

**COOPERATIVE CONTROL OF PERIPHERAL T HELPER CELL
TOLERANCE BY TGF- β AND PD-L1 SIGNALING IN
HOMEOSTASIS AND CANCER**

by

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DEDICATION

I would like to dedicate this dissertation to my parents, Kuanxian Gao and Chao Sheng, for their encouragement and unconditional support in this journey.

ABSTRACT

T helper (Th) cells dictate host defense responses against immunological challenges; yet, mistargeted Th cell responses can destruct healthy tissues and trigger immunopathology. Understanding the regulatory mechanisms governing Th cell tolerance is vital for developing novel therapeutics for immune-related disorders, such as autoimmune diseases and cancer. In this thesis project, I show that the regulatory cytokine transforming growth factor beta (TGF- β) and the immune coinhibitory ligand programmed death-ligand 1 (PD-L1) cooperatively control Th cell-intrinsic peripheral tolerance independent of regulatory T (Treg) cells. I found that mice with CD4⁺ T cell-specific ablation of TGF- β receptor 2 (TGFB2) succumbed to fatal autoimmunity on a genetic background deleted of PD-L1 or its receptor PD-1. The autoinflammatory response was marked by extensive liver infiltration of two subsets of chronically activated type 1 helper T (Th1) cells: one with follicular helper T (Tfh) cell characteristics and the other featuring a 'hyperactivated' effector phenotype with elevated IFN- γ production. Notably, induction of 'hyperactivated' Th1 cells and the lethal autoimmune phenotype could be recapitulated in a transfer model of TGFB2-deficient CD4⁺ T cells to mice with PD-L1 selectively deleted in macrophages. Additionally, the lethal autoinflammation was dependent on the costimulatory receptor CD40 that promoted expansion of a population of monocyte-derived macrophages with robust Th cell antigen-presenting cell capability. Furthermore, in a poorly immunogenic autochthonous hepatocellular carcinoma (HCC) model, targeting TGF- β signaling in Th cells combined with anti-PD-L1 inhibited tumor growth by enhancing the Th1 effector and monocyte-derived macrophage responses in a CD40-dependent manner, which also enhanced tumor-reactive CD8⁺ cytotoxic T lymphocyte (CTL) responses. Collectively,

these findings reveal that TGF- β and PD-L1 are crucial controllers of Th cell-intrinsic peripheral tolerance by functioning as 'tonic' and 'reactive' regulators, respectively. Dual genetic disruption of these pathways unleashes a positive feedback loop between Th cells and macrophages, causing uncontrolled autoimmunity. Conversely, pharmacological co-inhibition of both pathways rejuvenates protective immunity in cancer.

BIOGRAPHICAL SKETCH

Shengyu Gao, also known as Sherry Gao, was born and raised in Zaozhuang, Shandong Province, P.R. China. She attended The Chinese University of Hong Kong (CUHK) on a full-ride scholarship and graduated with a bachelor's degree in Cell and Molecular Biology in 2018, achieving first-class honors. During her time at CUHK, Shengyu completed her undergraduate capstone project in Prof. Liwen Jiang's laboratory, where she characterized the UFMylation modification of ATG6, a crucial protein in the autophagy pathway. Additionally, she spent a year as an exchange student at the University of California, Irvine, where she contributed to a research project on designing mechanosensitive CAR-T cells under the guidance of Prof. Weian Zhao. In 2018, Shengyu relocated to New York to commence her doctoral studies at the Louis V. Gerstner Jr. Graduate School of Biomedical Sciences at Memorial Sloan-Kettering Cancer Center. Concurrently, she pursued and completed a Master of Computer and Information Technology from the University of Pennsylvania through a virtual curriculum from 2021 to 2023.

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LIST OF ABBREVIATIONS

Ag: Antigen

APC: Antigen-presenting cells

BM: Bone marrow

CDR: Complementarity-determining regions

CTLA-4: Cytotoxic T-lymphocyte antigen 4

CTL: Cytotoxic T lymphocyte

CTV: Cell Trace Violet

HCC: Hepatocellular carcinoma

ICB: Immune checkpoint blockade

Mac_mono: Monocyte-derived macrophages

MHC: Major histocompatibility complex

PD-1: Programmed death-1

PD-L1: Programmed death-ligand 1

scRNA-seq: Single-cell RNA sequencing

TCR: T cell receptor

Tfh: T follicular helper

TGF- β : Transforming growth factor beta

TGFBR2: TGF- β receptor 2

Th: Conventional CD4⁺ T helper cell

Th1: Type 1 helper T cell

TNF: Tumor necrosis factor

Treg: Regulatory T cell

USA: Universal self-antigen

CHAPTER 1 INTRODUCTION AND OVERVIEW

1.1 Overview of T helper cell biology

The immune system has evolved to effectively defend against pathogens while minimizing harmful reactions to healthy tissues and commensal microorganisms. T lymphocytes, a pivotal part of the adaptive immune system, coordinate antigen-specific responses and sustain immunological memory. They are divided into two lineages, $\alpha\beta$ and $\gamma\delta$, based on their T cell receptor (TCR) chains, and their commitment occurs early in development. $\alpha\beta$ T cells recognize antigens presented by major histocompatibility complex (MHC) molecules on antigen-presenting cells (APC). They contain two functional subsets: $CD4^+$ and $CD8^+$ T cells, distinguished by their surface co-receptors. $CD4^+$ helper T cells facilitate B cell antibody production and regulate innate immune cells, such as macrophages. $CD8^+$ cytotoxic T cells are essential for pathogen clearance during bacterial and viral infections, and their differentiation and function require $CD4^+$ T cell assistance. Conventional $CD4^+$ T helper (Th) cells are indispensable in orchestrating a coordinated immune response against acute infections, play complex and crucial roles in the pathogenesis of autoimmune diseases and exhibit a diverse array of functions in chronic infection and cancer.

1.1.1 Molecular insights of T cell signaling

Immunological signals- antigen recognition (signal 1), co-stimulatory ligand engagement (signal 2), and cytokine stimulation (signal 3)- direct Th cell development, homeostasis, activation, and differentiation. These responses are highly coordinated and context-dependent, with T lymphocytes having adapted evolutionarily conserved signaling modules to interpret these environmental cues and orchestrate a diverse range of immune responses.

TCR Signaling

A CD4⁺ T cell surveys the body's tissues for immunogenic peptides using its TCR, a heterodimeric transmembrane receptor that binds short peptide fragments presented by MHC class II molecules on the surface of APCs. Each TCR chain has a constant region and a variable region. The constant region includes a short cytoplasmic tail without signaling capabilities, a transmembrane domain, and a membrane-proximal connecting peptide crucial for associating the TCR with the CD3 signaling complex. The variable region contains three complementarity-determining regions (CDRs), which interact with the sides (CDR1 and CDR2) and peptide-binding pocket (CDR3) of MHC molecules [1]. These loops determine the TCR's ability to bind peptide-MHC (pMHC) ligands, the strength (or affinity) of the TCR interaction, and the orientation of TCR binding to pMHC [2–4].

While the $\alpha\beta$ TCR alone is sufficient for initial pMHC binding, effective T cell signaling requires the formation of the immunological synapse (IS), a specialized membrane structure at the interface between a T cell and an APC [5]. The IS

comprises the TCR/CD3 complex, which triggers downstream signaling events; the coreceptors CD4 or CD8, which stabilize TCR-ligand interactions by binding to the sides of MHC class I and II molecules, respectively; cell adhesion molecules that maintain proximity between T cells and APCs; and co-stimulatory and co-inhibitory molecules that modulate T cell signaling, function, and survival. For a naïve CD4⁺ T cell, binding of pMHCII ligands leads to TCR crosslinking and Lck kinase recruitment to the IS, where it phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytoplasmic tails of CD3 subunits [6]. Phosphorylated ITAMs bind the Zap70 kinase, releasing it from autoinhibition and activating it via Lck phosphorylation [7]. Active Zap70 then propagates TCR signaling by recruiting LAT and Slp76, and activating the Ras/MAPK, PI3K, and calcium/NFAT pathways, which are essential for CD4⁺ T cell proliferation and differentiation [5]. This initial signaling is referred to as "Signal 1," which, along with "Signal 2" derived from CD28 co-stimulatory receptor ligation, is necessary for full T cell activation.

T cell Costimulatory and Coinhibitory Signaling Mechanisms

Optimal T cell activation and proliferation also require a second signal, provided by the colligation of additional cell surface receptors. Among these, CD28 delivers the most potent costimulatory signal upon binding to B7-1 and B7-2 (CD80 and CD86) expressed by APCs. CD28 ligand binding induces phosphorylation of its cytoplasmic tail, recruiting downstream proteins such as PI3K and ITK [5]. Since

these molecules are also recruited to the activated TCR complex, CD28 engagement enhances T cell signaling quantitatively rather than qualitatively.

In addition to CD28, other transmembrane proteins like inducible costimulator (ICOS), 4-1BB, and OX40 also provide costimulatory functions [8]. ICOS, a member of the CD28 superfamily, binds to the B7-H2 ligand and is induced on activated T cells, unlike CD28, which is constitutively expressed on both naive and activated T cells [9,10]. ICOS ligand engagement stimulates PI3K signaling. 4-1BB and OX40, members of the TNF receptor superfamily, transmit costimulatory signals through the TNFR-associated factor (TRAF) family of adapter proteins [11].

Conversely, a group of coinhibitory receptors restricts the expansion and activation of TCR-triggered cells. Cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed death-1 (PD-1) are two well-known coinhibitory receptors. CTLA-4 inhibits T cell responses through both intrinsic and extrinsic mechanisms by regulating the surface availability of its ligands, B7-1 (CD80) and B7-2 (CD86). CTLA-4 has a higher affinity for these ligands than CD28, acting as a competitive inhibitor. Disease in CTLA-4-deficient mice can be mitigated by deleting both B7-1 and B7-2 or modifying CD28 to disrupt its Lck phosphorylation site, suggesting disease arises from unopposed costimulation through CD28 [12,13]. Extrinsically, CTLA-4 is crucial for Treg cell function, as its deficiency impairs Treg suppression leading to autoimmune diseases [14]. While constitutive conditional loss of CTLA-4 on Treg cells results in a lethal autoimmune syndrome, inducible deletion in adults does not cause systemic autoinflammation [15], suggesting a critical role

during the perinatal period in thymic selection and central tolerance. In addition, CTLA-4 can remove B7-1 and B7-2 from APC surfaces through transendocytosis,, thereby decreasing their availability for CD28 and diminishing T cell activation [16,17]. This process allows Treg cells to control CD28-dependent activation/reactivation of T cells in the lymph node as well as in the tumor microenvironment [18–20]. Indeed, anti-CTLA-4 therapy enhances CD28 costimulation at least partly through depleting high CTLA-4-expressing Treg cells through Fc-mediated mechanisms [21]. Intrinsically, CTLA-4 recruits phosphatases like SHP-2 and PP2A upon ligand binding, inhibiting TCR and CD28 signaling, reducing T cell proliferation and IL-2 production [22].

PD-1 signaling is primarily driven by its interaction with its ligands, PD-L1 and PD-L2 [23,24]. When PD-1 binds these ligands, it undergoes phosphorylation at its ITSM and ITIM sites, recruiting phosphatases such SHP-2 [25]. These phosphatases dephosphorylate key signaling molecules downstream of the TCR, as well as components of CD28 signaling, ultimately leading to the inhibition of T cell activation and effector functions [26,27]. The expression of *Pdcd1* transcripts is regulated by epigenetic modifiers and transcription factor binding, varying with cell state. PD-1 is not present on naive CD4⁺ or CD8⁺ T cells but is rapidly upregulated by TCR signaling [28]. PD-1 levels remain high on under chronic antigen stimulation, partly mediated by the transcription factor TOX [29–31]. PD-1 signaling impacts Th cell metabolism by inhibiting the switch to glycolysis and promoting fatty acid oxidation, which can influence T cell survival and function [32]. PD-1 also affects the balance and function Tfh cells and follicular regulatory T (Tfr)

cells [33,34]. Of note, PD-1 has also been reported to limit Treg cells, blockade of the PD-1 pathway at homeostasis, results in enhanced Treg cell activity [35].

Cytokine signaling

Antigen recognition and costimulation are vital for T cell activation, but a third signal mediated by cytokines is crucial for T cell development, differentiation, and function. In conventional Th cells, the cytokine environment encountered by antigen-activated CD4⁺ T cells directs their differentiation into distinct effector lineages.

Cytokines are categorized into families based on their structures and downstream signaling mechanisms. A major class of cytokines, including interleukins, interferons (IFNs), and hematopoietins, utilize the Janus kinase (JAK)-signal transducers and activators of transcription (STAT) pathway [36]. These cytokine receptors lack intrinsic kinase activity and associate with the JAK family of tyrosine kinases. Ligand binding and receptor dimerization activate the associated JAKs. There are four JAK proteins—JAK1, JAK2, JAK3, and Tyk2—which selectively bind different receptor chains. Activated JAKs phosphorylate the receptor cytoplasmic domains, creating docking sites for SH2-containing signaling proteins, primarily the STATs. The STATs family of transcription factors consist of seven members (STAT1-4, 5a, 5b, and 6). Cytokines such as IL-2, IL-4, IL-7, IL-9, and IL-15 activate the JAK-STAT pathway by interacting with a shared receptor subunit, the common γ chain (γ C, CD132) [37]. All interferons signal through the JAK/STAT pathway, with type I IFNs (IFN- α , IFN- β) activating TYK2 and JAK1,

resulting in STAT1-2 heterodimerization, and type II IFN (IFN- γ) activating JAK1 and JAK2 to induce STAT1 homodimerization [38]. The JAK/STAT pathway is negatively regulated by the suppressors of cytokine signaling (SOCS) proteins, which inhibit JAK/STAT signaling by binding to phosphorylated JAKs to suppress their kinase activity or by interacting with cytokine receptors to block STAT recruitment [39].

Tumor necrosis factors (TNFs), such as TNF- α and lymphotoxin (LT), represent another key class of pro-inflammatory cytokines [40]. TNF is primarily produced as a trimeric type II transmembrane protein, with soluble TNF released via proteolytic cleavage. Both TNF and LT bind to two receptors, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2), which differ in structure and expression patterns. The intracellular domains of these receptors lack intrinsic enzyme activity and transduce signals by recruiting distinct signaling modules. TNFR1 contains a cytoplasmic death domain (DD) that recruits the adaptor molecule TNFR1-associated death domain protein (TRADD). TNFR2 lacks the DD sequence and binds TNFR-associated factor 1 (TRAF1) and TRAF2. Both TNFR1-TRADD and TNFR2-TRAF1/TRAF2 signaling pathways lead to NF- κ B activation [41].

TGF- β , a cytokine with pleiotropic functions, is part of a family of regulatory cytokines [42]. Three TGF- β proteins (TGF- β 1-3) are identified in mammals, with TGF- β 1 being the predominant form in the immune system. TGF- β is synthesized in an inactive form, composed of a TGF- β dimer associated with latency-associated protein (LAP), which is degraded by a TGF- β activator [43]. Active TGF- β initiates signaling by binding and bringing together type I and type II receptors

(TGF- β RI and TGF- β RII), which possess serine/threonine kinase activity. TGF- β RII phosphorylates and activates TGF- β RI, propagating the signal through phosphorylation of Smad proteins such as Smad2 and Smad3. Phosphorylated Smad2/3 interact with the co-Smad, Smad4, and translocate to the nucleus to regulate transcription of target genes. Additionally, TGF- β activates Smad-independent signaling pathways, including MAPK and PI3K/Akt signaling.

1.1.2 Conventional CD4⁺ T cell immunity

Effector CD4⁺ Th Cells in Infection

Complete activation of peripheral Th cells results in two key outcomes: clonal expansion and acquisition of effector function. TCR engagement and co-stimulation trigger the proliferation of the Th cell clone that has recognized the antigen, generating multiple daughter cells with the same TCR, capable of responding to the antigen. Given that the estimated clonal frequencies of naïve T cells specific to a given pMHC often fall below 100 cells per million, clonal expansion is vital for efficient antigen detection and clearance [44]. Th cells can differentiate into various lineages upon activation [45]. Each lineage expresses a master transcription factor, upregulates signature cytokines and phenotypic markers, and performs functions tailored to specific immune challenges. Briefly, Th1 cells express the transcription factor T-bet (encoded by *Tbx21*) and mediate the elimination of intracellular pathogens by secreting IFN- γ to activate macrophages and DCs. Th2 cells express Gata-3 and orchestrate protective responses against helminths, recruiting eosinophils, basophils, and other cells. Ror γ t⁺ Th17 cells use IL-17 and other cytokines to recruit and activate neutrophils to defend against extracellular pathogens. T follicular helper (Tfh) cells, express Bcl6, provide B cells with cytokines and costimulatory signals to develop potent antibody-mediated responses against viruses and bacteria. (The function of regulatory T, or Treg, cells is discussed later in this introduction). Th cells coordinate with other immune cell types to eliminate the antigenic threat.

After the antigen is eliminated, a contraction phase ensues, during which most antigen-specific Th cells undergo apoptosis, while a minor subset persists as

memory CD4⁺ T cells [46,47]. If the host encounters the same antigen again, memory CD4⁺ T cells are epigenetically and transcriptionally primed to respond more rapidly, enhancing antigen clearance and providing better protection.

CD4⁺ T Cell Help in Infection

CD4⁺ T cells are indispensable in orchestrating a coordinated immune response against infections, by offering support of other immune cells such as macrophages and CD8⁺ T cells, and promotion of humoral immunity through Tfh cell-mediated B cell help. The complex interplay of various CD4⁺ T cell subsets ensures a comprehensive defense against a wide array of pathogens, contributing to both immediate and long-term immune protection.

Activation of Macrophages: CD4⁺ T cells, particularly Th1 cells, are central to the activation of macrophages through the release of cytokines IL-2, IFN- γ , and TNF- α/β , as well as through the expression of CD40L [48]; this activation enhances the antimicrobial activities of macrophages, including increased phagocytosis, production of reactive nitrogen and oxygen intermediates, and upregulation of MHCII and costimulatory molecules, thereby enabling effective antigen presentation to T cells [49].

Support for CD8⁺ T Cells: CD4⁺ T cells are essential for the optimal function of CD8⁺ T cells; they provide help through cytokines such as IL-2 and IL-21 and through CD40L signaling [50–52], which enhance the expression of CD25 on CD8⁺ T cells and reduce PD-1 expression, thereby supporting the expansion, differentiation, and memory formation of CD8⁺ T cells.

Support for B Cells: Tfh cells are crucial for B cell responses and the generation of high-affinity antibodies [53]; Tfh cells promote the formation and maintenance of germinal centers, support memory B cell development, and are involved in the production of protective antibodies that neutralize pathogens and prevent their replication.

1.2 T helper cell tolerance at homeostasis

Upon pathogenic challenge, the adaptive immune system enables the mammals to amplify innate immune responses, mobilize dedicated effector modules tailored for different pathogen types and form immunological memory. However, it also poses additional challenges. B and T cells use germline-encoded receptors generated via V(D)J recombination to recognize virtually any antigen with high specificity. Yet, the stochastic nature of this mechanism, which confers unlimited pathogen recognition capacity, can also produce lymphocytes that respond to and attack self, commensal microorganisms, and innocuous dietary and environmental antigens. If unchecked, these cells could cause debilitating autoimmune, inflammatory, or allergic disorders. In addition, the powerful effector programs orchestrated by the adaptive immune system can cause collateral damage to host tissues, which the immune system must mitigate to ensure host survival and functionality. Therefore, the immune system has evolved mechanisms to ensure tolerance to self-antigens and harmless foreign substances while enabling effective responses against pathogens, with tightly regulated magnitude and timing to limit overactivity and hyperinflammation.

The T cell tolerance mechanism can be divided into central and periphery tolerance based on the stage of T cell development. Central tolerance occurs in the thymus, where TCR signaling triggers apoptosis of autoreactive thymocytes, preventing their maturation and migration to the periphery [54]. However, thymic deletion of self-reactive T cells is only approximately 60-70% efficient, allowing the peripheral naive T cell repertoire to contain a significant portion of low-avidity, self-

reactive T cells [55–57]. These cells are further curtailed by peripheral tolerance mechanisms, which include T cell-intrinsic processes and T cell-extrinsic processes dependent on other cell subsets, such as regulatory T cells (Treg cells) [58] and dendritic cells [59].

1.2.1 Central tolerance

During T cell development, T cell precursors are subjected to multiple developmental checkpoints to ensure the successful expression of a functional TCR capable of binding ligand and promoting signal transduction [60]. The evaluation of TCR function occurs in the thymus, which is populated by lymphoid progenitors derived from hematopoietic stem cells in the bone marrow [61]. The major stages of thymocyte development can be delineated by the expression of co-receptors, CD4 and CD8, which define the CD4⁻CD8⁻ (double-negative, DN), CD4⁺CD8⁺ (double-positive, DP), and CD4⁺CD8⁻ or CD4⁻CD8⁺ single-positive (SP) subsets. For $\alpha\beta$ T cells, recombination of the *Tcrb* locus occurs first at the DN stage, mediated by the recombination activating genes 1 (Rag1) and 2 (Rag2) [62]. Once a VDJ segment is formed, it is transcribed with the TCR β constant region and translated if in frame. The resulting TCR β chain is then paired with a germline-encoded pre-TCR α chain to form the pre-TCR, triggering the first developmental checkpoint known as β -selection.

To survive, a thymocyte must receive signal through the pre-TCR upon ligation of pMHC molecules expressed by cells within the thymic cortex [63]. Otherwise, the absence of TCR signaling at this stage leads to apoptosis, known as death by neglect [64]. TCR-mediated signals also prompt maturation to the DP

stage, and recombination of the *Tcra* locus, which yields a complete $\alpha\beta$ TCR. The $\alpha\beta$ TCR⁺ DP thymocytes then interact with thymic cortical epithelial cells (cTECs) and bone marrow-derived antigen-presenting cells such as DCs that express a high density of MHC class I and class II molecules associated with self-peptides [63]. The diversely rearranged TCRs recognize self-peptide MHC (self-pMHC) ligands at various intensities and durations, and the signaling mediated by this TCR-self-pMHC interaction dictates the fate of the DP thymocytes. Too little signaling results in delayed apoptosis (death by neglect), while too much signaling can promote elimination of the thymocyte by negative selection. The appropriate, intermediate level of TCR signaling initiates effective maturation into SP thymocytes, a process known as positive selection.

SP cells migrate from the cortex to the medulla, where they continue to sample antigen presented by DCs and medullary TECs (mTECs). Thymocytes that express TCRs binding self-pMHCI complexes become CD8⁺ T cells, whereas those with TCRs contacting self-pMHCII ligands develop into CD4⁺ T cells [48]. Negative selection happens when thymocytes receiving strong TCR signals upregulate pro-apoptotic BH3 effectors, such as Bim, leading to cell death [65,66]. As negative selection acts to remove specific clones that exhibit high extents of self-reactivity, this process is also referred to as clonal deletion.

Another fate for self-pMHCII-reactive thymocytes is differentiation into the Foxp3⁺ CD4⁺ regulatory T (Treg) cell lineage. Treg cells are essential throughout life for limiting inflammation, maintaining tissue homeostasis, and promoting tissue repair [67]. Moderate TCR signaling, in conjunction with CD28 co-stimulation,

induces upregulation of the Treg lineage-defining transcription factor Foxp3 and the high-affinity interleukin-2 (IL-2) receptor, CD25 (IL2R α) [68]. CD25 sensitizes Treg precursors to the survival and proliferative cues imparted by IL-2 signaling.

The thymic medulla is viewed as the key site for imposing central T cell tolerance [61]. mTECs express the transcriptional regulator Aire [69], which enables ectopic expression of tissue-specific antigens (TSA). Aire allows thymocytes to be "trained" on a wider range of self-peptides that would otherwise be absent in the thymus but may be encountered in the periphery. Aire deficiency in thymocytes during the neonatal period results in multiorgan autoimmunity, as seen in both mice and humans [70]. Lymphocytic infiltration of specific tissues in *Aire*^{-/-} individuals is associated with defective negative selection of CD4⁺ and CD8⁺ T cells exhibiting high reactivity to TSAs, as well as poor TSA-specific tTreg cell differentiation [71–73]. Taken together, central tolerance mechanisms limit thymic egress of self-reactive CD4⁺ T cell clones through negative selection or induction of the tTreg cell fate.

1.2.2 peripheral tolerance

Central tolerance mechanisms in the thymus are crucial for immune homeostasis, yet numerous studies have shown that self-reactive conventional CD4⁺ Th and CD8⁺ T cells exist in the periphery of healthy mice [72,74–76] and humans [77], indicating that while negative selection and tTreg differentiation minimize the development of self-reactive clones, they do not entirely prevent it. Given the potential risk posed by these clones for autoimmunity, peripheral tolerance mechanisms must compensate for imperfect central tolerance to avert

disease. Peripheral tolerance mechanisms employ several strategies to limit self-reactive Th cell responses, broadly categorized into passive and active mechanisms.

Passive tolerance mechanisms

Passive tolerance mechanisms include ignorance to antigen, peripheral deletion, and induction of anergy. Ignorance involves preventing self-reactive Th cells from encountering their cognate antigens due to physical barriers, such as the blood-brain barrier, or inefficient antigen processing or presentation [78]. However, these "ignorant" self-reactive Th cells are not functionally impaired and may become activated if pMHCII availability increases.

Peripheral deletion and anergy can occur when T cells are primed without proper co-stimulatory signaling on APCs upon antigen encounter. Costimulation through CD28, typically provided by mature DCs that have taken up exogenous antigens and received additional pro-inflammatory signals, is necessary for T cell survival by promoting the expression of anti-apoptotic Bcl-2 family proteins, such as Bcl-XL, to counterbalance pro-apoptotic signals upregulated downstream of TCR signaling [79]. In some cases, self-reactive Th cells experiencing TCR stimulation without costimulation enter a state of functional hyporesponsiveness known as T cell anergy. Evidence for T cell anergy comes from allograft transplantation studies [80,81], where the administration of an anti-CD3 antibody mimicked the provision of Signal 1 without Signal 2, leading to the depletion of most recipient CD4⁺ T cells, and impaired proliferation and cytokine production in

the remaining cells upon *ex vivo* re-stimulation. These observations were further supported by studies assessing TCR-transgenic CD4⁺ T cells reactive to defined foreign antigens after intravenous transfer of their cognate antigen [82]. Surviving antigen-experienced Th cells displayed reduced upregulation of activation markers, IL-2 secretion, and expansion compared to naïve cells. Functional defects in anergic Th cells are generally due to impairments in signaling pathways involving Ras, MAPK, and AP-1, following TCR-dependent upregulation of molecular mediators of T cell dysfunction[83] including transcription factors Egr2 and Egr3, E3 ubiquitin ligases Cbl and GRIL, and diacylglycerol (DAG) kinases responsible for breaking down the secondary messenger DAG. Notably, T cell anergy can be reversed upon antigen removal, costimulation through CD28 and/or OX-40, or IL-2 provision.

The 'T cell-intrinsic/ T cell-extrinsic' framework of active suppression mechanisms

Active suppression mechanisms act as a failsafe to limit instances where self-reactive Th cells receive both TCR stimulation and costimulation. Among these are the Th cell-extrinsic suppressions from Treg cells and toleragenic APCs, as well as Th cell-intrinsic suppressive programs mediated by immune inhibitory receptors, including those require extrinsic ligands.

Suppression *in trans* by Treg cells is a cornerstone of peripheral tolerance. Genetic inactivation of Foxp3 in mice (scurfy) and humans (IPEX) causes fatal lymphoproliferative and multiorgan autoimmune disease [84]. Similar diseases occur if Treg cells are selectively ablated or functionally impaired, leading to robust

lymphocytic infiltration and increased mortality, as observed in Foxp3DTR mice, which express the human diphtheria toxin (DT) receptor in Foxp3-expressing cells and experience Treg cell loss upon DT administration [85]. Studies have elucidated Treg suppressive modalities, including IL-2 depletion via CD25, disruption of CD28-B7 interactions via CTLA-4, and production of soluble anti-inflammatory mediators like IL-10, through Treg-specific deletion of the high-affinity IL-2 receptor [86], the coinhibitory receptor CTLA-4 [87], and the IL-10 receptor [88], respectively. Apart from tTreg cells originating in the thymus, another subset of Treg cells, known as peripherally induced Treg (pTreg) cells, arises from mature CD4⁺ T cells. pTreg cells can be generated from Foxp3⁻ CD4⁺ T cells that are exposed to factors, including TGF- β and IL-2, in peripheral tissues [89,90]. These pTreg cells accumulate mostly at barrier sites (such as the intestines) where they respond to innocuous antigens derived from food or commensal microbes, metabolites, and environmental factors [91].

Both CTLA-4 and PD-1 are negative regulators expressed on Th cells, acting as Th-cell intrinsic tolerance regulators whose function depends on extrinsic cues. Additionally, Cbl-b, an E3 ubiquitin ligase that marks signaling proteins for degradation, thereby dampening TCR signaling pathways and preventing inappropriate activation of T cells, also serves as a Th-cell intrinsic tolerance regulator [92]. Many other ubiquitin-ligases also fall into this category.

The regulatory cytokine TGF- β promotes Th cell tolerance from multiple perspectives [93–95]. It is debatable whether it plays critical role as a Th-cell intrinsic tolerance regulator, or mainly act via Treg cell-mediated immune

suppression. In a CD4⁺ T cell TCR-transgenic model of autoimmune diabetes, deletion of TGF- β receptor 2 (TGFB2) during thymic selection results in severe disease progression with higher penetrance and faster kinetics compared to mice lacking Treg cells [96]. These findings, along with earlier studies on mixed bone marrow chimera mice with a polyclonal TCR background [94], indicate that TGF- β predominantly acts as a Th cell-intrinsic tolerance mechanism [97]. However, constitutive or inducible deletion of TGFB2 in mature CD4⁺ T cells surprisingly did not induce spontaneous autoimmunity but led to immunopathology upon transfer to a lymphopenic host [98,99]. These observations suggest that Th cell-intrinsic mechanisms of peripheral tolerance control may have limited importance, potentially due to the prominent functions of Treg cell-mediated immune suppression. Yet, it is possible that the absence of an overt autoimmune phenotype in mice with blockade of TGF- β signaling in mature CD4⁺ T cells is due to induction compensatory tolerance mechanisms to repress self-reactive T cells [100], which still supports an active and critical role of TGF- β mediated Th-cell intrinsic tolerance mechanisms in the periphery.

The ‘tonic and reactive regulation’ framework of active suppression mechanisms

Apart from Th cell-extrinsic and -intrinsic regulatory programs, another angle to look at periphery Th cell tolerance is from two primary regulatory strategies: tonic regulation and reactive regulation [101,102].

Tonic regulators establish a threshold that a stimulus must surpass to elicit an immune response. These regulators act continuously to prevent unnecessary

activation. For instance, TGF- β is present in many tissues in an active form and raises the activation threshold of T cells to prevent weak self-stimuli from triggering a response [103]. This mechanism acts as a high-pass filter, eliminating 'noise' from weak inputs. In the case of TGF- β , it prevents responses to prevalent self-ligands involved in positive selection, which are known to generate tonic signals through the TCRs of naive cells [104], while not substantially limiting responses to more avid (foreign) ligands. Tonic regulation is akin to setting a baseline level of suppression to avoid unwarranted activation of immune cells, thereby maintaining homeostasis and reducing the risk of inflammatory responses due to minor, non-threatening stimuli.

Reactive regulators, on the other hand, respond to the emerging immune activity itself. These regulators are engaged once an immune response has begun, usually in a negative feedback manner, aiming to limit its intensity or duration. Treg cells, for example, increase their suppressive capacity in response to IL-2 produced by activated T cells, thus limiting the response after it has initiated [104,105]. CTLA-4 and PD-1 also function in a reactive manner to interfere with productive signaling from the TCR at the immunological synapse. The strength of TCR signaling controls the relocation of CTLA-4 to the cell surface, linking robust TCR signals to greater suppression [106]. Similarly, PD-1 expression is highest on the most antigen-reactive T cells, and its inhibition in chronically infected animals allows the re-emergence of active effector cells [107].

The model of tonic and reactive regulators underscores the immune system's sophisticated balance between activation and suppression. By employing

both preventive and responsive strategies, the immune system can effectively manage pathogens while minimizing self-damage, illustrating a finely tuned evolutionary adaptation for maintaining homeostasis.

1.3 T helper cells in chronic disease

Th cells play a pivotal role in the immune system, orchestrating responses against pathogens and maintaining immune homeostasis. In the context of chronic diseases such as autoimmunity, cancer, and chronic infections, Th cells exhibit complex and diverse functions. In autoimmune diseases, dysregulated Th cell responses contribute to the production of autoreactive B cells and CD8⁺ T cells, leading to tissue damage and disease progression. In cancer, Th cells, particularly Th1 and Th17 subsets, can both support antitumor immunity and promote tumor progression through inflammatory pathways. In chronic infections, Th cells help sustain immune responses over prolonged periods, although persistent antigen exposure can lead to T cell exhaustion, diminishing their efficacy. Understanding the multifaceted roles of Th cells in these chronic disease states is crucial for developing targeted immunotherapies that can modulate their activity to restore immune balance and achieve therapeutic outcomes.

1.3.1 Th cell response in autoimmunity

A healthy immune system maintains a delicate balance crucial for host homeostasis by protecting against infections while preventing self-reactivity. Disruption of this balance leads to autoimmune diseases, of which more than 80 types have been identified, affecting approximately 5-8% of the global population [108]. Autoimmune diseases can be systemic, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), or organ-specific, such as multiple sclerosis (MS) and Type 1 Diabetes (T1D). Despite the complexity and incomplete understanding of the underlying mechanisms, the role of autoreactive

Th cells in driving the immunopathogenesis of these diseases is well-established. Here we focus on Th1 and Tfh cells.

Th1 cells are critically involved in the pathogenesis of various autoimmune diseases. In experimental autoimmune encephalomyelitis (EAE), the most commonly used experimental model for MS, Th1 cells are the predominant CD4⁺ T cell subset in the central nervous system, with significant IFN- γ production observed in MS patients [109]. The transfer of Th1 cells alone is sufficient to induce EAE symptoms in mouse models [110]. Th1 cells contribute to neuropathology in the central nervous system (CNS) through interactions with microglia, the resident macrophages of the CNS, by promoting pro-inflammatory M1-like microglia differentiation [111].

Tfh cells are closely linked to various autoimmune diseases, contributing to both autoantibody-dependent and -independent conditions. Increased frequencies of circulating Tfh cells are observed in autoimmune disorders such as MS, RA, SLE, MG, Sjögren's syndrome, psoriasis, AD, autoimmune thyroid and hepatitis disease, IBD, and T1D [48,112]. The pathogenic activity of Tfh cells largely depends on IL-21, which promotes autoimmunity by aiding B cells and driving CD8⁺ T cell effector functions [113]. IL-21 signaling drives GC responses, B cell activation, plasma cell differentiation, and antibody class switching. IL-21R expression in CD8⁺ T cells induces pathogenic responses, crucial in T1D where CD8⁺ T cells mediate β cell destruction [114].

In conclusion, CD4⁺ T cells play complex and crucial roles in the pathogenesis of autoimmune diseases. Their ability to modulate both pro-

inflammatory and regulatory pathways underscores the importance of maintaining a balance between immune activation and tolerance to prevent autoimmunity.

1.3.2 Th cell response in chronic infection and cancer

In contrast to acute infections, antigen stimulation is persistent in chronic infection and cancer. It is now well-accepted that most T cells in such circumstances adopt a unique differentiation trajectory—exhaustion. Exhausted T (Tex) cells have been identified in many high-grade chronic viral infections, such as HIV, HBV, HCV, and LCMV-Clone 13 strain, and in almost every mouse and human cancer [115]. The discovery of stem-like progenitor CD8⁺ Tex (Tpex) cells, the main responders to immune checkpoint blockade (ICB), attracts significant attention in both preclinical and clinical research fields for developing next-generation cancer immunotherapies [116,117].

