCAAAGAACTTATAGCCCC-3′; and *Thpok* transgene copy number, 5′-TTGAGGCTGTGGTGGTGGCAGT-3′ and 5′-GGTGAGGAAGAAGAGGAGGA-3′.

Functional assays

Mature T cell subsets from spleen of OTI (CD8^{+}) and OTI mice expressing specific ThPOK transgene (CD4^{+}, CD8^{+}, and DN) mice were purified and cultured in the presence of irradiated (2500 rad) BL/6 splenocytes pulsed with cognate OVA peptide (SIINFEKL) for 5–7 d. Purified mature T cell subsets from WT (CD4^{+} and CD8^{+}) and ThPOK-H^{+}*MHCII*$^{-/-}$ (CD4^{+}, CD8^{+}, and DN) mice were stimulated with irradiated splenocytes obtained

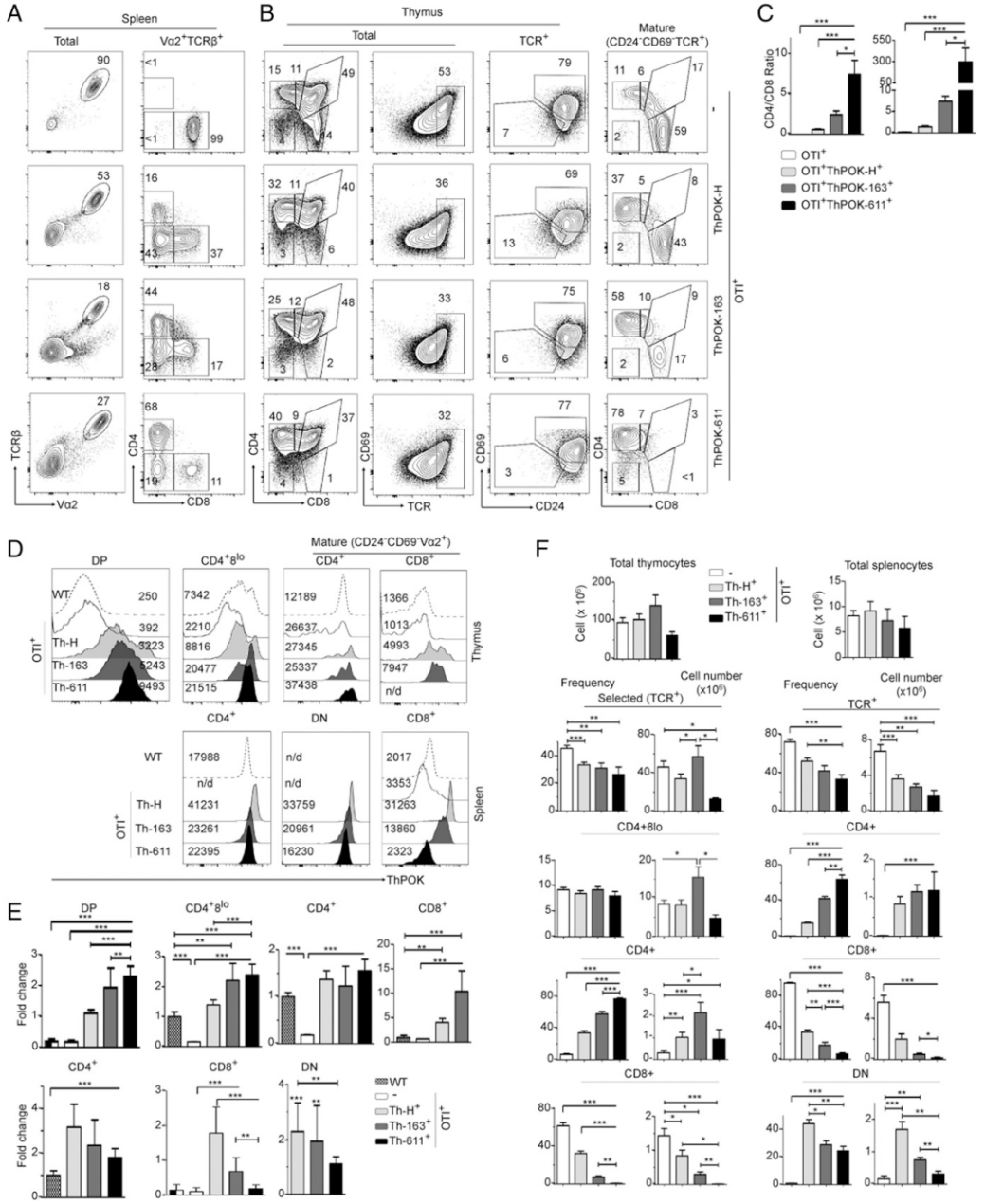

FIGURE 1. The ThPOK dose impacts the CD8 to CD4 helper lineage redirection. To assess the impact of individual ThPOK transgene on the CD8 to CD4 helper lineage redirection, each ThPOK transgenic line was introduced into OTI^{+}*Rag*$^{-/-}$ mice and T cells were analyzed by flow cytometry. (**A**) The frequency of mature T cells (TCRβ^{+}Vα2^{+}) and CD4/CD8 profiles of splenic T cells in OTI mice expressing the indicated ThPOK transgene are shown. (**B**) CD4/CD8 and CD69/TCR profile of total thymocytes, CD69/CD24 profile of TCR^{+} thymocytes, and CD4/CD8 profile of mature thymocytes (CD69-CD24-TCR^{+}) from the indicated strain of mice are shown. (**C**) The CD4/CD8 ratio for Vα2^{+} T cells from the spleen (left) and mature thymocytes (right) for the indicated strain of mice is shown. (**D**) ThPOK protein levels in DP, CD4^{+}8^{lo}, mature CD4^{+}, and CD8^{+} thymocytes (CD69^{-}CD24^{-}TCR^{+}; top histograms), and splenic T cell subsets (bottom histograms) from the indicated strain of mice are shown. Numbers in histograms represent the mean fluorescence intensity (MFI) values. (**E**) Compilations of ThPOK MFI for the indicated thymic and splenic T cell subsets from the indicated mice are shown. *(Figure legend continues)*

from BALB/c mice. In some cases, purified T cells were stimulated with plate-bound anti-CD3 and anti-CD28 (in suspension). The stimulated cells were stained with a combination of CD69, CD154, CD4, CD8, and TCRβ-specific Abs and analyzed by flow cytometry. For cytokine staining, the activated T cells were restimulated with PMA plus ionomycin in the presence of brefeldin for 4 h, surface stained, fixed in 2% paraformaldehyde, permeabilized, washed and stained with anti–IL-4 and anti–IFN-γ Abs, and analyzed by flow cytometry.

In vitro differentiation culture

FACS-sorted thymic subsets were cultured in 96-well flat-bottom plates at a concentration of 1×10^6 cells/ml. Cultures were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% (vol/vol) FBS, L-glutamine (2 mM), 2-ME (50 μM), streptomycin (100 mg/ml), penicillin (10 U/ml), and IL-7 (1 ng/ml). After 2 d of culture, cells were collected and analyzed by flow cytometry.

Luciferase reporter assay

For luciferase reporter assay, the promoter sequence of *Actin* and *Nur77* was cloned into the EcoRV and HindIII cut pGL4.17 vector (Promega). The promoter sequence was amplified using genomic DNA and the following primers: Nur7 promoter, 5′-TCGCCGGTCGACTCGATATCAGGAGATGGAGTTCGATGGCCC-3′ and 5′-GTCGCCTCTAGATCAAGCTTACCAAGCACCTTGCAGACCCTTC-3′; and Actin promoter, 5′-GGGGTGGCCGGTACCAGAGACACTAGCTAACGGCCC-3′ and 5′-GGGCCCGGGAAGCTTCTGGTGGCGGGTGTGGACCGG-3′.

The promoter-reporter DNA was cotransfected with either a ThPOK-YFP or control YFP plasmid (pMSCV) using the lipofectamine 2000 (Invitrogen) at a ratio of 3:1 (promoter-reporter to YFP) in 293T HEK cells. Twenty-four hours after transfection, an equal number of YFP-expressing cells were seeded in a 96 flat-bottom plate. *Socs1* promoter-driven luciferase plasmid was used as a positive control (kind gift of Hyun Park, National Institutes of Health). Luciferase activity was measured 48 h after transfection using the Luciferase Assay System (Promega).

Statistical analyses

Statistical analysis was performed using GraphPad Software or Microsoft Excel software. Data are displayed as a mean with SD error bar. Unpaired two-tailed Student *t* test was used for determining the statistical significance when thymic and splenic T cell subsets from different mice were compared. For experiments involving a comparison of T cell subsets isolated from the same mouse, a paired Student *t* test was used for evaluating the statistical significance. A p value ≤ 0.05 was considered statistically significant ($^*p \leq 0.05$, $^{**}p \leq 0.005$, and $^{***}p \leq 0.0005$).

Results

Characterization of ThPOK transgenic mice

To investigate if ThPOK-mediated suppression of the cytotoxic program in MHCI-signaled thymocytes is in itself sufficient for inducing the CD4 helper lineage program and the role of TCR signaling and MHC specificity in this process, we generated three independent ThPOK founder lines (ThPOK-H, ThPOK-163, and ThPOK-611) in which ThPOK expression is driven by human CD2 promoter/enhancer cassette (35). All the progenies of three ThPOK founders showed, in agreement with the previously published reports (23, 24), increased and decreased frequencies of $CD4^+$ and $CD8^+$ mature T cells, respectively, in the lymphoid organs (Supplemental Fig. 1). Although $CD8^+$ mature T cells in the spleen (TCR^+) and thymus ($CD24^-CD69^-TCR^+$) of ThPOK-611^+ and ThPOK-163^+ mice were almost completely absent, we consistently detected a small number of $CD8^+$ mature T cells in the lymphoid organs of ThPOK-H^+ mice (Supplemental Fig. 1A, 1B). Accordingly, compared with WT control, CD4/CD8 ratio of mature T cells increased by ∼20-fold in ThPOK-H^+ mice and >100-fold in ThPOK-163^+ and ThPOK-611^+ mice (Supplemental Fig. 1C).

To investigate the basis of differential CD4/CD8 phenotype of the three transgenic mice, we analyzed ThPOK protein expression by intracellular staining. In WT mice, the basal ThPOK staining observed in preselection DP thymocytes increased as signaled thymocytes matured into $CD4^+CD8^{lo}$ and $CD4^+$ thymocytes (Supplemental Fig. 1D). This ThPOK-specific staining pattern in WT thymic subsets is in agreement with ThPOK induction in MHCII- but not MHCI-signaled thymocytes and its continued expression in $CD4^+$ mature T cells (23). Importantly, compared with WT control, significantly higher ThPOK expression was observed in all the thymic subsets, including the preselection DP thymocytes from the three ThPOK transgenic mice (Supplemental Fig. 1E, 1G), which correlated with the observed CD4/CD8 phenotype in the thymus of these mice. Interestingly, ThPOK levels in DP thymocytes showed a hierarchical pattern with that in ThPOK-611 > ThPOK-163 > ThPOK-H; DP thymocytes from ThPOK-611^+ mice showed a significantly higher ThPOK level compared with ThPOK-H^+ DP thymocytes (Supplemental Fig. 1G). Similar to the thymic subsets, significantly higher ThPOK expressed was observed in the splenic $CD4^+$ mature T cells from the three transgenic mice compared with WT $CD4^+$ mature T cells (Supplemental Fig. 1F, 1G). The differential ThPOK staining in thymocytes from the three transgenic mice was not correlated with transgene copy number (Supplemental Fig. 1H). Interestingly, variegated ThPOK expression observed in the preselection DP thymocytes was lost as the signaled thymocytes matured as judged by largely uniform ThPOK staining in $CD4^+CD8^{lo}$ and $CD4^+$ thymocytes and $CD4^+$ splenic T cells (Supplemental Fig. 1E, 1F) from the three ThPOK transgenic mice; a small number of $CD4^+CD8^{lo}$ and $CD4^+$ thymocytes and mature T cells, particularly from ThPOK-H^+ and ThPOK-163^+ mice, showed a slightly lower ThPOK staining. At present, the reason for this change in ThPOK expression pattern in DP thymocytes versus mature T cells from these mice is not clear. Irrespective, we consistently observed ∼1.5–2-fold more ThPOK expression in $CD4^+$ mature T cells from the spleen of three transgenic mice compared with that in $CD4^+$ mature T cells from the spleen of WT mice.