Robust and functional CD4⁺ T cell responses are essential for effective pathogen clearance and tumor eradication. In cancer and chronic viral infection, CD8⁺ T cells undergo a progressive loss of cytokine production and cytotoxicity in a dysfunctional state of T cell ‘exhaustion’. Compared to the well-defined CD8⁺ exhausted T (Tex) cell differentiation, the cellular and functional signatures of CD4⁺ T cells in chronic disease settings are less characterized, particularly due to the complexity of multiple Th subsets. CD4⁺ T cells play multifaceted roles in chronic infection and tumors by constituting both favorable and deleterious subsets, enhancing CD8⁺ T cell function, and responding to immune checkpoint blockade [118,119].

The effects of persistent antigenic stimulation on CD4⁺ T cell phenotype, differentiation, and function remain less understood. Whether CD4⁺ T cells become "exhausted" during chronic infection has been a longstanding question. Controversial results were obtained as viral-specific CD4⁺ T cells lose effector function and produce reduced IFN- γ , TNF- α , and IL-2 during chronic infection [120,121], but the production of IL-10 and IL-21, the important cytokines in chronic infection for sustaining CD8⁺ T cell and B cell responses, are increased [122–124]. Transcriptional analysis of CD4⁺ T cells during chronic (LCMV-Clone 13) infection has demonstrated a unique exhaustion-associated molecular and transcriptional profile, which is distinct from CD8⁺ Tex cells and effector or memory CD4⁺ T cells in acute (LCMV-Armstrong) infection [125]. In addition to reduced cytokine production, CD4⁺ Tex cells express markedly upregulated inhibitory receptors including PD-1, CTLA-4, CD200, and BTLA, and costimulatory receptors OX40, CD27, and ICOS. Core transcription factors involved in CD4⁺ Tex cells include Eomes, Blimp-1, Helios, Klf4, and T-bet [125]. Different from TCF-1⁺ CD8⁺ progenitor Tex (Tpex) cells, TCF-1 expression in chronic virus-specific CD4⁺ T cells does not adequately define stem-like progenitor CD4⁺ T cells, rather marks and promotes Tfh cell development [119]. Recently, Xia et al. identified a population of memory-like TCF-1⁺Bcl-6^{lo/-} virus-specific CD4⁺ T cells that emerged as the progenitor cells that give rise to effector and Tfh cells, sustaining CD4⁺ T cell response in chronic infection. Importantly, such CD4⁺ progenitor cells play pivotal roles in antitumor response preferentially at the site of TdLNs [126]. Hence, CD4⁺ T cells display an exhausted yet functional phenotype in chronic infection.

Th cells are crucial for supporting effective CD8⁺ T cell responses in antitumor immunity. They aid CTLs in priming, clonal expansion, effector function, memory formation, and responsiveness to cancer immunotherapies. CD4⁺ T cells enhance DC activation and antigen-presenting capability via CD40/CD40L signaling, which is essential for fully priming the CTL response [127,128]. During CTL priming, CD4⁺ T cell help triggers the upregulation of CD80/CD86 and CD70 on cDC1s, which interact with CD28 and CD27 on CD8⁺ T cells, respectively. Other TNFR family members, such as 4-1BB, OX40, CD30, and GITR, also play important roles in mediating CD4⁺ T cell help [11,129,130]. Tfh cells expressing CXCL13 attract CXCR5⁺ CD8⁺ T cell migration in chronic infection and cancer [131]. CD4⁺ T cells also enhance CTL migratory capacity into tumor tissues by upregulating CXCR4 and CX3CR1 expression and promoting CTL extravasation at tumor sites [132].

In conclusion, Th cells exhibit a diverse array of functions in chronic infection and cancer, ranging from direct cytotoxicity to providing critical help to other immune cells. Their ability to modulate both innate and adaptive immune responses makes them indispensable in the control of persistent infections and in the orchestration of anti-tumor immunity. Understanding the complex roles and regulatory mechanisms of CD4⁺ T cells in these settings can inform the development of novel immunotherapeutic strategies aimed at harnessing their full potential to combat chronic infections and malignancies.

CHAPTER 2 DECODING PERIPHERAL T HELPER CELL-INTRINSIC TOLERANCE CONTROL BY TGF-B AND PD-L1 AT HOMEOSTASIS

2.1 Introduction

CD4⁺ T helper (Th) cells, utilizing T cell antigen receptors (TCRs) to recognize major histocompatibility complex II (MHCII)-bound peptides presented by antigen-presenting cells (APCs), play pivotal roles in orchestrating cellular and humoral immune responses [133]. Impaired Th cell immunity, as seen in individuals infected with human immunodeficiency virus (HIV), increases susceptibility to infections and certain malignancies [134]. Conversely, the breakdown of Th cell tolerance is a significant contributor to immunopathology, as evidenced by the association between MHCII haplotypes and autoimmune diseases [135,136]. Therefore, deciphering the molecular and cellular mechanisms underlying Th cell regulation is crucial not only for comprehending the fundamental principles of immune system function but also for guiding the development of therapeutics to rectify immunological disorders.

Deletion of self-reactive T cells during thymic development constitutes a central tolerance mechanism [137]; yet, some autoreactive CD4⁺ T cells can escape from thymic negative selection and require subsequent restraint in peripheral tissues [138–140]. Over the past three decades, studies have highlighted the critical role of immune suppression in maintaining Th cell self-tolerance in the periphery. Regulatory T (Treg) cells, characterized by the expression of the transcription factor FOXP3, represent a distinct CD4⁺ T cell lineage [67,141], operating as a Th cell-extrinsic peripheral tolerance mechanism

to prevent lethal autoimmunity throughout the lifespan of mice [85]. Additionally, several Th-cell intrinsic tolerogenic strategies have been postulated to act in preventing autoreactive Th cells from being activated and causing immunopathology in the periphery [78]. Among them, how Th cells are regulated by various intrinsic immune suppressive pathways remains poorly understood.

The regulatory cytokine transforming growth factor- β (TGF- β) promotes Th cell tolerance from multiple perspectives [93–95]. During T cell development, it controls Treg cell selection and differentiation [142,143], and induction of interleukin-7 (IL-7) receptor on T cells which later regulates naïve CD4⁺ T cell homeostasis in the periphery [144,145]. In a CD4⁺ T cell TCR-transgenic model of autoimmune diabetes, deletion of TGF- β receptor 2 (TGFB2) during thymic selection results in severe disease progression with higher penetrance and faster kinetics compared to mice lacking Treg cells [96]. These findings, along with earlier studies on mixed bone marrow chimera mice with a polyclonal TCR background [94], indicate that TGF- β predominantly acts as a Th cell-intrinsic tolerance mechanism [97]. However, constitutive or inducible deletion of TGFB2 in mature CD4⁺ T cells surprisingly did not induce spontaneous autoimmunity but led to immunopathology upon transfer to a lymphopenic host [98,99]. These observations suggest that Th cell-intrinsic mechanisms of peripheral tolerance control may have limited importance, potentially due to the prominent functions of Treg cell-mediated immune suppression. Yet, it is possible that the absence of an overt autoimmune phenotype in mice with blockade of TGF- β signaling in mature CD4⁺ T cells is due to induction compensatory tolerance mechanisms to repress

self-reactive T cells [100], which still supports an active and critical role of TGF- β mediated Th-cell intrinsic tolerance mechanisms in the periphery.

The balance between costimulatory and coinhibitory pathways is critical for regulating T cell activation and maintaining immune homeostasis. Programmed cell death protein 1 (PD-1), and its ligands programmed cell death-ligand 1 (PD-L1) and PD-L2 represents an important coinhibitory pathway in the B7:CD28 family [8]. PD-1 is not expressed on naive T cells, but rapidly upregulated in a TCR-dependent manner. TCR signaling is the primary driver of *Pdcd1* expression [28], accompanied by demethylation of the *Pdcd1* locus [146] and binding of the transcription factors NFATc1 and AP-1 [147]. PD-1 engagement with its ligands opposes TCR and CD28 signaling, resulting in inhibition of T cell proliferation, differentiation, and effector functions including IFN- γ , TNF- α , and IL-2 production [26,27]. Loss of PD-1, through genetic deletion or antibody blockade, leads to enhanced T cell activation and effector function [148,149]. In models of acute infection, *Pdcd1*^{-/-} mice exhibit a comparable or enhanced expansion of effector T cells in the early phase but decreased levels of memory T cells after antigen clearance [150–152]. Similarly, in mouse models of autoimmune disease, loss of PD-1 signaling, accelerates disease onset with increased numbers and function of pathogenic self-reactive T cells and decrease in memory cells [153]. However, the fundamental role of PD-1 in homeostatic Th cell tolerance, in particular its interactions with other tolerance mediators have not been well defined.

In this project, we uncovered a critical Th cell-intrinsic peripheral tolerance mechanism by cooperative TGF- β and PD-L1 signaling which inhibited type 1

cellular immunity driven by CD40-expressing myeloid APCs. TGF- β acted as a 'tonic' regulator by preventing priming and restimulation of self-reactive Th cells, while PD-L1 on macrophages functioned as a 'reactive' regulator to restrain further differentiation of pathogenic Th1 cells. The intricate regulation of Th cells mediated by the synergistic actions of TGF- β and PD-1 signaling, operating through sequential priming and reactivation of CD4⁺ T cells in secondary lymphoid organs and peripheral tissues, holds promise for the development of therapeutics aimed at modulating Th cell activity. Such advancements could potentially mitigate autoimmune disease pathology and enhance protective immunity in the contexts of chronic infections and cancer.

2.2 Results

2.2.1 Deficiency of TGF- β RII and PD-1 in peripheral CD4⁺ T cells leads to lethal autoimmunity.

To explore whether the absence of TGF- β signaling in mature CD4⁺ T cells induces compensatory mechanisms to repress self-reactive T cells, we generated mice with selective deletion of TGFBR2 in post-selection CD4⁺ T cells by crossing a floxed allele of the *Tgfb2* gene (*Tgfb2^{fl}*) [145] with *Thpok^{Cre}* mice [154]. Cre recombinase is expressed under the control of a *Cd4* enhancer-promoter locus associated with a *Thpok* silencer element. The *Thpok* silencer prevents *Cd4* enhancer-promoter activity in immature thymocytes and cells of the CD8 lineage, which results in Cre expression exclusively in mature CD4⁺ SP thymocytes committed to the helper T cell lineage [154]. As anticipated, *Thpok^{Cre}Tgfb2^{fl/fl}* mice appeared healthy without signs of autoimmunity [155]. However, they exhibited increased frequencies of conventional CD4⁺ T cells with an activated CD44^{hi} phenotype in secondary lymphoid organs and peripheral tissues such as the liver and the lung (Figure 2-1). Treg cells were not affected by TGFBR2 deletion and remained CD44^{hi} (Figure 2-1). Interestingly, PD-1 expression was higher in TGFBR2-deficient conventional CD4⁺CD44^{hi} Th cells than their wild-type (WT) counterparts, while similar frequencies of Treg cells constitutively expressed PD-1. Therefore, CD4⁺ conventional Th cells and Treg cells were differentially regulated by TGF- β signaling and showed different patterns of PD-1 expression upon TGFBR2 deletion, with Th cells exhibiting increased activation and PD-1 upregulation.

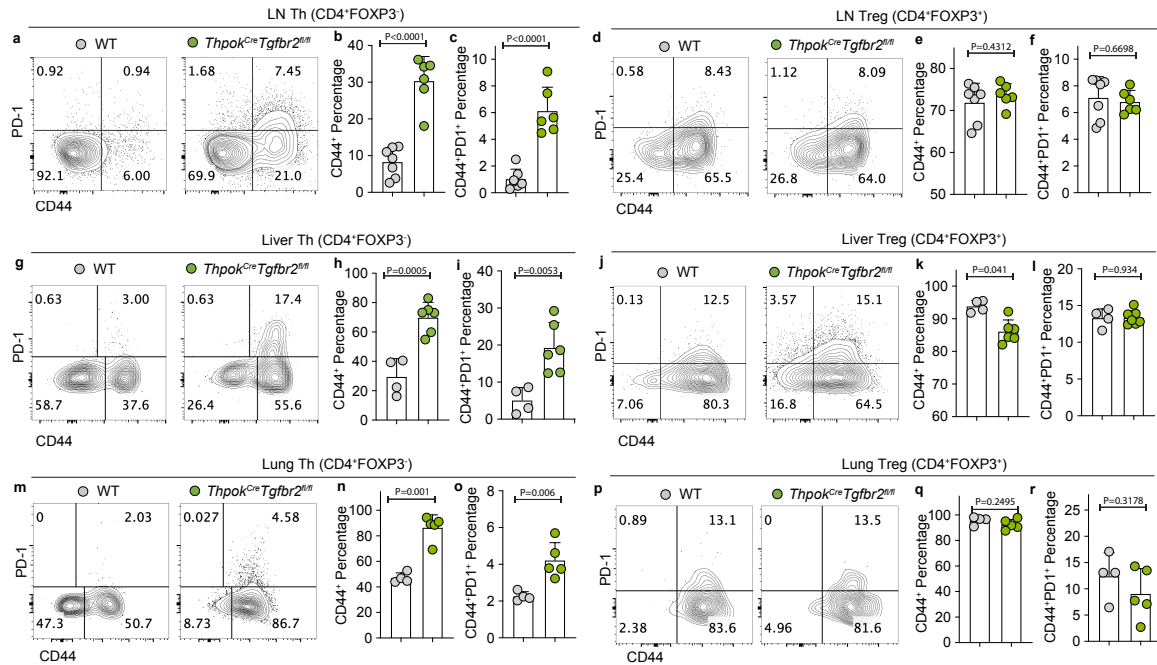


Figure 2-1 Increased activation and PD-1 upregulation in conventional CD4⁺ T cells but not Treg cells upon TGFR2 deletion.

Representative flow cytometry plots and quantification of CD44 and PD-1 expression in CD4⁺Foxp3⁻ T helper (Th) cells and CD4⁺Foxp3⁺ regulatory T (Treg) cells in peripheral lymph nodes (LN), liver, and lung from 3 to 4-week-old wild-type (WT) and *Thpok^{Cre}Tgfr2^{fl/fl}* mice.

Data are presented as the mean values $\pm$ s.d. with two-sided unpaired Student's t-test. Results represent at least three independent experiments.

We then crossed $\text{Thpok}^{\text{Cre}}\text{Tgfb}\beta^{\text{fl/fl}}$ mice onto a PD-1-deficient background [156] to further interrogate the role of PD-1 in repressing activated Th cells. Deletion of PD-1, in addition to the dampened TGF- β signaling in CD4^+ T cells, resulted in a fulminant neonatal lethal phenotype of $\text{Thpok}^{\text{Cre}}\text{Tgfb}\beta^{\text{fl/fl}}\text{Pdc}\delta^{\text{-/-}}$ mice (Figure 2-2, a), similar in severity to that observed in mice lacking Treg cells [157], while each of the single deletion controls survive normally. Pathology analysis showed increased leukocyte infiltration in multiple vital organs including the liver and the lung (Figure 2-2, b). Of note, $\text{Thpok}^{\text{Cre}}\text{Tgfb}\beta^{\text{fl/fl}}\text{Pdc}\delta^{\text{-/-}}$ mice had pronounced expansion of Th cells in peripheral tissues, with around 3.4-, 30.7- and 28.2-fold increase of Th cells in the lymph nodes, liver, and lung, respectively, compared to WT controls. Treg cells were also expanded, with around 7.2-, 12.2- and 5.0-fold in each of these compartments (Figure 2-3, a-b). Therefore, Th cell, but not Treg cell expansion was more prominent in the peripheral tissues compared to secondary lymphoid organs. This was further supported by the evidence that Treg cell percentage increased among total CD4^+ T cells in the lymph node, while decreased in both the liver and lung (Figure 2-3, c). Notably, blocking TGF- β signaling in CD4^+ T cells alone also enhanced proliferation of both conventional Th and Treg cells, but to a less extent. PD-1 deficiency alone did not affect Th or Treg cell number. Although the total CD45^+ cell number also increased in the lymph nodes, liver, and lung in $\text{Thpok}^{\text{Cre}}\text{Tgfb}\beta^{\text{fl/fl}}\text{Pdc}\delta^{\text{-/-}}$ mice (Figure 2-3, d), the fold change was less dramatic than the conventional Th cells. Taken together, blocking TGF- β signaling in CD4^+ T cells synergized with PD-1 deficiency,

causing lethal autoinflammation and skewed expansion of conventional Th cells in peripheral tissues.

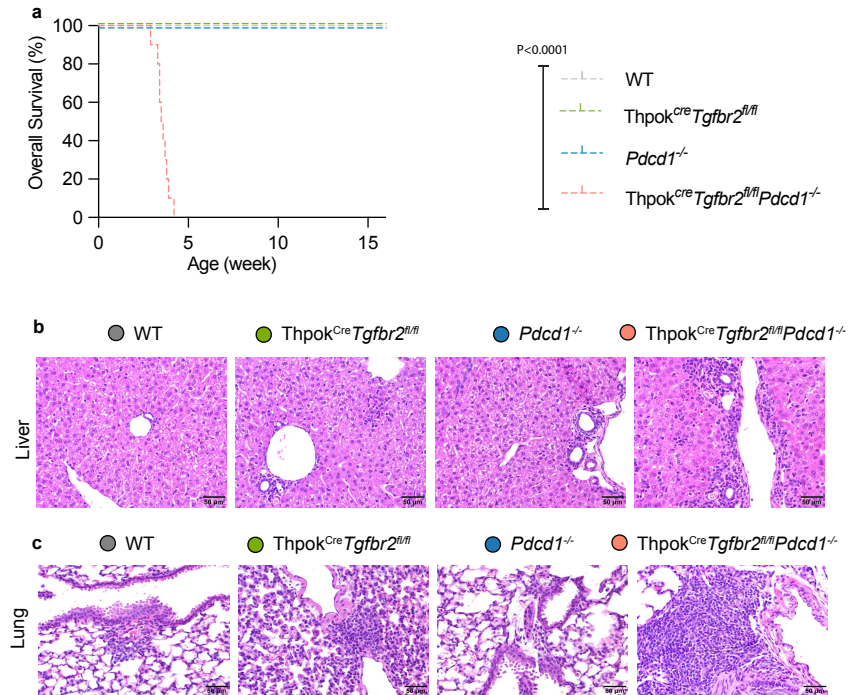


Figure 2-2 Deletion of TGF-βRII in peripheral CD4⁺ T cells on PD-1 deficient background resulted in lethal autoinflammation.

a: Kaplan-Meier survival analysis of mice of wild-type (WT), Thpok^{Cre}Tgfr2^{fl/fl}, Pdc1^{-/-} and Thpok^{Cre}Tgfr2^{fl/fl}Pdc1^{-/-} genotypes. n=10 mice for each group. Log-rank test compares all four groups.

b: Representative hematoxylin and eosin (H&E) sections of liver and lung from mice of indicated genotypes. The scale bar is 50 μm.

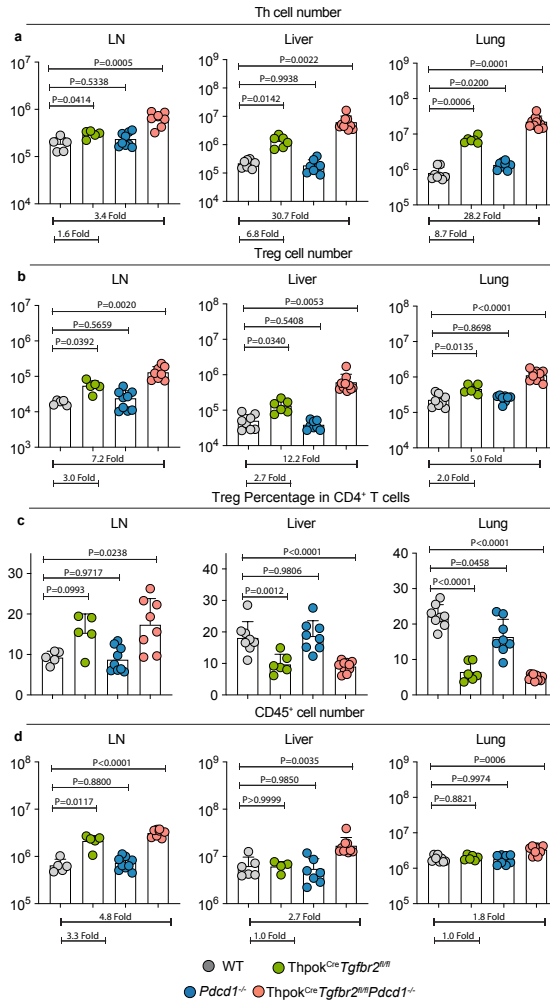


Figure 2-3 Skewed expansion of conventional CD4⁺ T cells but not Treg cells in the tissue in Thpok^{Cre}Tgfbf2^{fl/fl}Pdc1^{-/-} mice.

a, b, d: Quantification of Th (**a**), Treg (**b**) and total CD45⁺ (**d**) cell number in LN (per lymph node), liver (per gram of tissue), and lung (whole tissue) from wild-type (WT), Thpok^{Cre}Tgfbf2^{fl/fl}, Pdc1^{-/-} and Thpok^{Cre}Tgfbf2^{fl/fl}Pdc1^{-/-} mice of 3 to 4-week of age.

c: Quantification of Treg cell percentage in total CD4⁺ T cell population in LN, liver and lung from indicated genotypes.

Data are presented as the mean values $\pm$ s.d. One-way ANOVA with Dunnett T3 multiple comparison test correction. Results represent at least three independent experiments.

As the *Thpok^{Cre}* targets both conventional Th and Treg cells, to ascertain that the immunopathology stemmed from dysregulated Th cells rather than Treg cells, we generated bone marrow chimera mice using bone marrow cells isolated from *Thpok^{Cre}Tgfb^{fl/fl}Pdcd1^{-/-}* and/or WT mice, which were then transferred to congenically-mismatched WT recipients (Figure 2-4, a). Transferring *Thpok^{Cre}Tgfb^{fl/fl}Pdcd1^{-/-}* bone marrow caused lethal autoinflammation in recipient mice after 5 to 10 weeks, while addition of WT Treg cells in 1:1 mixed bone marrow chimera mice failed to alleviate the lethal autoimmune phenotype (Figure 2-4, b). This finding suggested that the lethal autoimmunity associated with *Thpok^{Cre}Tgfb^{fl/fl}Pdcd1^{-/-}* was not due to loss of function of Treg cells in the absence of TGF- β or PD-1 signaling. Interestingly, in the mixed bone marrow chimera mice, both Th and Treg cells from the *Thpok^{Cre}Tgfb^{fl/fl}Pdcd1^{-/-}* donor expanded prominently and dominated over the WT compartment (the endogenous and the WT donor-derived) in the tissue; while cells originated from *Thpok^{Cre}Tgfb^{fl/fl}Pdcd1^{-/-}* donor expanded to a similar extent as the WT ones in the lymph nodes (Figure 2-4, c-d). This finding aligned with our observation of more robust expansion of Th cells in the periphery tissue in *Thpok^{Cre}Tgfb^{fl/fl}Pdcd1^{-/-}* mice (Figure 2-3). Altogether, these findings underscored a pivotal Th cell-intrinsic peripheral tolerance mechanism mediated by cooperative TGF- β and PD-L1 signaling that prevented excessive Th cell expansion in peripheral tissues.

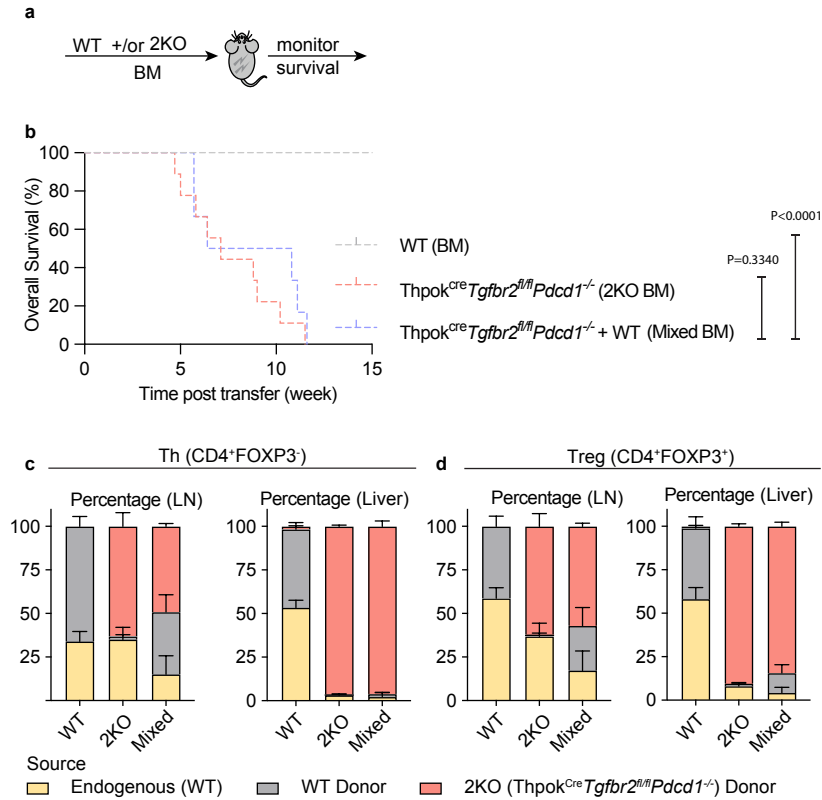


Figure 2-4 Lethal autoinflammation is not rescued by wild-type Treg cells.

a: Schematics of experiment design. Briefly, congenically mismatched wild-type (WT), Thpok^{cre}Tgfb2^{fl/fl}Pdcd1^{-/-} (2KO) or 1:1 mixed bone marrow (BM) of WT and 2KO are transferred to lethally irradiated WT recipients. The resulted BM chimera recipients are monitored for survival.

b: Kaplan-Meier survival analysis of recipient mice of the indicated groups. Log-rank test compares all three groups or the two groups excluding WT BM. Mice receiving WT BM: n=9, 2KO BM: n=9, mixed BM: n=6.

c, d: Percentage of Th and Treg cells of endogenous, WT donor and 2KO donor source in the BM chimera in LN and liver. Mice receiving WT BM: n=4 (for LN analysis), n=6 (for liver analysis); 2KO BM: n=6 (both LN and liver analysis); mixed BM: n=8 (both LN and liver analysis). Results represent at least three independent experiments.

2.2.2 TGF- β and PD-1 signaling cooperate to suppress CD4⁺ T Cell activation in SLO and pathogenic Th1 cell differentiation in peripheral tissues.

Th cells demonstrate phenotypic and functional diversity, with Th1, Th2, Th17, and follicular helper T (Tfh) cells representing major effector subsets [45,158,159]. Each of these populations have been revealed to contribute to autoimmune diseases when tolerance is breached [160]. To delineate the specific effector Th cell subsets induced and their roles in driving autoimmunity upon losing TGF- β and PD-1 suppression, we performed single-cell (sc) RNA- and TCR-sequencing experiments on CD4⁺ T cells from lymph nodes and peripheral tissues. We majorly characterized the liver as a model of peripheral organ, who has a rich and diverse immune population and a critical role in orchestrating the immunologic and metabolic adaptation of neonatal mice to the extra-uterine life is critical [161]. We isolated CD4⁺ T cells from the liver and liver-draining lymph nodes of wild-type, Thpok^{Cre}Tgfbr2^{fl/fl}, Pdc1^{-/-}, and Thpok^{Cre}Tgfbr2^{fl/fl}Pdc1^{-/-} mice for paired scRNA- and TCR-sequencing to characterize CD4⁺ T cell subsets, track their phenotypic change across genotypes, and infer clonal differentiation trajectories in association with their migration from secondary lymphoid organs to target tissues.

In total, we obtained 27,767 cells, which we grouped into 12 distinct clusters using PhenoGraph [162] as shown in the uniform manifold approximation and projection (UMAP) (Fig. 2-5, a-b). Cells in cluster 0 (C0) exhibited elevated expression of TCR β variable genes such as *Trbv3*, indicating recent thymic emigrant (RTE) 'naïve CD4 T cells', whereas C1 cells displayed similarly high levels of *Lef1*, *Tcf7*, *Ccr7*, and *Sell*, encoding transcription factors and lymph node-homing molecules [163], representing more mature 'naïve CD4 T cells'. Genes

associated with acute TCR stimulation, including *Nr4a1* and *Cd69* [164], were highly expressed in C2 cells, representing 'activated CD4 T cells'. C3 cells exhibited high expression of *Bcl6* and *Cxcr5*, signature genes for 'Tfh cells' [159]. C4 cells downregulated *Ccr7* and *Sell*, re-expressed the T cell egress marker genes *Klf2* and *S1pr1*, and began to express Th1 cell signature genes *Tbx21* and *Cxcr3* [165,166], representing 'precursor Th1 cells'. C5 cells expressed Tfh and Th1 cell marker genes *Bcl6*, *Cxcr5*, *Tbx21*, and *Cxcr3*, as well as *Il21* and *Ifng*, and were designated as 'Tfh-like Th1 cells'. Similar to C5 cells, C6 cells exhibited high expression of genes associated with persistent antigen stimulation, including *Tox*, *Ctla4*, *Lag3*, *Tigit*, and *Havcr2*, but downregulated *Tcf7* and upregulated *Ifng*, and were termed 'hyperactivated Th1 cells'. C7 and C8 cells displayed high levels of *Tbx21* and *Cxcr3*, but downregulated *Tox*, *Ctla4*, and *Lag3*. This suggests a halt of antigen stimulation and the acquisition of memory-like Th1 phenotypes. C7 cells expressed high levels of *Cxcr6* and *Itga1* [167], representing '(resident memory-like) T_{RM}-like Th1 cells', whereas C8 cells showed the highest expression of *Klrg1*, as well as *Cx3cr1* and *S1pr5* [168,169], indicating circulating '(effector memory-like) T_{EM}-like Th1 cells'. C9 cells exhibited high levels of *Tbx21* and *Cxcr3*, as well as *Cxcr6* and *Itga1*, and were characterized by induction of the innate lymphocyte marker gene *Fce1rg* [170], representing 'miscellaneous T cells'. C10 cells showed high expression of *Foxp3* and *Il2ra*, signature genes for 'Treg cells' [141], whereas C11 cells upregulated *Mki67* and *Top2a*, indicating their proliferative state.

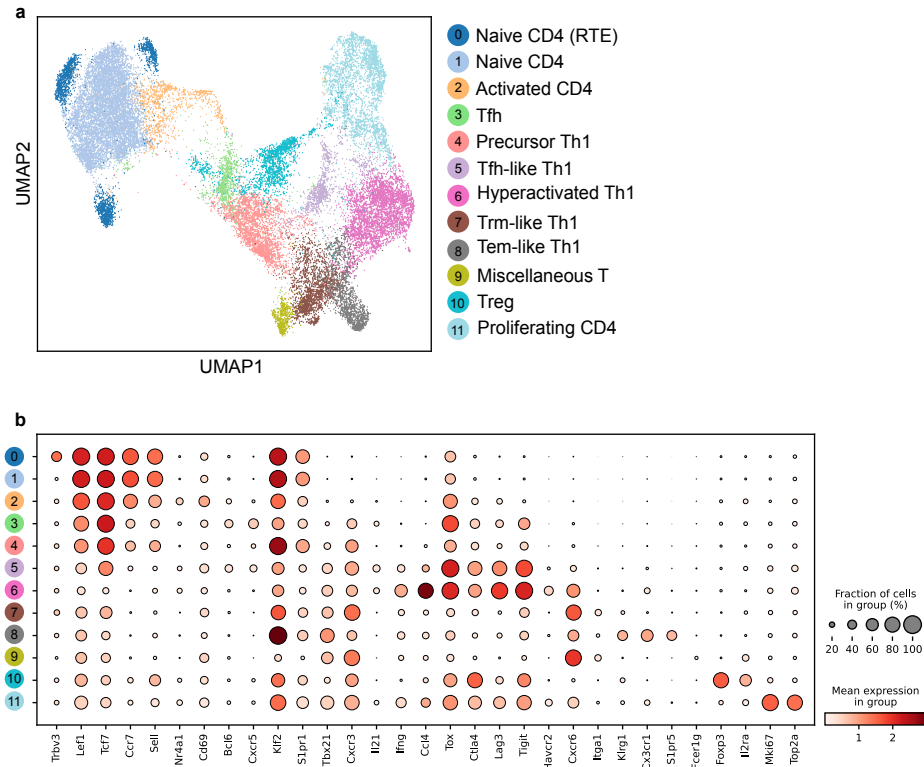


Figure 2-5 Single-cell RNA- and TCR-sequencing experiments on CD4⁺ T cells in the liver and the liver draining lymph nodes.

a: Uniform manifold approximation and projection (UMAP) plot (27,767 cells) representing CD4⁺ T cell populations isolated from the liver and liver draining lymph nodes (LN) of mice of 3-week of age from the following genotypes: wild-type, *Thpok^{cre}Tgfb^{fl/fl}*, *Pdcd1^{-/-}*, *Thpok^{cre}Tgfb^{fl/fl}Pdcd1^{-/-}*. The data represent analysis of one mouse per group colored by cell subtype clusters.

b: Dotplot showing the relative expression levels of key marker genes across identified cell clusters. Dot color represents the mean expression level within the cluster and dot size reflects fraction of cells expressing the gene within the cluster.

In liver-draining lymph nodes, TGFBR2 deficiency led to increased frequency of ‘proliferating CD4 T cells’, accompanied by a diminished frequency of ‘naïve CD4 T cells’, whereas PD-1 deficiency did not substantially affect T cell activation (Figure 2-6, a). TGFBR2 and PD-1 double deficiency led to further proliferation and markedly increased frequencies of ‘Tfh cells’ and ‘precursor Th1 cells’ (Figure 2-6, a). To gain deeper insights into the cellular changes upon losing TGF- β and PD-1 suppression, we used Milo [171] to quantify changes in abundance of cell subtype clusters by comparing each single knockout with WT, and double knockout with each single knockout (Figure 2-6, b-e). In line with the cluster distribution, we found a shift in the naïve cluster after TGFBR2 deletion (Figure 2-6, b). While PD-1 deficiency alone did not cause pronounced phenotypic shifts (Figure 2-6, c), PD-1 deficiency combined with TGFBR2 deletion resulted in a further reduction in the naïve population and their differentiation into ‘Tfh’ cells and ‘precursor Th1’ cells (Figure 2-6, d). Similarly, comparing *Thpok^{Cre}Tgfb2^{fl/fl}Pdcd1^{-/-}* to *Pdcd1^{-/-}* also revealed dramatic expansion of the two clusters (Figure 2-6, e).

We next confirmed these findings using flow cytometry analysis of CD4⁺ T cells from the liver draining lymph node (Figure 2-7), using markers identified in the scRNA-sequencing analysis. We found decreased proportion of naïve cells within the conventional Th cells gated as CD44⁺ICOS⁻ in *Thpok^{Cre}Tgfb2^{fl/fl}* mice, which further diminished in *Thpok^{Cre}Tgfb2^{fl/fl}Pdcd1^{-/-}* mice, in parallel to a robust expansion of the Tfh and precursor Th1 subset gated on CXCR5⁺BCL6⁺ and CD44⁺CXCR5⁻TCF1⁺T-bet⁺, respectively.

TCR clonality analysis [172] revealed a similar trend in clonal expansion as the proliferation and differentiation across genotypes (Figure 2-8, a). TCR clones were defined by identical TCR $\alpha\beta$ CDR3 sequences. Notably, TGFB β 2 deficiency led to increased clonal expansion, which was further enhanced by loss of PD-1 suppression, as shown by the decreased value of Normalized Shannon Entropy (Figure 2-8, a). These observations suggested PD-1 function is prominently manifested under the condition of TGF- β signaling blockade, which aligns with the induction of PD-1 expression in TGFB β 2-deficient conventional CD4 $^{+}$ T cells (Figure 2-1).

Th1 and Tfh cell bifurcation instructs cellular and humoral immune responses to type 1 pathogens [47,173–178]. In tolerance regulation, a recent study demonstrated that the self-reactive CD4 $^{+}$ T cell clones in the periphery adopt a Tfh-like phenotype at steady state and are highly proliferative in lymphoid organs [76]. To examine whether the clonally expanded cells in Thpok Cre Tgfb β 2 $^{fl/fl}$ Pdcd1 $^{-/-}$ also exhibit preference in Tfh differentiation, we examined the cell subtype cluster distribution in the top 20 expanded clones in the lymph node, ranged from 6 to 26 cells per clone (Figure 2-8, b). We found a majority of the top expanded clones span both the Tfh and Th1-related clusters. Therefore, following TGFB β 2 and PD-1 deletion, at least some clones of self-reactive Th cells can differentiate into both Tfh and Th1 precursor cells in secondary lymphoid organs.

Consistent with these observations, we found the transition of CD4 $^{+}$ T cells from 'naïve' to 'activated', 'Tfh', and 'precursor Th1', states was associated with diminished expression of TGF- β target genes, such as Ski [179]. The naïve

clusters in $\text{Thpok}^{\text{Cre}}\text{Tgfb}\beta^{\text{fl/fl}}$ also upregulated CD69, an activation marker downstream of TCR signaling, as shown in differentially expressed gene analysis (DEG), indicating more active priming compared to WT controls. PD-1 was induced in 'activated' and 'Tfh', but not 'precursor Th1', states (Figure 2-9, a). The lack of PD-1 expression on 'precursor Th1' indicated they ceased TCR signaling from interaction with APCs and were in transition to migration. Thus, TGF- β acted before naïve T cell activation to prevent priming, while PD-1 exerting effects on activated Th cells to limit their further differentiation (Figure 2-9, b). The two pathways worked cooperatively to regulate Th cell tolerance in secondary lymphoid organs.

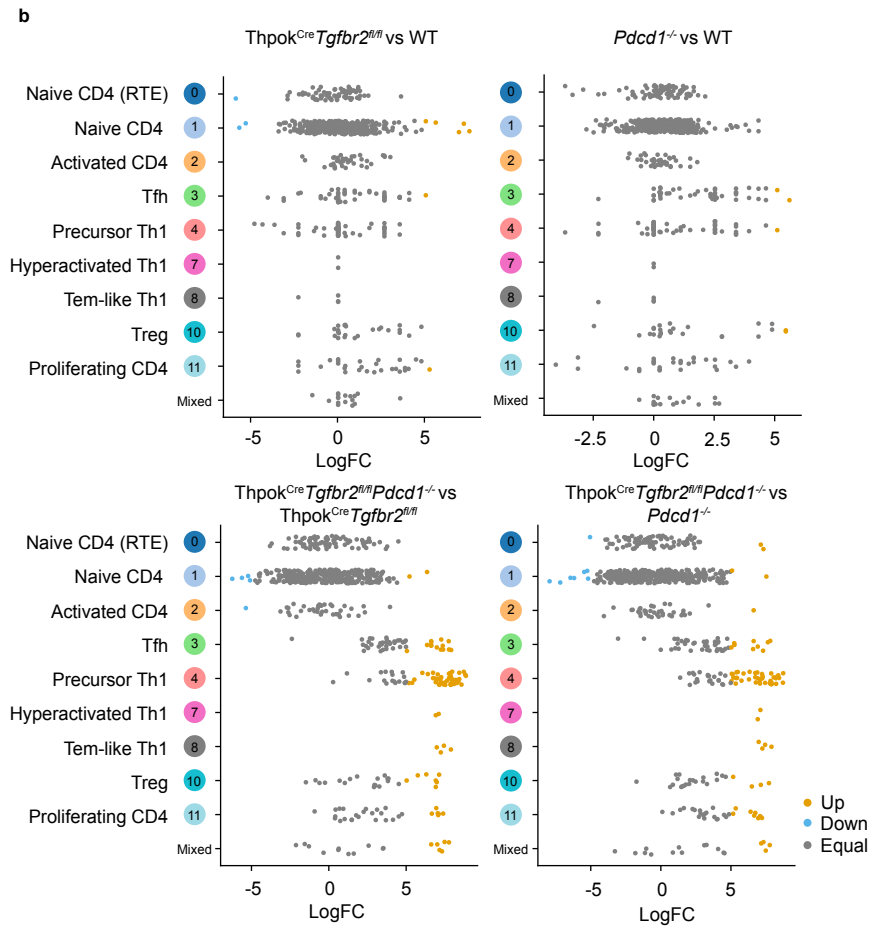
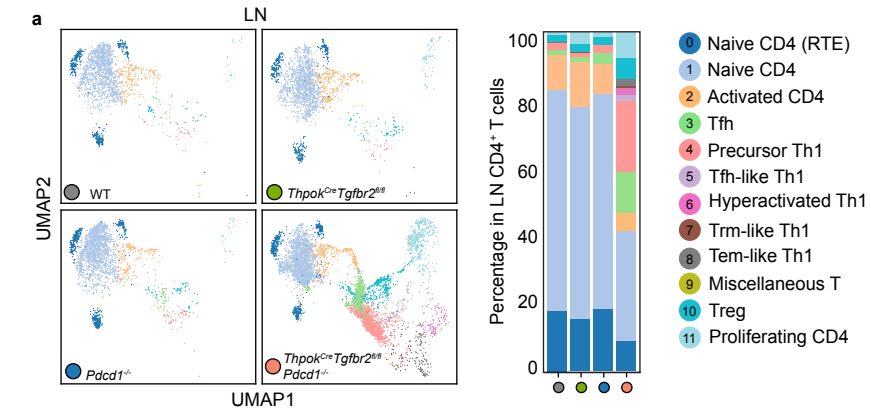


Figure 2-6 Increased activation and differentiation into follicular T helper cells and precursor Th1 cells in the SLO of *Thpok^{Cre}Tgfbr2^{fl/fl}Pdcd1^{-/-}* mice in scRNA-sequencing.

a: (left) UMAP plots representing CD4⁺ T cell populations isolated from the liver-draining lymph nodes (LN) colored by cell clusters across genotypes: wild-type (WT) (1,935 cells), *Thpok^{Cre}Tgfbr2^{fl/fl}* (2,011 cells), *Pdcd1^{-/-}* (2,403 cells), *Thpok^{Cre}Tgfbr2^{fl/fl}Pdcd1^{-/-}* (6,004 cells). (Right) Distribution of cell clusters within each genotype.

b: Swarm plots depicting the log fold change of differential cell type abundance in the pair of indicated comparisons in each neighborhood across different cell subtypes in the LN samples. Each dot represents a neighborhood computed using MiloR and is color-coded by the magnitude of the log fold change with a cutoff value of 5. A neighborhood is classified as a cell type if it comprises at least 50% of cells in the neighborhood, otherwise it is called 'mixed'.

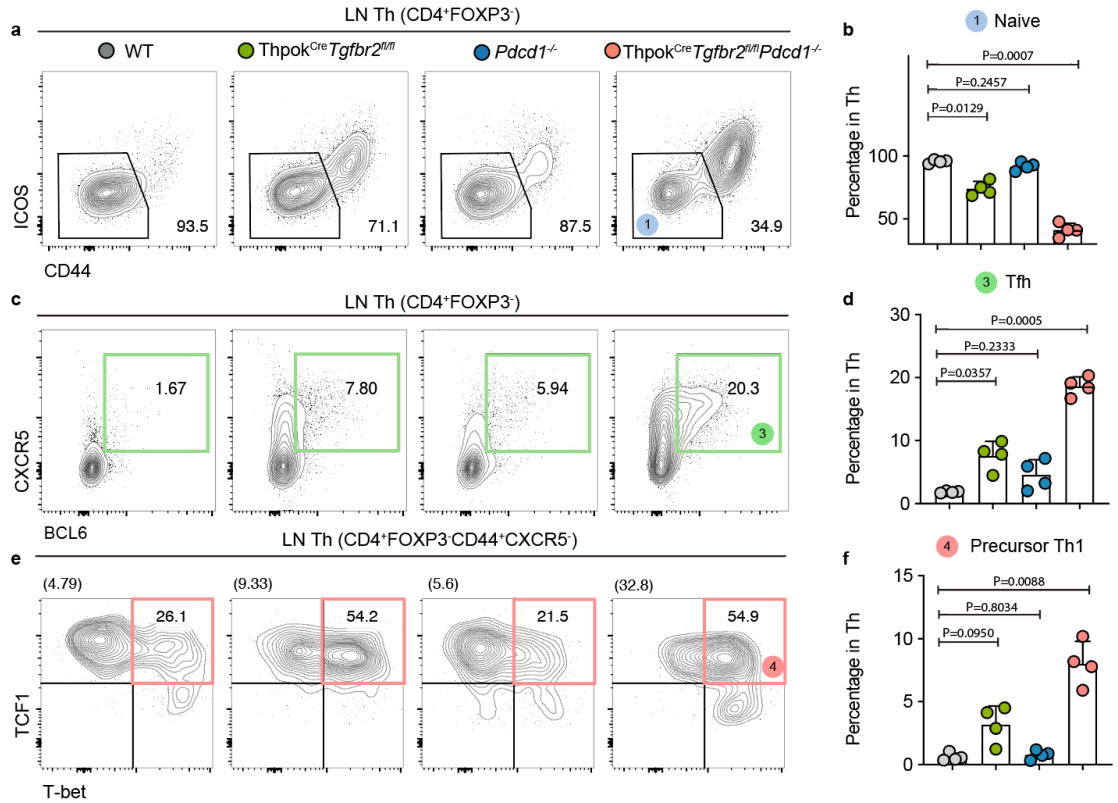


Figure 2-7 Increased activation and differentiation into follicular T helper cells and precursor Th1 cells in the SLO of *Thpok^{Cre}Tgfb2^{fl/fl}Pdc1^{-/-}* mice shown by flow cytometry analysis.

a, b: Representative plots (**a**) and quantification (**b**) of naïve cells gated by ICOS and CD44 expression within CD4⁺Foxp3⁺ T helper (Th) cells of indicated genotypes in peripheral lymph nodes (LN).

c, d: Representative plots (**c**) and quantification (**d**) of follicular T helper (Tfh) cells gated by CXCR5 and BCL6 expression within CD4⁺Foxp3⁺ Th cells of indicated genotypes in LN.

e, f: Representative plots (**e**) and quantification (**f**) of type 1 T helper (Th1) precursor cells gated by TCF1 and T-bet expression within CD4⁺Foxp3⁺ CD44⁺CXCR5⁺ Th cells in LN. Percentage of the parent population for each genotype is labeled at the top.

Data are mean ± s.d. One-way ANOVA with Dunnett T3 multiple comparison test correction. Results represent at least three independent experiments.

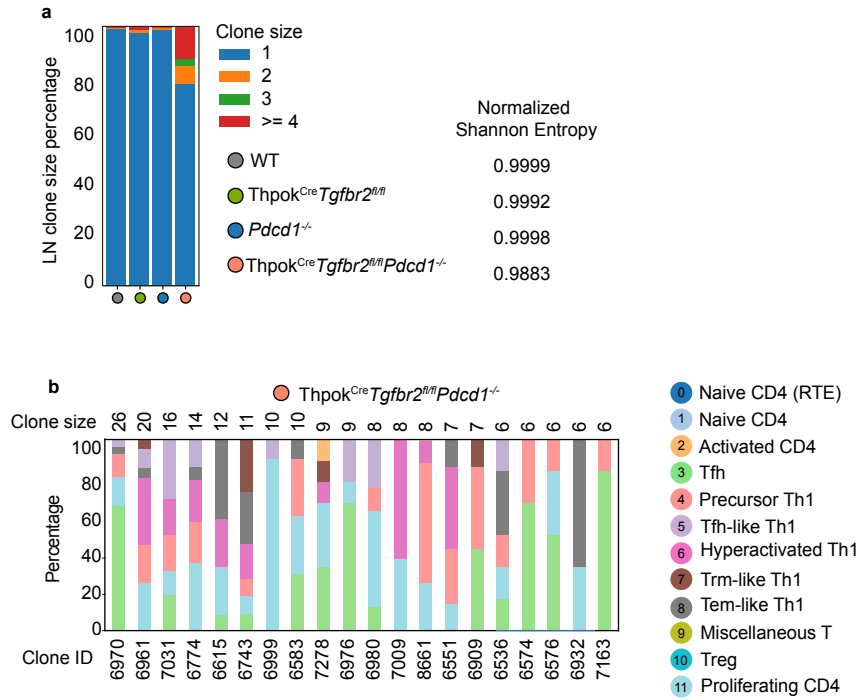


Figure 2-8 Clonal expansion in the SLO of Thpok^{Cre}Tgfb2^{fl/fl}Pdcd1^{-/-} mice.

a: Distribution of clones of different sizes within each indicated genotype in liver draining lymph node samples, with normalized Shannon entropy.

b: Distribution of cell subtypes within each clone in the top 20 expanded clones by size in the LN sample of Thpok^{Cre}Tgfb2^{fl/fl}Pdcd1^{-/-}, arranged by clone sizes.

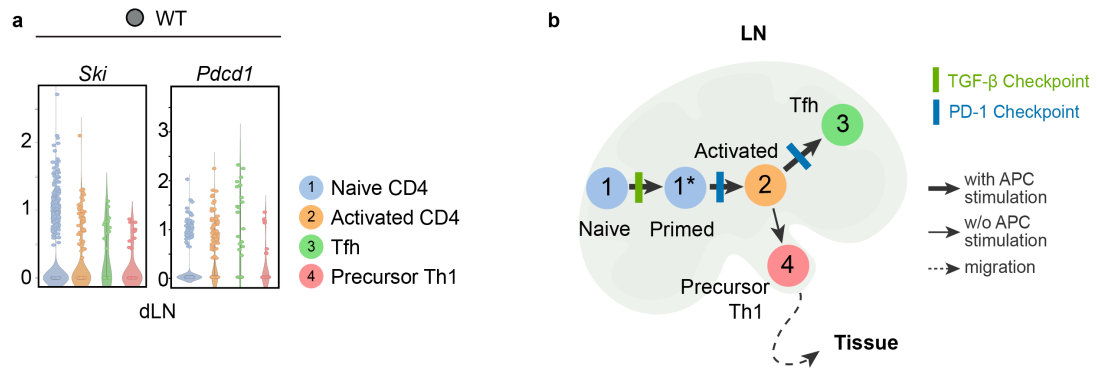


Figure 2-9 Model of cooperative regulation of Th cell differentiation in the SLO by TGF- β and PD-1.

a: Expression levels of *Ski* and *Pdccl1* in various CD4⁺ T cell subsets from wild-type (WT) mice in draining lymph nodes (dLN).

b: Model of cooperative regulation of Th cell differentiation in the SLO by TGF- β and PD-1.

In the liver, CD4⁺ T cell population comprised Th1-related clusters, including 'precursor Th1', 'Tfh-like Th1', 'hyperactivated Th1', 'T_{RM}-like Th1' and 'T_{EM}-like Th1' cells, as well as Treg cells, 'miscellaneous T cells' and 'proliferating CD4⁺ T cells'. Notably, 'activated CD4 T cells' and 'Tfh cells' were present in liver-draining lymph nodes, but not in the liver (Figure 2-10, a), while 'Tfh-like Th1 cells' were more prevalent in the tissue. The subset 'precursor Th1' cells were present in both the lymphoid organ and the liver, suggesting that this was the population that seeded peripheral tissues after activation, consistent with their expression of the T cell egress marker *S1pr1* (Figure 2-5, b).

TGFBR2 deficiency led to increased frequencies of 'T_{RM}-like Th1 cells' and 'T_{EM}-like Th1 cells', accompanied by a diminished frequency of 'precursor Th1 cells', while PD-1 deficiency expanded 'Treg cells' and 'miscellaneous T cells', but had minimal effects on conventional Th cells (Figure 2-10, a). Remarkably, TGFBR2 and PD-1 double deficiency caused massive expansion of 'hyperactivated Th1' cells and 'Tfh-like Th1' cells (Figure 2-10, a). TGFBR2 deficiency also promoted proliferation, further manifested by PD-1 deficiency. These observations were supported by Milo analysis to quantify changes in abundance of cell subtype clusters across genotypes (Figure 2-10, b-e).

We then validated the presence and distribution of various Th1-related effector populations using flow cytometry analysis in each of the genotypes (Figure 2-11). Similar to what we found in scRNA-sequencing, 'Tfh-like Th1 cells' gated by TOX⁺TCF1⁺ and 'hyperactivated Th1 cells' gated by TOX⁺TCF1⁻ dominated the Thpok^{Cre}*Tgfb₂*^{fl/fl}*Pdcd1*^{-/-} mice, while TOX⁻CXCR6⁺ 'T_{RM}-like Th1 cells' and TOX⁻

KLRG1⁺ 'T_{EM}-like Th1 cells' were the major effector population expanded in Thpok^{Cre}Tgfb^{fl/fl} mice (Figure 2-11, a-d). We also confirmed that the two unique populations in Thpok^{Cre}Tgfb^{fl/fl}Pdcd1^{-/-} mice, 'Tfh-like Th1 cells' and 'hyperactivated Th1 cells' had more potent effector function demonstrated by higher level of IFN- γ expression upon restimulation, compared to 'T_{RM}-like Th1 cells' and 'T_{EM}-like Th1 cells' (Figure 2-11, e), consistent with the findings in transcriptomic analysis (Figure 2-5, b).

TCR clonality analysis revealed a similar trend with more pronounced magnitude in clonal expansion as in the draining-lymph nodes across genotypes (Figure 2-12, a). TGFB β 2 deficiency increased clonal expansion, which was further enhanced by loss of PD-1 suppression shown by the decreased value of Normalized Shannon Entropy. Of note, the clone sizes of top expanded clones in both Thpok^{Cre}Tgfb^{fl/fl} and Thpok^{Cre}Tgfb^{fl/fl}Pdcd1^{-/-} liver sample were much higher than those in the lymph nodes (Figure 2-12, a), with the top clone comprising around 10% of the total identified cells within each of the sample (data not shown). This was consistent with our previous observation that conventional Th cells expansion was more robust in the periphery tissue following TGF- β signaling depletion compared to the SLO (Figure 2-3). Furthermore, analysis of cell cluster distribution in the top 20 expanded clones in the liver samples of each genotype revealed that in WT and Pdcd1^{-/-} tissue, the top expanded clones are limited in size and mostly span the 'precursor Th1 cells' and "miscellaneous T cells", indicating restrained restimulation at homeostasis. In contrast, the top clones in absence of TGFB β 2 were mostly spanning the 'T_{RM}-like Th1', and 'T_{EM}-like Th1'

cell clusters, while most of the top clones in $\text{Thpok}^{\text{Cre}}\text{Tgfbr2}^{\text{fl/fl}}\text{Pdc1}^{-/-}$ liver sample spanning ‘hyperactivated Th1’ and ‘Tfh-like Th1’ cell cluster (Figure 2-12, b). Interestingly, there were also highly expanded clones in $\text{Thpok}^{\text{Cre}}\text{Tgfbr2}^{\text{fl/fl}}\text{Pdc1}^{-/-}$ liver sample with a similar distribution pattern as the ones found in $\text{Thpok}^{\text{Cre}}\text{Tgfbr2}^{\text{fl/fl}}$ liver, such as #8500, #6970 and #18847 (Figure 2-12, b). The absence of the ‘hyperactivated Th1’ cell cluster in these clones suggested that it represented a more terminally differentiated state achieved by some, but not all, antigen specificities. Moreover, this indicated that apart from TGFBR2 and PD-1, effector T cell fates were partially determined by antigen specificity. Additionally, cell from both the liver and liver-draining lymph nodes were detected in most of the top expanded clones (data not shown), suggesting that pathogenic T cells underwent priming in secondary lymphoid organs before migration to target tissues.

TGFBR2 and PD-1 double deficiency also promoted Treg expansion in the tissue, although not as prominent as conventional Th cells (Figure 2-3, b). However, TCR clonality analysis highlighted TCR overlap only among conventional Th cell clusters, but not between Th cell clusters and the Treg cell cluster (Figure 2-12, c), suggesting that the Treg cells were less likely to be converted from the conventional Th cell pool.

Next, to investigate the potential developmental relationships between Th1-related clusters in the tissue, we performed trajectory inference [180] analysis using the scRNA-seq data from $\text{Thpok}^{\text{Cre}}\text{Tgfbr2}^{\text{fl/fl}}\text{Pdc1}^{-/-}$ liver sample, which contains all the Th1 clusters in abundance. We filtered the data to include only Th1 cells from any of the ‘precursor Th1’, ‘Tfh-like Th1’, ‘hyperactivated Th1’, ‘T_{RM}-like

Th1' and 'T_{EM}-like Th1' clusters. The start point was chosen from the 'precursor Th1' cells. A terminal state along the pseudotime was identified in each of the 'Tfh-like Th1', 'hyperactivated Th1', 'T_{RM}-like Th1' and 'T_{EM}-like Th1' clusters (Figure 2-13, a), indicating each of these effector population possibly transitioned directly from 'precursor Th1' cells. Remarkably, *Tox* expression was higher at the beginning in 'Tfh-like Th1' and 'hyperactivated Th1' cells (Figure 2-13, b), indicating stronger TCR signaling among these cells. *Tox* expression, as an indicator of TCR signaling strength, was shaped by both the intrinsic TCR specificity, as well as the TGF- β and PD-1 signaling. This indicated that some 'precursor Th1' cells received strong and persistent TCR stimulation in Thpok^{cre}*Tgfbr2*^{fl/fl}*Pdcd1*^{-/-} mice, allowing them to reach 'Tfh-like Th1' and 'hyperactivated Th1' state, while those with lower intrinsic TCR affinity in Thpok^{cre}*Tgfbr2*^{fl/fl}*Pdcd1*^{-/-} mice, or those with PD-1 to compromise TCR signaling in Thpok^{cre}*Tgfbr2*^{fl/fl} mice developed into T_{RM}-like Th1' or 'T_{EM}-like Th1' cells instead. Therefore, the differentiation choice of convention Th cells at the precursor state was accumulatively dictated all these factors, and TGF- β and PD-1 worked collaboratively to prevent the differentiation of the most pathogenic effector Th1 cells.

In the liver, expression of TGF- β target genes, such as *Ski* was higher in 'precursor Th1' cells, but downregulated in 'Tfh-like Th1', 'hyperactivated Th1', 'T_{RM}-like Th1', and 'T_{EM}-like Th1', states (Figure 2-14, a). Notably, after TGFBR2 deletion, the 'precursor Th1' cells upregulated *Ly6a*, *Cxcr3* while down regulated *Klf2*, *S1pr1* and *Tcf7* shown by DEG analysis, shifting them to the more mature

Th1 state along the spectrum of differentiation. This indicated that reactivation of 'precursor Th1' cells upon tissue entry was regulated by TGF- β . Meanwhile, PD-1 was induced in 'Tfh-like Th' and 'hyperactivated Th1', but not 'T_{RM}-like Th1' or 'T_{EM}-like Th1', states, indicating the former two subsets were actively engaging with APCs and receiving TCR stimulation (Figure 2-14, a). Therefore, TGF- β acted on newly recruited 'precursor Th1' cells to prevent their reactivation, while PD-1 restrained TCR signaling of the restimulated Th1 cells to prevent them from developing into the most pathogenic effectors (Figure 2-14, b) in the tissue. Taken together, the two pathways worked cooperatively to regulate Th cell tolerance in secondary lymphoid organs and peripheral tissues by preventing the priming, restimulation, and differentiation of potent 'Tfh', 'Tfh-like Th1' and 'hyperactivated Th1' cells at homeostasis.

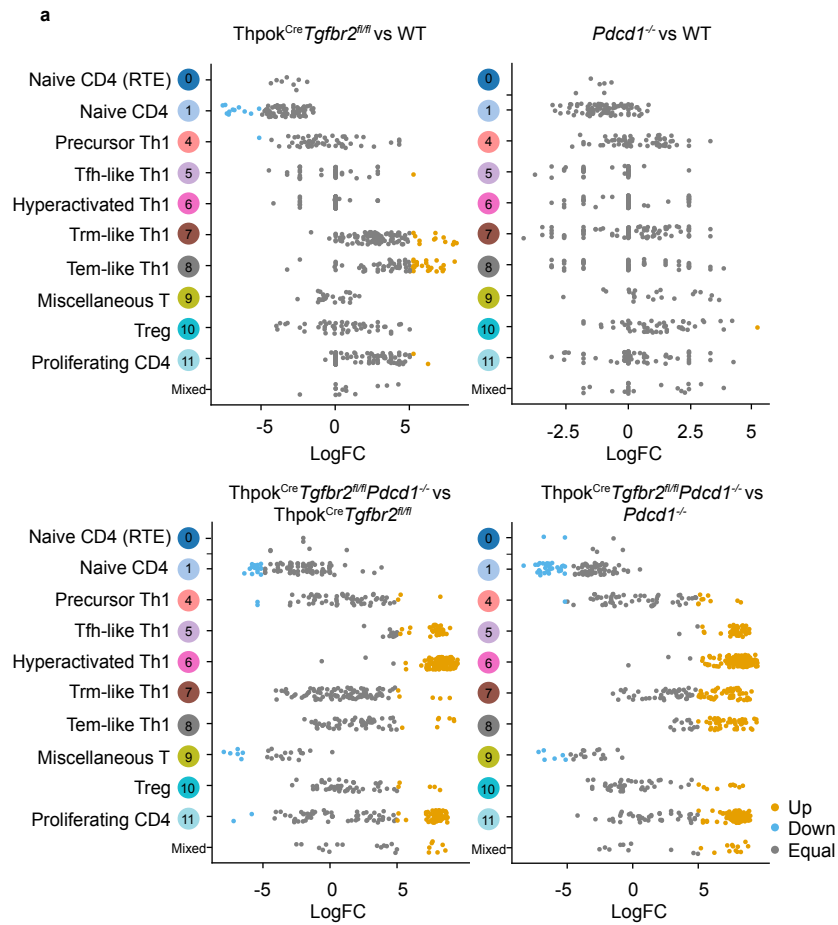
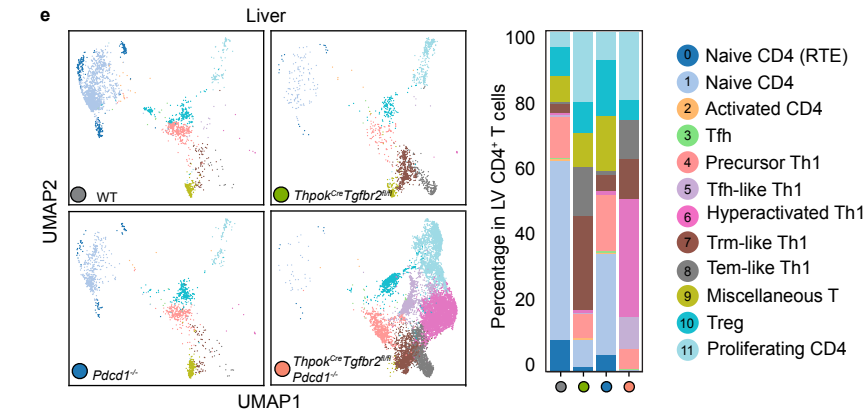


Figure 2-10 Increased activation and differentiation Tfh-like and hyperactivated Th1 cells in the liver of *Thpok^{Cre}Tgfb β 2^{fl/fl}Pdcd1^{-/-}* mice in scRNA-sequencing.

a: (Left) UMAP plots representing CD4⁺ T cell populations isolated from the liver colored by cell clusters across genotypes: WT (2,465 cells), *Thpok^{Cre}Tgfb β 2^{fl/fl}* (1,400 cells), *Pdcd1^{-/-}* (1,317 cells), *Thpok^{Cre}Tgfb β 2^{fl/fl}Pdcd1^{-/-}* (10,232 cells).

b: Swarm plots depicting the log fold change of differential cell type abundance in the pair of indicated comparisons in each neighborhood across different cell subtypes in the liver samples. Each dot represents a neighborhood computed using MiloR and is color-coded by the magnitude of the log fold change with a cutoff value of 5. A neighborhood is classified as a cell type if it comprises at least 50% of cells in the neighborhood, otherwise it is called 'mixed'.

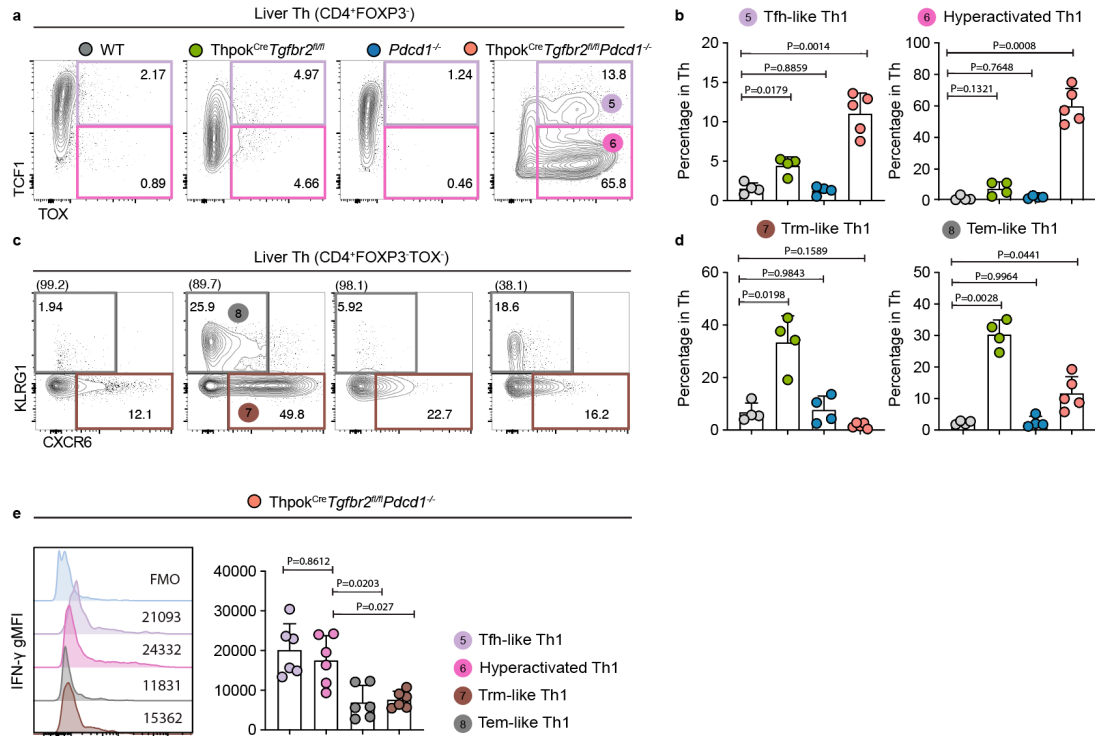


Figure 2-11 Increased activation and differentiation Tfh-like and hyperactivated Th1 cells in the liver of Thpok^{Cre}Tgfb2^{fl/fl}Pdcd1^{-/-} mice in flow cytometry.

a, b: Representative plots (**a**) and quantification (**b**) of Tfh-like Th1 cells and hyperactivated Th1 cells gated by TCF1 and TOX expression within CD4⁺Foxp3⁺ T helper (Th) cells of indicated genotypes the liver from mice of 3 to 4 week-of-age.

c, d: Representative plots (**c**) and quantification (**d**) of resident memory like (Trm-like) Th1 cells and effector memory like (Tem-like) Th1 cells gated by CXCR6 and KLRG1 expression within CD4⁺Foxp3⁺TOX⁻ Th cells in the liver. Percentage of the parent population for each genotype is labeled at the top.

e: Representative plots and quantification of IFN-γ expression using geometric mean of fluorescence intensity (gMFI) after ex vivo restimulation of Th1 cell population, including Tfh-like Th1, hyperactivated Th1, Trm-like Th1 and Tem-like Th1 isolated from the liver of Thpok^{Cre}Tgfb2^{fl/fl}Pdcd1^{-/-} mice of 3 to 4 week-of-age. The baseline fluorescence intensity represented by fluorescence minus one (FMO) control is subtracted in the quantification.

Data are mean ± s.d. One-way ANOVA with Dunnett T3 multiple comparison test correction. Results represent at least three independent experiments.

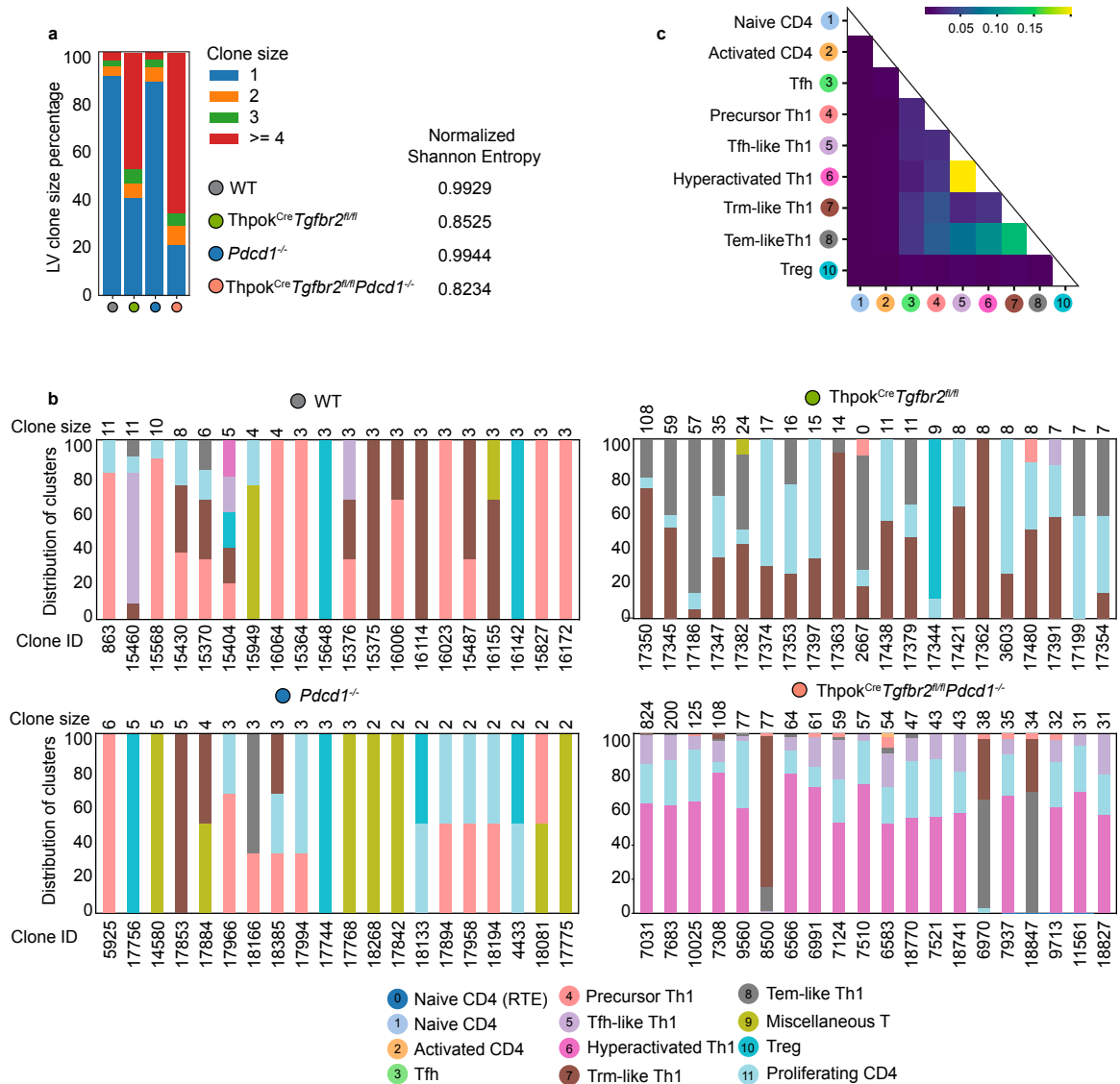


Figure 2-12 Clonal expansion in the liver of *Thpok^{Cre}Tgfb2^{fl/fl}Pdcd1^{-/-}* mice.

a: Distribution of clones of different sizes within each indicated genotype in the liver samples, with normalized Shannon entropy.

b: Distribution of cell clusters within each clone in the top 20 expanded clones by size in the liver sample of each indicated genotype, arranged by clone sizes.

c: Heatmap showing the pairwise comparison of T cell receptor (TCR) repertoire overlaps among the indicated cell clusters, calculated using 1-Jaccard Index. Each square in the heatmap represents the dissimilarity between groups, with values closer to 1 indicating lower overlap and values near 0 indicating higher overlap of TCR sequences.