Impact of ThPOK dose on the CD4 helper lineage choice of MHCI-signaled thymocytes

To evaluate the impact of differential ThPOK levels on the CD8 to CD4 helper lineage redirection, we bred the three ThPOK transgenic lines to mice expressing MHCI-restricted OTI-TCR ($V\beta5^+V\alpha2^+$; all mice $Rag^{-/-}$). In these mice, intrathymic signaling in MHCI-specific thymocytes does not induce endogenous ThPOK expression and thus allows us to study the role of transgenic ThPOK expression in the CD8 to CD4 helper lineage redirection. Indeed, we observed a ThPOK dose-dependent impact on the CD8 to CD4 helper lineage redirection in OTI mice expressing each ThPOK transgene as judged by the hierarchical

For DP thymocytes, ThPOK MFI is relative to that in DP thymocytes from ThPOK-H. For all other thymic subsets, ThPOK MFI is relative to the corresponding WT thymic subset. ThPOK MFI for splenic subsets is relative to that in splenic $CD4^+$ mature T cells from WT mice. ThPOK MFI for $CD4^+$ mature T cells from OTI mice and DN mature T cells from OTI and WT mice are ND because of the lack of a substantial number of these cells. (**F**) The frequency and absolute number of TCR^+ and $CD4^+CD8^{lo}$ subsets in total thymocytes and $CD4^+$ and $CD8^+$ mature thymocytes ($CD24^-CD69^-TCR^+$) in the indicated mice are shown. Also shown are the total splenocytes and the frequency and number of splenic T cells and T cell subsets from OTI mice expressing or not the indicated ThPOK transgene ($n > 12$). Data are representative examples of four or more independent experiments (A, B and D). $^*p \leq 0.05$, $^{**}p \leq 0.005$, $^{***}p \leq 0.0005$. n/d, not determined.

pattern of the CD4$^+$ mature T cell frequency in the thymus and spleen of these mice (Fig. 1A, 1B) with that in OTI$^+$ThPOK-611$^+$ > OTI$^+$ThPOK-163$^+$ > OTI$^+$ThPOK-H$^+$ mice. Thus, there were only 16% Vα2$^+$CD4$^+$ mature T cells in the spleen of OTI$^+$ThPOK-H$^+$ mice, whereas it was 44% in OTI$^+$ThPOK-163$^+$ and 68% in OTI$^+$ThPOK-611$^+$ mice compared with <1% in OTI control mice (Fig. 1A). An increase in the CD4$^+$ mature T cell frequency observed in the spleen was also observed in the thymus of these mice, indicating an efficient lineage redirection in OTI$^+$ThPOK-611$^+$ mice compared with partial lineage redirection in OTI$^+$ThPOK-163$^+$ and OTI$^+$ThPOK-H$^+$ mice (Fig. 1B). As expected, the frequency of CD8$^+$ mature T cells in the thymus and spleen showed an opposing pattern (Fig. 1A, 1B), resulting in the CD4/CD8 ratio in OTI$^+$ThPOK-611$^+$ mice significantly higher than that in OTI$^+$ThPOK-163$^+$ or OTI$^+$ThPOK-H$^+$ mice (Fig. 1C). We also noticed a significant number of DN mature T cells in OTI$^+$ThPOK-H$^+$ and OTI$^+$ThPOK-163$^+$ mice; as many as 25–40% of total splenic T cells were DN in these mice (Fig. 1A). The DN mature T cells in OTI$^+$ThPOK-H$^+$ mice did not express NK1.1 and were CD62L^{hi}CD44^{lo}, indicating that they were not innate or memory T cells (Supplemental Fig. 2A) (36, 37).

Similar to non-TCR transgenic background, ThPOK-specific staining in DP thymocytes was hierarchical with that in OTI$^+$ThPOK-611$^+$ > OTI$^+$ThPOK163$^+$ > OTI$^+$ThPOK-H$^+$ cells and was substantially higher compared with similar subsets from OTI control (Fig. 1D, 1E). Similar to the analysis of ThPOK transgenic mice with WT background, variegated ThPOK expression observed in DP thymocytes was lost in a majority of the redirected CD4$^+$ mature T cells from the three ThPOK transgenic mice expressing OTI-TCR and was ~1.5–2-fold higher compared with ThPOK expression in CD4$^+$ mature T cells from WT mice (Fig. 1D, 1E). Interestingly, DN and CD8$^+$ mature T cells from the spleen of OTI$^+$ThPOK-H$^+$ or OTI$^+$ThPOK-163$^+$ mice continued to express a significant amount of ThPOK compared with ThPOK levels in CD4$^+$ mature T cells from WT mice (Fig. 1D, bottom panels, Fig. 1E). Although total thymocytes in OTI mice expressing or not individual ThPOK transgene were comparable, the frequency and number of selected thymocytes were reduced in ThPOK-expressing mice likely because of impaired Runx3 expression (Fig. 1F; Refs. 11, 38, 39) see below)). As expected, the frequency and number of CD4$^+$ single-positive thymocytes was significantly increased, whereas that of CD8$^+$ single-positive thymocytes decreased in all three ThPOK transgenic OTI lines compared with control. Similarly, the frequency and number of TCR$^+$ splenic cells was reduced in ThPOK-expressing OTI mice compared with control, likely reflecting a reduced thymic maturation and survival/expansion of the redirected T cells in the periphery (Fig. 1F; Refs. 23, 24). Nevertheless, the frequency and cell number compilation data show a significant increase in CD4$^+$ and/or DN mature T cells and a decrease in CD8$^+$ mature T cells in all OTI mice expressing individual ThPOK transgene compared with control (Fig. 1F). Additionally, the DN mature T cells appeared to be mostly derived from CD4$^+$ thymocytes in OTI$^+$ThPOK-H$^+$ mice (Supplemental Fig. 2B).

To rule out the possibility that the ThPOK-H–mediated partial CD8 to CD4 helper lineage redirection was not specific to the OTI model, we introduced the ThPOK-H transgene into mice expressing MHCI-specific monoclonal TCR (P14-TCR) or polyclonal TCRs (*MHCII*$^{-/-}$). Similar to OTI$^+$ThPOK-H$^+$ mice, P14$^+$ThPOK-H$^+$ mice also showed partial CD8 to CD4 helper lineage redirection as judged by the presence of CD4$^+$, DN, and CD8$^+$ mature T cells in the spleen of these mice (Supplemental Fig. 3A). Importantly, the introduction of ThPOK-H transgene in *MHCII*$^{-/-}$ mice also resulted in the partial CD8 to CD4 helper lineage redirection (Fig. 2A, 2B) and a significant increase in the CD4/CD8 ratio in the thymus and spleen of ThPOK-H$^+$*MHCII*$^{-/-}$ mice compared with *MHCII*$^{-/-}$ mice (Fig. 2C). Similar to the OTI model, we noted a decrease in thymic selection and mature splenic T cell frequency and number in ThPOK-H$^+$*MHCII*$^{-/-}$

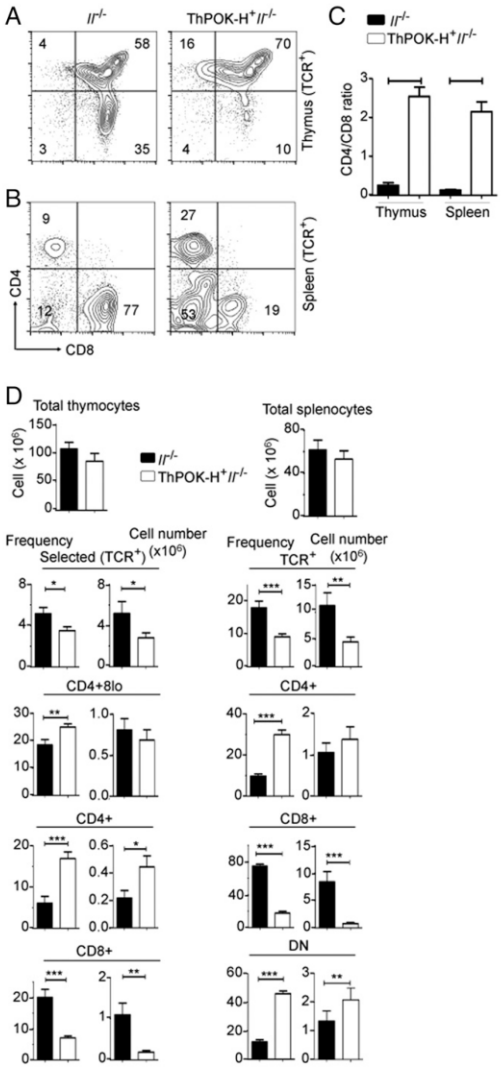

FIGURE 2. ThPOK induces partial CD8 to CD4 helper lineage redirection in *MHCII*$^{-/-}$ mice. The CD4/CD8 profiles of TCR$^+$ thymocytes (**A**) and splenocytes (**B**) from *MHCII*$^{-/-}$ (left) and ThPOK-H$^+$*MHCII*$^{-/-}$ (right) mice are shown. (**C**) The CD4/CD8 ratio in the thymus and spleen of *MHCII*$^{-/-}$ (black bars) and ThPOK-H$^+$*MHCII*$^{-/-}$ (white bars) mice is shown. (**D**) The frequency and absolute number of TCR$^+$ and CD4$^+$CD8^{lo} subsets in total thymocytes and CD4$^+$ and CD8$^+$ mature thymocytes (TCR$^+$) in *MHCII*$^{-/-}$ expressing or not ThPOK-H are shown. Also shown are the total splenocytes and the frequency and number of splenic T cells and T cell subsets from *MHCII*$^{-/-}$ mice expressing or not ThPOK-H transgene ($n > 8$). Data are representative of six or more independent experiments (A and B). *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$.

mice (Fig. 2D). In the thymus of ThPOK-H$^+$*MHCII*$^{-/-}$ mice, the frequency and number of CD4$^+$ thymocytes was significantly increased, whereas that of CD8$^+$ thymocytes was significantly decreased compared with control mice (Fig. 2D). In the spleen, we observed a similar pattern except that the number of CD4$^+$ mature T cells were only slightly higher in ThPOK-H$^+$*MHCII*$^{-/-}$ mice compared with control mice likely due to their differentiation into DN mature T cells in ThPOK-H$^+$*MHCII*$^{-/-}$ mice (Fig. 2B, 2D). Together, the generation of a small number of CD4$^+$ mature T cells and the presence of a substantial number of DN mature T cells with impaired cytotoxic function but lack of activation of helper function (see below) suggests that ThPOK-H induces partial CD8 to CD4 helper lineage redirection of thymocytes expressing MHCI-specific monoclonal or polyclonal TCRs.