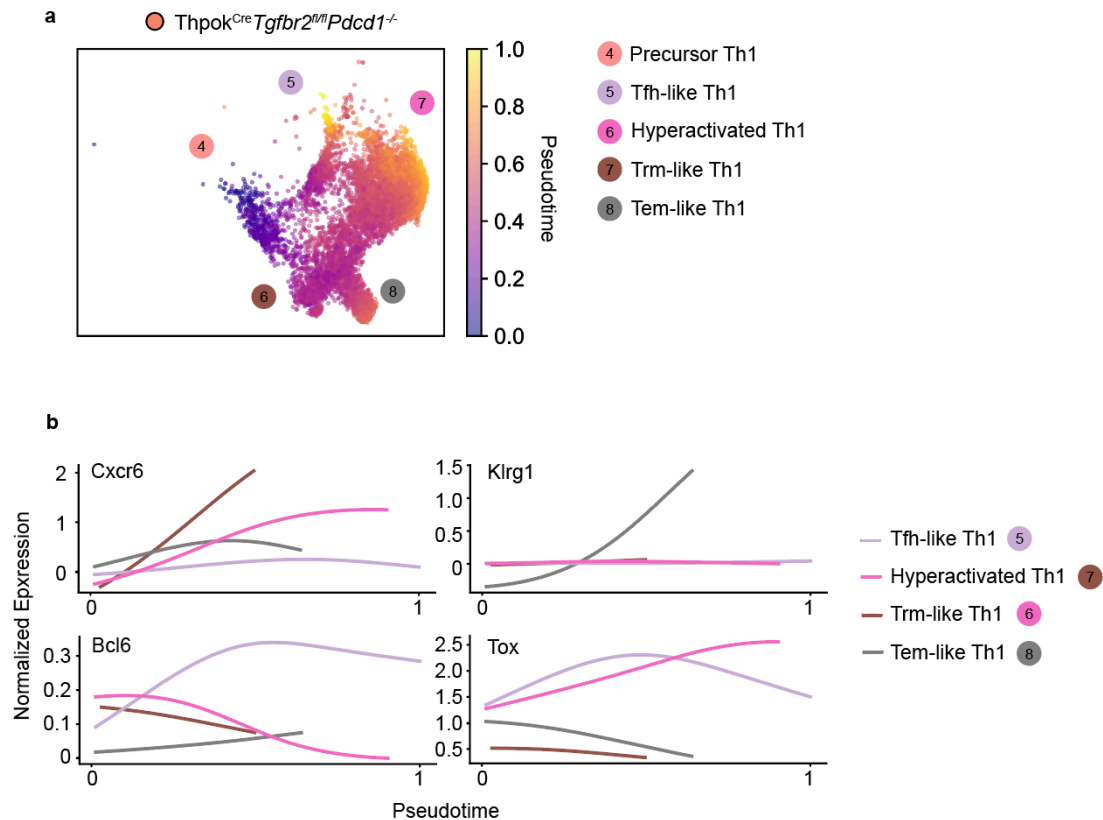


Figure 2-13 Trajectory analysis of liver Th1 cells.

a: UMAP plot colored by pseudotime. Pseudotime analysis was performed using the Palantir algorithm to identify differentiation trajectories within the type 1 T helper (Th1) cell population in the liver sample of $\text{Thpok}^{\text{Cre}}\text{Tgfb}\beta 2^{\text{fl/fl}}\text{Pdc}\delta 1^{-/-}$ mice, including precursor Th1 (cluster 4), Tfh-like Th1 (cluster 5), hyperactivated Th1 (cluster 6), Trm-like Th1 (cluster 7) and Tem-like Th1 (cluster 8).

b: Line plots depicting the normalized expression profiles of key marker genes (*Cxcr6*, *Klrg1*, *Bcl6*, *Tox*) on the y axis across pseudotime on the x axis. Each line represents a distinct trajectory identified in pseudotime analysis.

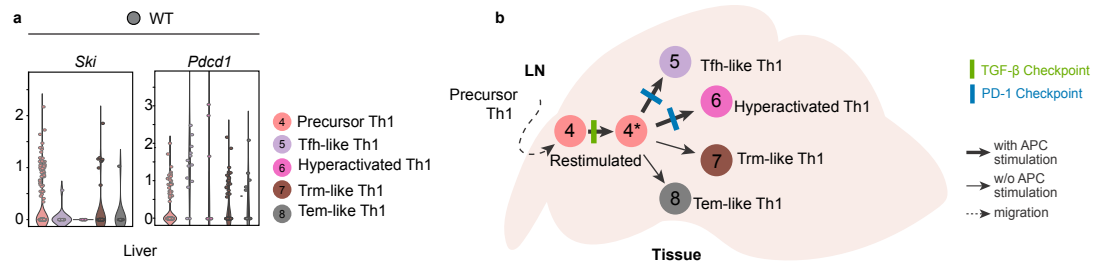


Figure 2-14 Model of cooperative regulation of Th cell differentiation in the liver by TGF- β and PD-1.

a: Expression levels of *Ski* and *Pdc1* in various CD4⁺ T cell subsets from wild-type (WT) mice in the liver.

b: Model of cooperative regulation of Th cell differentiation in the liver by TGF- β and PD-1.

2.2.3 PD-L1 on macrophages cooperates with TGF- β to promote peripheral Th cell tolerance in the tissue.

PD-1 has two known ligands, programmed death ligand-1 (PD-L1) and PD-L2. PD-L1 exhibits a broader expression pattern than PD-L2 [181]. To investigate whether PD-L1 is the major ligand interacting with PD-1 suppress TGFB2-deficient autoreactive Th cells, we bred Thpok^{Cre}Tgfb2^{fl/fl} mice onto a PD-L1-deficient (Cd274^{-/-}) background. Thpok^{Cre}Tgfb2^{fl/fl}Cd274^{-/-} mice developed similar lethal autoimmunity around 3 to 4 week-of age (Figure 2-15, a), phenocopying Thpok^{Cre}Tgfb2^{fl/fl}Pdcd1^{-/-} mice. Moreover, pathology analysis revealed increased leukocyte infiltration in multiple vital organs including the liver and the lung (Figure 2-15, b). Similar to Thpok^{Cre}Tgfb2^{fl/fl}Pdcd1^{-/-} mice, TGFB2 deficiency on the PD-L1 deficient background had pronounced expansion of Th cells in peripheral tissues, with around 2.7- and 16.0 -fold increase of Th cells in the lymph nodes and liver, respectively, compared to WT controls (Figure 2-16, a).. Treg cells were also expanded, however, with around 5.0-fold in both compartments (Figure 2-16, b). As a result, Treg cell percentage was increased among total CD4⁺ T cells in the lymph node, but decreased in the liver (Figure 2-16, c). PD-L1 deficiency alone did not affect Th or Treg cell number. Although the total CD45⁺ cell number increased in the lymph nodes and liver in Thpok^{Cre}Tgfb2^{fl/fl}Cd274^{-/-} mice (Figure 2-16, d), the fold change was not comparable to conventional Th cells. Thus, we reached the same conclusion that Th cell, but not Treg cell expansion was more prominent in the peripheral tissues compared to secondary lymphoid organs upon blocking the two pathways.

We went on to examine the cellular phenotype of Th cells in *Thpok^{Cre}Tgfb β 2^{fl/fl}Cd274^{-/-}* mice using flow cytometry. In liver-draining lymph nodes of 3-week-old mice, we found significant reduction of the naïve population, accompanied by a robust expansion of the Tfh and precursor Th1 subset, mirroring *Thpok^{Cre}Tgfb β 2^{fl/fl}Pdcd1^{-/-}* mice (Figure 2-17, a). In the liver, the TOX⁺TCF1⁺ ‘Tfh-like Th1 cells’ and TOX⁺TCF1⁻ ‘hyperactivated Th1 cells’ were also found in *Thpok^{Cre}Tgfb β 2^{fl/fl}Cd274^{-/-}* mice but not the controls (Figure 2-17, b), indicating differentiation of the more potent effector population without the regulation of TGF- β and PD-L1 signaling. Thus, we concluded that PD-L1 is the functional ligand to interact with PD-1 and restrain autoreactive Th cell differentiation in the absence of TGF- β signaling.

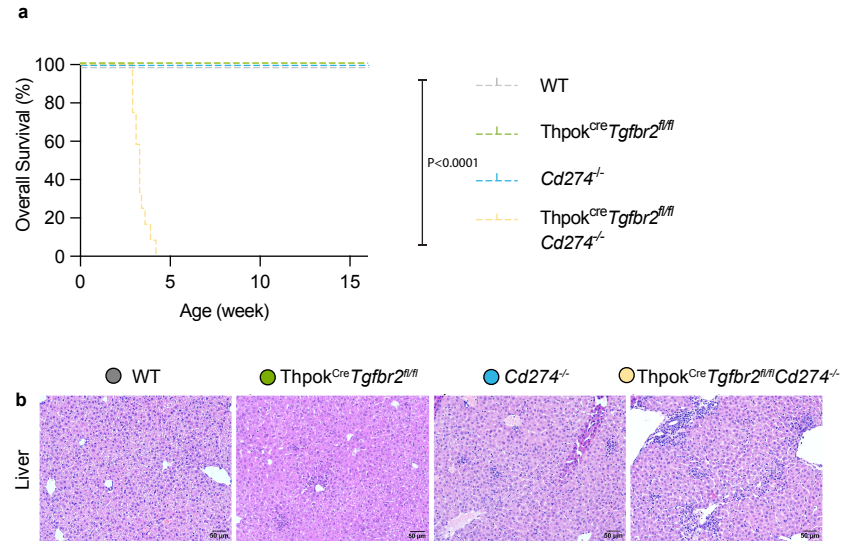


Figure 2-15 Deletion of TGF- β RII in peripheral CD4⁺ T cells on PD-L1 deficient background resulted in lethal autoinflammation.

a: Kaplan-Meier survival analysis of mice of wild-type (WT), Thpok^{Cre}Tgfb2^{fl/fl}, Pdccl1^{-/-} and Thpok^{Cre}Tgfb2^{fl/fl}Cd274^{-/-} genotypes. n=10 mice for each group. Log-rank test compares all four groups.

b: Representative hematoxylin and eosin (H&E) sections of liver and lung from mice of indicated genotypes. The scale bar is 50 μ m.

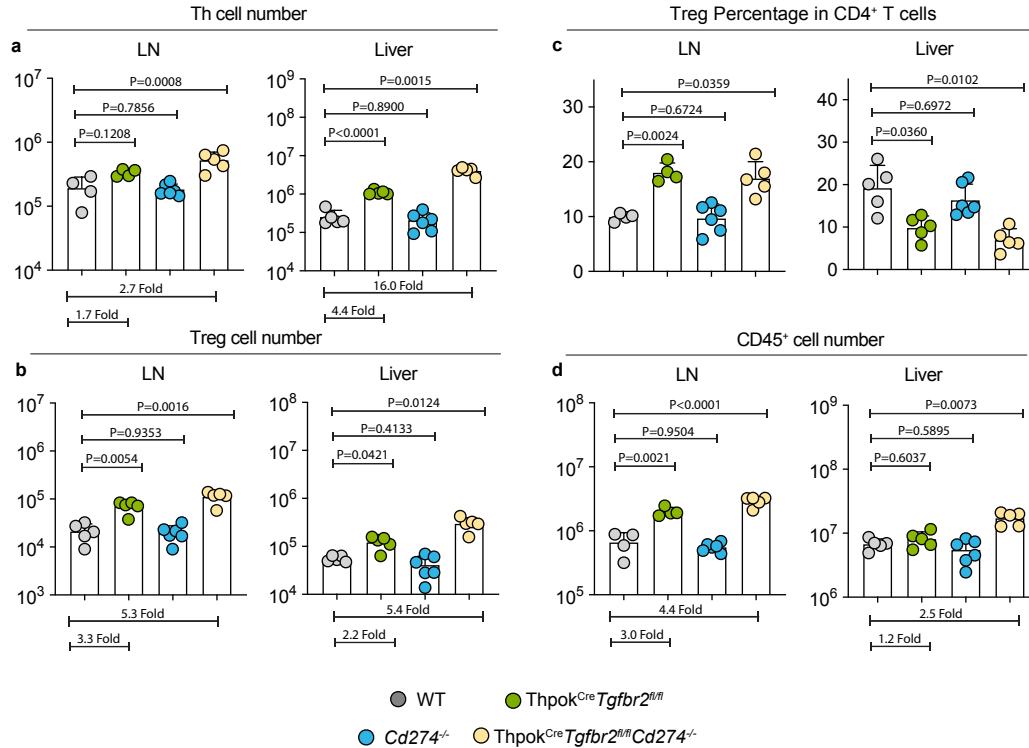


Figure 2-16 Skewed expansion of conventional CD4⁺ T cells but not Treg cells in the tissue in Thpok^{Cre}Tgfb2^{fl/fl}Cd274^{-/-} mice.

a, b, d: Quantification of Th (**a**), Treg (**b**) and total CD45⁺ (**d**) cell number in LN (per lymph node), liver (per gram of tissue), and lung (whole tissue) from wild-type (WT), Thpok^{Cre}Tgfb2^{fl/fl}, *Pdcd1*^{-/-} and Thpok^{Cre}Tgfb2^{fl/fl}Cd274^{-/-} mice of 3 to 4-week of age.

c: Quantification of Treg cell percentage in total CD4⁺ T cell population in LN, liver and lung from indicated genotypes.

Data are presented as the mean values $\pm$ s.d. One-way ANOVA with Dunnett T3 multiple comparison test correction. Results represent at least three independent experiments.

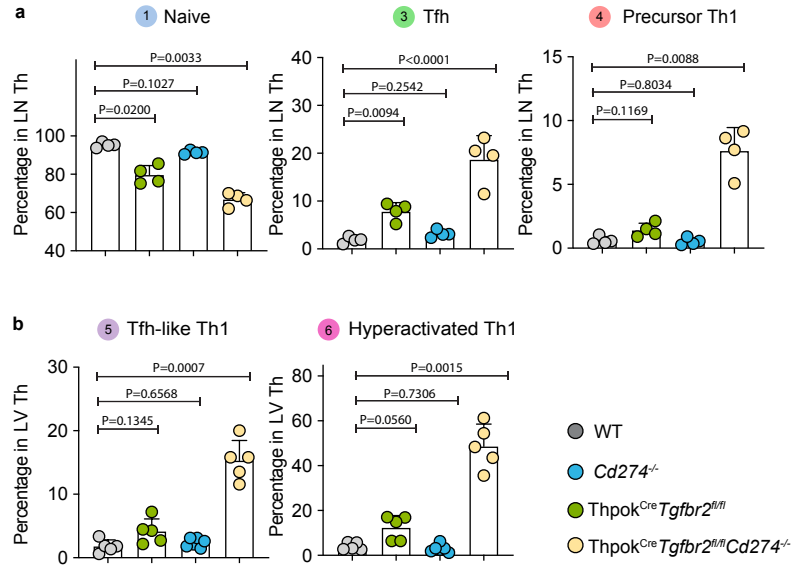


Figure 2-17 Th cell cellular phenotype in SLO and liver of $Thpok^{Cre}Tgfb2^{fl/fl}Cd274^{-/-}$ mice shown by flow cytometry analysis.

a: Quantification of naïve cells, follicular T helper (Tfh) cell, type 1 T helper (Th1) precursor cells within conventional Th cells in peripheral lymph nodes (LN).

b: Quantification of Tfh-like Th1 cells and hyperactivated Th1 cells within conventional Th cells in the liver.

Data are mean $\pm$ s.d. One-way ANOVA with Dunnett T3 multiple comparison test correction. Results represent at least three independent experiments.

Moreover, we discovered that CD4⁺ T cells isolated from Thpok^{Cre}Tgfbr2^{fl/fl} mice induced widespread autoinflammation upon transfer to sublethal irradiated Cd274^{-/-} mice (Figure 2-20, c), revealing that PD-L1 acts *in trans* to suppress Th cell-mediated immunopathology. To investigate whether hematopoietic sources of PD-L1 mediate tolerance control, we utilized bone marrow cells from WT and/or Cd274^{-/-} mice to reconstitute WT mice, followed by transfer of TGFBR2-deficient CD4⁺ T cells after sublethal irradiation (Figure 2-18, a). We found that lacking PD-L1 in hematopoietic lineage cells triggered lethal autoimmunity, which took place even in the presence of a WT hematopoietic compartment (Figure 2-18, b).

Among the hematopoietic cells, PD-L1 is constitutively expressed on B cells, macrophages, and dendritic cells (DCs) [181], and is further up-regulated upon activation [8]. In the liver APC compartment, we found that PD-L1 was highly induced in each of these three cell types in Thpok^{Cre}Tgfbr2^{fl/fl}Pdcd1^{-/-} mice (Figure 2-19), potentially due to the high degree of ongoing inflammation. Of note, up-regulation of PD-L1 under the condition of Th cell TGFBR2 single deficiency was most prominent on macrophages (Figure 2-19, b), indicating potential roles in constraining self-reactive Th cells in target tissues.

To test whether PD-L1 expression on macrophages was essential to mediate Th cell peripheral tolerance upon losing TGF- β signaling inhibition, we generated mouse models with selective deletion of PD-L1 in macrophages. For macrophage targeting, we used pan-macrophage-directed *Fcgr1*^{Cre} mice [182] to cross with a mouse strain carrying a floxed allele of PD-L1 (*Cd274*^{fl}) [33]. We confirmed the reduction of PD-L1 surface expression on liver macrophages in

Fcgr1^{Cre}Cd274^{fl/fl} mice (Figure 2-20, a). However, PD-L1 surface expression on liver DCs were also slightly reduced, possibly due to non-specific expression of *Fcgr1* in the DC lineage. To address this problem, we also generated conditional PD-L1 knockout mice in pan-DCs using the *Zbtb46^{Cre}* [183]. *Zbtb46^{Cre}Cd274^{fl/fl}* exhibited more specific PD-L1 reduction on DCs with a slight decrease on macrophages (Figure 2-20, a). We isolated total CD4⁺ T cells from *Thpok^{Cre}Tgfb²^{fl/fl}* mice and transferred to congenically marked, sublethal irradiated *Fcgr1^{Cre}Cd274^{fl/fl}* and *Zbtb46^{Cre}Cd274^{fl/fl}* recipients, with *Cd274^{-/-}* mice as a positive control (Figure 2-20, b). We found that deletion of PD-L1 from macrophages, but not DCs, resulted in a lethal inflammatory phenotype in recipients of TGFBR2-deficient T cells, mimicking PD-L1 total knockout recipients (Figure 2-20, c). *Fcgr1^{Cre}Cd274^{fl/fl}* recipients also phenocopied *Cd274^{-/-}* mice in terms of expansion of donor Th cells in lymph nodes and the liver (Figure 2-20, d). Interestingly, in terms of donor effector cell differentiation, *Zbtb46^{Cre}Cd274^{fl/fl}* recipients, but not *Fcgr1^{Cre}Cd274^{fl/fl}* recipients phenocopied *Cd274^{-/-}* recipients in the lymph node, while *Fcgr1^{Cre}Cd274^{fl/fl}* recipients phenocopied *Cd274^{-/-}* recipients in the liver (Figure 2-21). This included decreased naïve population, expanded Tfh and Th1 precursor cells in the lymph node in *Zbtb46^{Cre}Cd274^{fl/fl}* recipients, and expansion of ‘hyperactivated Th1’ cells in the liver in *Fcgr1^{Cre}Cd274^{fl/fl}* recipients. Different from the genetic deficiency of both the TGFBR2 and PD-L1/PD-1 pathway, we did not observe increase in the ‘Tfh-like Th1’ cells in the liver to be associated with lethal autoinflammation in the transfer experiments, indicating that the ‘hyperactivated Th1’ cells were more likely to be the driver for immunopathology.

Of note, adoptive transfer of conventional CD4⁺ T cells into lymphopenic mice in the absence of Treg cells can induce multi-organ autoimmunity [138] and colitis [139,140] in mice, therefore, we transferred total CD4⁺ T cells rather than conventional CD4⁺ T cells alone.

Altogether, we found there was a distinct role for each myeloid APC subset in restraining TGFBR2-deficient CD4⁺ T cells in secondary lymphoid organs and peripheral tissues. While deletion of PD-L1 in DCs promoted priming of TGFBR2-deficient T cells and their differentiation into Tfh and precursor Th1 in SLO, this was not sufficient to cause autoimmunity. In contrast, local tissue APCs, particularly macrophages, were crucial regulators of peripheral Th cell tolerance by providing PD-L1 to prevent differentiation of pathogenic Th1 cells. These observations aligned with a previous report indicating that PD-L1/PD-1 signaling constitutes a major negative feedback control circuit to desensitize Th cell restimulation in peripheral tissues [184]. In summary, PD-L1 on macrophages cooperated with TGF- β to promote peripheral Th cell tolerance in the tissue.

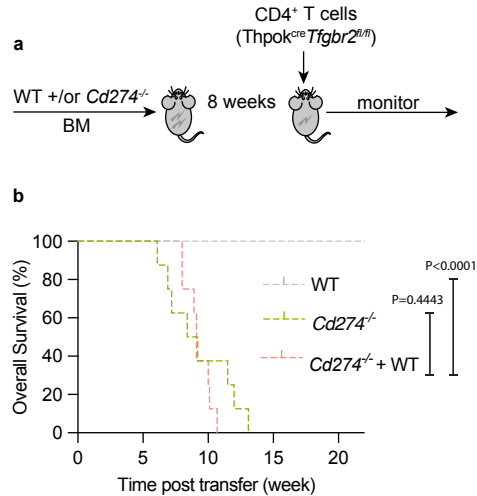


Figure 2-18 Hematopoietic sources of PD-L1 mediate tolerance control.

a: Schematics of experiment design. Briefly, congenically mismatched WT, Cd274^{-/-} or 1:1 mixed bone marrow (BM) of WT and Cd274^{-/-} are transferred to lethally irradiated WT recipients. After 8 weeks, 5,000,000 Thpok^{cre} Tgfr2^{fl/fl} CD4⁺ T cells are adoptively transferred to the recovered bone marrow chimera mice. The resulted BM chimera recipients are monitored for survival.

b: Kaplan-Meier survival analysis of recipient mice of the indicated groups. n=8 mice for each group. Log-rank test compares all three groups or the two groups excluding WT donor BM.

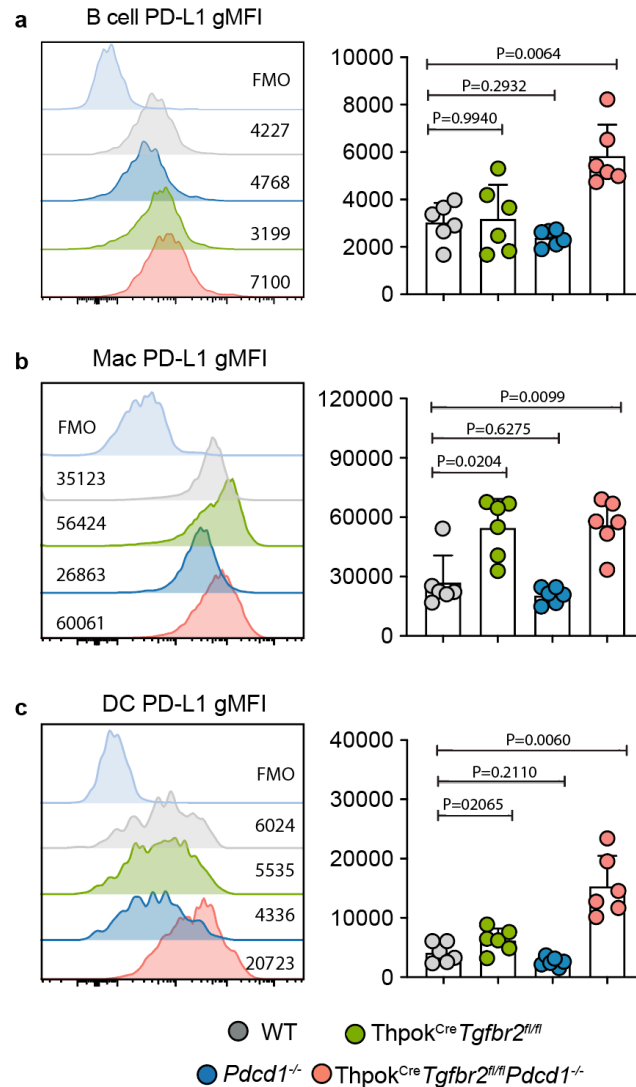


Figure 2-19 PD-L1 is upregulated in professional antigen presentation cells in Thpok^{Cre}Tgfb2^{fl/fl}Pdc1^{-/-} mice.

a-c: Representative plots and quantification of PD-L1 expression using geometric mean of fluorescence intensity (gMFI) in B cells, macrophages (Mac) and dendritic cells (DC) isolated from the liver of Thpok^{Cre}Tgfb2^{fl/fl}Pdc1^{-/-} mice of 3 to 4 week-of-age. The baseline fluorescence intensity represented by Cd274^{-/-} control is subtracted in the quantification.

Data are mean $\pm$ s.d. One-way ANOVA with Dunnett T3 multiple comparison test correction. Results represent at least three independent experiments.

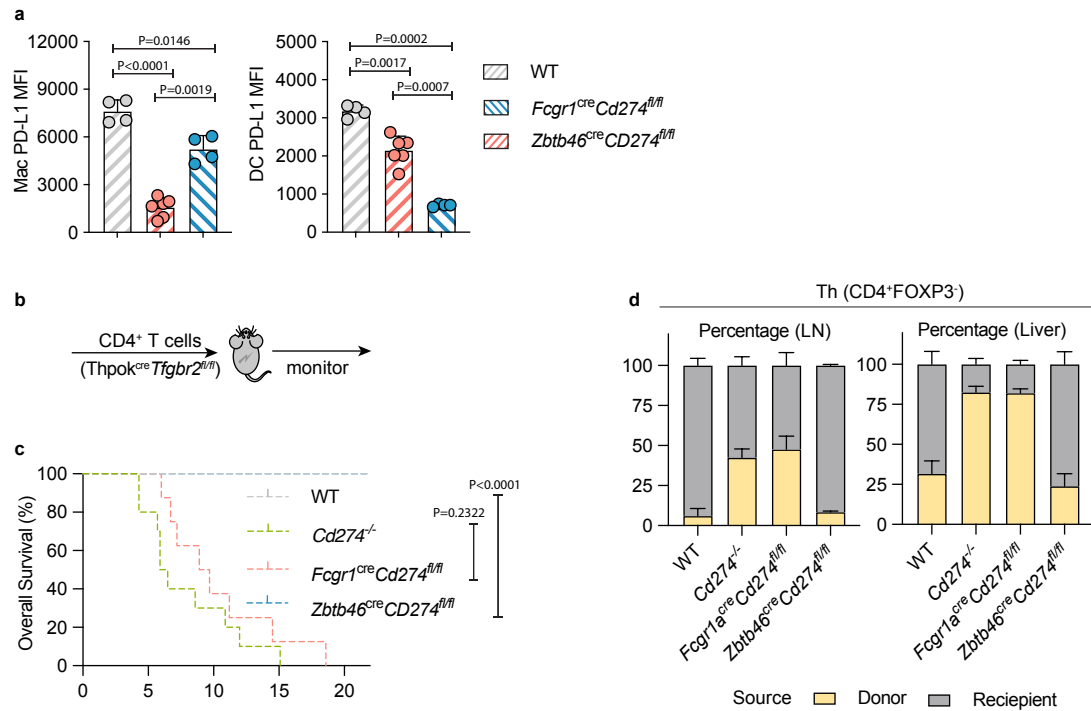


Figure 2-20 PD-L1 expression on macrophages mediates tolerance control.

a: Quantification of PD-L1 expression using geometric mean of fluorescence intensity (gMFI) in macrophages (Mac) and dendritic cells (DC) isolated from the liver of indicated genotypes of 8 to 12 week-of-age. The baseline fluorescence intensity represented by *Cd274^{-/-}* control is subtracted in the quantification.

b: Schematics of experiment design. Briefly, 5,000,000 Thpok^{cre}*Tgfr2^{fl/fl}* CD4⁺ T cells are adoptively transferred to congenically mismatched recipient mice if indicated genotype. The mice are monitored for survival.

c: Kaplan-Meier survival analysis of recipient mice of the indicated groups. WT: n=9; *CD274^{-/-}*: n=10; *Fcgr1^{cre}Cd274^{fl/fl}*: n=8; *Zbtb46^{cre}Cd274^{fl/fl}*: n=9. Log-rank test compares all four groups or the two groups including *CD274^{-/-}* and *Fcgr1^{cre}Cd274^{fl/fl}*.

d: Percentage of Th of donor or recipient origin in lymph node (LN) and liver. WT: n=4 (for LN analysis), n=5 (for liver analysis); all other groups: n=4 (both LN and liver analysis).

Data are mean $\pm$ s.d. One-way ANOVA with Dunnett T3 multiple comparison test correction. Results represent at least three independent experiments.

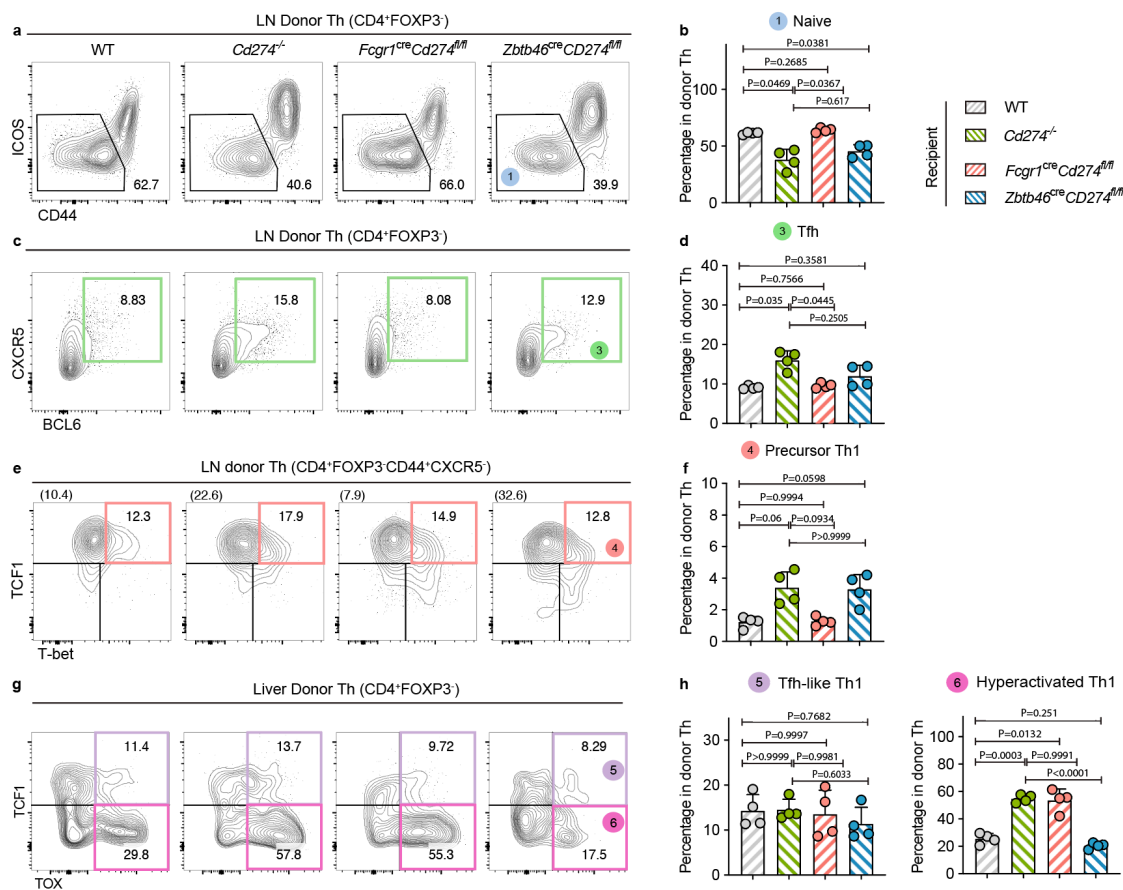


Figure 2-21 Cellular phenotype TGFBR2-deficient Th cells in PD-L1 conditional deletion recipients shown by flow cytometry analysis.

a-f: Representative plots and quantification of naïve cells, follicular T helper (Tfh) cell, type 1 T helper (Th1) precursor cells within conventional Th cells in peripheral lymph nodes (LN).

g-h: Quantification of Tfh-like Th1 cells and hyperactivated Th1 cells within conventional Th cells in the liver.

Data are mean $\pm$ s.d. One-way ANOVA with Dunnett T3 multiple comparison test correction. Results represent at least three independent experiments.

2.2.4 Monocyte-derived macrophages with an M1-like phenotype and potent antigen presenting function are recruited to the inflamed tissue.

In models of infection, APC sensing of microbial components and activities constitutes a prominent mechanism of innate immune control of adaptive T cell immunity. However, the regulation of pathological autoimmune Th cell responses by APCs remains less well understood. The pathogenic ‘hyperactivated Th1’ cells upregulated markers associated chronic TCR stimulation (Figure 2-5, b). As CD4⁺ T cell activation and differentiation requires APC guidance, we next interrogated the contribution to the unrestrained stimulation of TGFB2- and PD-1-deficient Th cells from the APC compartment. Deletion of B cells in Thpok^{Cre}Tgfb2^{fl/fl}Pdcd1^{-/-} mice by breeding with the *Ighm*^{-/-} (muMt⁻) mice failed to rescue the lethal autoimmunity (Figure 2-22). Therefore, we focused on the myeloid APC compartment.

To delineate the changes in the myeloid APCs and their roles in driving autoimmunity with TGFB2- and PD-1-deficient Th cells, we sorted CD45⁺ MHCII⁺ lineage⁻ (TCRβ⁻CD90⁻NK1.1⁻CD19⁻Siglec-F⁻Ly6G⁻) mononuclear phagocytes, from the liver of WT, Thpok^{Cre}Tgfb2^{fl/fl}, *Pdcd1*^{-/-} and Thpok^{Cre}Tgfb2^{fl/fl}*Pdcd1*^{-/-} mice for scRNA-seq profiling. In total, we obtained 7,359 cells after pre-processing, which we grouped into 5 distinct clusters and annotated using expression of known markers into major cell types [185], including two macrophage population- the liver resident Kupffer cells and the monocyte-derived macrophages (Mac_mono), as well as three DC population- type 1 DCs (DC1), type 2 DCs (DC2) and a small subset expressing high levels of *Ccr7* (DC_Ccr7) (Figure 2-23, a-b). Remarkably, TGFB2 and PD-1 double deficiency was associated with massive recruitment of

monocyte-derived macrophage at the price of decreased frequency of DC1 and Kupffer cells (Figure 2-23, c).