To determine the basis for the presence of CD4$^+$, CD8$^+$, and DN mature T cells in OTI$^+$ThPOK-H$^+$ mice, we assessed *Thpok*, *Runx3d*, *Socs1*, and *Nur77* levels in each T cell subset purified from the same mouse. *Socs1* is positively regulated by ThPOK (21) and *Nur77*, which influences CD8$^+$ mature T cell development by modulating *Runx3* expression (40), is suggested to be preferentially expressed in CD4$^+$ mature T cells (41). QPCR and flow cytometric analysis of mature T cells from OTI$^+$ThPOK-H$^+$ mice showed graded ThPOK expression levels with that in CD4$^+$ > DN > CD8$^+$ mature T cells (Fig. 3A), which is in agreement with staining data (Fig. 1D, 1E). In agreement with ThPOK expression analysis, the expression of *Runx3* from a distal promoter (*Runx3d*) was completely abolished in CD4$^+$ and DN mature T cells and reduced in CD8$^+$ mature T cells from OTI$^+$ThPOK-H$^+$ mice compared with CD8$^+$ mature T cells from OTI mice (Fig. 3B). Similarly, *Socs1* was mostly expressed in CD4$^+$ mature T cells (Fig. 3C), whereas *Nur77* expression was directly proportional to ThPOK levels in the three mature T cell subsets from OTI$^+$ThPOK-H$^+$ mice (Fig. 3D). Nur77 expression was significantly higher in CD4$^+$ mature T cells ($p = 0.0032$) but not in DN or CD8$^+$ mature T cells from OTI$^+$ThPOK-H$^+$ mice compared with CD8$^+$ mature T cells from OTI mice. Comparable CD5 levels [a surrogate marker for TCR signal strength (42)] in CD4$^+$ and CD8$^+$ mature T cells (Supplemental Fig. 3B) from OTI$^+$ThPOK-H$^+$ and

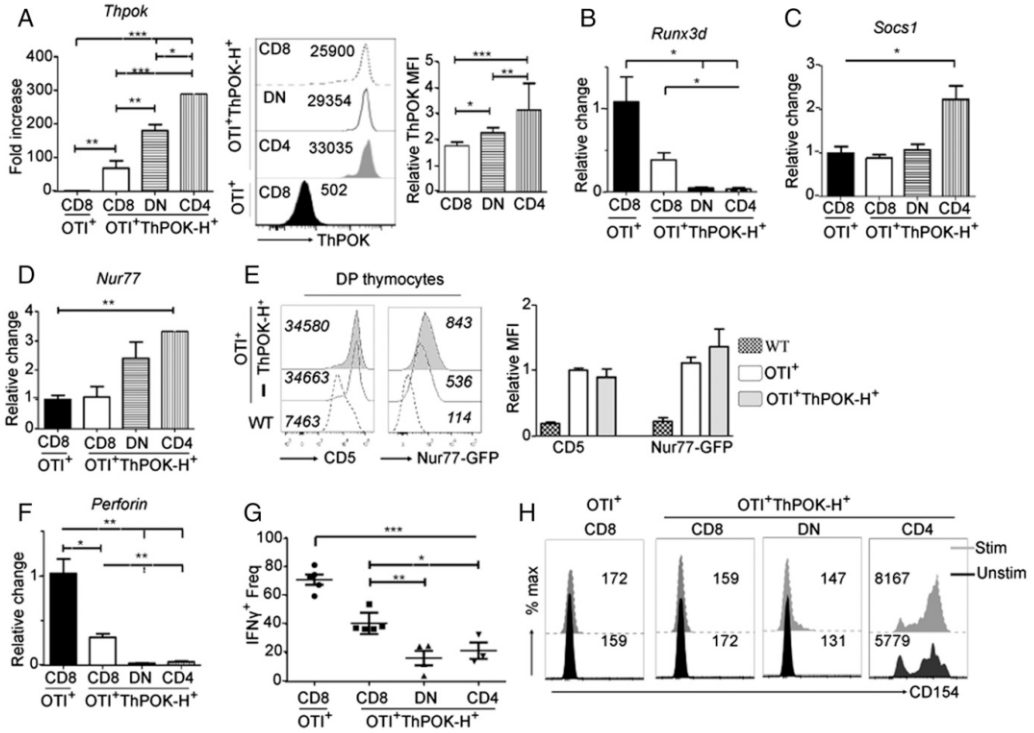

FIGURE 3. ThPOK modulates lineage-specific gene expression in T cell subsets. CD4$^+$, CD8$^+$, and DN mature T cells from the spleen of OTI$^+$ThPOK-H$^+$ mice were isolated and the expression levels of *Thpok* (**A**, left), *Runx3d* (**B**), *Socs1* (**C**), and *Nur77* (**D**) were evaluated by QPCR and were compared with that in CD8$^+$ mature T cells from OTI mice (normalized to *Hprt* expression). Data depicts the average of triplicate values with SD and are expressed as fold increase over the expression of individual genes in control CD8$^+$ mature T cells from OTI mice. (A, right) ThPOK protein levels in the indicated splenic T cell subsets from OTI$^+$ThPOK-H$^+$ or OTI$^+$ mice are shown. Also shown is ThPOK mean fluorescence intensity (MFI) compilation for the indicated T cell subsets (relative to WT CD4$^+$ mature T cells). (**E**) MFI of CD5 and Nur77-GFP expression in DP thymocytes from OTI$^+$ThPOK-H$^+$, OTI, and WT mice are compared (left) and compiled (right; relative to OT1). (**F**) shows perforin expression levels by QPCR in CD4$^+$, DN, and CD8$^+$ mature T cells from the spleen of OTI$^+$ThPOK-H$^+$ mice compared with CD8$^+$ mature T cells from OTI control mice (normalized to *Hprt* expression). (**G**) Purified T cell subsets from OTI mice expressing or not ThPOK-H were activated for 5–7 d in the presence of irradiated splenocytes from BL/6 mice pulsed with OTI peptide (SIINFEKL) and then restimulated with PMA/ionomycin in the presence of brefeldin for the analysis of IFN-γ expression. (**H**) CD154 expression in the cognate-peptide activated indicated T cell subsets from OTI$^+$ and OTI$^+$ThPOK-H$^+$ mice is shown. Data are representative of two to six independent experiments. $^*p \le 0.05$, $^{**}p \le 0.005$, $^{***}p \le 0.0005$.

OTI control suggest that the differential *Nur77* expression observed in mature T cells may be due to differential ThPOK expression (32). We then evaluated Nur77-GFP reporter expression in DP thymocytes (to exclude the influence of intrathymic signaling on Nur77 expression) from OTI$^+$ThPOK-H$^+$ and control mice. Comparable CD5 levels but higher GFP expression was detected in DP thymocytes from OTI$^+$ThPOK-H$^+$ mice compared with OTI control mice expressing Nur77-GFP reporter (Fig. 3E); however, an increase in Nur77-GFP expression in the presence of transgenic ThPOK did not appear to be significant. In cell transfection studies, we did not observe any increase in *Nur77* promoter-driven luciferase expression in the presence of ThPOK (Supplemental Fig. 3C). These data suggest that ThPOK may not be involved in regulating Nur77 expression.

Functionality of mature T cell subsets in OTI$^+$ThPOK-H$^+$ mice

Because ThPOK is proposed to suppress the cytotoxic program in mature T cells (28), we wondered about the functionality of the three T cell subsets, particularly CD8$^+$ and DN mature T cells that expressed a significant amount of ThPOK and reduced levels of Runx3. To this end, we evaluated the expression of genes involved in cytotoxic and helper function in these T cell subsets. In agreement with Runx3 and ThPOK expression analysis, we observed, compared with CD8$^+$ mature T cells from OTI mice, almost complete ablation of perforin and severely reduced IFN-γ expression in CD4$^+$ and DN mature T cells (Fig. 3F, 3G). Interestingly, perforin and IFN-γ expression was significantly reduced in CD8$^+$ mature T cells isolated from OTI$^+$ThPOK-H$^+$ mice as well (Fig. 3F, 3G). Upon activation, CD4$^+$, but not DN or CD8$^+$, mature T cells from OTI$^+$ThPOK-H$^+$ mice upregulated CD154, a CD4 helper lineage marker (Fig. 3H). This was also observed in mature T cells subsets isolated from ThPOK-H$^+$*MHCII*$^{-/-}$ mice; activated CD4$^+$ mature T cells from ThPOK-H$^+$*MHCII*$^{-/-}$ mice expressed CD154 and IL-4 (Supplemental Fig. 3D, 3E), whereas DN and CD8$^+$ mature T cells from ThPOK-H$^+$*MHCII*$^{-/-}$ mice continued to express IFN-γ with DN mature T cells expressing lower amounts. Together, these data suggest that a ThPOK level sufficient to suppress the cytotoxic program does not activate the helper program (phenotype of DN mature T cells) and that a higher amount of ThPOK is required to redirect MHCI-signaled thymocytes into the CD4 helper lineage.

Role of endogenous ThPOK in the CD8 to CD4 helper lineage redirection in OTI$^+$ThPOK-H$^+$ mice

ThPOK is proposed to form a positive autoregulatory loop (27). Hence, we wondered if the transgenic ThPOK induced the expression of endogenous ThPOK in the signaled thymocytes and whether this contributed to the CD8 to CD4 helper lineage redirection in OTI$^+$ThPOK-H$^+$ mice. To address this, we first evaluated, using specific QPCR primers, the endogenous ThPOK expression in various T cell subsets from OTI$^+$ThPOK-H$^+$ mice. Indeed, we observed a significant increase in endogenous ThPOK expression in the splenic CD4$^+$ mature T cells of OTI$^+$ThPOK-H$^+$ mice (Fig. 4A). To assess if this endogenous ThPOK induction played any role in the CD8 to CD4 helper lineage redirection in OTI$^+$ThPOK-H$^+$ mice, we analyzed CD4/CD8 phenotype of OTI$^+$ThPOK-H$^+$ mice expressing endogenous ThPOK or not. Surprisingly, we did not observe any significant changes in the frequency of CD4$^+$ and CD8$^+$ thymocytes in OTI$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice compared with OTI$^+$ThPOK-H$^+$*Thpok*$^{+/+}$ control (Fig. 4B). A slight decrease in CD4$^+$ and increase in CD8$^+$ mature T cell frequency in the spleen of OTI$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice compared with OTI$^+$ThPOK-H$^+$*Thpok*$^{+/+}$ mice was noticed; however, it did not result in any significant change in the CD4/CD8 ratio in these mice [0.41 ± 0.09 in OTI$^+$ThPOK-H$^+$*Thpok*$^{+/+}$ versus 0.28 ± 0.035 in OTI$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice; (Fig. 4C; relative to OTI control)]. The frequency/number of various thymic and splenic T cell subsets in OTI$^+$ThPOK-H$^+$ mice were comparable irrespective of the presence or absence of endogenous ThPOK (Fig. 4D; some mice were in *Rag*$^{+/-}$ background, which did not affect the CD4/CD8 phenotype). These data suggest that endogenous ThPOK played an insignificant role in the CD8 to CD4 helper lineage redirection in OTI$^+$ThPOK-H$^+$ mice.

Evaluating role of ThPOK-H in the CD4 helper lineage choice of MHCII-specific thymocytes

The presence of a large number of DN and CD8$^+$ mature T cells in ThPOK-H$^+$ mice expressing MHCI-specific TCR (OTI, P14, or MHCII$^{-/-}$), despite expressing ∼1.5–2-fold more transgenic ThPOK compared with endogenous ThPOK levels in CD4$^+$ mature T cells from WT mice, suggests that differential amount of ThPOK may be required for the CD4 helper lineage choice of MHCI- and MHCII-specific thymocytes. Alternately, the observed phenotype of OTI$^+$ThPOK-H$^+$ mice could be because of the ThPOK-H transgene–specific effect. To address these questions, we evaluated the impact of ThPOK-H on the rescue of CD4 helper lineage development of MHCII-specific thymocytes in ThPOK-deficient mice. To this end, we generated OTII$^+$*Thpok*$^{-/-}$ mice expressing or not ThPOK-H transgene. In OTII$^+$*Thpok*$^{+/+}$ mice, >95% of the Vα2$^+$ mature T cells are CD4$^+$, which are directed into CD8 lineage in the absence of ThPOK (Fig. 5A). Indeed, the introduction of ThPOK-H transgene into OTII$^+$*Thpok*$^{-/-}$ mice completely rescued CD4 helper lineage commitment; >95% of Vα2$^+$ mature T cells were CD4$^+$ in the thymus and spleen of OTII$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice, which was similar to that in OTII$^+$*Thpok*$^{+/+}$ mice (Fig. 5A, 5B). ThPOK-specific staining of the mature CD4$^+$ thymocytes and splenic T cells showed a ∼2-fold higher expression compared with endogenous ThPOK expression in control CD4$^+$ mature T cells from OTII$^+$*Thpok*$^{+/+}$ mice (Fig. 5C). The frequency and absolute cell numbers in the thymus and spleen of OTII$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice were comparable to that in littermate control OTII$^+$ThPOK-H$^+$*Thpok*$^{+/+}$ mice (Fig. 5D).

To further support the observation that the same level of ThPOK differentially influences the CD4 helper lineage choice of MHCI- and MHCII-specific thymocytes, we introduced each of the three ThPOK transgene into *Thpok*$^{-/-}$ mice expressing polyclonal TCR repertoire. In *Thpok*$^{-/-}$ mice, positively selected MHCII-specific thymocytes are redirected into the CD8 lineage, and thus, the peripheral CD8$^+$ mature T cell population consists of MHCI- and MHCII-specific T cells. Indeed, each of the ThPOK transgene rescued CD4 helper development and impaired CD8 cytotoxic development in *Thpok*$^{-/-}$ mice (Supplemental Fig. 4A–C). Together, these data strongly suggest that, compared with CD4 helper lineage choice of MHCII-specific thymocytes, an efficient CD8 to CD4 helper lineage redirection of MHCI-specific thymocytes requires a higher amount of ThPOK, and the partial CD8 to CD4 helper lineage redirection in OTI$^+$ThPOK-H$^+$ mice is unlikely because of the ThPOK-H transgene–specific effect.

Impact of augmented TCR signal strength on the ThPOK-induced CD4 helper lineage choice of MHCI-signaled thymocytes

The partial CD8 to CD4 helper lineage redirection of MHCI-signaled thymocytes but complete rescue of CD4 helper lineage choice of MHCII-signaled thymocytes prompted us to ask if differential TCR signaling played a role in the ThPOK-H–mediated CD4 helper lineage choice of MHCI- and MHCII-specific thymocytes. We considered the possibility that weak TCR signaling in MHCI-specific thymocytes, compared with that in MHCII-specific

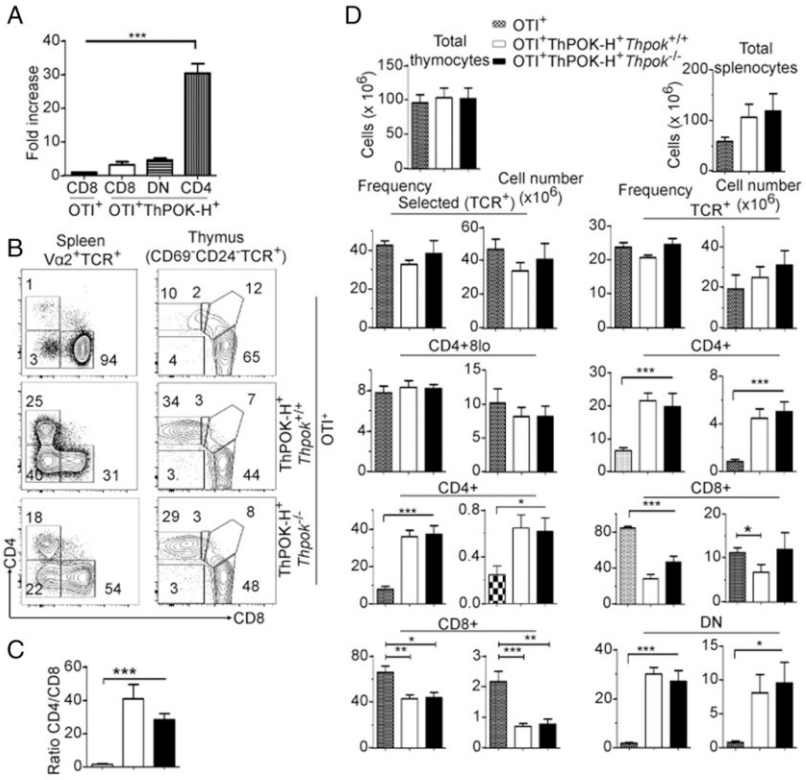

FIGURE 4. Insignificant contribution of endogenous ThPOK in the CD8 to CD4 helper lineage redirection by ThPOK-H. (**A**) To assess endogenous ThPOK levels, various T cell subsets from OTI$^+$THPOK-H$^+$ mice were purified for QPCR analysis. Data show endogenous ThPOK levels in the indicated splenic T cell subsets from OTI$^+$ThPOK-H$^+$ mice compared with that in CD8$^+$ mature T cells from OTI mice (normalized to *Hprt* expression). Data depicts the average of triplicate values with SD and are expressed as fold increase over endogenous ThPOK levels in control CD8$^+$ mature T cells from OTI mice. To determine the impact of endogenous ThPOK expression on ThPOK-H–mediated CD4 helper lineage choice OTI$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice were generated. (**B**) shows the CD4/CD8 profiles of the spleen and thymus isolated from OTI$^+$, OTI$^+$ThPOK-H$^+$*Thpok*$^{+/+}$, and OTI$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice. (**C**) The CD4/CD8 ratio in the spleen of indicated mice relative to OTI is shown. (**D**) The frequency and absolute number of TCR$^+$ and CD4$^+$CD8^{lo} subsets in total thymocytes and CD4$^+$ and CD8$^+$ mature thymocytes (CD24$^-$CD69$^-$TCR$^+$) in the indicated mice are shown ($n > 6$). Also shown are total splenocytes and the frequency and number of splenic T cells and T cell subsets in these mice. Data are representative of three or more independent experiments (A and B). $*p \leq 0.05$, $**p \leq 0.005$, $***p \leq 0.0005$.

thymocytes, may be responsible for an inefficient CD4 helper lineage choice of MHCI-specific thymocytes expressing ThPOK-H transgene. If so, we reasoned that increasing the TCR signal strength may enhance the efficiency of ThPOK-H–mediated CD4 helper lineage choice of MHCI-specific thymocytes. To test this notion, we introduced constitutively active Lck encoding transgene, dLGF, (16, 17, 33) into OTI$^+$ThPOK-H$^+$ mice. We bred mice to obtain OTI$^+$dLGF$^+$ThPOK-H$^+$ triple transgenic mice with a *Rag*$^{-/-}$ background and analyzed CD4/CD8 development in these mice. As reported previously (16), increased TCR signaling due to constitutively active Lck led to an increase in the frequency of Vα2$^+$CD4$^+$ and a decrease in the frequency of Vα2$^+$CD8$^+$ mature T cells in the thymus and spleen of OTI$^+$dLGF$^+$ mice compared with control mice (Fig. 6A). Importantly, analysis of mature T cells in the thymus and spleen of OTI$^+$dLGF$^+$ThPOK-H$^+$ mice showed a significant increase in the frequency and number of Vα2$^+$CD4$^+$ T cells, whereas that of Vα2$^+$CD8$^+$ mature mature T cells was significantly reduced compared with OTI$^+$ThPOK-H$^+$ mice (Fig. 6A, 6B). About 70% of mature T cells were CD4$^+$ in the spleen and thymus of triple transgenic mice, resulting in a significantly higher CD4/CD8 ratio compared with OTI$^+$ThPOK-H$^+$ or OTI$^+$dLGF$^+$ mice (Fig. 6C). Of note, the frequency and absolute number of DN mature T cells were also reduced in the spleen of triple transgenic mice compared with OTI$^+$ThPOK-H$^+$ mice but was still higher compared with OTI$^+$dLGF$^+$ mice (Fig. 6A, 6B).

To ascertain that the efficient CD8 to CD4 helper lineage redirection was not specific to the introduction of dLGF transgene into OTI$^+$ThPOK-H$^+$ mice, we analyzed the CD4/CD8 phenotype of OTI$^+$dLGF$^+$ThPOK-163$^+$ mice as well. Indeed, an increase in the CD4$^+$ and a decrease in the CD8$^+$ mature T cell frequency was observed in the thymus and spleen of OTI$^+$dLGF$^+$ThPOK-163$^+$ mice compared with OTI$^+$ThPOK-163$^+$ mice (Supplemental Fig. 4D). We then evaluated the expression of *St8sia6* and *St3gal2*, the helper lineage-associated genes (31, 41), and *Cxxc5*, a ThPOK target gene that negatively regulates CD154 and is highly expressed in CD8$^+$ mature T cells (43). Indeed, QPCR analysis showed a significantly elevated expression of *St8sia6* and *St3gal2* and a decreased expression of *Cxxc5* in CD4$^+$ mature T cells from

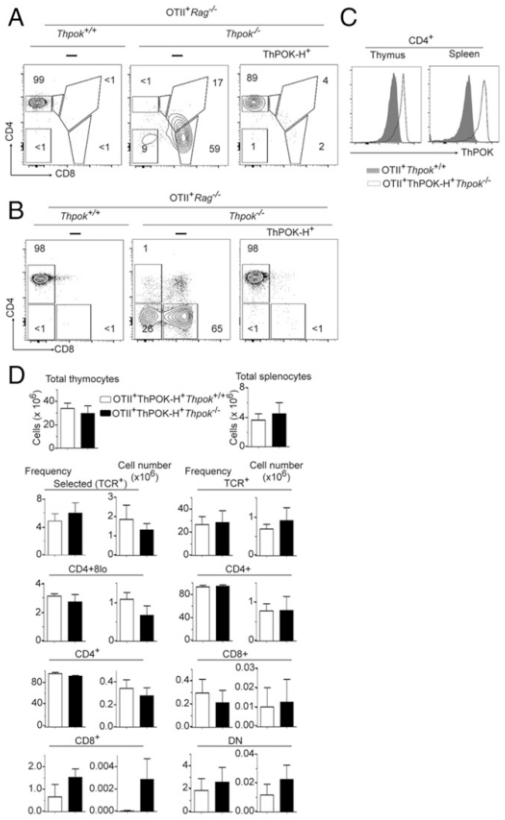

FIGURE 5. ThPOK-H completely rescues CD4 helper lineage development in OTII$^+$*Thpok*$^{-/-}$ mice. To evaluate the ability of ThPOK-H transgene to rescue CD4 helper lineage development of MHCII-specific thymocytes, the transgene was introduced into OTII$^+$*Thpok*$^{-/-}$ mice. (**A**) shows the CD4/CD8 profile of mature thymocytes (CD24$^-$CD69$^-$TCR$^+$), and (**B**) shows the CD4/CD8 phenotype of splenic T cells from the indicated mice. (**C**) ThPOK protein levels in CD4$^+$ mature T cells from the thymus and spleen of OTII$^+$*Thpok*$^{+/+}$ (shaded histogram) and OTII$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ (open histogram) mice are shown. (**D**) The frequency and absolute number of TCR$^+$ and CD4$^+$CD8^{lo} subsets in total thymocytes and CD4$^+$ and CD8$^+$ mature thymocytes (CD24$^-$CD69$^-$TCR$^+$) in OTII$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ and littermate OTII$^+$ThPOK-H$^+$*Thpok*$^{+/+}$ (all *Rag*$^{-/-}$) control mice are shown. Also shown are total splenocytes and the frequency and number of splenic T cells and T cell subsets from the same mice ($n > 4$). Data are representative of three or more independent experiments (A–C).

the triple transgenic mice, which was similar to the expression of these genes in CD4$^+$ mature T cells but opposite to their expression in CD8$^+$ mature T cells from WT mice (Fig. 6D). The DN mature T cells from the triple transgenic mice showed a significantly lower *St8sia6* and *St3gal2* expression compared with CD4$^+$ mature T cells from the same mice. Interestingly, DN mature T cells, which upon activation failed to upregulate CD154, expressed very little *Cxxc5*, suggesting a possible complex regulation of CD154 expression in the activated CD4$^+$ mature T cells (43). Upregulation of *St8sia6* and *St3gal2* and suppression of *Cxxc5* was also observed in the redirected CD4$^+$ mature T cells isolated from OTI$^+$ThPOK-163$^+$ and OTI$^+$ThPOK-611$^+$ mice as well (Supplemental Fig. 4E). Together, these data strongly suggest that an elevated TCR signal strength and transgenic ThPOK act synergistically in redirecting MHCI-signaled thymocytes into the CD4$^+$ helper T cell lineage.