During inflammation, classical, “migratory” Ly6C^{hi}CCR2^{hi} monocytes enter the tissue to become classically activated (M1, proinflammatory) macrophages and, over time, transit to alternatively activated (M2, reparative) macrophages and/or integrate into a longer-lasting resident macrophage pool [186–188]. During monocyte maturation, they acquire MHCII and upregulate F4/80 and CD64 to attain the phenotype of mature macrophages [189,190]. We first confirmed the presence and expansion of MHCII⁺F4/80⁺ monocyte-derived macrophages in the liver of double-deficient mice by flow cytometry (Figure 2-23, d). To directly examine their function in antigen presentation to Th cells, we adopted an *in vivo* endogenous antigen presentation assay, using *Alb^{cre}USA^{LSL}* mice models in which a universal self-antigen (USA) expression cassette containing multiple model antigen sequences, including the OVA₃₂₃₋₃₃₉ peptide of ovalbumin, is driven by the CAG promoter downstream of an LSL sequence [191] controlled by the Albumin-cre [192] targeting hepatocytes. The *Alb^{cre}USA^{LSL}* mice were lethally irradiated and reconstituted with bone marrow cells from *Thpok^{cre}Tgfb²^{fl/fl}Pdcd1^{-/-}* mice to induce tissue inflammation and recruitment of monocyte-derived macrophages (Figure 2-24, a). We then sorted the MHCII⁺F4/80⁺Ly6C⁺ monocyte-derived macrophages loaded with OVA₃₂₃₋₃₃₉ peptides and co-cultured them with CellTrace Violet (CTV)-labeled OT-II T cells. After three days of co-culture, OT-II T cell proliferation was assessed based on CTV dilution. As expected, these monocyte-derived macrophages were potent antigen presenting cells capable of inducing the

proliferation of antigen-specific Th cells (Figure 2-24, b). These discoveries prompted us to further examine the reciprocal interaction between the monocyte-derived macrophages and the conventional Th cells.

Analysis of differentially expressed genes (DEG) and pathways between the *Thpok^{cre}Tgfb^{2fl/fl}Pdcd1^{-/-}* samples and the rest within the 'Mac_mono' population revealed that they adopted an M1-like phenotype. This included upregulation of genes downstream of interferon signaling, such as *Isg15*, *Ifitm3*, *Ifi205*, and *Irf7*, consistent with the high expression of IFN- γ from the largely expanded 'hyperactivated Th1' cells in the inflamed liver (Figure 2-5, a). The monocyte derived macrophages highly upregulated genes in antigen processing and presentation, including *H2-Aa*, *H2-Ab1*, *H2-Eb1* and *Cd74*, possibly in response to IFN- γ signaling [193]. This aligned with their potent APC functions (Figure 2-24, b), explaining the features of chronic activation observed in 'hyperactivated Th1' cells (Figure 2-5, b). Expression of and signaling from the tumor necrosis factor (TNF) superfamily was also upregulated in both cell types, including the ligand receptor pair *Tnfsf9* on macrophages and *Tnfrsf9* on T cells, and *Tnfrsf1a*, *Tnfrsf1b*, *Cd40* on macrophages, as well as *Tnfrsf4* (OX40) and *Tnfrsf18* (GITR) on T cells. We also found co-enrichment of chemokines and their reciprocal receptors on 'mac_mono' and 'hyperactivated Th1' cells, including *Cxcl9* (MIG) and *Cxcl10* (IP-10) on macrophages and *Cxcr3* on T cells; *Cxcl16* on macrophages and *Cxcr6* on T cells; *Ccl3*, *Ccl4*, *Ccl5* and their receptors *Ccr1* and *Ccr5* on both macrophages and T cells. This indicated cooperation and feedback between the Th1 cells and macrophages in recruitment and tissue homing of

additional effector cells. Notably, *Nos2*, the gene encoding the inducible nitric oxide synthase (iNOS) that functions in formation of nitric oxide (NO) was also upregulated, indicating that these monocyte-derived macrophages may directly contribute to tissue damage. It is worth noting that the power of DEG and Gene Set Enrichment Analysis were limited as the control groups did not have substantial numbers of monocyte-derived macrophages. Nonetheless, we found that monocyte-derived macrophages with an M1-like phenotype were recruited to the inflamed tissue in *Thpok^{cre}Tgfb²^{fl/fl}Pdcd1^{-/-}* mice, were potent APCs and engaged with the 'hyperactivated Th1' cells to amplify inflammation and promote tissue damage.

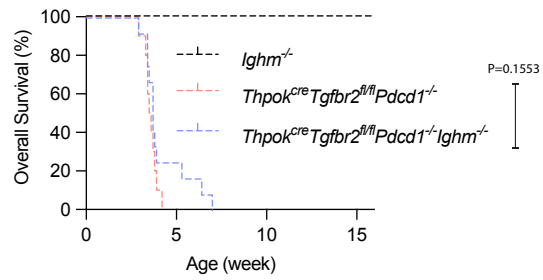


Figure 2-22 Lethal autoimmunity is not rescued by B cell deletion.

a: Kaplan-Meier survival analysis of mice of indicated genotypes. n=10 mice for each group. Log-rank test compares all four groups.

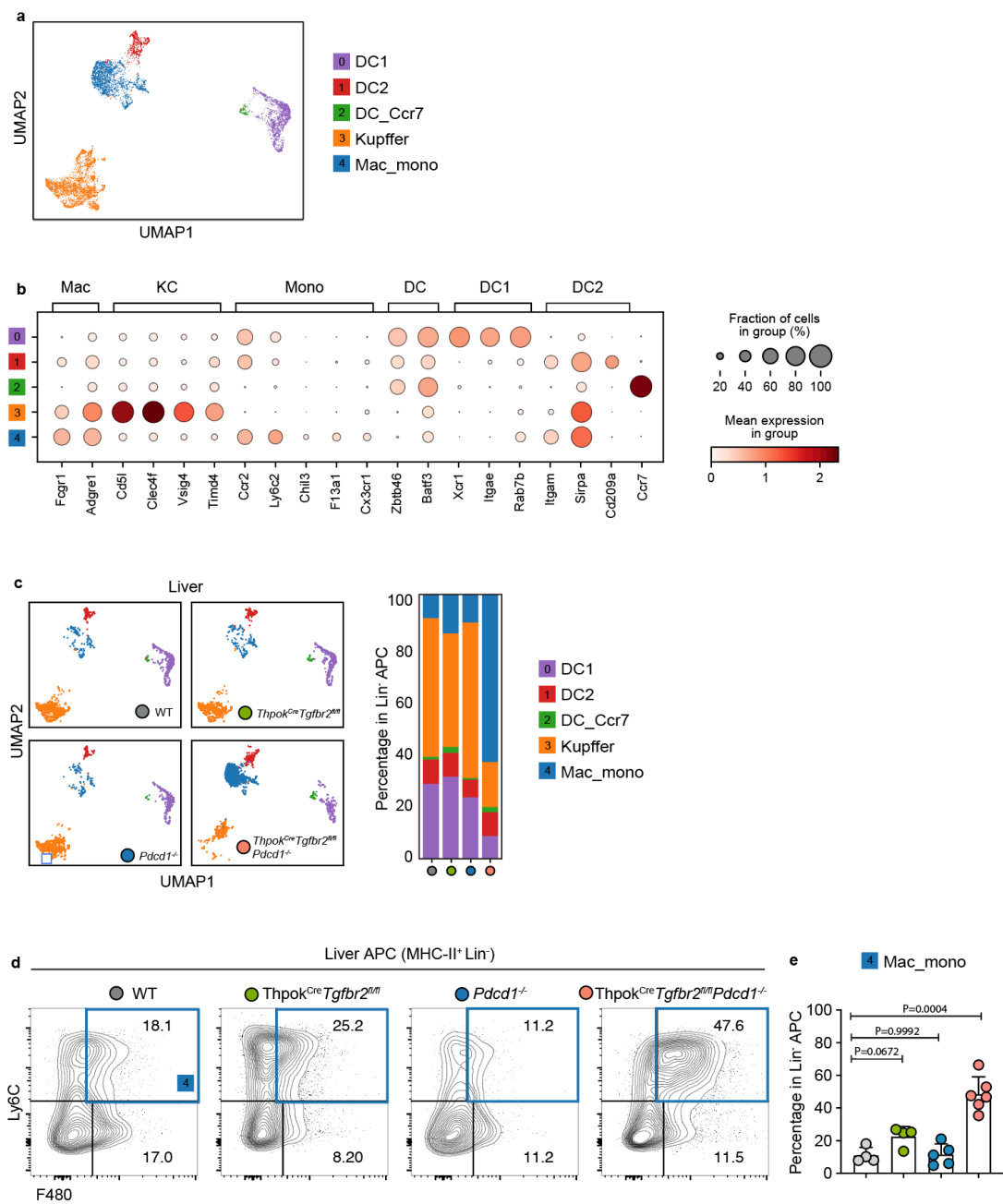


Figure 2-23 Increased recruitment of monocyte-derived macrophages in the liver of *Thpok^{Cre}Tgfb β 2^{fl/fl}Pdcd1^{-/-}* mice in scRNA-sequencing.

a: Uniform manifold approximation and projection (UMAP) plot (7,359 cells) representing MHCII⁺ myeloid antigen presenting cell (APC) populations isolated from the liver of mice of 3-week of age from the following genotypes: wild-type, *Thpok^{Cre}Tgfb β 2^{fl/fl}*, *Pdcd1^{-/-}*, *Thpok^{Cre}Tgfb β 2^{fl/fl}Pdcd1^{-/-}*. The data represent analysis of one mouse per group colored by cell subtype clusters.

b: Dotplot showing the relative expression levels of key marker genes across identified cell clusters. Dot color represents the mean expression level within the cluster and dot size reflects fraction of cells expressing the gene within the cluster.

c: (left) UMAP plots representing MHCII⁺ APC populations isolated from the liver colored by cell clusters across genotypes. (Right) Distribution of cell clusters within each genotype.

d, e: Representative plots (**d**) and quantification (**e**) of monocyte-derived macrophages gated by F/480 and Ly6C within MHCII⁺ lineage⁻ (TCR β ⁻CD90⁻NK1.1⁻CD19⁻Siglec-F⁻Ly6G⁻) APC population in the liver.

Data are presented as the mean values $\pm$ s.d. One-way ANOVA with Dunnett T3 multiple comparison test correction (**d,e**). Results represent at least three independent experiments (**d,e**).

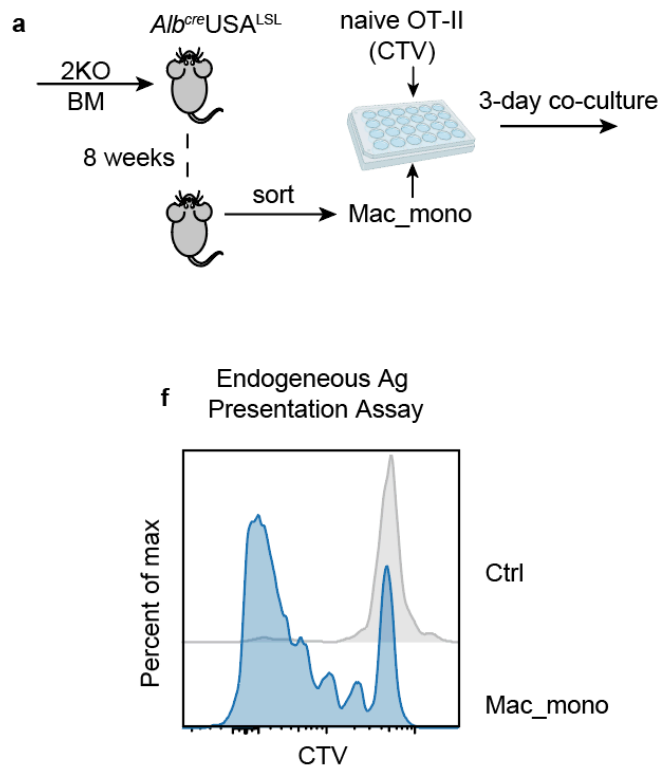


Figure 2-24 Monocyte-derived macrophages induces antigen-specific CD4+ T cell proliferation in an endogenous antigen presentation assay.

a, b: Schematics of experiment design. Briefly, Bone marrow (BM) of *Thpok^{cre}Tgfb^{r2}fl/flPdc^{d1}-/-* (2KO) is transferred to lethally irradiated *Alb^{cre}USA^{LSL}* recipients. After 8 weeks, monocyte derived macrophages (Mac_mono) are sorted from the recovered bone marrow chimera mice and cocultured with CellTrace Violet (CTV)-labeled naive OT-II T cells for 3 days before flow cytometry analysis. **b:** Representative plots of CTV fluorescence intensity in OT-II cells w/o Mac_mono coculture in the endogenous antigen (Ag) presentation assay. Results represent at least three independent experiments.

2.2.5 CD40 mediates a feedback circuit between antigen presenting cells and pathogenic Th cells to drive inflammation.

To elucidate the factors driving pathogenic Th cell responses in the absence of TGFBR2 and PD-1 signaling, particularly Th cell interaction with the monocyte-derived macrophages, we focused on CD40, a known mediator of Th cell and APC communications. The CD40 pathway was initially recognized as a critical regulator of CD4 T cell help of humoral immunity [194,195]. Later, it was also identified to offer essential help to myeloid APC (DC and macrophage)-mediated cellular immunity [196,197]. CD40 are expressed on APCs and subjected to tight transcriptional regulation, and its ligand CD40L (CD154, encoded by *Cd40lg*) is limited to the Th cell lineage [126] and is constitutively expressed in naïve Th cells [198]. CD40L has been shown to be stored in secretory lysosomes and released to the cell surface following antigen stimulation in effector and memory Th cells [199,200]. Notably, both CD40 gene and surface protein expression (Figure 2-25) are induced in monocyte-derived macrophages in *Thpok^{cre}Tgfb^{fl/fl}Pdcd1^{-/-}* mice compared to the controls. To test the functional contribution of the CD40 pathway in driving lethal autoimmunity, we crossed *Thpok^{cre}Tgfb^{fl/fl}Pdcd1^{-/-}* mice to *Cd40^{-/-}* background. Surprisingly, CD40 deficiency reversed tissue pathology (Figure 2-26, b) and led to a complete rescue of early lethality of *Thpok^{Cre}Tgfb^{fl/fl}Pdcd1^{-/-}* mice (Figure 2-26, a).

Remarkably, CD40 deficiency largely reduced the excessive recruitment of monocyte-derived macrophages in the liver, as shown by the decrease in population frequency in the scRNA-seq data (Figure 2-27, a) and flow cytometry quantification (Figure 2-27, b). CD40 deficiency also set back the gene programs

related to M1-like macrophage polarization (Figure 2-27, c). In the T cell compartment, scRNA-seq (Figure 2-28 , a-b) and flow cytometry experiments (Figure 2-29, a-b) revealed that CD40 deficiency reversed the expansion of ‘Tfh-like Th1’ and ‘hyperactivated Th1’ cells in the liver. Clonal expansion was also reduced in *Thpok^{cre}Tgfb²^{fl/fl}Pdcd1^{-/-}Cd40^{-/-}* mice (Figure 2-28, d). These findings demonstrated that the CD40L/CD40 signaling axis was crucial to fuel a feedback circuit between macrophages and effector Th1 cells that led to unrestrained inflammation in the tissue.

Interestingly, conventional Th cells in the CD40 deficient background phenotypically resembled those found in *Thpok^{cre}Tgfb²^{fl/fl}* mice as shown in neighborhood enrichment analysis (Figure 2-28, c), with more pronounced expansion of ‘T_{RM}-like Th1’ cells and ‘T_{EM}-like Th1’ cells (Figure 2-28, a) confirmed by flow cytometry (Figure 2-29, b). This suggested that the PD-L1/PD-1 signaling blocked TGFBR2-deficient Th cell differentiation into pathogenic Th1 effectors via antagonizing the CD40L/CD40 axis between Th cells and APCs. Indeed, previous studies showed that CD40L is stored in secretory lysosomes in effector and memory Th cells [199,200]. The transfer of CD40L from Th cells to APCs through trans-synaptic vesicles happens in a contact-, CD40- and antigen-dependent manner [201,202], and is inhibited by PD-L1 trans-presentation [203]. Given that PD-L1 expression on macrophages was highly induced following TGFBR2 deletion and functionally required to keep those Th cells in check as demonstrated in the previous section, we concluded that PD-L1 on macrophages and TGF- β act in cooperation in the tissue to maintain peripheral Th cell tolerance by breaking a

feedback circuit with myeloid APCs, most likely macrophages, steered by CD40 signaling.

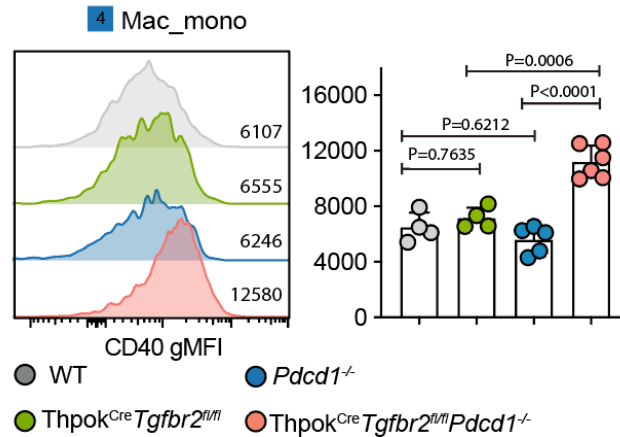


Figure 2-25 CD40 is upregulated in monocyte-derived macrophages in *Thpok*^{Cre}*Tgfr2*^{fl/fl}*Pdc1*^{-/-} mice.

Representative plots and quantification of CD40 expression using geometric mean of fluorescence intensity (gMFI) monocyte-derived macrophages (Mac_mono) isolated from the liver of *Thpok*^{Cre}*Tgfr2*^{fl/fl}*Pdc1*^{-/-} mice of 3 to 4 week-of-age. Data are mean ± s.d. One-way ANOVA with Dunnett T3 multiple comparison test correction. Results represent at least three independent experiments.

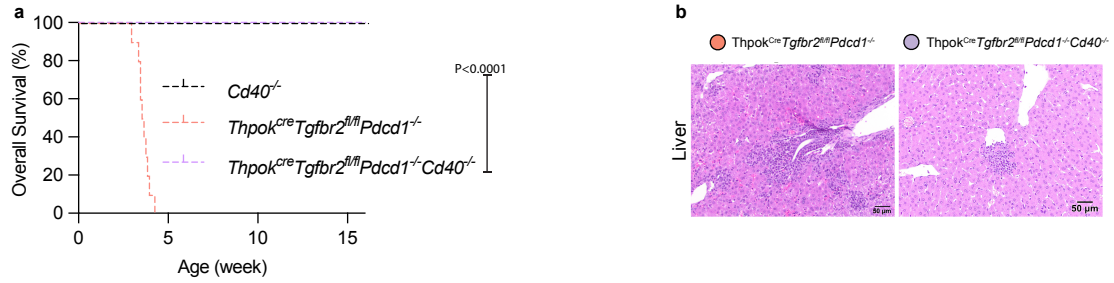


Figure 2-26 CD40 deletion rescues lethal autoimmunity in $Thpok^{Cre}Tgfb2^{fl/fl}Pdcd1^{-/-}$ mice.

a: Kaplan-Meier survival analysis of mice of indicated genotypes. n=10 mice for each group. Log-rank test compares all four groups.

b: Representative hematoxylin and eosin (H&E) sections of liver and lung from mice of indicated genotypes. The scale bar is 50 μ m.

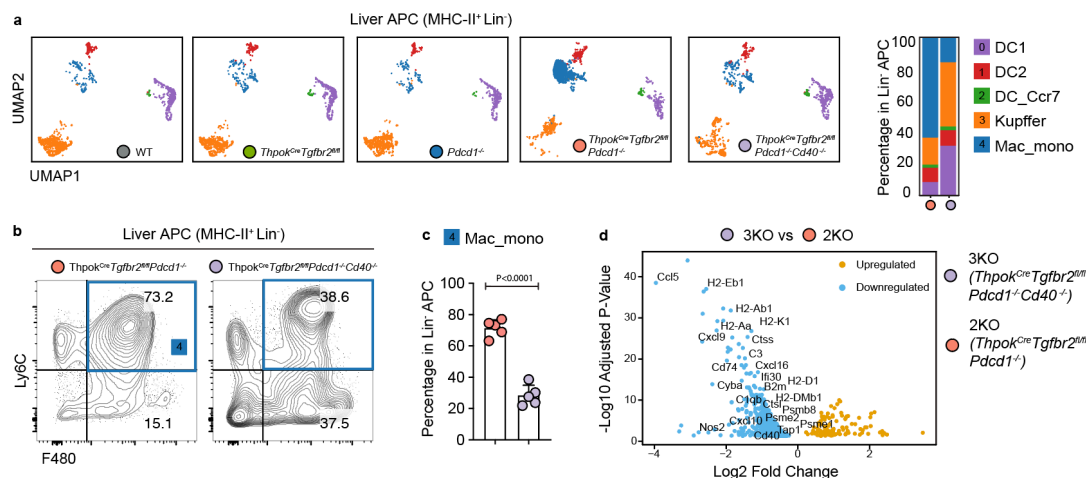


Figure 2-27 CD40 deficiency reverts expansion and polarization of monocyte-derived macrophages.

a: (left) UMAP plots representing MHCII⁺ APC populations isolated from the liver colored by cell clusters across genotypes. (Right) Distribution of cell clusters within each genotype.

b, c: Representative plots (**b**) and quantification (**c**) of monocyte-derived macrophages gated by F/480 and Ly6C within MHCII⁺ lineage⁻ (TCRβ⁻CD90⁻NK1.1⁻CD19⁻Siglec-F⁻Ly6G⁻) APC population in the liver.

d: (left) Volcano plot showing the differential expression analysis results of genes between Mac_mono of Thpok^{cre}Tgfr2^{fl/fl}Pdc1^{-/-} and Thpok^{cre}Tgfr2^{fl/fl}Pdc1^{-/-}Cd40^{-/-}. Key antigen presentation genes and effector genes are labeled.

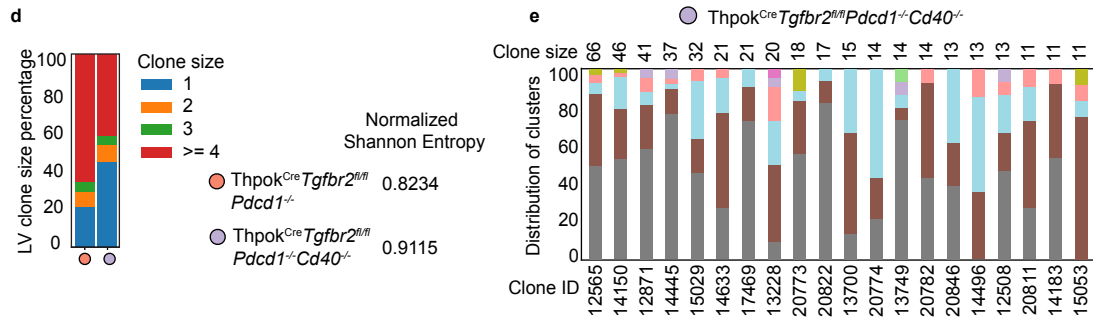
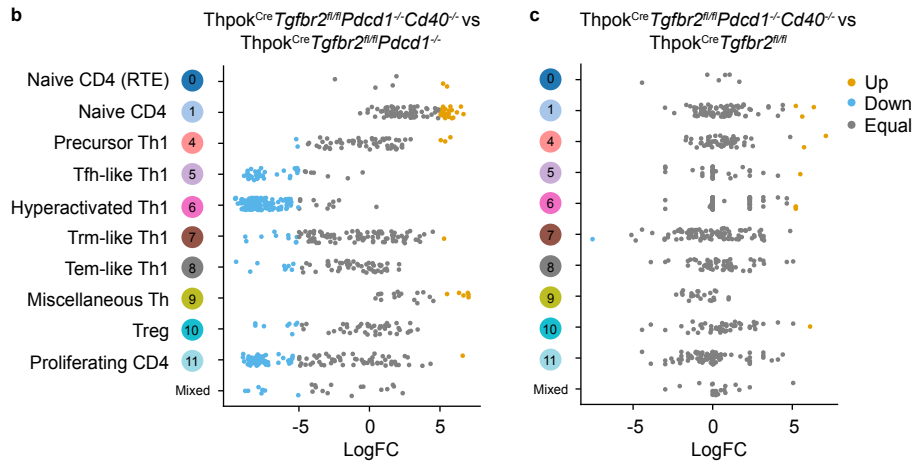
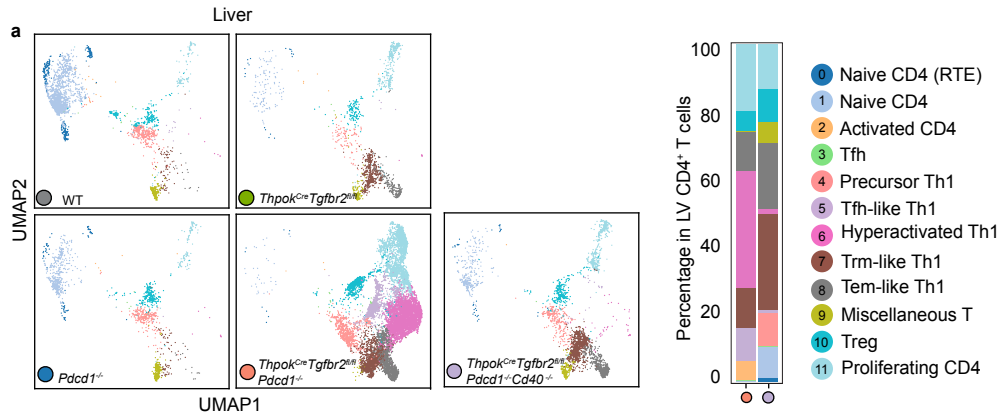


Figure 2-28 Th cells in the CD40 deficient background phenotypically resembled those found in $\text{Thpok}^{\text{cre}}\text{Tgfb}\beta 2^{\text{fl/fl}}$ mice shown by scRNA analysis.

a: (left) UMAP plots representing CD4^+ T cell populations isolated from the liver colored by cell clusters across genotypes. (Right) Distribution of cell clusters within each genotype.

b, c: Swarm plots depicting the log fold change of differential cell type abundance in the pair of indicated comparisons in each neighborhood across different cell subtypes in the liver samples. Each dot represents a neighborhood computed using MiloR and is color-coded by the magnitude of the log fold change with a cutoff value of 5. A neighborhood is classified as a cell type if it comprises at least 50% of cells in the neighborhood, otherwise it is called 'mixed'.

d: Distribution of clones of different sizes within each indicated genotype in the liver samples, with normalized Shannon entropy.

e: Distribution of cell clusters within each clone in the top 20 expanded clones by size in the liver sample of each indicated genotype, arranged by clone sizes.

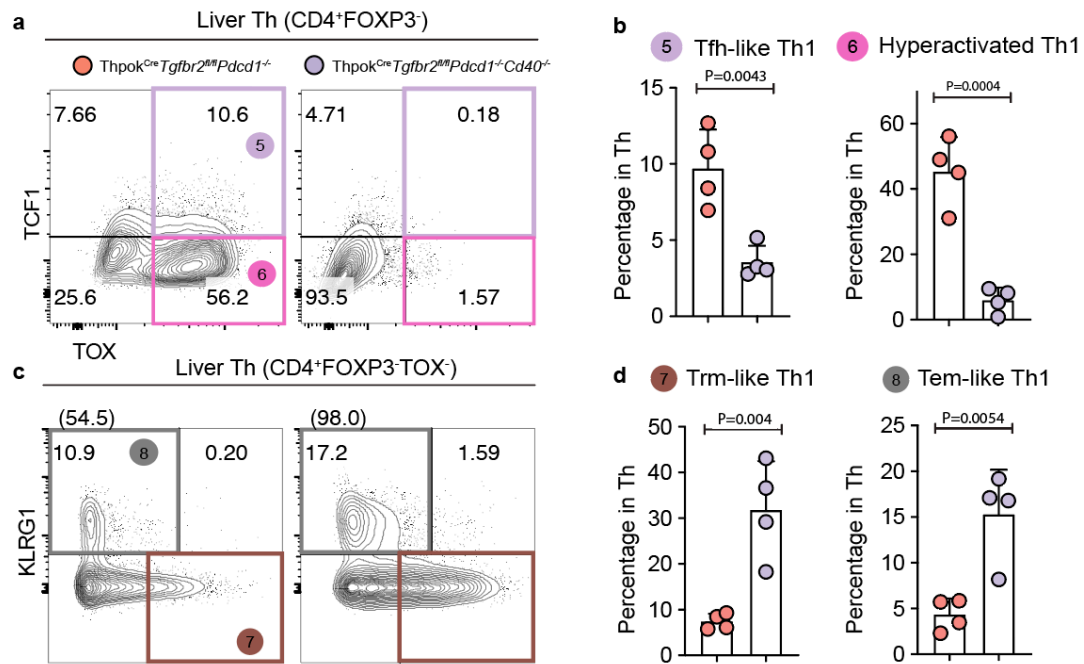


Figure 2-29 Th cells in the CD40 deficient background phenotypically resembled those found in *Thpok^{Cre}Tgfb²^{fl/fl}* mice shown by flow cytometry analysis.

a, b: Representative plots (**a**) and quantification (**b**) of Tfh-like Th1 cells and hyperactivated Th1 cells gated by TCF1 and TOX expression within CD4⁺Foxp3⁻ T helper (Th) cells of indicated genotypes the liver from mice of 3 to 4 week-of-age. **c, d:** Representative plots (**c**) and quantification (**d**) of resident memory like (Trm-like) Th1 cells and effector memory like (Tem-like) Th1 cells gated by CXCR6 and KLRG1 expression within CD4⁺Foxp3⁻TOX⁻ Th cells in the liver. Percentage of the parent population for each genotype is labeled at the top. Data are mean ± s.d. One-way ANOVA with Dunnett T3 multiple comparison test correction. Results represent at least three independent experiments.

2.2.6 Pronounced differentiation of potent cytotoxic T lymphocytes are dispensable for lethal autoimmunity.

In autoimmunity, CD4⁺ T cells are considered the primary mediators of disease. However, similar to infection, CD4⁺ T cells can 'help' CD8⁺ T cells by upregulating B7 costimulatory molecule expression on APCs to augment T cell activation and by secreting multiple immunoregulatory cytokines, such as IL-2, to support CD8⁺ T cell proliferation. CD8⁺ T cells mediate critical cytotoxic effector functions in autoimmunity to damage host tissues and augment inflammation. To further delineate the effector response following TGFBR2-conditional deletion in PD-1 deficiency, we examined the cellular changes of CD8⁺ T cells across genotypes. Similar to conventional Th cells, CD8⁺ T cell number was increased in both the SLO and peripheral tissue, but to a less magnitude and without skewed expansion in the tissue, with only around 5-fold increment in the lymph node, liver and lung (Figure 2-30, a). Of note, PD-1 total deficiency alone did not promote CD8⁺ T cell expansion.

The synergy in cell expansion seen in Thpok^{cre}Tgfr2^{fl/fl}Pdcd1^{-/-} mice are likely to be secondary to the enhanced activation and proliferation of conventional Th cells. Therefore, we focused on assessing CD8⁺ T cells in the tissue, where they can cause direct damage and exacerbate autoimmunity. Similar to Th cells, CD8⁺ T cell downregulate TCF1 and upregulate TOX during chronic antigen stimulation [29,204]. However, while in cancer and chronic viral infection, CD8⁺ T cells undergo a progressive loss of cytokine production and cytotoxicity in a dysfunctional state of T cell 'exhaustion', autoreactive CD8⁺ T cells retain the capacity to effectively mediate the destruction of host tissues in autoimmunity

despite chronic antigen exposure [205]. Therefore, we first characterized the function of each of the population within the quadruple gate of TCF1⁺TOX⁻, TCF1⁻TOX⁻, TCF1⁺TOX⁺ and TCF1⁻TOX⁺ of CD8⁺ T cells in Thpok^{cre}Tgfbr2^{fl/fl}Pdcd1^{-/-} mice by assessing granzyme B expression (Figure 2-30, b-c). The terminally differentiated TCF1⁻TOX⁺ population exhibited the highest levels of granzyme B expression, followed by the TCF1⁻TOX⁻ intermediate effector populations. The TCF1⁺TOX⁺ 'stem-like' progenitor cells showed lower levels, while the least expression was observed in the less activated naïve TCF1⁺TOX⁻ cells. Remarkably, the most potent TCF1⁻TOX⁺ was significantly expanded at the expense of TCF1⁺TOX⁻ cells in Thpok^{cre}Tgfbr2^{fl/fl}Pdcd1^{-/-} mice, with a slight expansion in Pdcd1^{-/-} mice and no changes in Thpok^{cre}Tgfbr2^{fl/fl} mice (Figure 2-30, d).

We then asked whether the prominently expanded monocyte-derived macrophages with upregulated transcriptional programs in antigen cross presentation could be facilitating CTL differentiation. As the Alb^{cre}USA^{LSL} mice also express the SIINFEKL peptide of ovalbumin, we again performed the endogenous antigen presentation assay by sorting monocyte-derived macrophages and co-cultured them with CTV-labeled OT-I cells (Figure 2-31, a). The monocyte-derived macrophages induced OT-I proliferation during the 3-day co-culture period (Figure 2-31, b), validating their capability in cross presentation to CTLs. Consistent with this observation, Thpok^{cre}Tgfbr2^{fl/fl}Pdcd1^{-/-}Cd40^{-/-} mice without the expanded monocyte-derived macrophage population failed to induce CD8⁺ T cell differentiation into effector TCF1⁻TOX⁺ CTLs (Figure 2-31, c).

However, despite the pronounced expansion of effector CTL population, genetic deletion of CD8⁺ T cells did not rescue lethal autoimmunity, with only minimal extension of the lifespan (Figure 2-31, d). This indicated that the enhanced effector CTL response was likely to be an outcome rather than cause of the rampant autoinflammation in *Thpok^{cre}Tgfb^{fl/fl}Pdcd1^{-/-}* mice. It was possible that while the deficiency of the two pathways synergized to enable a feedback circuitry between pathogenic ‘hyperactivated Th1’ cells and monocyte-derived macrophages via CD40 signaling, the unrestrained activation of both cells also feedforwarded to further promote CD8⁺ T cell effector function, potentially through antigen cross presentation by highly functional macrophages polarized toward M1 type (Figure 2-32). However, removing CTLs from the system had minimal effect on the feedback circuitry of Th and macrophages, thus failed to rescue the lethal autoimmunity. These discoveries further supported our idea that Th cells were the primary drivers of autoimmunity and that TGFBR2 and PD-1 served as conventional Th cell-intrinsic tolerance regulators in cooperation.

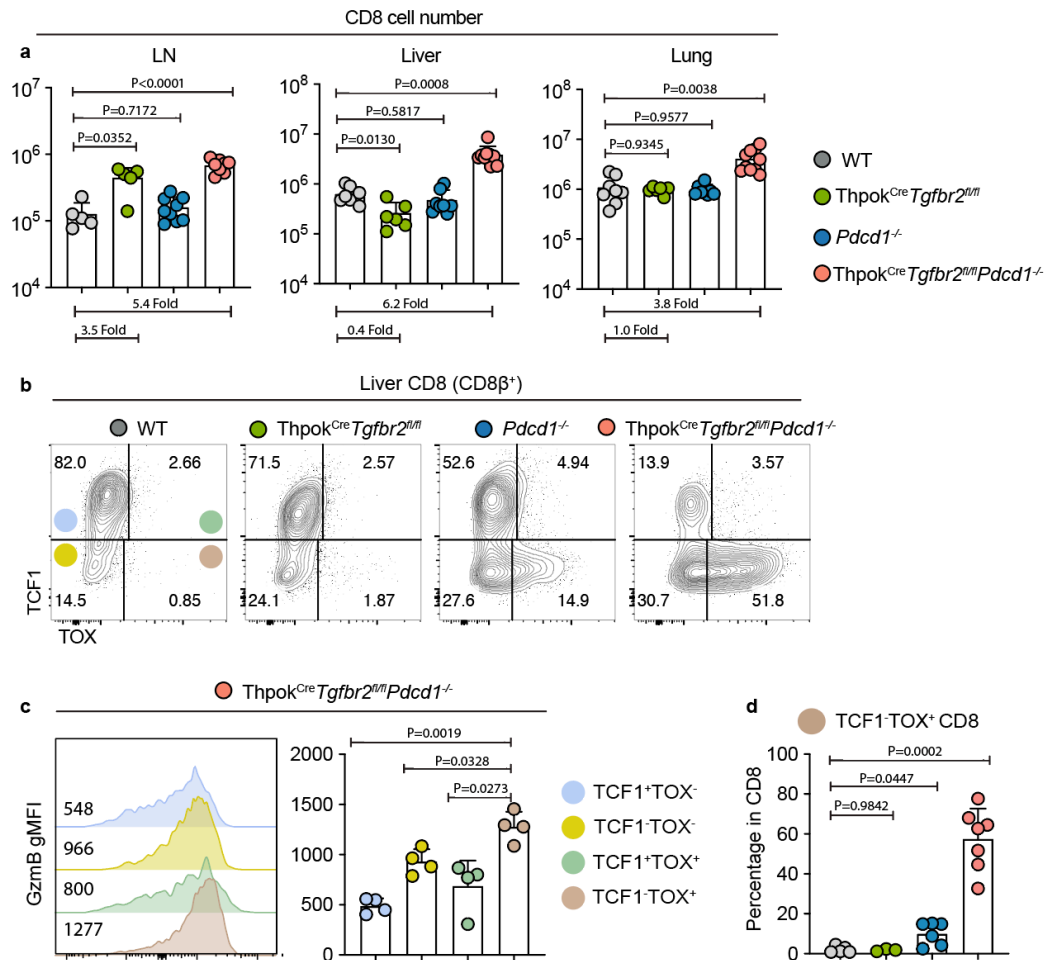


Figure 2-30 CD8⁺ T cells exhibit enhanced effector function in *Thpok^{Cre}Tgfb2^{fl/fl}Pdc1^{-/-}* mice.

a: Quantification of CD8⁺ T cell number in LN (per lymph node), liver (per gram of tissue), and lung (whole tissue) from wild-type (WT), *Thpok^{Cre}Tgfb2^{fl/fl}*, *Pdc1^{-/-}* and *Thpok^{Cre}Tgfb2^{fl/fl}Pdc1^{-/-}* mice of 3 to 4-week of age.

b, d: Representative plots (**b**) and quantification (**d**) of CD8⁺ T cell population gated by TCF1 and TOX expression in the liver of indicated genotypes of 3 to 4 week-of-age.

c: Representative plots and quantification of granzyme B expression using geometric mean of fluorescence intensity (gMFI) of CD8⁺ T cell population gated by TCF1 and TOX isolated from the liver of *Thpok^{Cre}Tgfb2^{fl/fl}Pdc1^{-/-}* mice of 3 to 4 week-of-age.

Data are mean ± s.d. One-way ANOVA with Dunnett T3 multiple comparison test correction. Results represent at least three independent experiments.

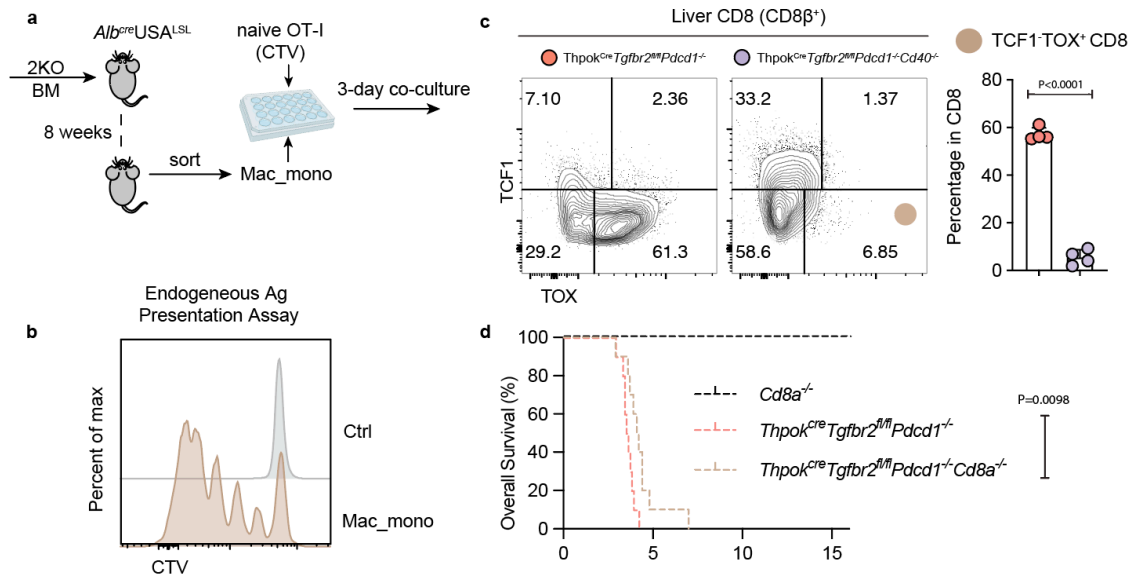


Figure 2-31 CD8⁺ T cells are dispensable for lethal autoimmunity.

a, b: Schematics of experiment design. Briefly, Bone marrow (BM) of *Thpok^{cre}Tgfb2^{fl/fl}Pdcd1^{-/-}* (2KO) is transferred to lethally irradiated *Alb^{cre}USA^{LSL}* recipients. After 8 weeks, monocyte derived macrophages (Mac_mono) are sorted from the recovered bone marrow chimera mice and cocultured with CellTrace Violet (CTV)-labeled naive OT-I T cells for 3 days before flow cytometry analysis.

b: Representative plots of CTV fluorescence intensity in OT-I cells w/o Mac_mono coculture in the endogenous antigen (Ag) presentation assay. Results represent at least three independent experiments.

c: Representative plots and quantification of CD8⁺ T cell population gated by TCF1 and TOX expression in the liver of indicated genotypes of 3 to 4 week-of-age.

d: Kaplan-Meier survival analysis of mice of indicated genotypes. n=10 mice for each group. Log-rank test compares all four groups.

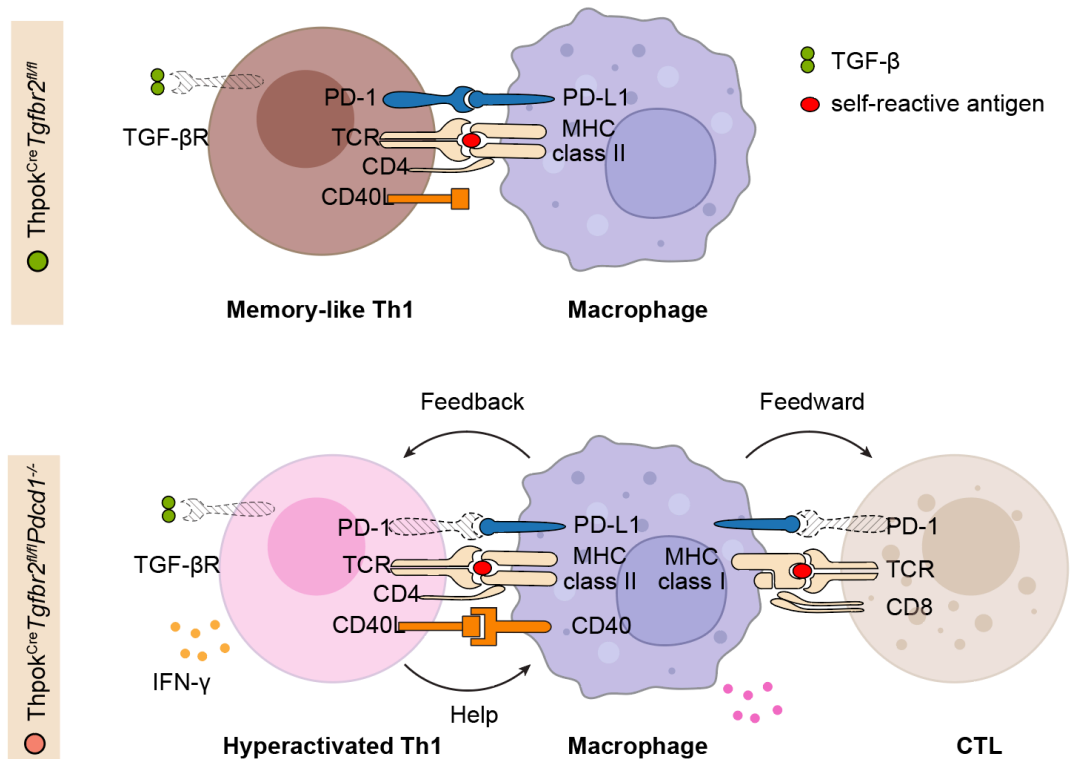


Figure 2-32 Model of feedback-feedforward loop between CD4⁺ T cells, myeloid antigen presenting cells and CD8⁺ T cells.

(Upper) TGFBR2-deficient self-reactive Th cells are inhibited by PD-L1 signaling on macrophages.

(Lower) TGFBR2 and PD-1 double deficient self-reactive Th cells enter a feedback loop with myeloid antigen presenting cells, likely M1-like monocyte-derived macrophages through CD40L-CD40 signaling. The unrestrained activation of both cells feedforwards to further promote CD8⁺ cytotoxic lymphocyte (CTL) effector function, potentially through antigen cross presentation.

2.3 Discussion

The adaptive immune system is distinguished by its unlimited antigen recognition specificity, amplification of innate immune responses, novel effector functions, and memory formation, providing mammals with a versatile anticipatory defense against rapidly evolving infectious agents. However, this superior protection comes with a significant fitness constraint due to the risk of autoimmune inflammation, which arises from the inherent self-reactivity of T cells. Conventional Th cells orchestrate host defense responses against immunological challenges, whereas self-reactive Th cells that escape negative selection require multiple layers of tolerance regulation to prevent immune pathology. The regulatory cytokine TGF- β promotes Th cell tolerance from multiple perspectives [93–95]. Previous studies have demonstrated that TGF- β promotes Th cell self-tolerance largely independent of Treg cells in transgenic models of autoimmune diabetes [96]. However, the significance of TGF- β signaling in regulating peripheral Th cell tolerance on a polyclonal TCR background remains contentious. This uncertainty stems from observations that mice with a blockade of TGF- β signaling initiated before, but not after, thymic selection exhibit rampant autoimmunity [98,99]. Here, we showed that the absence of an overt autoimmune phenotype in mice with blockade of TGF- β signaling in mature CD4⁺ T cells was due to induction compensatory tolerance mechanisms to repress self-reactive T cells. The collaborative action of TGF- β and PD-L1 inhibitory pathways constitutes a Th cell-intrinsic peripheral tolerance mechanism, rivaling the significance of Treg cell-mediated Th cell-extrinsic controls.

Of note, although $\text{Thpok}^{\text{cre}}$ spares TGFBR2 deletion in thymic Tregs, absence of TGF signaling might impede peripheral Treg differentiation and Treg cell function [142,206]. However, transferring 1:1 mixed bone marrow of $\text{Thpok}^{\text{Cre}}\text{Tgfb}r2^{\text{fl/fl}}\text{Pdc}d1^{-/-}$ mice and WT mice failed to alleviate the lethal autoimmune phenotype in the presence of WT Treg cells, suggesting that the lethal autoimmunity was likely not due to loss of function of Treg cells in the absence of TGF- β or PD-1 signaling, but rather gain of function of conventional Th cells. Indeed, we found Treg proliferation was enhanced with TGFBR2 deficiency, consistent with previous reports [99,207]. Moreover, CD4^+ T cells isolated from $\text{Thpok}^{\text{Cre}}\text{Tgfb}r2^{\text{fl/fl}}$ mice, but not WT mice, induced widespread lethality upon transfer to $\text{Cd}274^{-/-}$ mice that had even a functionally enhanced Treg cell compartment [35]. These findings underscored a pivotal Th cell-intrinsic peripheral tolerance mechanism mediated by TGF- β and PD-L1 signaling.

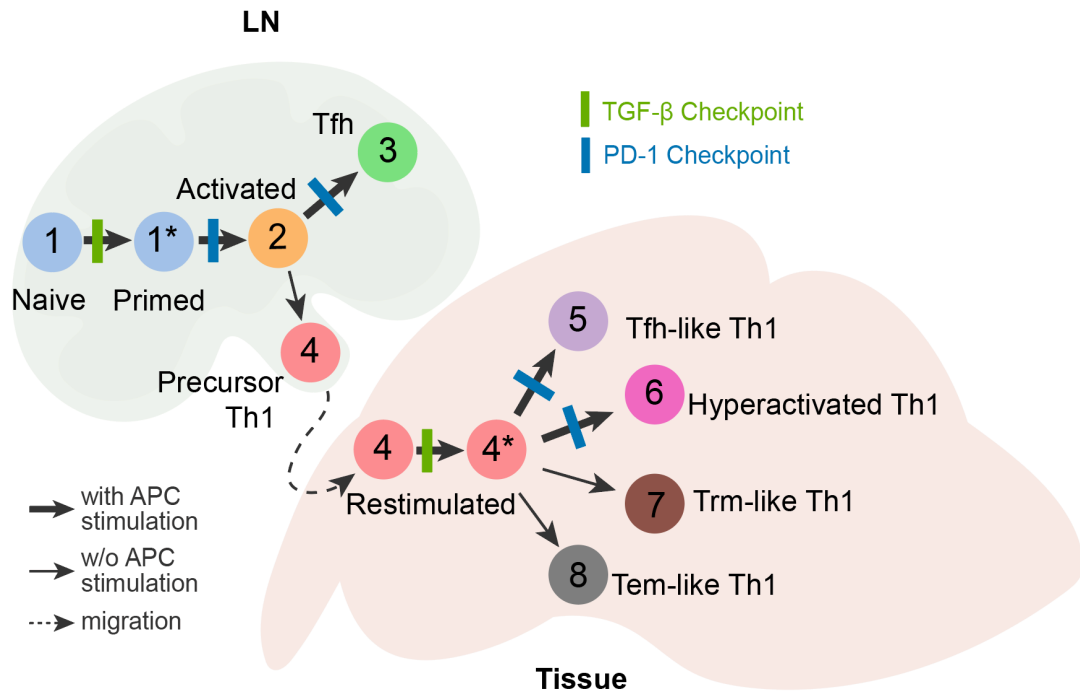


Figure 2-33 Model of cooperative regulation of Th cell by TGF- β and PD-1.

A schematic to depict TGF- β and PD-1 checkpoints in Th cell tolerance control. TGF- β inhibits 'naïve T' cell priming in secondary lymphoid organs and 'precursor Th1' cell re-stimulation in the liver, whereas PD-1 inhibits later stages of Th cell activation and differentiation to 'Tfh', 'Tfh-like Th1', and 'hyperactivated Th1' cells, all in the context of antigen presenting cell stimulation.

In the SLO, TGF- β directly inhibited priming of autoreactive CD4⁺ T cells, while PD-1 suppressed functional activation and downstream differentiation of Tfh cells, both of which required robust interaction with APCs (Figure 2-32). Some of the activated autoreactive cells then adopted Th1 effector program and seeded the peripheral tissues as precursor Th1 cells. In the tissue, TGF- β prevented reactivation of these cells, while PD-1 signaling blocked their further differentiation into pathogenic Th1 effectors, although the effector T cell fates was also partially determined by antigen specificity. In both the lymphoid organ and the tissue, PD-1 function was prominently manifested under the condition of TGF- β signaling blockade, which aligned with the induction of PD-1 expression in TGFBR2-deficient conventional CD4⁺ T cells (Figure 2-1). Previous studies have indicated that TGF- β inhibits proximal TCR signaling via the inhibitory tyrosine kinase CSK to prevent T cell priming or reactivation [208,209]. Conversely, PD-1-mediated inhibition primarily involves recruitment of the tyrosine phosphatase Shp2 to impair TCR, and potentially CD28, signaling [26,27,210–214], providing the molecular basis for the collaborative function of the two pathways. These findings supported the premise that the TGF- β and PD-L1/PD-1 coinhibitory pathways serve as ‘tonic’ and ‘reactive’ regulators, respectively [102], working together to promote peripheral Th cell tolerance by inhibiting effector ‘Tfh’, ‘Tfh-like Th1’, and ‘hyperactivated Th1’ cell differentiation.

The latter two processes occur within target tissues, expanding beyond the one-step Th cell bifurcation model observed in secondary lymphoid organs [47]. Interestingly, we found that blocking TGF- β signaling in CD4⁺ T cells synergized

with PD-1 deficiency to drive a skewed expansion of conventional Th cells in peripheral tissues, resulting in a more dramatic fold increase and clonal expansion. Consistently, we observed that PD-L1 on macrophages, a tissue APC, cooperated with TGF- β to promote peripheral Th cell tolerance within the tissue. Indeed, each myeloid APC subset played a distinct role in restraining TGFBR2-deficient CD4⁺ T cells in SLOs and peripheral tissues. Conditional PD-L1 deletion in DCs promoted the priming of TGFBR2-deficient T cells and their differentiation into Tfh and precursor Th1 cells in SLOs but was not sufficient to cause autoimmunity. In contrast, local tissue APCs, particularly macrophages, were crucial regulators of peripheral Th cell tolerance by providing PD-L1 to prevent the differentiation of pathogenic Th1 cells. These observations emphasized a major negative feedback control circuit via PD-L1/PD-1 signaling to desensitize Th cell restimulation in peripheral tissues that was crucial peripheral tolerance, as supported by previous discoveries [184].

In the lethal autoimmunity associated with transferring TGFBR2-deficient CD4⁺ Th into PD-L1 deficient or macrophage-specific PD-L1 conditional knockout mice, we only observed increase of the 'hyperactivated Th1' cells but not 'Tfh-like Th1' cells in the liver, indicating that the 'hyperactivated Th1' cells were more likely to be the driver for immunopathology. These pathogenic cells upregulated markers associated chronic TCR stimulation, indicating active interactions with APCs. Concomitantly, we found that monocyte-derived macrophages with an M1-like phenotype were recruited to the inflamed tissue, differentiated into potent APCs and engaged with the 'hyperactivated Th1' cells to amplify inflammation and

promote tissue damage. CD40 deficiency largely reduced the excessive recruitment of monocyte-derived macrophages in the liver, while reversing the expansion of 'hyperactivated Th1' cells, in parallel with rescued lethal autoimmunity. These findings pointed to a feedback loop between macrophages and effector Th1 cells fueled by the CD40L/CD40 signaling axis that finally led to irreversible tissue damage. However, while we confirmed through an *ex vivo* endogenous antigen presentation assay that these monocyte-derived macrophages could efficiently present antigen to CD4⁺ and even CD8 T⁺ cells, we did not have direct evidence to prove that CD40 signaling, and the feedback circuitry between Th cells and myeloid APCs, were exclusive to macrophages or monocyte-derived macrophages. In fact, CD40 expression was upregulated in all the APC compartment, including B cells, DCs and Kupffer cells (data not shown) other than the monocyte-derived macrophages. In an attempt to address whether macrophages were the functional effectors of CD40 signaling, we transferred CD4⁺ T cells from Thpok^{cre}Tgfb^{2^{fl/fl}}Pdcd1^{-/-} mice into sublethally irradiated mice with CD40 conditional deletion [128] in macrophages or DCs, with WT and CD40^{-/-} recipients as negative and positive controls. We predicted that Th cells deficient for both TGFBR2 and PD-1 could induce lethal autoimmunity only in WT, but not CD40^{-/-} or macrophage-specific CD40 deficient mice. However, this experiment system failed to address our questions, as CD40^{-/-} recipients also died after transfer (data not shown). This was possibly due to the overt inflammatory environment created by tissue damage following irradiation, the TGFBR2 and PD-1- double deficient Th cells were too potent, or the number of transferred cells were

too high. Nonetheless, to address this question in future experiments, one can resume to the system that we have successfully established by transferring TGFB β 2-deficient CD4 $^{+}$ T cells from Thpok cre Tgfb β 2 $^{fl/fl}$ donors into sublethally irradiated mice with conditional double deficiency of PD-L1 and CD40 in macrophages. The prediction will be that further CD40 deficiency rescues the lethal autoimmunity and the expansion of ‘hyperactivated Th1’ cells seen in macrophage-specific PD-L1 conditional deletion recipients.

Beyond the feedback circuitry between effector Th1 cells and myeloid APCs, we also found evidence for feedforward stimulation of CD8 $^{+}$ T cells in Thpok cre Tgfb β 2 $^{fl/fl}$ Pdcd1 $^{-/-}$ mice, including pronounced expansion of effector CTL population capable of high level of granzyme production. However, the CTLs likely promoted tissue damage at the late stage of, or had minor effect on autoinflammation, as deletion of CD8 $^{+}$ T cells only minimally extended mice survival. It also indicated that the PD-L1/PD-1 co-inhibitory signaling prominently targeted self-reactive Th cells to maintain homeostatic tolerance, despite the receptor was deleted on both CD4 $^{+}$ and CD8 $^{+}$ T cells. Indeed, CD8-specific deletion of TGFB β 2 on Pdcd1 $^{-/-}$ did not cause severe autoimmunity as seen in Thpok cre Tgfb β 2 $^{fl/fl}$ Pdcd1 $^{-/-}$ mice (data not shown). This discovery further emphasized the role of Th cells as primary drivers of autoimmunity.

Two outstanding questions remain for this project. The first is to determine whether the lethal inflammatory disease observed in Thpok cre Tgfb β 2 $^{fl/fl}$ Pdcd1 $^{-/-}$ mice is contingent upon neonatal lymphopenia. Previous research argued that lymphopenia predisposes TGFB β 2-deficient Th cell-induced immunopathology

primarily through Treg cell-independent mechanisms [98,99]. This predisposition likely contributes to lethal autoimmunity in neonatal mice deleted of TGFBR2 in developing T cells, as TGF- β regulates thymic T cell selection to promote survival of low-affinity TCR CD4⁺ T cells [145]. The absence of this regulation would exacerbate the lymphopenic environment in neonatal mice. Similarly, although PD-1-deficient mice develop a lupus-like disease later in life [215], the transfer of PD-1-deficient thymocytes into lymphopenic recipients leads to a MHCII-dependent inflammatory disease independent of Treg cells [216–218]. Among the mechanisms governing peripheral T cell tolerance, Treg cell-mediated suppression stands out as a crucial pathway for restraining autoreactive Th cells in both neonatal and adult mice [85]. To address whether the Th cell-intrinsic TGF- β and PD-L1 signaling controls peripheral self-tolerance throughout life, rather than specific to the neonatal lymphopenic period, one can use the tamoxifen-inducible ThPOK^{CreERT2.hCD2} mice [219] with floxed alleles of *Tgfb2* and *Pdcd1* to delete the two receptors on peripheral Th cells in adult mice. If these mice also develop lethal autoinflammation due to excessive activation of Th cells upon tamoxifen treatment, it will generalize the function of Th cell-intrinsic TGF- β and PD-L1 signaling in peripheral tolerance maintenance throughout life.

The second question is to ascertain that the massively expanded effector Th1 cells causing rampant inflammation in Thpok^{cre}*Tgfb2*^{fl/fl}*Pdcd1*^{-/-} mice were self-reactive. To test this hypothesis, as we had identified the top expanded clones in the liver in one of those mice from scTCR-seq, one can clone some of these TCRs and establish ‘retrogenic’ bone-marrow chimera mice with these TCRs to

study their reactivity [170]. We anticipate that TCRs from clonally expanded liver-infiltrating T cells will exhibit self-reactivity during coculture experiments with DCs from liver-draining lymph nodes.

Overall, in this project, we defined a crucial peripheral tolerance mechanism which primarily operated through inhibiting Th cell-intrinsic type 1 cellular immunity driven by CD40-expressing myeloid APCs. We provided a deeper comprehension of Th cell regulation by cooperative TGF- β and PD-1 signaling that can potentially aid in the development therapeutics targeting Th cells to ameliorate pathology associated with autoimmune diseases and bolster protective immunity in chronic infections and cancer.

2.4 Experimental Procedures

2.4.1 Mice

Experiments in this study were approved by the Sloan Kettering Institute (SKI) Institutional Animal Care and Use Committee under protocol 07-06-008 and conducted in compliance with institutional ethics guidelines. Mice were housed at the SKI animal facility under specific pathogen-free (SPF) conditions on a 12-hour light/dark cycle with free access to water and regular chow diet. The average ambient temperature is 21.5°C and the average humidity is 48%. All control and experimental animals were age-matched, and littermates were used as controls when possible. Unless mentioned otherwise, both male and female mice were included. Investigators were not blinded to group allocation and outcome assessment. No statistical methods were used to predetermine sample size and the experiments were not randomized.

OT-I, OT-II, *Ighm*^{-/-} (muMt) and *Cd274*^{-/-} mice were purchased from Jackson Laboratory. *Tgfb β 2*^{fl} [145], *Thpok*^{Cre} [154], *Pdcd*^{-/-} [156], *Cd274*^{fl} [33], *Zbtb46*^{Cre} [183], *Fcgr1*^{Cre} [182], *Alb*^{cre} [192], *USA*^{LSL} [191] mice were previously reported.

2.4.2 Reagents and antibodies

The following antibodies and streptavidin were used in this study for flow cytometry, with clones, vendors, catalog numbers, and dilutions indicated in the parentheses: anti-Siglec-F (E50-2440, BD, 562681, 400), anti-I-A/I-E (M5/114.15.2, BioLegend, 107641, 1000), anti-NK1.1 (PK136, ThermoFisher, 47-5941-82, 200), anti-CD45 (30-F11, BioLegend, 103136, 600), anti-CD11b (M1/70, BioLegend, 101257, 800), anti-CD3 ϵ (17A2, BioLegend, 100237, 500), anti-Ly-6C (HK1.4, BioLegend, 128037, 1000), anti-CD90.2 (30-H12, BioLegend, 105331, 1000), anti-Foxp3 (FJK-16s, ThermoFisher, 17-5773-82, 400), anti-CD19 (6D5, BD, 563557, 400), anti-Ly-6G (1A8, BioLegend, 127618, 500), anti-TCR β (H57-597, ThermoFisher, 61-5961-82, 400), anti-F4|80 (BM8, ThermoFisher, 61-4801-82, 200), anti-CD90.1 (HIS51, ThermoFisher, 17-0900-82, 200), anti-CD4 (RM4-5, BioLegend, 100553, 400), anti-CD8 α (53-6.7, BioLegend, 100759, 400), anti-GITR (DTA-1, ThermoFisher, 48-5874-82, 400), anti-CD73 (TY/11.8, BioLegend, 127215, 400), anti-CD44 (IM7, BD, 563971, 400; ThermoFisher, 48-0441-82, 400; BioLegend, 103049, 400), anti-CD103 (M290, BD, 564322, 200), anti-CD62L (MEL-14, BioLegend, 104438, 1600; BioLegend, 104441, 400), anti-CTLA4 (UC10-4B9, BioLegend, 106314, 600), anti-Helios (22F6, BioLegend, 137218, 400), anti-Ki-67 (16A8, BioLegend, 652420, 400), anti-CD25 (PC61.5, ThermoFisher, 47-0251-82, 400; ThermoFisher, 17-0251-82, 400), anti-PD-1 (29F.1A12, BioLegend, 135225, 400), anti-ICOS (7E.17G9, ThermoFisher, 12-9942-82, 400), anti-CD45.1 (A20, BioLegend, 110738, 100), anti-CD45.2 (104, BD, 612778, 200), anti-IL-2 (JES6-5H4, BioLegend, 503818, 400), anti-IL-17A (17B7,

ThermoFisher, 48-7177-82), anti-IFN γ (XMG1.2, ThermoFisher, 17-7311-82, 500), anti-IL-4 (BVD6-24G2, ThermoFisher, 25-7042-82, 400), anti-GARP (YGIC86, ThermoFisher, 25-9891-82, 200), anti-CD119 (2E2, BD, 550482, 100; GR20, BD, 740032, 100), anti-EpCAM (G8.8, BioLegend, 118227, 100; BD, 740281, 100), anti-CD124 (mIL4R-M1, BD, 742172, 100), anti-TCR V β 5.1, 5.2 (MR9-4, BioLegend, 139508, 400), anti-TCR V β 6 (RR4-7, ThermoFisher, 46-5795-82, 300), anti-TCR V β 8 (F23.1, BD, 553860, 400), streptavidin (BioLegend, 405225, 4000; ThermoFisher, 61-4317-82, 4000), anti-CD45R/B220 (RA3-6B2, ThermoFisher, 47-0452-82, 400). CellTrace Violet was purchased from Thermo Fisher Scientific.

2.4.3 Methods

Isolation of cells from lymphoid organs, livers and lungs

For flow cytometric analyses, animals were perfused with a total of 10 mL 2 mM PBS-EDTA through portal vein or right ventricles immediately after euthanasia. Cells were retrieved from peripheral (axillary and inguinal) lymph nodes and livers by meshing the organs through a 70 μ m strainer (Corning, 07-201-432) with a syringe plunger for lymphocyte analysis. For myeloid cell analysis, liver was manually dissociated using a razor blade and digested with Collagenase D (1 mg/ml; Sigma-Aldrich) for 30 min at 37°C in RPMI. The lung was manually dissociated using a razor blade and digested for 1 hour in Collagenase Type III (1 mg/ml; Worthington) and DNase I (4 μ g/ml; Sigma-Aldrich) in Hanks' balanced salt solution at 37°C with periodic vortex. After collagenase treatment, cells isolated from the liver and lung were filtered through a 70- μ m cell strainer, layered in a 44 and 66% Percoll gradient (Sigma-Aldrich), and centrifuged at 1900g for 30 min without brake. Cells at the interface were collected and analyzed by flow cytometry. Erythrocytes in the liver and lung samples were lysed with ACK lysis buffer [150 mM NH₄Cl (Sigma-Aldrich, A9434), 10 mM KHCO₃ (Sigma-Aldrich, P7682), 0.1 mM Na₂EDTA, pH 7.4].

For bone marrow, bones were collected from limbs of mice, cleaned, sterilized, mashed with a mortar and pestle, and passed through a 70- μ m cell strainer before transfer.

Flow Cytometry

Cells were stained with Ghost Dye Violet 510 (Tonbo, 13-0870), Ghost Dye Red 780 (Tonbo, 13-0865), or Zombie NIR Flexible Viability Kit (Biolegend, 423106) in PBS for 10 minutes at 4°C to help identify dead cells. Cell surface staining was performed by incubating cells with antibody cocktail for 30 min on ice in the presence of purified anti-Mouse CD16/CD32 (2.4G2, Tonbo, 70-0161) in staining buffer [0.5% (w/v) BSA, 2 mM EDTA, 10 mM HEPES, 0.02% NaN₃ (Sigma-Aldrich, S2002) in 1x PBS]. CCR7 staining was incubated in 37 °C for 30 min prior to cell surface staining. For intracellular antibodies, the Tonbo or ThermoFisher Transcription Factor Fix/Perm Kit was used to fix and permeabilize cells for 30 min on ice, followed by intracellular staining for 30 min on ice. Secondary antibodies with fluorochrome-conjugation were used for the staining of purified antibodies.

Samples were recorded on an LSR II cytometer (BD) using the BD FACS DIVA software v8.0 or an Aurora cytometer (Cytek) using the SpectroFlo software v2.2.0.3 and analyzed with FlowJo v10.6.1 (BD).

Stimulation for cytokine assessment

To measure cytokine production after *ex vivo* restimulation, single cell suspensions were incubated at 37°C for 3-4 hours with 5% CO₂ in 96-well flat-bottom plates in the presence of 50 ng/mL phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich, P8139) and 500 ng/mL ionomycin (Sigma-Aldrich, I0634) with 1 µg/mL brefeldin A (Sigma-Aldrich, B6542) and 2 µM monensin (Sigma-Aldrich, M5273) to inhibit ER and Golgi transport.

FACS cell sorting

Immune cells isolated from tissues were resuspended in MACS buffer (1x PBS with 1% FBS and 100 U/ml Penicillin G and 0.1 mg/ml Streptomycin) with appropriate cell surface marker antibodies and incubated for 30 minutes on ice. Cells were washed 2x in MACS buffer and stained with LIVE/DEAD kit (Invitrogen) or Zombie Live/Dead kit (BioLegend) to exclude dead cells. Cells were sorted using the BD FACS Aria Cell Sorter. Samples were collected in RPMI with 10% FBS and processed immediately after sorting.

Histopathology

Mice were euthanized by CO₂ asphyxiation and cardiac exsanguination. Complete necropsies were performed at the Laboratory of Comparative Pathology (MSK, the Rockefeller University, and Weill Cornell Medicine). Representative sections from all organ systems including heart, thymus, lungs, liver, gallbladder, kidneys, pancreas, stomach, duodenum, jejunum, ileum, cecum, colon, lymph nodes (mandibular, mesenteric), salivary glands, skin (trunk and head), urinary bladder, uterus, cervix, vagina, ovaries, oviducts, testes, epididymes, seminal vesicles, prostates, adrenal glands, spleen, thyroid gland, esophagus, trachea, spinal cord, vertebrae, sternum, femur, tibia, stifle joint, skeletal muscle, nerves, skull, nasal cavity, oral cavity, teeth, ears, eyes, pituitary gland, and brain were fixed in 10% neutral-buffered formalin, processed in alcohol and xylene, embedded in paraffin, sectioned (5- μ m-thick) and stained with hematoxylin and eosin. The skull, spinal column, sternum, and hindlimb were decalcified in a formic acid and

formaldehyde solution (Surgipath Decalcifier I, Leica Biosystems, Wetzlar, Germany) before processing. All tissues were examined by at least two board-certified veterinary pathologists (SEC, RM, MA). Representative images of tissues were captured using a brightfield BX45 microscope with a DP26 camera and cellSens (version 1.18) Dimension software (Olympus America, Center Valley, PA).

Adoptive transfer of T cells

Naïve CD4⁺ T cells from Thpok^{cre}Tgfb²^{fl/fl} (CD45.1/CD45.2 or CD45.2/CD45.2) mice were purified from the spleen and peripheral (brachial, axillary, and inguinal) lymph nodes using EasySep™ Mouse Naïve CD4⁺ T Cell Isolation Kit (STEMCELL™ TECHNOLOGIES) according to manufacturer's instructions. The isolated cells were then transferred to congenically mismatched, sublethally irradiated (450 Gyz) recipients via intravenous injection. A total number of 5×10⁶ cells were transferred into each recipient. Liver and lymph nodes were analyzed 6 to 8 weeks post transfer.

Bone marrow chimera

Recipient mice (8 to 12 weeks old) were irradiated with 900 Gyz. Eighteen hours after irradiation, bone marrow was harvested from donor mice and transferred intravenously into recipient mice. In mixed bone marrow chimera setting, the harvested bone marrow cells from Thpok^{cre}Tgfb²^{fl/fl}Pdcd1^{-/-} mice (CD45.2/CD45.2) and wild-type mice (CD45.1/CD45.1 or CD45.1/CD45.2) were depleted for erythrocytes with ACK lysis buffer [150 mM NH₄Cl (Sigma-Aldrich,

A9434), 10 mM KHCO₃ (Sigma-Aldrich, P7682), 0.1 mM Na₂EDTA, pH 7.4], counted, and mixed at 1:1 ratio, before co-transferring intravenously into wild-type recipient mice congenically mismatched for both donors. Mice were analysed 6 to 8 weeks after transfer.

Endogenous antigen presentation assay

Naïve OT-I or OT-II cells were isolated from the spleen or peripheral lymph nodes of OT-I or OT-II TCR-transgenic mice, using negative selection with magnetic beads, as per kit's instructions. T cells were stained with CellTrace Violet and plated in 96 U-bottom wells (15,000 T cells/well), co-cultured with various ratios of monocyte-derived macrophages sorted from the liver of chimeric mice in T cell media (RPMI supplemented with 10% FCS, 1 mM sodium pyruvate, non-essential amino acids [Gibco], 10 mM Hepes, 55 µM 2-Mercaptoethanol, 100 U/mL Penicillin G and 0.1 mg/ml Streptomycin) for 72 h at 37°C. Cells were then analyzed by flow cytometry.