Evaluating the contribution of transgenic and endogenous ThPOK in the CD8 to CD4 helper lineage redirection in the presence of augmented TCR signaling

An efficient CD8 to CD4 helper lineage redirection of MHCI-specific thymocytes in the presence of augmented TCR signaling and ~2-fold more transgenic ThPOK protein (compared with endogenously expressed ThPOK in WT CD4$^+$ mature T cells) in OTI$^+$dLGF$^+$ThPOK-H$^+$ and OTI$^+$dLGF$^+$ThPOK-163$^+$ mice could be because of two overlapping possibilities: augmented TCR signaling 1) induces endogenous ThPOK that contributes to this lineage redirection or 2) plays a role in the CD4 helper lineage choice of MHCI-specific thymocytes that is independent of ThPOK. To investigate these possibilities, we first measured endogenous ThPOK levels in the positively selected thymocytes from OTI$^+$dLGF$^+$ mice. As expected, we detected a significant ThPOK induction in the CD4$^+$CD8^{lo} thymocytes from OTI$^+$dLGF$^+$ mice compared with OTI control (Fig. 7A); ThPOK induction was essential for the generation of CD4$^+$ mature T cells in these mice as indicated by the absence of these cells in the thymus and spleen of OTI$^+$dLGF$^+$*Thpok*$^{-/-}$ mice (Fig. 7B). Thus, it was conceivable that the induction of endogenous ThPOK due to increased TCR signal strength substantially contributed to the increased frequency of CD4$^+$ mature T cells in OTI$^+$dLGF$^+$ThPOK-H$^+$ mice. Therefore, to evaluate the relative contribution of the two sources of ThPOK (transgenic and endogenous) in the CD4 helper lineage choice in triple transgenic mice, we ablated ThPOK expression in these mice (all mice *Rag*$^{-/-}$). We predicted that if endogenous ThPOK induced by augmented TCR signaling primarily contributed to the increased frequency of CD4$^+$ mature T cells in OTI$^+$dLGF$^+$ThPOK-H$^+$ mice, then ablating endogenous ThPOK in these mice would result in a CD4 frequency that would be lower compared with the CD4 frequency in OTI$^+$dLGF$^+$ThPOK-H$^+$ThPOK$^{+/+}$ mice, but it would be comparable to the CD4 frequency in OTI$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice (Fig. 4B). Indeed, ablating the endogenous ThPOK expression resulted in a small but significant decrease ($p < 0.02$) in the splenic CD4$^+$ mature T cell frequency in OTI$^+$dLGF$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice compared with ThPOK-sufficient control mice; >50% of mature T cells were still CD4$^+$ in the thymus and spleen of OTI$^+$dLGF$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice compared with >60% in triple transgenic ThPOK-sufficient mice (Fig. 7C, 7D). Importantly, despite expressing only the transgene-encoded ThPOK, the CD4$^+$ mature T cell frequency (52%) in the spleen of OTI$^+$dLGF$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice was still significantly higher than the CD4$^+$ mature T cell frequency (20%) observed in the spleen of OTI$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice (Fig. 7C, 7D; $p < 0.0001$). The frequency and number of DN and CD8$^+$ splenic T cell subsets were comparable in triple transgenic mice expressing endogenous ThPOK or not (Fig. 7D).

To ascertain the observed differential CD4 frequency in OTI$^+$dLGF$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice, we analyzed the CD4/CD8 phenotype of OTI$^+$dLGF$^+$ThPOK-163$^+$*Thpok*$^{-/-}$ mice as well. Indeed, we observed only a small decrease in the splenic CD4$^+$ mature T cell frequency in OTI$^+$dLGF$^+$ThPOK-163$^+$*Thpok*$^{-/-}$ mice (48%) compared with OTI$^+$dLGF$^+$ThPOK-163$^+$*Thpok*$^{+/+}$ mice (54%), but it was higher compared with that in OTI$^+$ThPOK-163$^+$*Thpok*$^{-/-}$ mice (29%; Supplemental Fig. 4D). Of note, ThPOK expression analysis showed a slightly higher frequency of ThPOK^{lo} DP thymocytes from OTI$^+$dLGF$^+$ThPOK-H$^+$*Thpok*$^{-/-}$

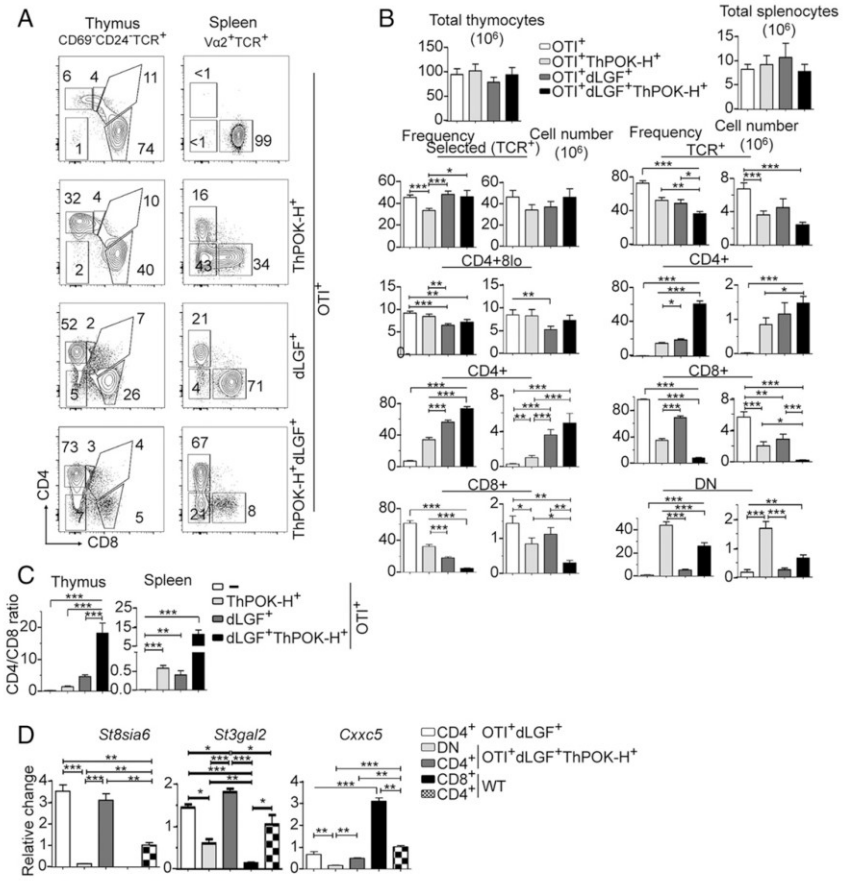

FIGURE 6. Augmenting TCR signal strength enhances ThPOK-induced CD8 to CD4 helper lineage redirection. The role of increased TCR signal strength in promoting CD8 to CD4 helper lineage redirection in OTI$^+$ThPOK-H$^+$ mice was investigated by introducing dLGF transgene into OTI$^+$ThPOK-H$^+$ mice. (**A**) shows a representative example of the CD4/CD8 profile of mature thymocytes (left) and splenic T cells (right) from the indicated mice. (**B**) The frequency and absolute number of TCR$^+$ and CD4$^+$CD8^{lo} subsets in total thymocytes and CD4$^+$ and CD8$^+$ mature thymocytes (CD24$^-$CD69$^-$TCR$^+$) as well as splenic T cells and subsets from the indicated mice are shown ($n > 6$). (**C**) The CD4/CD8 ratio of mature thymic and splenic T cells in the indicated mice is shown. (**D**) Mature T cells from the spleen of WT (CD4$^+$ and CD8$^+$), OT1$^+$dLGF$^+$ (CD4$^+$), and OTI$^+$dLGF$^+$ThPOK-H$^+$ (CD4$^+$ and DN) mice were isolated, and the expression of *St8sia6*, *St3gal2*, and *Cxxc5* was evaluated by QPCR. Data depict the average of triplicate values with SD and are expressed as fold increase over the expression of individual genes in control CD4$^+$ mature T cells from WT mice (normalized to *Hprt* expression). Data are representative of >6 independent experiments (A) and two experiments (D). $*p \leq 0.05$, $**p \leq 0.005$, $***p \leq 0.0005$.

mice compared OTI$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ control, suggesting a possible influence of augmented TCR signaling on the transgenic ThPOK levels in DP thymocytes (Fig. 7E). Significantly higher phospho-Src staining in DP and CD4$^+$CD8^{lo} thymocytes confirmed augmented TCR signaling in OTI$^+$dLGF$^+$ mice expressing ThPOK-H or not compared with OTI or WT control (Fig. 7F, 7G). We also observed elevated, albeit insignificant, phospho-CD3ζ levels in DP and CD4$^+$CD8^{lo} thymocytes from these mice compared with OTI control [it was significantly higher compared with similar subsets from WT mice (Fig. 7F, 7G)]. The increased pSrc and pCD3ζ staining observed in DP thymocytes in OTI mice expressing dLGF transgene became less pronounced in CD4$^+$CD8^{lo} thymocytes, reflecting a possible impact of intrathymic signaling and/or the limit of sensitivity of phospho-specific Ab staining. We then evaluated the expression of CD4 helper lineage genes in purified T cells from the triple transgenic *Thpok*$^{-/-}$ mice. Indeed, the expression pattern of *St8sia6*, *St3gal2*, and *Cxxc5* in CD4$^+$ mature T cells from OTI$^+$dLGF$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice was similar to that in CD4$^+$ mature T cells from WT mice, which is upregulation of *St8sia6* and *St3gal2* and downregulation of *Cxxc5* (Fig. 7H). Collectively, these data strongly suggest that augmenting TCR signal strength in MHCI-specific thymocytes significantly promotes the ThPOK-induced CD8 to CD4 helper lineage redirection. These data also suggest that TCR signaling plays a role in CD4 helper lineage choice that may be independent of ThPOK.