Library Preparation and Single cell RNA sequencing

Library preparation and sequencing for the paired scRNA-Seq and scTCR-seq were performed using 10X genomics Chromium Single Cell 5' Library & Gel bead Kit V3 according to manufacturer's protocol. Cells, with a mean viability of 75%, were encapsulated in microfluidic droplets at a dilution of ~60 cells/µL with the multiplet fraction being 3.5%. After the RT step, the barcoded-cDNA was purified with DynaBeads, followed by 12-cycles of PCR-amplification (98°C for 180

s; [98°C for 15 s, 67°C for 20 s, 72°C for 60 s] x 12-cycles; 72°C for 60 s). Next, 50 ng of PCR-amplified barcoded cDNA was fragmented with the reagents provided in the kit and purified with SPRI beads to obtain an average fragment size of 600 bp. The fragmented DNA was ligated to sequencing adapters which was then indexed with PCR (98°C for 45 s; [98°C for 20 s, 54°C for 30 s, 72°C for 20 s] x 10 cycles; 72°C for 60 s). The resulting DNA library was double-size purified (0.6-0.8X) with SPRI beads and sequenced on an Illumina NovaSeq 6000 System (R1 – 26 cycles, i7 – 8 cycles, R2 – 96 cycles) at a depth of 210 million reads per sample (average reads per single cell being 31,000 and average reads per transcript 3.96 – 4.10), with a median sequencing saturation of 74%.

Data Pre-processing

Fastq files were processed using Cell Ranger v3.0 (10x Genomics) and reads were aligned to the mouse genome mm10 from ENSEMBL GRCm38 that was modified to include sequences corresponding to the coding region. Cells containing over 5% mitochondria-derived transcripts were filtered out. Cells with total molecule counts below 1000, as determined by the lower mode of the distribution of total molecules per cell, were additionally filtered out to remove putative empty droplets. Genes that were expressed in more than 10 cells were retained for further analysis. The resulting count matrices were normalized to median library size, where library size is defined as total molecule counts per cell. The normalized data are then log transformed as $\log(\text{counts} + 1)$ for downstream analysis.

Principal component analysis

For dimensionality reduction and visualization of data, we further excluded genes with very low or very high expression of transcripts (log average expression <0.02 or >3 and dispersion >0.15), and principal component analysis was then performed on the log-transformed normalized data. Using 40 principal components, where the number of principal components was determined by the knee-point in eigenvalues, yielded a good representation of the variability in the data.

Clustering and downstream analysis

The following methods were used in the analysis of scRNA-seq and scTCR-seq: PhenoGraph [162] for clustering, Milo [171] for neighborhood enrichment analysis, Palantir [180] for trajectory inference, and Scirpy for TCR clonality analysis [172].

CHAPTER 3 TARGETING PERIPHERAL T HELPER CELL-INTRINSIC TOLERANCE CONTROL BY TGF- β AND PD-L1 IN CANCER IMMUNOTHERAPY

3.1 Introduction

Recent advances in cancer immunotherapy- ranging from immune checkpoint blockade therapy to adoptive cellular therapy and vaccines- have revolutionized cancer treatment paradigms, yet the variability in clinical responses to these agents has motivated intense interest in developing new therapeutic strategies to target effector populations with more specificity as our understanding of the tumor immune landscape grows.

TGF- β signaling is associated with non-response to immune checkpoint blockade in patients with advanced cancers [43]. However, the pleiotropic roles of TGF- β in the context of cancer make it a prominent, but complex, target for therapy. Although TGF- β can promote cancer cell invasion and dissemination, its tumor suppressor functions can be observed in advanced cancers [220]. Furthermore, while mesenchymal stromal cell TGF- β signaling inhibits T cell infiltration into tumors [221–224], blockade of TGF- β signaling in fibroblasts induces forestomach carcinoma as a consequence of excessive inflammation-induced DNA damage [225,226]. Additionally, systemic blockade of TGF- β signaling can trigger cardiac damage [227], limiting safety profiles of some drug programs. Despite these constraints, several TGF- β -targeting programs are being developed for cancer immunotherapy. Combining checkpoint blockade with inhibitors of TGF- β have demonstrated promising preclinical findings and inspired multiple clinical trials of

therapeutic agents that target TGF- β [228–231] in combination with checkpoint inhibition.

Robust Th cell help is crucial for protective immunity against cell transformation and microbial infection by assisting both humoral and cellular immunity, including that mediated by CTLs. They have been increasingly appreciated as a direct target of immune checkpoint blockade therapy [232]. In the second chapter, I presented our findings that TGF- β and PD-L1 redundantly controlled peripheral Th cell tolerance by suppressing effector Th cell responses, making Th cell TGF- β signaling an attractive therapeutic target to be used in combination with checkpoint blockade targeting the PD-L1/PD-1 pathways. Previously, we have developed a therapeutic approach to blockage of TGF- β signaling in CD4⁺ T cells using a bispecific receptor decoy [233]. The bispecific antibody, namely CD4 TGF- β Trap (4T-Trap), contains ibalizumab, a non-immunosuppressive CD4 antibody, linked to the TGFBR2 extracellular domain. The pharmacological properties, targeting specificity and efficacy of 4T-trap had been evaluated as a single therapeutic agent in preclinical breast cancer models using transgenic mice expressing human CD4 (hCD4), as ibalizumab does not cross react to mouse CD4 [233]. However, whether it can synergize with anti-PD-L1 or anti-PD-1 to enhance antitumor immunity through Th cell-mediated responses remains unknown.

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related mortality worldwide [234]. The emergence of combination therapy, atezolizumab (anti-PD-L1) and bevacizumab (anti-VEGF) has revolutionized the management

of HCC. Despite this breakthrough, the best overall response rate with first-line systemic therapy is only about 30% [235], owing to intra-tumoral heterogeneity, complex tumor microenvironment and relatively low mutational burdens [236]. Thus, there remains a large unmet medical need for novel and effective therapeutic strategies against HCC. Noticeably, CD4⁺ T cells bear the potential to be targeted for immunotherapy in low mutation-tumors lacking neoantigens recognizable by CTLs, as they can mobilize the innate immune effectors [237]. However, the function of Th cells is poorly understood in HCC.

In the second part of my thesis, we examined the efficacy of a combination therapy using 4T-Trap and anti-PD-L1 in enhancing Th cell immunity and inhibiting tumor growth in a poorly immunogenic HCC model. Despite the protective role of the TGF- β and PD-L1 signaling-controlled Th cell-intrinsic tolerance module in preventing autoimmunity under homeostatic conditions, we found that targeting this module can enhance protective immunity against cancer. Our results showed an increase in Th1 effector responses and monocyte-derived macrophage activity in a CD40-dependent manner, which also boosted the tumor-reactive CTL response. Thus, pharmacological co-inhibition of both pathways revitalizes protective immunity in cancer.

3.2 Results

3.2.1 4T-Trap and anti-PD-L1 cotreatment inhibits tumor development in an HCC model unresponsive to checkpoint blockade.

HCC is the predominant form of primary liver cancer, with limited response to immune checkpoint blockade therapies [238]. Two of the most frequently altered genes in patients with HCC are the oncogene *MYC* (amplified in 17% of HCCs) and the tumor suppressor *TP53* (deleted or mutated in 33% of HCCs) [239]. The complexity of TGF- β -regulated immune cell circuitries, as well as the contextual roles of TGF- β signaling in cancer cells and tumor stromal cells, necessitates the use of rigorous experimental systems that closely recapitulate human cancer, such as autochthonous tumor models [43], to uncover the underlying immunobiology. To study HCC, we have established a murine autochthonous HCC model through somatic gene engineering [239] (Figure 3-1, a), using vectors depleting the tumor suppressor gene *p53* and inducing stable expression of the oncogene *MYC* via the *SB* transposon system, delivered through tail vein hydrodynamic injection targeting hepatocytes. The transposon vector expressing *MYC* also expresses luciferase (*MYC-Luc*), which is only mildly immunogenic [240], allowing for monitoring tumor progression with luciferase imaging. In this low mutational HCC model, C57BL/6J mice can survive around 30 to 60 days post induction without treatment, and fail to respond to anti-PD-1 therapy as demonstrated previously [239]. We found that luciferase signal peaked around 5 days post tumor induction, potentially due to high level of transient expression of the plasmid, and remained low until around day 20 (Figure 3-1, b), when luciferase signal started to rise again until the mice succumb to death. This indicated that cell transformation with stable integration of

the oncogenes happened between day 5 and 20, when the immune system failed to clear the actively growing tumor. Thus, we started cotreatment with 4T-Trap and anti-PD-L1 within this time window at day 14 and continued until week 6, when the control mice started to die. As 4T-trap bears a human CD4 paratope, we used the transgenic hCD4 mice to evaluate its function. We checked luciferase signal at day 5 to ensure similar injection and efficiency and vector expression before treatment group allocation. hCD4 mice had no differences in tumor growth compared with controls (data not shown).

Cotreatment with 4T-Trap and anti-PD-L1 effectively inhibited tumor development, as shown by bioluminescence imaging at day 45 (Figure 3-1, c). Single treatment of each arm did not render similar therapeutic effect, suggesting a synergy between TGF- β and PD-L1 blockade. Of note, 4T-Trap and anti-PD-L1 cotreatment in WT mice without hCD4 to allow targeted inhibition of TGF- β pathway on CD4⁺ T cells failed to generate equal response, suggesting that the synergy between blocking the two pathways required targeting TGF- β inhibition to CD4⁺ T cells. Consistent with this discovery, we also found that Bintrafusp alfa, a bispecific antibody targeting TGF- β via anti-PD-L1 [241] (Figure 3-1, a), failed to induce protective synergy of the two pathways (Figure 3-1, d). Therefore, we found targeted blockade of TGF- β on CD4⁺ T cells synergized with anti-PD-L1 in antitumor immunity in a low mutational burden autochthonous HCC model.

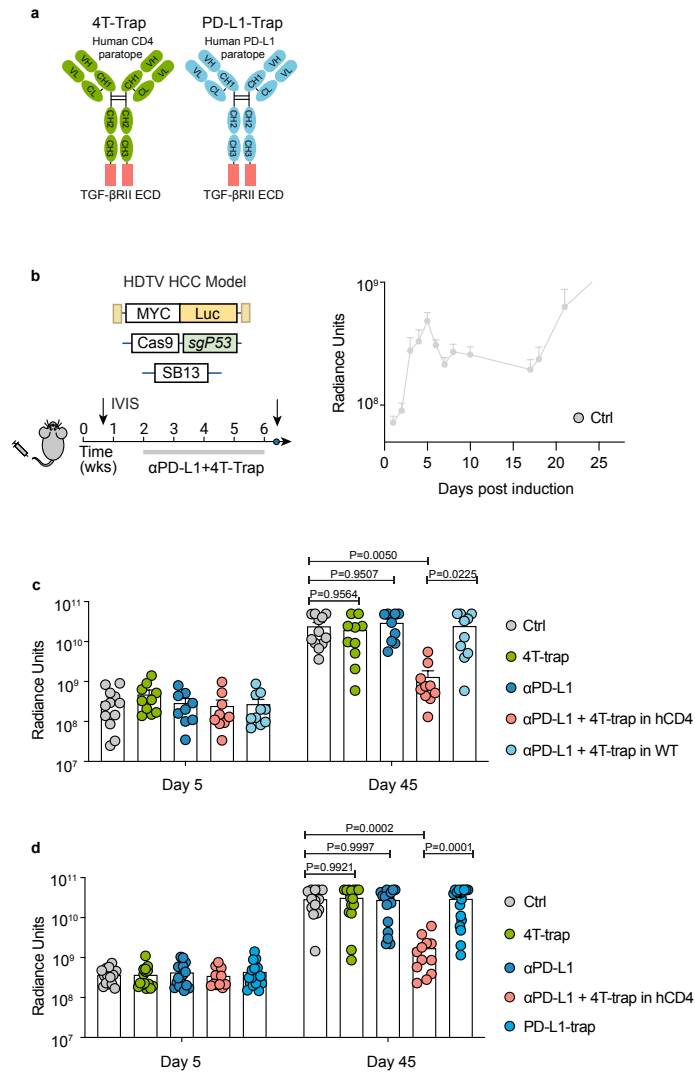


Figure 3-1 4T-Trap and anti-PD-L1 cotreatment inhibits tumor development in HCC.

a: Antibody structures for 4T-Trap, PD-L1-Trap.

b: (left) A schematic to depict a hydrodynamic injection (HDTV) model of HCC induced by overexpression of the MYC oncogene and deletion of the P53 tumor suppressor gene. (Right) Luciferase signal in the first 25 days post injection.

c, d: Tumor burden in mice treated with the indicated antibodies at Day 5 (before treatment) and Day 45, measured by luciferase signal.

Data are presented as the mean values $\pm$ s.d. One-way ANOVA with Dunnett T3 multiple comparison test correction. Results are pooled from at least three independent experiments.

3.2.2 Expanded effector Th1 cells with a hyperactivated phenotype inhibited tumor progression depending on IFN- γ .

Next, we investigated the effector mechanism under the therapeutic efficacy of combined treatment with 4T-Trap and anti-PD-L1 in HCC. The requirement of CD4 targeting of TGF- β pathway prompted us to examine the effector phenotypes of T helper cells first. As discussed in the previous chapter, TGFBR2 and PD-1 served as 'tonic' and 'reactive' regulators, respectively, working together to promote peripheral Th cell tolerance by inhibiting 'Tfh-like Th1' and 'hyperactivated Th1' cell differentiation in the tissue. The self-reactive 'hyperactivated Th1' cells acted as potent effector cells, driving the immunopathology observed upon the disruption of immune tolerance. To determine if a similar effector Th1 response was induced by dual treatment, we analyzed the tumor microenvironments approximately 10 days post-treatment. At this juncture, tumors were just beginning to become microscopically apparent with minimal physiological burden to the tumor-bearing mice. This contrasted with the later stages of the experiment, where significant variability in tumor sizes between cotreatment and control groups presented additional challenges in interpreting cellular changes.

We found that tumor suppression under cotreatment was associated with Th cell differentiation along the Th1 pathway, with increased proportion of TCF1⁺ TOX⁺ 'hyperactivated Th1' cells among the conventional Th cell compartment (Figure 3-2, a-b). Notably, cotreatment of 4T-trap and anti-PD-L1 in WT mice failed to expand 'hyperactivated Th1' cells as seen in hCD4 mice, in line with our observation that the synergy of cotreatment relied on targeting TGF- β pathway on CD4⁺ T cells. Indeed, applying depleting anti-CD4 antibody before treatment

reversed the therapeutic effect (Figure 3-2, c), confirming the essential role of CD4⁺ T cells in mediating the superior antitumor response under cotreatment.

Furthermore, the ‘hyperactivated Th1’ cells found in the tumor upregulated IFN- γ production compared to each of the control groups (Figure 3-2, d), suggesting enhanced effector function despite chronic antigen stimulation in the tumor microenvironment. IFN- γ signaling had been associated with favorable responses to anti-PD-L1 therapy in urothelial bladder and non-small cell lung cancer patients [222,242,243], and anti-PD-1 therapy in melanoma, head and neck, and gastric cancer patients [244]. To test if IFN- γ was required to mediate antitumor response, we performed cotreatment with IFN- γ neutralization. The addition of anti-IFN- γ antibodies abolished the therapeutic efficacy of combined therapy (Figure 3-2, e). Therefore, blocking TGF- β signaling and synergized with anti-PD-L1 via expansion of potent effector Th1 cells and augmented IFN- γ responses.

Of note, different from genetic deletion of TGFBR2 on PD-1-deficient background, we only observed expansion of TCF1⁺TOX⁺ ‘Tfh-like Th1’ cells in some mice under pharmacological blockade of the two pathways (Figure 3-2, b). We also failed to detect a pronounced change in Tfh differentiation in the tumor draining lymph nodes (data not shown). This pointed to the differential regulation of Th cell fate by TGF- β and PD-L1 during autoinflammation and tumor progression. We confirmed the dispensable role of the humoral arm in antitumor response by depleting B cells in conjunction with combined treatment (Figure 3-3,). Taken together, TGF- β and PD-L1 signaling likely work in synergy to suppress the

effector Th1 cells through inhibiting IFN- γ responses in cellular immunity arm during HCC development.

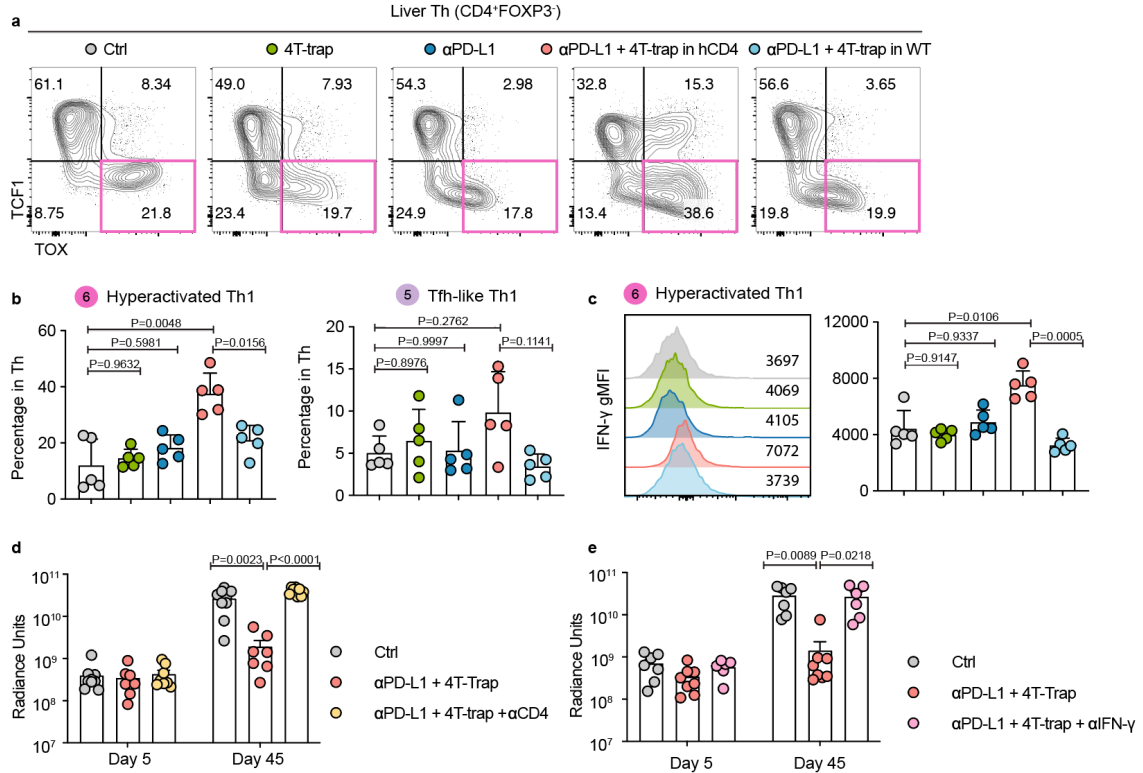


Figure 3-2 Expanded effector Th1 cells with a hyperactivated phenotype inhibited tumor progression depending on IFN- γ .

a, b: Representative plots (**a**) and quantification (**b**) of Tfh-like Th1 cells and hyperactivated Th1 cells gated by TCF1 and TOX expression within CD4⁺Foxp3⁻ T helper (Th) cells of indicated treatment arms 3-4 weeks post tumor induction.

c: Representative plots and quantification of IFN- γ expression using geometric mean of fluorescence intensity (gMFI) after *ex vivo* restimulation of hyperactivated Th1 cells in each indicated treatment arms 3-4 weeks post tumor induction.

d, e: Tumor burden in mice treated with the indicated antibodies at Day 5 (before treatment) and Day 45, measured by luciferase signal.

Data are mean $\pm$ s.d. One-way ANOVA with Dunnett T3 multiple comparison test correction. Results represent at least three independent experiments.

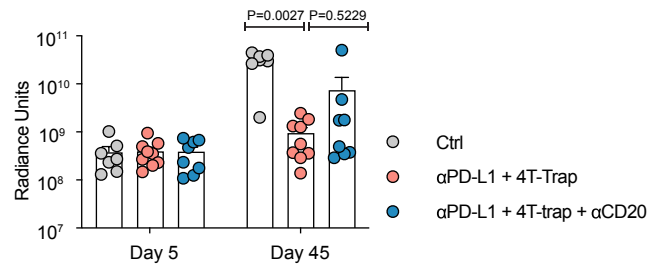


Figure 3-3 Dispensable role of the B cells in combined treatment.

Tumor burden in mice treated with the indicated antibodies at Day 5 (before treatment) and Day 45, measured by luciferase signal. Data are mean $\pm$ s.d. One-way ANOVA with Dunnett T3 multiple comparison test correction. Results represent at least three independent experiments.

3.2.3 Cotreatment elicits CD40-mediated cancer immunity through a Th cell and macrophage feedback circuit.

To elucidate how effector Th1 cells orchestrated tumor suppression under 4T-Trap and anti-PD-L1 cotreatment, we next focused on the myeloid APC compartment, which cooperates with CD4⁺ T cells in mediating cellular immunity. We found enhanced recruitment of monocyte-derived macrophages following cotreatment (Figure 3-4, a-b), in parallel with ‘hyperactivated Th1’ cell expansion. However, the function of macrophages in antitumor immunity is multifaceted due to its functional diversity and plasticity, with evidence suggesting both immunosuppressive and tumor-reactive roles during tumor progression [245]. We found depleting the total macrophage population through an anti-CSF1 antibody during combined treatment reverted the tumor suppressive effect (Figure 3-4, c), highlighting the critical contribution from macrophages in tumor control in HCC.

Similar to the M1-like polarization of the monocyte-derived macrophages following TGFBR2 and PD-1 genetic double deficiency, we found they upregulated CD40 expression under combined therapy, indicating better effector functions (Figure 3-5, a-b). In tumor immunity, activation of macrophages and other APCs via CD40-targeting therapies has been shown to revert the suppressive tumor microenvironment [246,247], with growing clinical evidence supporting the benefits of immunotherapy with agonistic CD40 antibodies, in particular as combination with chemotherapy [248]. CD40 signaling was also indispensable for the antitumor response in HCC model, as combination therapy of 4T-trap and anti-PD-L1 lost therapeutic advantage on the hCD4 in the CD40-deficient background (hCD4 *Cd40*^{-/-}) (Figure 3-5, b). Cotreatment in hCD4 *Cd40*^{-/-} mice failed to expand

'hyperactivated Th1' cells and monocyte-derived macrophages (Figure 3-5, c-d). Altogether, we discovered that 4T-Trap and anti-PD-L1 cotreatment elicited CD40-mediated cancer immunity through a Th cell and macrophage feedback circuit.

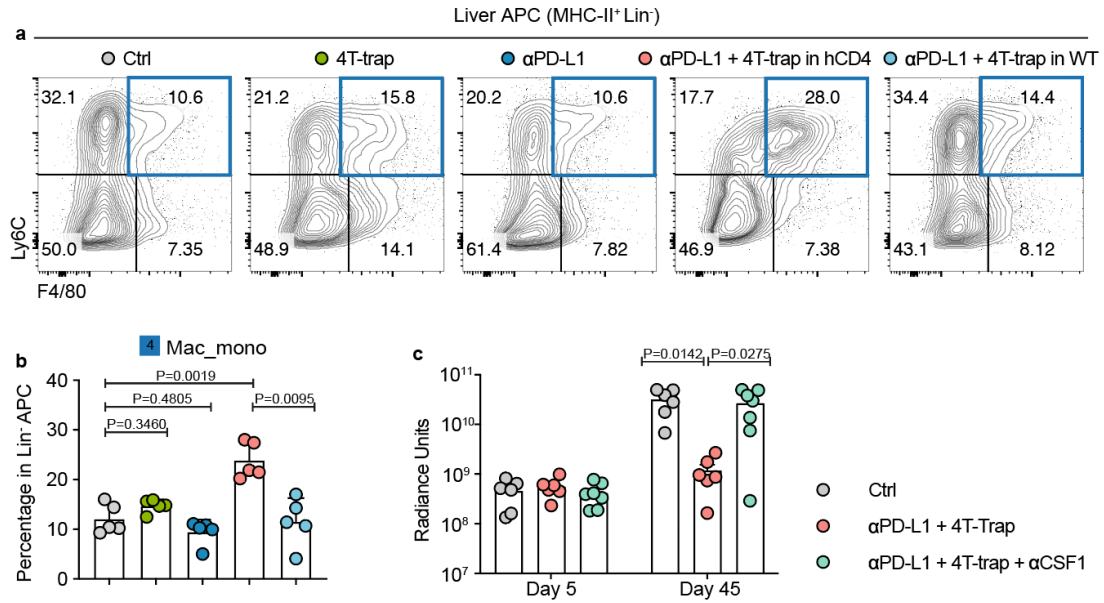


Figure 3-4 M1-like macrophages are recruited to the tumor.

a, b: Representative plots (**d**) and quantification (**e**) of monocyte-derived macrophages gated by F4/80 and Ly6C within MHCII⁺ lineage⁻ (TCRβ⁻CD90⁻ NK1.1⁻CD19⁻Siglec-F⁻Ly6G⁻) APC population in the liver in each indicated treatment arms 3-4 weeks post tumor induction.

c: Tumor burden in mice treated with the indicated antibodies at Day 5 (before treatment) and Day 45, measured by luciferase signal.

Data are mean ± s.d. One-way ANOVA with Dunnett T3 multiple comparison test correction. Results represent at least three independent experiments.

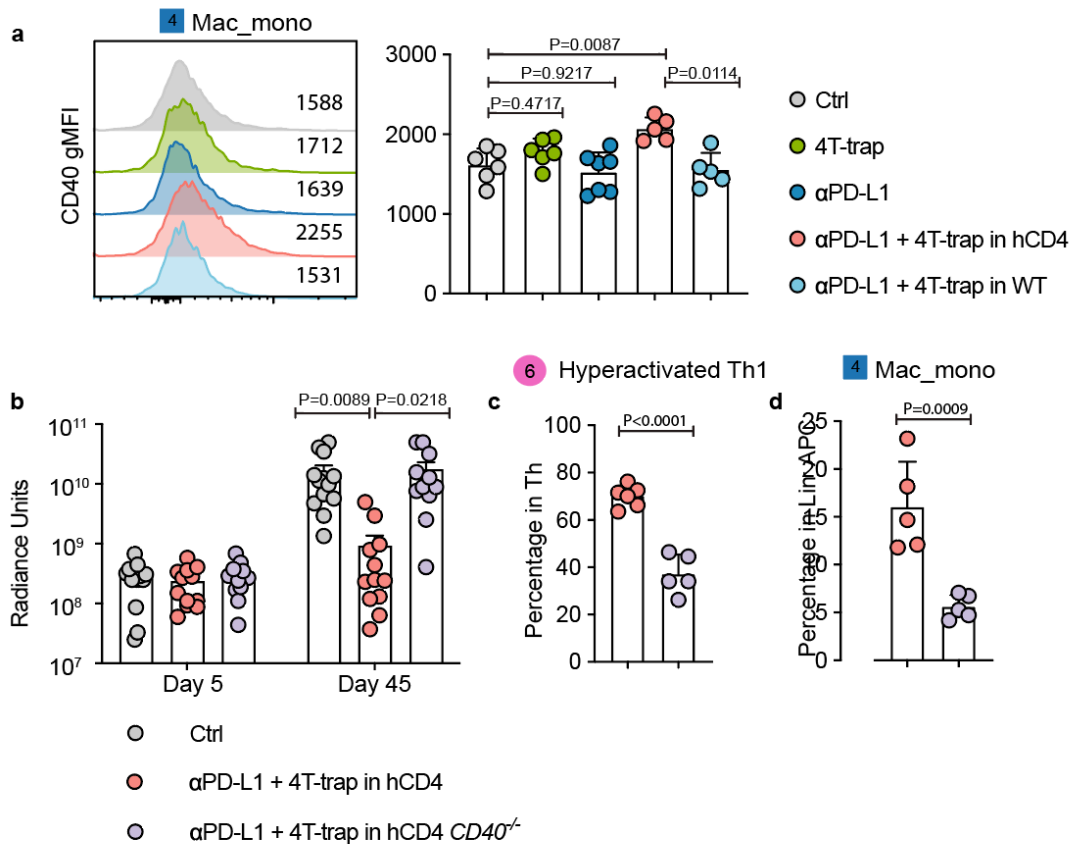


Figure 3-5 CD40 is required for tumor control and expansion of hyperactivated Th1 and monocyte-derived macrophages.

a: Representative plots and quantification of CD40 expression using geometric mean of fluorescence intensity (gMFI) in liver monocyte-derived macrophages in each indicated treatment arms 3-4 weeks post tumor induction.

b: Tumor burden in mice treated with the indicated antibodies at Day 5 (before treatment) and Day 45, measured by luciferase signal.

c, d: Quantification of hyperactivated Th1 cells or monocyte-derived macrophages in each indicated treatment arms 3-4 weeks post tumor induction.

Data are mean $\pm$ s.d. One-way ANOVA with Dunnett T3 multiple comparison test correction. Results represent at least three independent experiments.

3.2.4 Enhanced effector functions of cytotoxic lymphocytes are essential for tumor control.

CD8⁺ T cells are critical mediators of cytotoxic effector function in infection, cancer and autoimmunity. In cancer and chronic viral infection, CD8⁺ T cells undergo a progressive loss of cytokine production and cytotoxicity, a state termed T cell exhaustion [29,249–251]. CD8⁺ T cells had been demonstrated to be essential for suppressing an HCC model with introduced model antigens [239], yet, its role on low mutational burden HCC is unknown.

We went on to examine the cellular changes of CD8⁺ T cells in the tumor microenvironment. Different from the autoimmune model (Figure 1-30, b), we did not see pronounced expansion of the terminally differentiated TCF1-TOX⁺ CTL population with combination therapy (Figure 3-6, a), as the baseline level of TCF1-TOX⁺ CTL within the control groups was already elevated compared to the steady state, a sign of T cell exhaustion due to chronic inflammation in the growing tumor. However, the effector function of the TCF1-TOX⁺ CTLs were boosted following combined therapy, as demonstrated by increased production of IFN- γ and granzyme B (Figure 3-6, b-c). To test the role of CD8⁺ T cells in tumor control, we depleted CD8⁺ T cells during cotreatment and found the benefit of combinatorial therapy of 4T-trap and anti-PD-L1 diminished without CTLs (Figure 3-6, d). Thus, CD8⁺ T cells are crucial to achieve the therapeutic efficacy of dual treatment. Although we cannot rule out the possibility that 4T-trap acted directly to inhibit TGF- β signaling on CD8⁺ T cells and synergized with anti-PD-L1 treatment, it is most likely that the synergy happened on conventional Th as we observed with genetic double deficiency, and the enhanced function of conventional Th and

macrophages feedforwarded to offer better 'help' to CTLs, preventing them from functional exhaustion under chronic antigen stimulation in tumor. Overall, 4T-trap and anti-PD-L1 dual treatment enhanced effector functions of cytotoxic lymphocytes that were essential for tumor control during HCC progression.

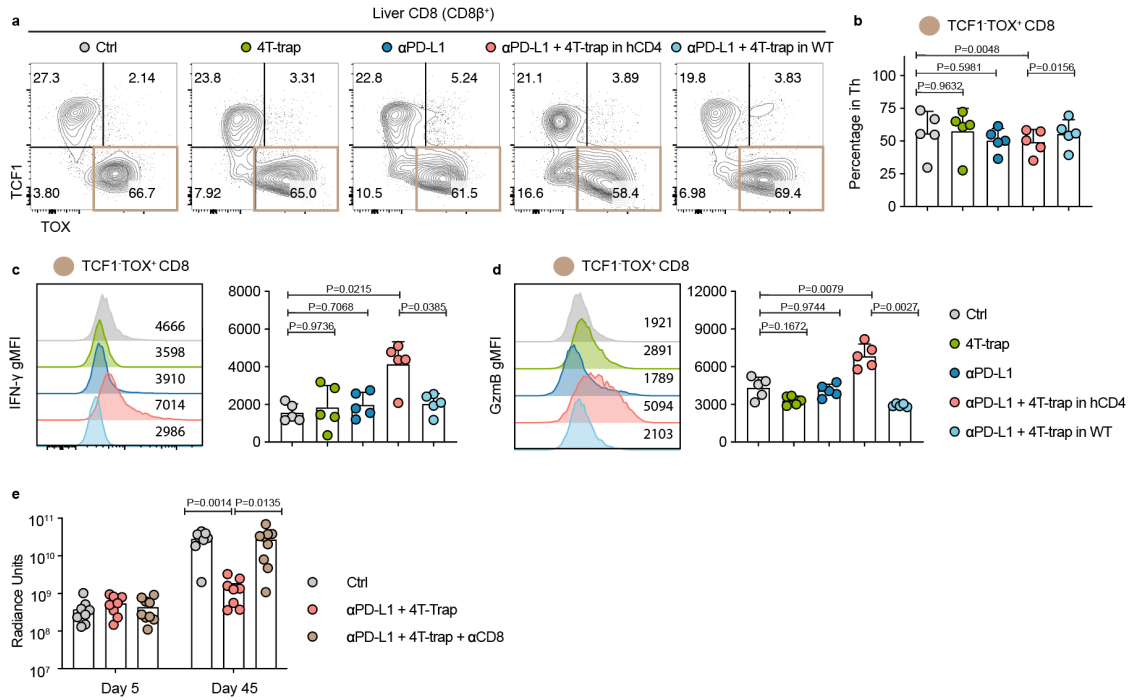


Figure 3-6 Enhanced effector functions of cytotoxic lymphocytes are essential for tumor control.

a, b: Representative plots (**a**) and quantification (**b**) of liver CD8⁺ T cell population gated by TCF1 and TOX expression in each indicated treatment arms 3-4 weeks post tumor induction.

c, d: Representative plots and quantification of IFN-γ (**c**) and granzyme B (**d**) expression using geometric mean of fluorescence intensity (gMFI) of CD8⁺ T cell population gated by TCF1 and TOX isolated from the liver in each indicated treatment arms 3-4 weeks post tumor induction.

e: Tumor burden in mice treated with the indicated antibodies at Day 5 (before treatment) and Day 45, measured by luciferase signal.

Data are mean ± s.d. One-way ANOVA with Dunnett T3 multiple comparison test correction. Results represent at least three independent experiments.

3.3 Discussion

The redundancy of TGFBR2 and PD-1 signaling in Th cell-intrinsic peripheral tolerance regulation inspired us to explore whether targeting TGF- β signaling on CD4⁺ T cells can synergize with anti-PD-L1 immune checkpoint in cancer therapy. We found 4T-trap, the TGF- β receptor decoy directed to CD4⁺ T cells, effectively inhibited tumor development in combination with anti-PD-L1 in a low mutational burden murine autochthonous HCC model. This combination therapy induced a robust Th1 response, characterized by the expansion of 'hyperactivated Th1' cells and enhanced IFN- γ production, in addition to enhanced recruitment and activation of monocyte-derived macrophages. Moreover, the combined treatment boosted the effector functions of CD8⁺ cytotoxic lymphocytes, increasing their IFN- γ and granzyme B production to contribute to tumor control. Similar to the autoimmunity model, CD40 exerted an indispensable role in the heightened T cell response in both chronic inflammatory conditions. Of note, the synergy of cotreatment relied on targeting the TGF- β pathway on CD4⁺ T cells. Therefore, we propose the following model to elucidate the effector mechanism under dual blockade (Figure 3-7): CD4⁺ T cell immunity is suppressed by TGF- β in the tumor microenvironment and PD-L1 signaling on APCs during tumor development. Alleviating these inhibitory pathways enables conventional Th cells to differentiate into potent effector Th1 cells, establishing a feedback loop with macrophages to promote effective antitumor immunity via CD40 signaling. This circuitry also sustains CTL effector functions, allowing them to maintain high levels of IFN- γ and granzyme B production. Taken together, targeting TGF- β signaling

on CD4⁺ T cells and PD-L1 blockade synergize to induce a feedback-feedforward loop in between Th cells, M1-polarized macrophages, and CTLs that effectively prevents tumor progression.