The CD4$^+$ mature T cell frequency in OTI$^+$dLGF$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice, although higher than the CD4$^+$ mature T cell frequency in OTI$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice, was still significantly lower than the CD4$^+$ mature T cell frequency in OTII$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice (Fig. 8A; $p < 0.0005$). CD4$^+$ mature T cells in all these three mouse strains expressed the same amount of ThPOK but received differential intrathymic signaling

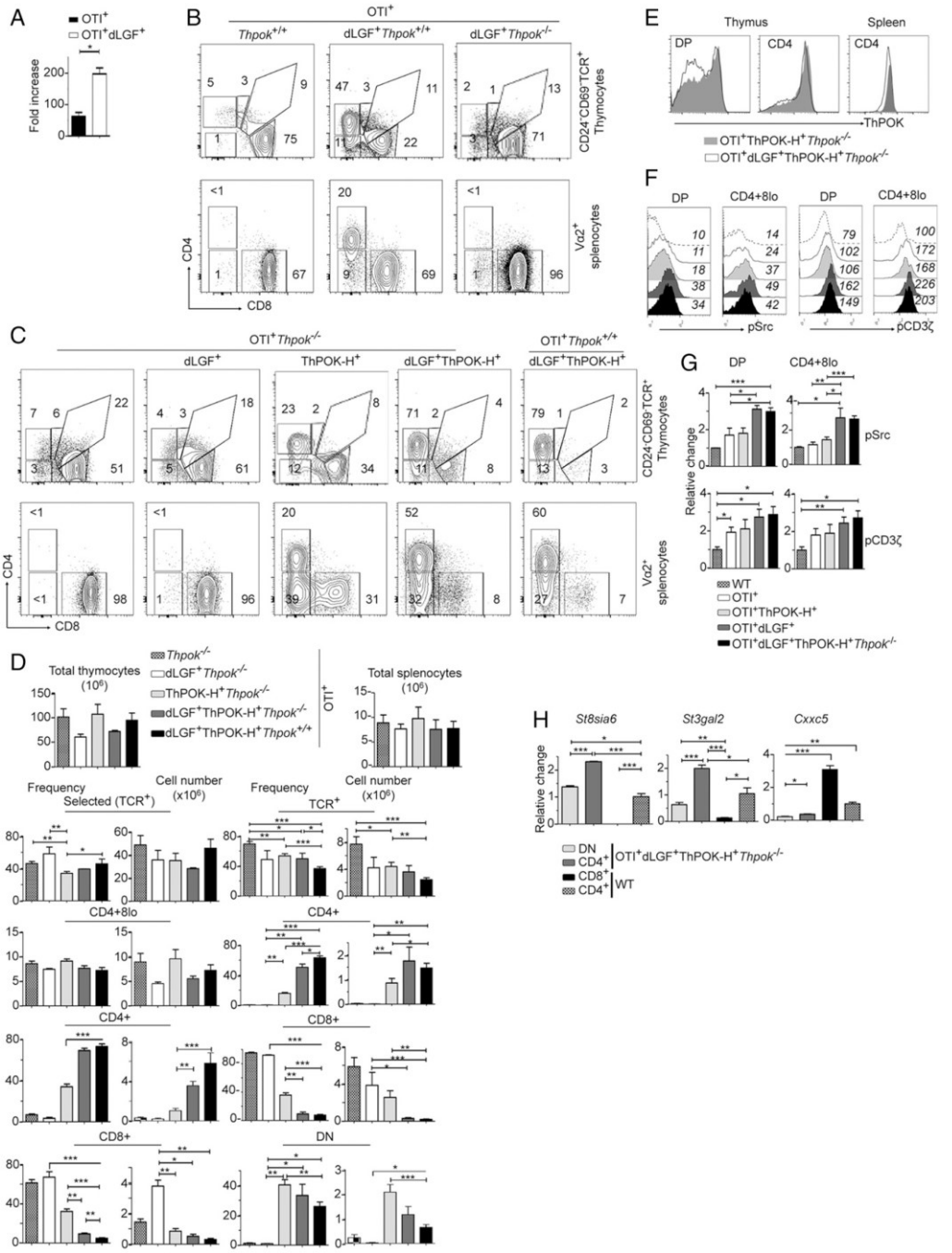

FIGURE 7. Evaluating the contribution of endogenous and transgenic ThPOK in CD4 helper lineage choice in OTI$^+$dLGF$^+$ThPOK-H$^+$ mice. (**A**) The CD4$^+$CD8^{lo} thymocytes were purified from OTI$^+$dLGF$^+$ and control OTI mice, and endogenous ThPOK expression was analyzed by QPCR. A representative example shows the average of triplicate values ± SD and are expressed as relative fold increase over preselection DP thymocytes from OTI control mice. (**B**) shows the CD4/CD8 profiles of the mature thymocytes (CD69$^-$CD24$^-$Vα2$^+$) and splenic *(Figure legend continues)*

(MHCI-induced signaling, MHCI-induced signaling combined with augmented TCR signaling, and MHCII-induced signaling). Thus, it was possible that TCR signaling in MHCI-specific CD4$^+$ mature T cells from OTI$^+$dLGF$^+$ThPOK-H$^+$ mice, although elevated compared with that in MHCI-specific CD4$^+$ mature T cells from OTI$^+$ThPOK-H$^+$ mice, may still be lower than that in MHCII-specific CD4$^+$ mature T cells from OTII mice. Therefore, we compared CD5 levels in the thymic and splenic T cells from OTI mice expressing or not dLGF with that from OTII mice to assess their TCR signal strength. In each experiment, we calculated CD5 levels in the thymocytes and mature T cell subsets from various mice relative to CD5 levels in the relevant thymic subsets or CD8$^+$ mature T cells from OTI mice. As expected, DP and CD4$^+$CD8^{lo} thymocytes from OTI mice expressing dLGF transgene showed significantly higher CD5 levels compared with the OTII subset (Fig. 8B). Interestingly, CD5 levels in CD4$^+$ thymocytes from the two mice were quite comparable (Fig. 8C), likely reflecting stronger intrathymic signaling transduced in OTII$^+$ thymocytes, but became significantly higher in the CD4$^+$ splenic T cells from OT1$^+$dLGF$^+$ mice compared with that from OTII mice (Fig. 8D). In OTI$^+$dLGF$^+$ThPOK-H$^+$ mice, we observed a similar trend in CD5 expression levels in DP, CD4$^+$CD8^{lo} thymocytes (Fig. 8B, 8C), and CD4$^+$ splenic T cell (Fig. 8D) subsets compared with similar subsets from OTII mice. Of note, CD5 levels were lower in CD4$^+$ mature thymocytes and splenic T cells from OTI$^+$dLGF$^+$ThPOK-H$^+$ mice compared with CD5 levels in similar subsets from OTI$^+$dLGF$^+$ mice. As well, CD5 levels in DN and/or CD8$^+$ mature T cells from OTI$^+$dLGF$^+$ or OTI$^+$ThPOK-H$^+$ mice were lower compared with that in CD8$^+$ mature T cells from OTI mice. An analysis of CD5 levels in the splenic CD4$^+$ mature T cells from OTI$^+$dLGF$^+$ThPOK-163$^+$ mice also showed a similar trend. CD5 levels in CD4$^+$ mature T cells from OTI$^+$dLGF$^+$ThPOK-163$^+$ mice were significantly higher compared with that in CD4$^+$ mature T cells from OTII mice, and they were slightly lower compared with CD5 levels in CD4$^+$ mature T cells from OTI$^+$dLGF$^+$ mice (Supplemental Fig. 4F). It is unclear whether differential intrathymic signaling, which influences CD5 levels and correlates with mature T cell function (44, 45), may be responsible for the altered CD5 levels in CD4$^+$ or CD8$^+$ mature T cells in the presence of transgenic ThPOK in OTI$^+$dLGF$^+$ mice. Nevertheless, these data support the notion that TCR signaling in MHCI-specific OTI$^+$ thymocytes expressing dLGF transgene is significantly higher than that in MHCII-specific OTII$^+$ thymocytes. Collectively, our in-depth analysis of CD4/CD8 lineage choice of MHCI-specific thymocytes with or without augmented TCR signaling and of MHCII-specific thymocytes in the presence of the same amount of ThPOK strongly suggest that ThPOK-induced CD4 helper lineage choice of developing thymocytes is critically influenced by quantitative as well as differential TCR signaling.

Discussion

In the present manuscript, we have investigated the impact of ThPOK levels on the CD4 helper lineage choice of MHCI- and MHCII-specific thymocytes and the role of TCR signaling in it. Specifically, we have evaluated the impact of ThPOK levels on the CD4 helper lineage choice of thymocytes with differential TCR signaling. Our data strongly suggest that MHCI-restricted thymocytes require a higher level of ThPOK in preselection thymocytes for an efficient CD8 to CD4 helper lineage redirection (ThPOK-611 mice), whereas relatively low/moderate levels (still higher than ThPOK levels in WT CD4$^+$ mature T cells) result in the partial CD8 to CD4 helper lineage redirection (ThPOK-H and ThPOK-163 mice). The lower frequency of mature T cells observed in the spleen of OTI$^+$ThPOK-163$^+$ and OTI$^+$ThPOK-611$^+$ mice likely reflects a reduced thymic maturation and/or the effect of mismatched coreceptor expression (CD4$^+$ mature T cells) or lack of coreceptor expression (DN mature T cells) on survival and/or homeostatic expansion of the redirected T cells (23, 24). A consequence of partial lineage redirection is that a substantial number of CD8$^+$ and DN mature T cells are detected in OTI$^+$ThPOK-H$^+$ or OTI$^+$ThPOK-163$^+$ mice. It is interesting that the DN mature T cells in OTI$^+$ThPOK-H$^+$ mice fail to maintain CD4 expression despite almost complete suppression of Runx3 expression indicating a complex regulation of *Cd4* expression requiring sustained TCR signaling in developing thymocytes (46). In vitro differentiation culture data supports such a notion; disrupting intrathymic TCR signaling in an in vitro culture of purified CD4$^+$ thymic subsets from OTI$^+$ThPOK-H$^+$ mice results in the loss of CD4 expression in a significant number of cells, leading to the generation of DN mature T cells. Importantly, DN and CD8$^+$ mature T cells, particularly the former, continued to express a substantial amount of ThPOK that compromised the cytotoxic function but still failed to activate the helper program in these cells. A simple explanation would be that activation of the helper program in MHCI-signaled thymocytes requires a higher amount of ThPOK than that required for suppression of the cytotoxic program (phenotype of DN mature T cells). However, it was paradoxical that the ThPOK level in DN mature T cells from OTI$^+$ThPOK-H$^+$ or OTI$^+$ThPOK-163$^+$ mice was significantly higher compared with endogenous ThPOK levels in MHCII-restricted CD4$^+$ mature T cells from WT mice and yet failed to redirect them into the CD4 helper lineage. The inability of ThPOK-H to induce an efficient CD8 to CD4 helper lineage redirection is unlikely because of the variegated expression as the same ThPOK-H transgene completely rescued CD4 helper development in *Thpok*$^{-/-}$ mice expressing or not OTII-TCR.

We propose two mutually nonexclusive possibilities that may explain the ability of the same amount of transgenic ThPOK to completely rescue the CD4 helper development in *Thpok*$^{-/-}$ or OTII$^+$*Thpok*$^{-/-}$ mice but induce an inefficient CD8 to CD4 helper lineage redirection of MHCI-specific thymocytes (in OTI$^+$, P14$^+$,

T cells from OTI$^+$dLGF$^+$ mice expressing or not endogenous ThPOK. (**C**) The contribution of endogenous and transgenic ThPOK in the CD4 helper lineage choice of MHCI-specific thymocytes with augmented TCR signaling was evaluated by assessing the CD4/CD8 phenotype of the mature thymocytes (top) and splenic T cells (bottom) isolated from the indicated mice. (**D**) The frequency and absolute number of TCR$^+$ and CD4$^+$CD8^{lo} subsets in total thymocytes and CD4$^+$ and CD8$^+$ mature thymocytes (CD24$^-$CD69$^-$TCR$^+$) as well as splenic T cells and subsets from the indicated mice are shown ($n > 6$). (**E**) ThPOK-specific staining in DP thymocytes and CD4$^+$ mature thymocytes and splenic T cells from the indicated mice is shown. (**F**) Histograms show phospho-Src (left panels) and phospho-CD3ζ (right panels) expression levels in DP and CD4$^+$CD8^{lo} thymocytes from the indicated mice. (**G**) The compilation of mean fluorescence intensity (MFI) data for pSrc and pCD3ζ for DP and CD4$^+$CD8^{lo} subsets from the indicated mice are shown and are expressed relative to MFI values in WT subsets. (**H**) The expression of *St8sia6*, *St3gal2*, and *Cxxc5* was evaluated by QPCR in mature T cell subsets purified from the spleen of WT (CD4$^+$ and CD8$^+$) and OTI$^+$dLGF$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ (CD4$^+$ and DN) mice. Data depict the average of triplicate values with SD and are expressed as fold increase over the expression of individual genes in control CD4$^+$ mature T cells from WT mice (normalized to *Hprt* expression). Data are representative of two to six independent experiments (A–C, E, F, and H). *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$.

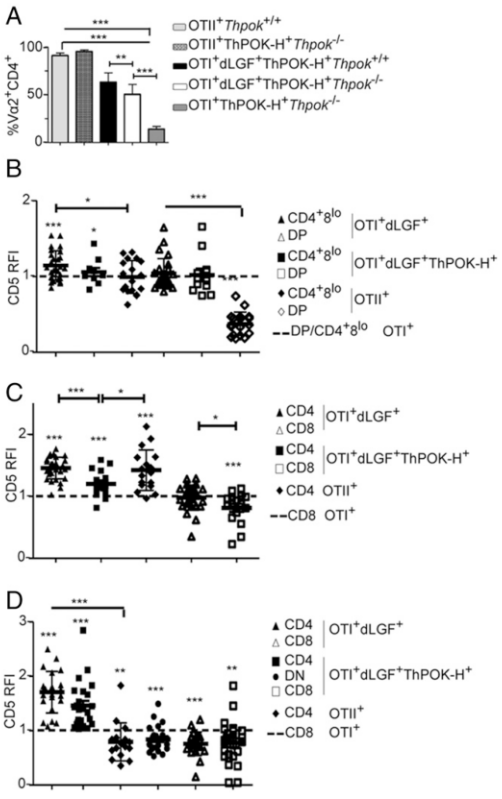

FIGURE 8. Comparison of CD4$^+$ mature T cell frequency and TCR signal strength in MHCI- and MHCII-specific thymic and splenic T cells from control mice and ThPOK-H mice expressing or not dLGF transgene. (**A**) The efficiency of ThPOK-H–mediated CD4 helper lineage choice of thymocytes with differential TCR signaling was evaluated by comparing the CD4$^+$ splenic T cell frequencies in the indicated mice. To assess relative TCR signal strength, CD5 levels in various thymic subsets and mature T cells from OTI$^+$, OTI$^+$dLGF$^+$, OTI$^+$dLGF$^+$ThPOK-H$^+$, and OTII$^+$ mice were compared. (**B**) CD5 levels in DP and CD4$^+$CD8^{lo} thymocytes from the indicated mice were normalized to CD5 levels in the relevant thymic subsets from OTI mice. CD5 levels in the mature CD4$^+$ and CD8$^+$ thymocytes (**C**) and splenocytes (**D**) were normalized to CD5 levels in mature CD8$^+$ thymocytes and splenocytes from OTI mice. Each symbol represents one mouse. *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$.

or MHCII$^{-/-}$ mice). It is possible that genes responsible for activating the helper program in MHCI-specific thymocytes, because of weak or shorter duration of TCR signaling, are epigenetically modified in such a way that they are inaccessible or accessible for a shorter time for ThPOK-mediated regulation, and in such a case, a significantly higher amount of ThPOK (than the one required for CD4 helper lineage choice of MHCII-specific thymocytes) would be required to override this constrain on CD4 helper lineage choice of MHCI-specific thymocytes. A significantly higher CD4$^+$ mature T cell frequency in OTI$^+$dGLF$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice compared with OTI$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice, both expressing the same amount of ThPOK but differing in their TCR signal strength, strongly suggests that TCR signal strength plays a critical role in establishing the ThPOK-mediated CD4 helper lineage choice. We propose that augmented TCR signal strength, although critical for ThPOK induction, promotes the CD4 helper lineage choice by extending the window of lineage choice during which the target gene loci are accessible readily or for a longer time for ThPOK-mediated modulation. Any MHCI-signaled thymocytes expressing ThPOK at levels comparable to that induced in MHCII-signaled thymocytes but remaining outside this temporal lineage commitment window will differentiate into CD8$^+$ mature T cells with a compromised cytotoxic function. Such a possibility is supported by the observation that although ThPOK induction in MHCI-signaled thymocytes, because of a compound deficiency of Runx1 and Runx3 or Tle1/3/4 or MAZR and Runx3, upregulates helper lineage genes including *Cd4*, it fails to completely suppress the expression of cytotoxic lineage genes including *Cd8*, resulting in the generation of a large number of CD4$^+$CD8$^+$ mature T cells of undefined functional potential (29–31). Compromised cytotoxic function, but failure to upregulate CD4 or secrete IL-4 following retroviral-mediated ThPOK expression in the peripheral CD8$^+$ mature T cells, also supports such a notion (47). These data are in agreement with the signal strength model of CD4/CD8 lineage commitment; irrespective of MHC specificity, stronger TCR signaling may alter the chromatin structure such that not only the CD4 helper lineage–specifying genes such as *Gata3*, *Tox*, or *c-Myb* are induced (48–51) but the accessibility of the target gene loci by ThPOK is enhanced as well, leading to the suppression of the CD8 cytotoxic lineage choice and imprinting of the CD4 helper lineage choice in these cells. These data also suggest that stronger TCR signaling may be sufficient for the CD4 helper lineage commitment even in the absence of ThPOK, provided those critical for the CD8 cytotoxic lineage commitment are suppressed (22).

Although strong TCR signaling is critical for the CD4 helper lineage choice, the MHC specificity of developing thymocytes appears to play an equally important role in the process. Comparing the CD4 helper development of MHCI-specific thymocytes in OTI$^+$dLGF$^+$ThPOK-H$^+$Thpok$^{-/-}$ and MHCII-specific thymocytes in OTII$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice provides some insight into this issue. A significantly higher frequency of CD4$^+$ mature T cells in OTII$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice (MHCII-specific) compared with OTI$^+$dLGF$^+$ThPOK-H$^+$*Thpok*$^{-/-}$ mice (MHCI-specific), both expressing the same amount of ThPOK, cannot simply be explained by the TCR signal strength model as thymic subsets, and CD4$^+$ mature T cells from OTII$^+$ mice show a significantly lower TCR signaling compared with that in similar subsets from OTI$^+$dLGF$^+$ mice expressing or not ThPOK transgene. Our data then suggest that TCR signaling in MHCI- and MHCII-specific thymocytes are likely to be different not only quantitatively but qualitatively as well, and introducing constitutively active Lck in MHCI-specific thymocytes mimics the quantitative aspect. We propose that continuous TCR signaling in the positively selected MHCII-specific thymocytes not only results in a stronger TCR signal that keeps the lineage commitment window "open" for a longer time but also induces the expression of CD4 helper lineage–establishing genes whose continued expression likely requires ThPOK.

Based on these data, we propose a model that links TCR signaling to the CD4/CD8 lineage choice of MHCI- and MHCII-signaled thymocytes. We propose that qualitatively distinct and stronger TCR signaling opens the window of lineage commitment during which the CD4 helper lineage–specifying genes are induced in MHCII-signaled thymocytes. It is conceivable that during the CD4 helper lineage specification phase, *Gata3* induced by TCR signaling functions, for instance, as a "pioneer" transcription factor that remodels the chromatin landscape, which then facilitates the ability of other transcription factors to access the target

gene loci in association with or independently of the pioneering factor (52–54). The persistent TCR signaling, along with Gata3 expression, would then initiate ThPOK induction (25, 55) in MHCII-signaled thymocytes, which collectively play a role in the CD4 helper lineage commitment and maintenance. The induction of the helper program in MHCI-signaled thymocytes but the inability to sustain it (56) may be due to inadequate chromatin alterations, leading to the insufficient induction of ThPOK and/or its residency at the target gene loci. In such a case, very high ThPOK expression would be necessary for the efficient redirection of the MHCI-signaled thymocytes into the CD4 helper lineage.

In conclusion, considerably different efficiency of CD4 helper lineage choice in three different mouse models expressing the same amount of ThPOK but different modes of TCR signaling (OTI^{+}ThPOK-H^{+}*Thpok*$^{-/-}$, OTI^{+}dLGF^{+}ThPOK-H^{+}*Thpok*$^{-/-}$, and OTII^{+}ThPOK-H^{+}*Thpok*$^{-/-}$) provides a critical insight into the mechanism of the CD4 helper lineage choice of developing thymocytes. Our data link stronger TCR signaling to ThPOK induction and strongly suggest that the CD4 helper lineage choice by a defined amount of ThPOK is critically influenced by the TCR signal strength and MHC specificity of developing thymocytes during a temporal window of lineage commitment.

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Disclosures

The authors have no financial conflicts of interest.

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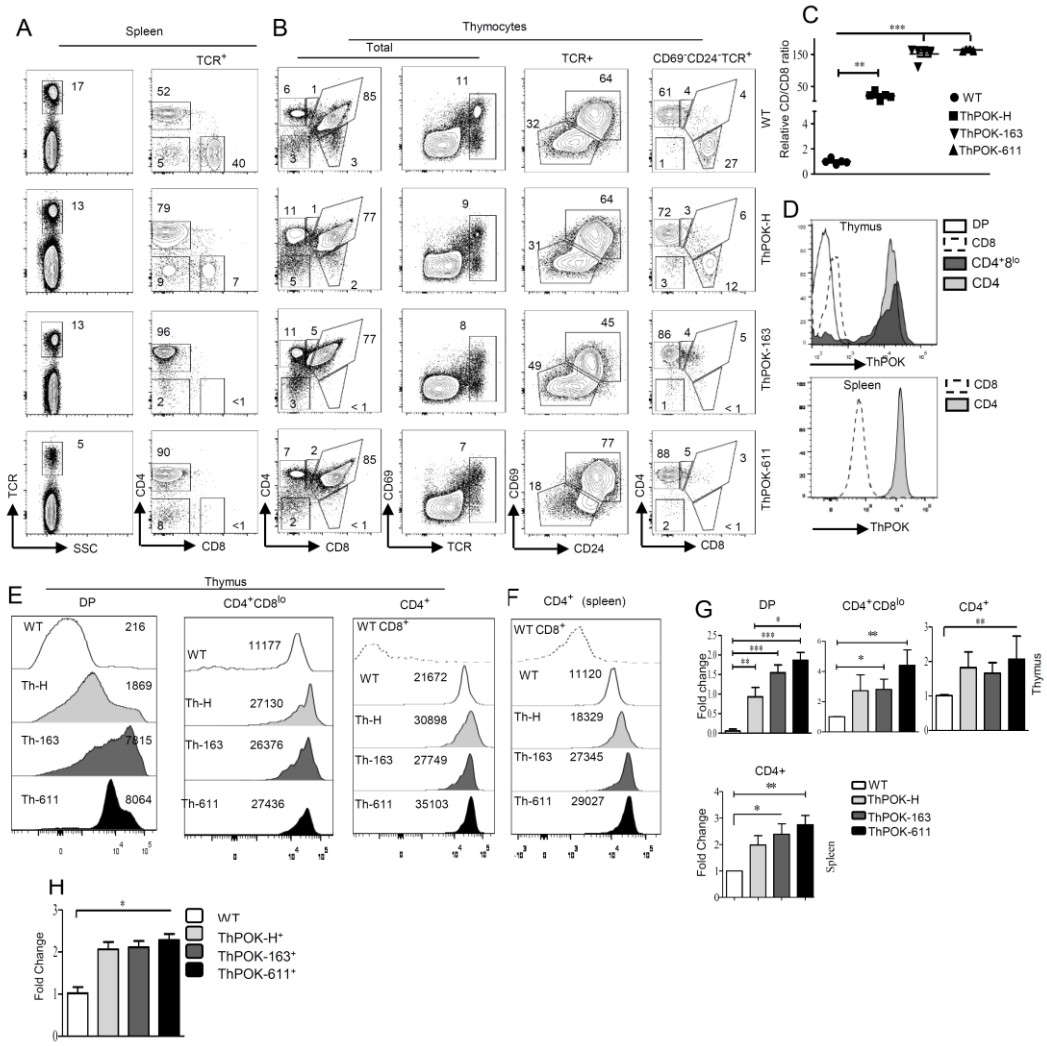

Figure S1: Characterization of ThPOK transgenic mice

Three independent transgenic ThPOK founder lines (ThPOK-H, ThPOK-611, and ThPOK-163) were generated and CD4/CD8 T cell lineage choice was determined by flow cytometry analysis of thymocytes and splenic T cells isolated from the 5 to 7 week old indicated strain of mice. The CD4/CD8 profiles of TCR^+ cells in the spleen (A) and mature ($CD69^-CD24^-TCR^+$) thymocytes (B) are shown. Numbers in each FACS plot represent the frequency of the specific population. (C) shows the CD4/CD8 ratio in the blood of WT and ThPOK transgenic lines. Each symbol represents an individual mouse. (D) Thymocytes from WT mice were stained for CD4, CD8α, TCRβ, CD24 and CD69 followed by intranuclear staining for ThPOK. ThPOK staining histograms for the indicated thymic susbets (top) and the splenic CD4+ and CD8+ T cells (bottom) from WT mice are shown, which concords with ThPOK expression profile in MHCII-signaled cells (ref 27). ThPOK expression in the indicated thymic subsets (E) and $CD4^+$ splenic T cells (F) from WT and the indicated ThPOK transgenic mice was assessed by flow cytometry. $CD4^+$ and $CD8^+$ T cells from WT thymus and spleen serve as controls. Numbers in the histogram represent Mean Fluorescent Intensity (MFI) values. (G) shows comparison of MFI for ThPOK staining for thymic subsets (top) and $CD4^+$ splenic T cells (bottom) from the indicated mice. ThPOK staining in DP thymocytes is relative to that in ThPOK-H mice, while for all other subsets it is relative corresponding WT subset. (H) Transgene copy number was determined by QPCR of genomic DNA and expressed as fold increase over WT mice. Data shown are representative examples of four or more experiments (A, B, D-F) or an average of at least three independent experiments (G, H).

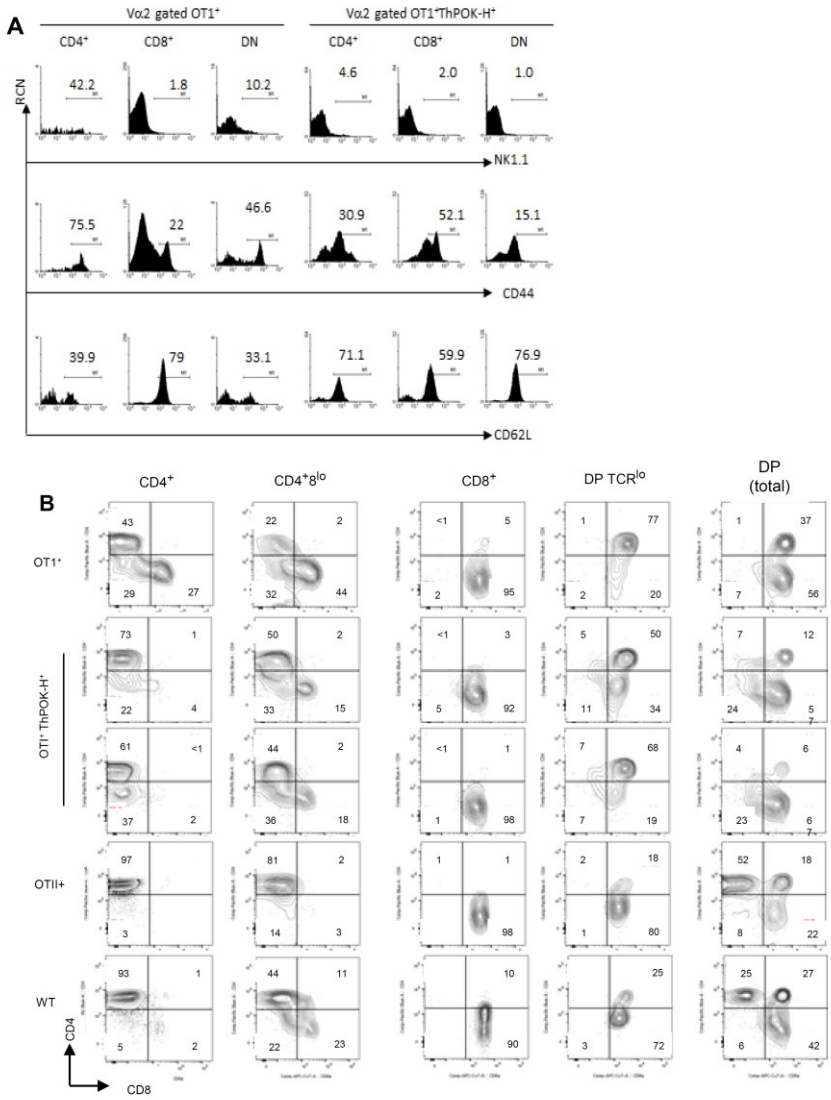

Figure S2: Mature DN T cells in OT1^{+}ThPOK-H^{+} mice are not innate like T cells and transdifferentiate mostly from CD4+ thymocytes.

(**A**) Innate like phenotype of various splenic T cell subsets from OT1 and OT1^{+}ThPOK-H^{+} mice was assessed by evaluating expression of NK1.1, CD44, and CD62L. Single color histograms for NK1.1, CD44, and CD62L for the indicated T cell subsets from the two strains of mice are shown. Data representative of two or more experiments. (**B**) The indicated thymocyte populations were purified from WT, OTII^{+}, OTI^{+} or OTI^{+}ThPOK-H^{+} mice, and cultured in the presence of IL-7 for two days and then analyzed for the CD4 and CD8 surface expression. Data are a representative example of two independent experiments.

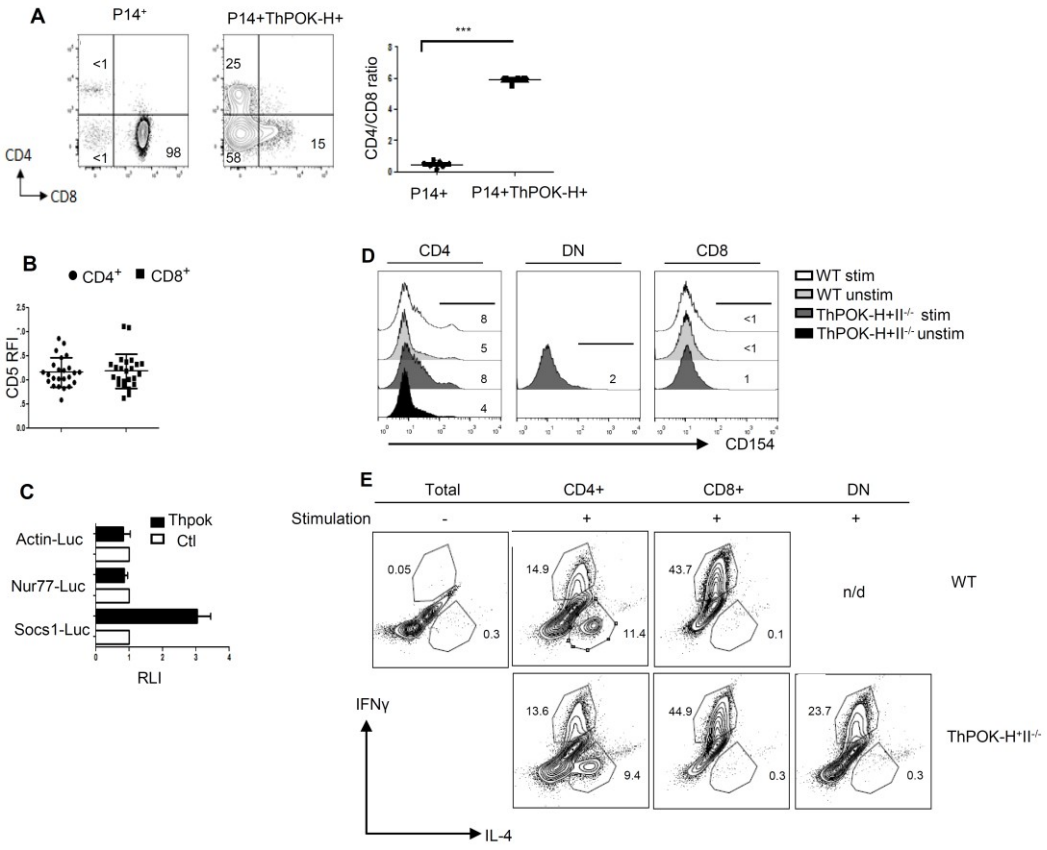

Figure S3: Impact of ThPOK-H on lineage choice in P14 mice, Nur77 reporter and function of mature T cells (A) Flow cytometric analyses of Vα2+ splenic T cells and the CD4/CD8 ratio of splenic T cells for P14^{+}ThPOK-H^{+} and P14 control mice are shown. Panel (B) compares CD5 levels on the CD4^{+} and CD8^{+} mature T cells from OTI^{+}ThPOK-H^{+} mice. Each symbol represents an individual mouse. (C) To evaluate if Nur77 was directly regulated by ThPOK, Nur77 promoter sequence was cloned into luciferase reporter plasmid pGL4.19 (Nur77-Luc). Reporter plasmid was transfected in 293T cells in the presence or absence of ThPOK expressing plasmid and luciferase activity was measured at 48 hours post-transfection. Luciferase expression under the control of Actin (Actin-Luc) and Socs1 (Socs1-Luc) promoters serve as negative and positive controls, respectively. RLI, Relative Luminescence Intensity. Purified mature T cell subsets from WT (CD4^{+} and CD8^{+}) and ThPOK-H^{+}Il$^{-/-}$ (CD4^{+}, CD8^{+} and DN) were stimulated in the presence of irradiated Balb/c splenocytes. Five to seven days later cells were stained for CD154 (D) or restimulated with PMA/Ionomycin and stained for intracellular IFNγ and IL-4 (E). Unstimulated T cells from WT mice served as a control. Data are representative of two or more independent experiments (A, C-E). n/d not determined.