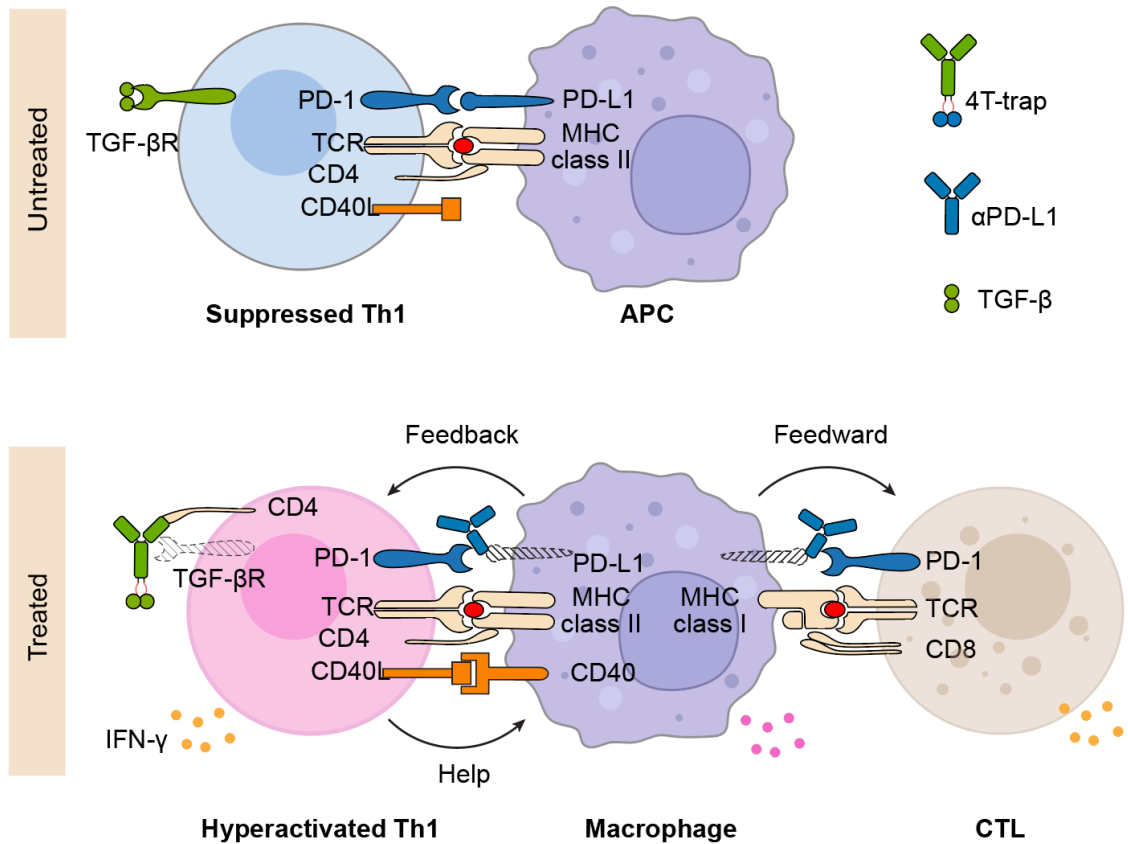


Figure 3-7 Model for the feedback-feedforward loop between CD4⁺ T cells, myeloid antigen presenting cells and CD8⁺ T cells under dual blockade.

(Upper) Effector Th cells are inhibited by TGF-β and PD-L1 signaling on in the tumor microenvironment.

(Lower) Dual inhibition of TGF-β and PD-L1 signaling on Th cells by 4T-trap and anti-PD-L1 enabled Th cells to enter a feedback loop with myeloid antigen presenting cells, likely M1-like monocyte-derived macrophages through CD40L-CD40 signaling. This loop feedforwards to CD8⁺ T cells and promote their effector function.

While we cannot completely dismiss the possibility that 4T-trap directly inhibits TGF- β signaling in CD8⁺ T cells, leading to synergy with anti-PD-L1 treatment, the evidence suggests that the primary synergy occurs in conventional Th cells, as seen with genetic double deficiency. Indeed, despite abundant evidence exists on CD8⁺ T cell-dependent antitumor immunity in preclinical models in response to TGF- β inhibition [43], the currently limited clinical success of TGF- β inhibitors highlights the need for a deeper understanding of this pathway's impact on CD8⁺ T cells, as well as their interplay with various cell types within the tumor microenvironment. It is known that TGF- β engages a number of gene expression programmes to attenuate CTL differentiation and function, including inhibiting expression of T-bet and EOMES, which are transcription factors crucial for CTL differentiation [252], and inhibition of CTL effector molecules, such as IFN- γ and granzyme B [253]. TGF- β also enhances PD-1 expression in a SMAD3-dependent manner via SMAD3 recruitment to the *Pdcd1* promoter region [254]. However, TGF- β also been shown to play a critical function in the establishment of CTL epithelial tissue residency [255,256], an important program to mediate immunosurveillance within the epithelial cancer microenvironment [257,258]. Thus, the effect of TGF- β signaling blockade on CD8⁺ T cell is complicated, with supporting evidence mostly coming from transplantation models where the function of resident memory-like CTL is largely ignored, and less evidence from genetic models of solid tumors [43]. Our findings on improved CTL functions in an autochthonous HCC model highlighted that CD8⁺ T cells can be revitalized following targeted TGF- β inhibition on CD4⁺ T cells downstream of enhanced

myeloid functions, adding new perspective to immunotherapies targeted at the TGF- β pathways.

Several preclinical studies as well as early clinical data have shown that CD40 agonists in combination with immune checkpoint inhibitor can provide synergistic activity and overcome PD-1 resistance some tumor types [259–262]. It is worth noting that CD40 targeting therapies have been shown to significantly impact tumor-associated macrophages [263,264]. CD40 stimulation favors M1-like human macrophage development *in vitro* [265] and skews tumor-infiltrating macrophages toward a pro-inflammatory M1 phenotype with tumoricidal effects in mice [266–268], thereby sensitizing tumors to chemotherapy [246,247]. However, the first generation of CD40 agonists were associated with dose-limiting toxicities, including mild to moderate cytokine release syndrome (CRS), likely mediated by elevations in IL-6 [269]. Therefore, people have been seeking novel strategies to target the CD40 pathway with limited additional toxicities. The synergy of 4T-trap and anti-PD-L1 enhanced Th1-mediated cellular immunity via the CD40L-CD40 axis, allowing for elevated CD40 responses in the tumor microenvironment while circumventing toxicities from systemic CD40 targeting. This provides a unique therapeutic opportunity to target this pathway in antitumor immunity.

Apart from promoting the function of macrophages, CD40 has also been shown to ‘licensing’ CTL-mediated cellular immunity through DCs following immunization [270] and in tumor [128,271,272]. This formed the classical ‘three-cell-type’ model, where CD4⁺ T cells stimulated conventional DC1s via CD40L-CD40 signaling, upregulate their cross-antigen presentation ability to further

stimulate CD8⁺ T cells. Such models have also been extended to the tumor immunity cycle [271,273]. While macrophages do not typically exhibit robust cross-presentation function to stimulate CTLs, we demonstrated that the M1-polarized macrophages from the monocyte origin can become potent stimulators for CD8⁺ T cells in the autoinflammation enabled by effector Th1 cells (Figure 2-24). In the tumor context, we observed a similar type I response being established after synergistic inhibition of the TGF- β and PD-L1 pathways, accompanied by elevated monocyte-derived macrophages and enhanced CTL functions in a CD40-dependent manner. This indicated that macrophages also could participate in such a 'three-cell-type' model together with Th cells and CTLs to promote antitumor responses. Our findings align with a recent report showing that antigen-specific Th1 cells instructs monocyte differentiation into anti-tumor macrophages mitigating CD8 T cell exhaustion via non-redundant functions of IFN- γ and CD40 [274]. In addition, several groups have described spatially organized intratumoral T cell-rich immune hubs in immune checkpoint blockade-responsive human tumors such as mismatch repair-deficient colorectal cancer [275], hepatocellular carcinoma [276], head and neck squamous cell carcinoma [277], and non-small cell lung cancer [278]. These intratumoral immune clusters are enriched for putative tumor-reactive CXCL13⁺PD-1⁺CD4⁺ and CXCL13⁺PD-1⁺CD8⁺ T cells and myeloid cells expressing interferon-stimulated gene signatures including antigen presentation genes and T cell-attracting chemokines. Our data provided insights on how such an immune niche could promote the function of each of the cellular immunity compartments and enhance antitumor immunity.

There are two outstanding questions left to be explored in future studies. First, an obvious concern for the combined 4T-trap and anti-PD-L1 therapy is whether it induces/elevates the immune-related adverse events (irAEs) as seen in immune checkpoint therapies [279–281], especially given the vital roles of the two pathways in peripheral Th cell tolerance demonstrated in the previous chapter. While within the 6-week treatment window we did not observe severe signs of autoimmunity in the murine HCC model, one can address this concern in scrutiny by measuring serum aspartate transaminase (AST) and alanine transaminase (ALT) levels on mice treated with combination therapy at steady state.

Secondly, the antigen specificity of the activated effector Th1 cells remains unknown in this model. T cells can recognize two types of epitopes in the tumor, tumor specific antigens (TSA), including neoantigens and viral oncoproteins that are genetically encoded only in tumor cells; and tumor associated antigens (TAA), that are encoded by unmutated genes and shared between the genomes of cancer and normal cells [282]. TSAs can elicit protective CTL responses, while the antigen specificity of effective Th response remains debatable. While in some context, cytotoxic Th cells can directly eradicate MHCII⁺ tumors powered by neoantigen recognition [283], this was less likely to be the case in our HCC model, as we did not observe granzyme expression among CD4⁺ T cells (data not shown). Evidence from the autoimmunity model suggested that TGF- β and PD-L1 act as ‘tonic’ and ‘reactive’ regulators of peripheral Th cell tolerance, respectively. Therefore, it is possible that the revigorated Th cells following combination blockade contains self-reactive populations. The alleviation of TGF- β signaling on Th cells may lift the

check on TCR activation threshold, thus allowing Th cells with suboptimal antigen stimulation to achieve functional activation/reactivation.

To address this question, one can leverage the potentially self-reactive MHCII-restricted TCRs we identified from the scTCR-seq in the autoimmunity model, create 'retrogenic' mice expressing these TCRs (discussed in Chapter 2), isolate the self-reactive Th cells and study their function under 4T-trap and anti-PD-L1 dual inhibition after transferring to CD4-deficient tumor bearing mice. We hypothesize that some of the self-reactive Th cells are capable of creating an inflammatory environment supportive for cellular-immunity and contribute to antitumor responses. This posts an exciting opportunity to further delineate CD4⁺ T cell biology in tumor immunity can provide insights for therapeutic development, such as tumor vaccine designs.

In summary, this section of my thesis presents compelling evidence that the pharmacological co-inhibition of the TGF- β and PD-L1 pathways revitalizes protective immunity in cancer, providing a promising new strategy for improving cancer treatment outcomes, while contributing to a deeper understanding of the mechanisms underlying immune suppression in the tumor microenvironment.

3.4 Experimental Procedures

3.4.1 Mice

Experiments in this study were approved by the Sloan Kettering Institute (SKI) Institutional Animal Care and Use Committee under protocol 07-06-008 and conducted in compliance with institutional ethics guidelines. Mice were housed at the SKI animal facility under specific pathogen-free (SPF) conditions on a 12-hour light/dark cycle with free access to water and regular chow diet. The average ambient temperature is 21.5°C and the average humidity is 48%. All control and experimental animals were age-matched, and littermates were used as controls when possible. Unless mentioned otherwise, both male and female mice were included. Group size was determined on the basis of the results of preliminary experiments, and no statistical method was used to predetermine sample size. Group allocation for treatments was performed to ensure equivalent luciferase signal, and outcome assessment was not performed in a blinded manner.

hCD4 mice were previously reported [233]. CD40^{-/-} mice were purchased from Jackson Laboratory.

3.4.2 Reagents and antibodies

The following antibodies and streptavidin were used in this study for flow cytometry, with clones, vendors, catalog numbers, and dilutions indicated in the parentheses: anti-Siglec-F (E50-2440, BD, 562681, 400), anti-I-A/I-E (M5/114.15.2, BioLegend, 107641, 1000), anti-NK1.1 (PK136, ThermoFisher, 47-5941-82, 200), anti-CD45 (30-F11, BioLegend, 103136, 600), anti-CD11b (M1/70, BioLegend, 101257, 800), anti-CD3 ϵ (17A2, BioLegend, 100237, 500), anti-Ly-6C (HK1.4, BioLegend, 128037, 1000), anti-CD90.2 (30-H12, BioLegend, 105331, 1000), anti-Foxp3 (FJK-16s, ThermoFisher, 17-5773-82, 400), anti-CD19 (6D5, BD, 563557, 400), anti-Ly-6G (1A8, BioLegend, 127618, 500), anti-TCR β (H57-597, ThermoFisher, 61-5961-82, 400), anti-F4|80 (BM8, ThermoFisher, 61-4801-82, 200), anti-CD90.1 (HIS51, ThermoFisher, 17-0900-82, 200), anti-CD4 (RM4-5, BioLegend, 100553, 400), anti-CD8 α (53-6.7, BioLegend, 100759, 400), anti-GITR (DTA-1, ThermoFisher, 48-5874-82, 400), anti-CD73 (TY/11.8, BioLegend, 127215, 400), anti-CD44 (IM7, BD, 563971, 400; ThermoFisher, 48-0441-82, 400; BioLegend, 103049, 400), anti-CD103 (M290, BD, 564322, 200), anti-CD62L (MEL-14, BioLegend, 104438, 1600; BioLegend, 104441, 400), anti-CTLA4 (UC10-4B9, BioLegend, 106314, 600), anti-Helios (22F6, BioLegend, 137218, 400), anti-Ki-67 (16A8, BioLegend, 652420, 400), anti-CD25 (PC61.5, ThermoFisher, 47-0251-82, 400; ThermoFisher, 17-0251-82, 400), anti-PD-1 (29F.1A12, BioLegend, 135225, 400), anti-ICOS (7E.17G9, ThermoFisher, 12-9942-82, 400), anti-CD45.1 (A20, BioLegend, 110738, 100), anti-CD45.2 (104, BD, 612778, 200), anti-IL-2 (JES6-5H4, BioLegend, 503818, 400), anti-IL-17A (17B7,

ThermoFisher, 48-7177-82), anti-IFN γ (XMG1.2, ThermoFisher, 17-7311-82, 500), anti-IL-4 (BVD6-24G2, ThermoFisher, 25-7042-82, 400), anti-GARP (YGIC86, ThermoFisher, 25-9891-82, 200), anti-CD119 (2E2, BD, 550482, 100; GR20, BD, 740032, 100), anti-EpCAM (G8.8, BioLegend, 118227, 100; BD, 740281, 100), anti-CD124 (mIL4R-M1, BD, 742172, 100), anti-TCR V β 5.1, 5.2 (MR9-4, BioLegend, 139508, 400), anti-TCR V β 6 (RR4-7, ThermoFisher, 46-5795-82, 300), anti-TCR V β 8 (F23.1, BD, 553860, 400), streptavidin (BioLegend, 405225, 4000; ThermoFisher, 61-4317-82, 4000), anti-CD45R/B220 (RA3-6B2, ThermoFisher, 47-0452-82, 400). CellTrace Violet was purchased from Thermo Fisher Scientific.

3.4.3 Methods

HCC tumor induction and imaging

A sterile 0.9% NaCl solution/plasmid mix was prepared containing the following DNA: 11.4 µg of *pT3-EF1a-MYC-IRES-luciferase (MYC-luc)*, 10 µg of *px330-sg-p53 (sg-p53)* and a 4:1 ratio of transposon to *SB13* transposase-encoding plasmid. Mice were injected with the 0.9% NaCl solution/plasmid mix into the lateral tail vein with a total volume corresponding to 10% of body weight in 5 to 7 seconds. Vectors for hydrodynamic delivery were produced using the QIAGEN plasmid *PlusMega* kit (QIAGEN). Equivalent DNA concentration between different batches of DNA was confirmed to ensure reproducibility among experiments.

Treatments

Treatments were initiated two weeks after the hydrodynamic delivery of the plasmids. 4T-trap was constructed and synthesized in house as previously described [233]. 4T-trap was administered at 10 mg per kg twice weekly through intravenous injection for 4 weeks. Anti-PD-L1 (6E11) with mouse IgG2a and LALAPG mutation was obtained from Genentech (Roche) and administered as described in [284]. In brief, the first dose was administered at 10 mg per kg intravenously, subsequent antibody doses were administered via intraperitoneal injection at the fourth mammary fat pad twice weekly for 4 weeks at 5 mg per kg. For the cell depletion experiments, mice were injected intraperitoneally with anti-CD4 (200 µg, clone GK1.5, BioXcell), anti-CD8 (200 µg, clone 2.43, BioXcell), or IgG (200 µg, IgG2b, clone LTF-2, BioXcell) at days 7, 9, 11, and 13, and then once weekly until the end of the experiment.

Luciferase Detection

In vivo bioluminescence imaging was performed using an IVIS Spectrum system (Caliper LifeSciences) to quantify liver tumor burden before being evenly assigned to various treatment study cohorts. Mice were imaged directly after intravenous injection with fresh D-luciferin (150 mg/kg; Thermo Fisher Scientific). Luciferase signal was quantified using Living Image software (Caliper LifeSciences). Normalized luciferase signal was calculated by subtracting the background signal. Each treatment cohort had equivalent average luciferase signal before treatment started. The mice with a luciferase signal a log of magnitude lower than the average signal were excluded from the study.

Isolation of cells from the liver draining lymph nodes and liver

For flow cytometric analyses, the liver was perfused with 2 mM PBS-EDTA from the portal vein immediately after euthanasia, chopped using a razor blade, and enzymatically digested using a buffer composed of HBSS (Corning) supplemented with 1 mg/ml collagenase IV (SIGMA) and DNase I 0.02 mg/ml (SIGMA) for 30 minutes at 37 °C. The digestion was neutralized with HBSS and the liver suspension was strained through a 70 µm nylon mesh (Corning) and centrifuged at 800 g at room temperature for 6 minutes. After centrifugation, the pellet was re-suspended in 36% HBSS-percoll and centrifuged at 800 g at 4°C for 20 minutes. The resultant pellet was incubated with ACK lysing buffer (Gibco) to lyse erythrocytes. For draining lymph nodes, cells were retrieved by meshing the

organs through a 70 µm strainer (Corning, 07-201-432) with a syringe plunger for lymphocyte analysis.

Flow Cytometry

Cells were stained Zombie NIR Flexible Viability Kit (Biolegend, 423106) in PBS for 10 minutes at 4°C to help identify dead cells. Cell surface staining was performed by incubating cells with antibody cocktail for 30 min on ice in the presence of purified anti-Mouse CD16/CD32 (2.4G2, Tonbo, 70-0161) in staining buffer [0.5% (w/v) BSA, 2 mM EDTA, 10 mM HEPES, 0.02% NaN₃ (Sigma-Aldrich, S2002) in 1x PBS]. For intracellular antibodies, the Tonbo or ThermoFisher Transcription Factor Fix/Perm Kit was used to fix and permeabilize cells for 30 min on ice, followed by intracellular staining for 30 min on ice. Secondary antibodies with fluorochrome-conjugation were used for the staining of purified antibodies.

Samples were recorded on an LSR II cytometer (BD) using the BD FACS DIVA software v8.0 or an Aurora cytometer (Cytex) using the SpectroFlo software v2.2.0.3 and analyzed with FlowJo v10.6.1 (BD).

Stimulation for cytokine assessment

To measure cytokine production after *ex vivo* restimulation, single cell suspensions were incubated at 37°C for 3-4 hours with 5% CO₂ in 96-well flat-bottom plates in the presence of 50 ng/mL phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich, P8139) and 500 ng/mL ionomycin (Sigma-Aldrich, I0634) with 1

μg/mL brefeldin A (Sigma-Aldrich, B6542) and 2 μM monensin (Sigma-Aldrich, M5273) to inhibit ER and Golgi transport.

3.4.4 Statistics

Statistical significance was determined using tests indicated in the respective figure legends. P-values for *t*-test on flow cytometric data were calculated with GraphPad Prism 9. P-values for ANOVA had been corrected for multiple hypothesis testing using Dunnett T3 multiple comparison test corrections (according to Prism's recommendations). Throughout the entire study, error bars represent mean ± standard deviation, and the P-values were labeled directly on the graph when applicable.

CONCLUSIONS

The adaptive immune system is characterized by its broad antigen recognition specificity, providing mammals with a versatile defense against rapidly evolving pathogens. However, this protection is accompanied by a significant fitness cost due to the risk of autoimmune inflammation, which arises from the self-reactivity of T cells. Th cells orchestrate host defense responses against immunological challenges, while self-reactive Th cells that escape negative selection require multiple layers of peripheral tolerance programs to prevent immune pathology. In this project, we found a critical two-tier regulatory module of Th cell peripheral tolerance with TGF- β and PD-L1/PD-1 as intrinsic proactive and reactive regulators, respectively. Such a cooperative Th cell control circuit in homeostasis and cancer immunity is unique from the other regulatory models composed of reactive regulators, such as PD-1 and Lag3 [285]. The proactive and reactive regulation operates in the lymphoid organs and the tissue. While conditional deletion of TGFBR2 in mature CD4⁺ T cells promoted homeostatic activation and restimulation, the induced expression of PD-L1 on macrophages and PD-1 on Th cells kept the potentially pathogenic Th cells in tolerogenic resting memory-like states, thereby preventing robust amplification of effector cells. In the absence of both regulators, a tolerogenic to immunogenic state switch characterized by excessive autoinflammation is accompanied by Th cells differentiation into Tfh-like Th1 cells and hyperactivated Th1 cells, which engage in a positive feedback loop with antigen-presenting macrophages driven by CD40. Of note, disruption of such a pivotal Th cell-intrinsic tolerance control circuit via the

CD4 T cell-targeted TGF- β blockade and simultaneous PD-L1 inhibition can also reprogram the tumor tissue microenvironment and promote antitumor immunity, achieved by CD40-mediated type 1 immunity mediated by CD4 T cells and macrophages.

TGF- β is present in many tissues, including the tumor, in an active form and raises the activation threshold of T cells to prevent weak self-stimuli from triggering a response [103]. We found that at steady state, TGF- β receptor is expressed on naïve cells and downregulated following activation and differentiation. TGF- β signaling restrains the priming process of Th cells in lymphoid organs, and the restimulation of precursor Th1 cells in the tissue. As a tonic regulator, it filters 'noise' from weak inputs. It is known that naïve T cells continuously receives tonic signals through their TCRs, which harbor a potential to respond to self-ligands involved in positive selection. Tonic regulators establish a baseline level of suppression to avoid unwarranted activation of immune cells, thereby maintaining homeostasis and reducing the risk of inflammatory responses due to minor, non-threatening stimuli. Reactive regulators, on the other hand, respond to the emerging immune activity itself. They are engaged once an immune response has begun, usually in a negative feedback manner, aiming to limit its intensity or duration. PD-1, for example, is not expressed on naïve T cells and only induced following activation. Previous study has shown that PD-1 plays a role in inhibiting autoreactive peripheral Th cells at homeostasis [286–288], but in general, as a reactive regulator, PD-1 genetic deficiency alone does not trigger systemic inflammation [215]. To break tolerance, its absence requires additional mutations

which promote Th cell priming and activation, as previously demonstrated in mice lacking PD-1 and AIRE [289], or with additional CTLA4 haploinsufficiency [290], or in mice with PD-1 deficiency in autoimmune prone genetic background [291–294]. Here we also observed that PD-1 deficiency alone barely induces phenotypic changes in Th cells in either lymphoid organs or the tissue at homeostasis. However, PD-1 function was prominently manifested under the condition of TGF- β signaling blockade to prevent the further activation and differentiation of effector populations, which aligns with the induction of PD-1 expression in TGFBR2-deficient conventional CD4⁺ T cells. Previous studies demonstrated that TGF- β inhibits proximal TCR signaling via the inhibitory tyrosine kinase CSK, while PD-1-mediated inhibition primarily involves recruitment of the tyrosine phosphatase Shp2 to impair TCR, and potentially CD28, signaling, providing the molecular basis for the collaborative function of these two pathways.

Therefore, the absence of tonic regulators, which potentially broadens the repertoire of T cells able to reach functional activation at homeostasis, can synergize with additional deficiency of reactive regulators to cause systemic autoimmune responses.

Interestingly, the tonic regulation and reactive regulation framework [101,102] can also be applied to explain the essential role of Treg cells in peripheral tolerance regulation. Treg cells can prevent unnecessary T cell activation toward self-peptide presented on MHCs via proactive mechanisms, such as continuous stripping of peptide-MHC complexes [295], or competing for costimulatory signal via CTLA4 [20,296]; they can also constrain T cells activated by self-antigens in a

negative feedback manner through paracrine signaling of IL-2 [86]. A defective Treg cell compartment, as demonstrated in genetic inactivation of *Foxp3* in mice (scurfy) and humans (IPEX), causes fatal lymphoproliferative and multiorgan autoimmune disease [84]. However, disruption of each of the proactive/reactive aspect of Treg function alone, if uncoupled from Treg cells development and survival, seems to develop less aggressive autoimmune conditions compared to *Foxp3* deficiency [86,297], indicating the synergistic effects of disrupting of both the proactive and reactive modules of peripheral tolerance. Therefore, the model of tonic and reactive regulators underscores the immune system's sophisticated balance between activation and suppression. By employing both preventive and responsive strategies, the immune system can effectively manage pathogens while minimizing self-damage, illustrating a finely tuned evolutionary adaptation for maintaining homeostasis.

Notably, although mice with Th cell TGF- β signaling deficiency in the PD-1 knockout background develop severe systemic inflammation paralleling *Foxp3* deficient mice, we did not observe lymphadenopathy and splenomegaly typically associated with the fatal lymphoproliferative disorder of the scurfy mouse. Previously, both TGF- β and PD-1 have been reported to have non-overlapping functions with FOXP3. In a CD4 T cell TCR-transgenic model of autoimmune diabetes, deletion of TGFBR2 results in severe disease progression with higher penetrance and faster kinetics compared to mice lacking Treg cells [96]. Additionally, mice with partial *Foxp3* insufficiency develop early-onset lymphoproliferation and lethal autoimmune pancreatitis only when PD-1 is absent

[298]. In $\text{Thpok}^{\text{Cre}}\text{Tgfb}r2^{\text{fl/fl}}\text{Pdc}d1^{-/-}$ mice, we confirmed that the break of tolerance is not due to a defective Treg cell compartment. As Treg cells barely shared clonality with the expanded effector Th1 subsets in the absence of both pathways, we also rule out the possibility that peripheral pathogenic Th cells may be tolerized by redirecting to a Treg cell fate. This suggests that the Th cell-intrinsic tolerance module defined by TGF- β and PD-1, and the Th cell-extrinsic tolerance module exerted by Treg cells, may have non-overlapping functions in peripheral tolerance. Interestingly, experimental and computational modeling evidence revealed that the Treg tolerance module seems to be highly sensitive to modest perturbations [287]. But it was observed in experiments that at least some self-antigen-specific Th cells can remain in a non-responsive state in the absence Treg cells [299], implying that T cell-intrinsic tolerance mechanisms may take on different responsibility to maintain robust peripheral tolerance control in addition to Treg cells. At homeostatic state, Treg-mediated suppression of self-reactive Th cells appears to be primarily in lymphoid organs [76,287]. Along this line, we observed that Treg cells majorly expanded in the lymphoid organs in $\text{Thpok}^{\text{Cre}}\text{Tgfb}r2^{\text{fl/fl}}\text{Pdc}d1^{-/-}$ mice, while Th cell expansion was more dominant in the tissue, which was associated with the lethal immunopathology. These findings underscore the complementary yet distinct roles of Th cell-intrinsic and Treg cell-mediated tolerance mechanisms in maintaining immune homeostasis and preventing systemic autoimmunity.

In both lymphoid organs and tissues, PD-1 function becomes prominent when TGF- β signaling is blocked. Interestingly, different APC subsets seem to play a distinct role in restraining potentially pathogenic, yet activated, TGFBR2-deficient

CD4⁺ T cells in each compartment. Conditional PD-L1 deletion in DCs promotes the priming of TGFBR2-deficient T cells and their differentiation into Tfh and precursor Th1 cells in secondary lymphoid organs but is insufficient to cause autoimmunity. In contrast, tissue APCs, particularly macrophages, are crucial regulators of peripheral Th cell tolerance by providing PD-L1 to prevent the differentiation of pathogenic Th1 cells. This local checkpoint in the tissue is indispensable to prevent the fetal autoimmunity. Of note, phosphorylation of PD-1 by Lck is required for interaction with SHP-2 and downstream suppressive functions, indicating that PD-L1/PD-1 predominantly exert its inhibitory function at antigen recognition [25,300,301]. In the tissue, antigen recognition reduces T cell motility and promote stable Th cell-APC interaction. This process is negatively regulated by PD-L1/PD-1 signaling [184,211]. Therefore, it is likely that PD-1 keeps the potentially pathogenic TGFBR2-deficient CD4⁺ T cells in a tolerized, resting, memory-like state likely by restraining persistent CD4-APC interaction. Further disruption of the PD-L1/PD-1 checkpoint thus causes the excessive clonal expansion and differentiation of chronically stimulated hyperactivated Th1 cells and expansion of monocyte-derived macrophages. These potent APCs engage with hyperactivated Th1 cells to form a positive feedback loop driven by CD40. Previous studies have shown that CD40L is stored in secretory lysosomes in effector and memory Th cells [199,200], and its transfer to APCs through trans-synaptic vesicles occurs in an antigen-dependent manner [201,202]. Notably, this process is inhibited by PD-L1 trans-presentation [203]. Given that PD-L1 expression on hepatic macrophages was induced in Thpok^{cre}Tgfbr2^{fl/fl} mice, and

the macrophage source of PD-L1 was critical for preventing the differentiation of 'hyperactivated Th1' cells, it is likely that PD-L1 functions by blocking CD40L-CD40 engagement between Th cells and APCs. Thus, the cooperative function of TGF- β and PD-L1/PD-1 in peripheral tissue Th cell tolerance control likely operates through engaging the PD-L1/PD-1 axis as a negative feedback loop to prevent the establishment of a positive feedback loop between Th cells and antigen-presenting macrophages, mediated by CD40 signaling.

The two-tier Th cell intrinsic regulatory module defined by the TGF- β and PD-1 duet also operates in cancer immune suppression. We demonstrated that 4T-trap, a TGF- β receptor decoy targeting CD4⁺ T cells, effectively inhibited tumor development when combined with anti-PD-L1 in a low mutational burden murine autochthonous HCC model. This combination therapy induced a robust Th1 response, marked by the expansion of hyperactivated Th1 cells and increased IFN- γ production, alongside enhanced recruitment and activation of monocyte-derived macrophages. Additionally, the combined treatment bolstered CD8⁺ cytotoxic lymphocyte effector functions, resulting in elevated IFN- γ and granzyme B production, thereby contributing to tumor control. CD40 also played a crucial role in the heightened T cell response, by driving a feedback loop of effector Th1 cells and macrophages. This interaction also feedforwarded to CTL effector functions, maintaining high levels of IFN- γ and granzyme B production. Collectively, targeting TGF- β signaling in CD4⁺ T cells and PD-L1 blockade synergizes to induce a tolerogenic to immunogenic state switch that effectively preventing tumor progression.

The current combination ICB therapies, including additional blockade of CTLA4 or Lag3 to the PD-1 pathway [302,303], target primarily the reactive regulators. TGF- β signaling is associated with non-response to immune checkpoint blockade in patients with advanced cancers [43], making it a compelling yet complex additional target for therapy. While TGF- β can promote cancer cell invasion and dissemination, it also exhibits tumor suppressor functions [220]. Moreover, while mesenchymal stromal cell TGF- β signaling inhibits T cell infiltration into tumors [221–224], its blockade in fibroblasts can induce carcinoma due to excessive inflammation-driven DNA damage [225,226]. Systemic blockade of TGF- β signaling poses additional risks, such as cardiac damage [227], which limits the safety profiles of some drug programs. As our understanding of the tumor immune landscape deepens, the search for new strategies that target effector populations with greater specificity has been motivated to address the variability in clinical responses to currently available ICB therapies. Here we demonstrated that co-targeting the tonic regulator TGF- β on Th cells and the reactive regulator PD-L1/PD-1 synergize to reprogram the tumor microenvironment and enhance tumor immunity, highlighting a novel strategy for combination cancer immunotherapy.

Notably, previous efforts have been made in combining inhibitors of TGF- β with checkpoint blockade [228–231]. Despite promising preclinical findings, the clinical outcome was limited [304,305]. In those preclinical models, CD8⁺ T cell-dependent antitumor immunity was highlighted in response to TGF- β inhibition [43]. Yet, the limited clinical success underscores revealed a more complex view of this pathway's impact on CD8⁺ T cells and their interactions with other cell types within

the tumor microenvironment. TGF- β is known to engage gene expression programs that attenuate CTL differentiation and function, including inhibition of transcription factors T-bet and EOMES, which are crucial for CTL differentiation [252], and suppression of effector molecules such as IFN- γ and granzyme B [253]. Additionally, TGF- β enhances PD-1 expression via SMAD3 recruitment to the *Pdcd1* promoter region [254]. However, TGF- β also plays a critical role in establishing CTL epithelial tissue residency [255,256], a key program for immunosurveillance within the epithelial cancer microenvironment [257,258]. Therefore, the supporting evidence for the beneficial effect of TGF- β signaling blockade on CD8⁺ T cells mostly derived from transplantation models that largely overlook the function of resident memory-like CTLs, and less from genetic models of solid tumors. Our findings on enhanced CTL functions in an autochthonous HCC model highlight that CD8⁺ T cells can be revitalized following targeted TGF- β inhibition on CD4⁺ T cells, without disrupting the CTL residency programs, offering new perspectives on immunotherapies targeting the TGF- β pathway.

Several preclinical studies and early clinical data have demonstrated that CD40 agonists combined with immune checkpoint inhibitors can provide synergistic activity and overcome PD-1 resistance in some tumor types [259–262]. CD40 stimulation favors M1-like human macrophage development *in vitro* [265] and skews tumor-infiltrating macrophages toward a pro-inflammatory M1 phenotype with tumoricidal effects in mice [266–268], thereby sensitizing tumors to chemotherapy [246,247]. However, the first generation of CD40 agonists was associated with dose-limiting toxicities, including mild to moderate cytokine release

syndrome, likely mediated by elevations in IL-6 [269]. Therefore, novel strategies are being sought to target the CD40 pathway with reduced additional toxicities. The synergy of 4T-trap and anti-PD-L1 treatment enhanced Th1-mediated cellular immunity via the CD40L-CD40 axis, facilitating elevated CD40 responses in the tumor microenvironment while avoiding the toxicities associated with systemic CD40 targeting. This presents a unique therapeutic opportunity to target this pathway in antitumor immunity.

Beyond promoting macrophage function, CD40 has also been shown to "license" CTL-mediated cellular immunity through DCs following immunization [270] and in the tumor [128,271,272], forming the classical "three-cell-type" model where CD4⁺ T cells stimulate conventional DC1s via CD40L-CD40 signaling, enhancing their cross-antigen presentation ability to further stimulate CD8⁺ T cells. Such models have also been extended to the tumor immunity cycle [271,273]. While macrophages typically do not exhibit robust cross-presentation to stimulate CTLs, we demonstrated that M1-polarized macrophages of monocyte origin can become potent stimulators for CD8⁺ T cells in the autoinflammation driven by effector Th1 cells. In the tumor context, a similar type I response was established following the synergistic inhibition of the TGF- β and PD-L1 pathways, accompanied by elevated monocyte-derived macrophages and enhanced CTL functions in a CD40-dependent manner. This indicates that macrophages can participate in a "three-cell-type" model alongside Th cells and CTLs to promote antitumor responses. Our findings align with recent reports showing that antigen-specific Th1 cells instruct monocyte differentiation into anti-tumor macrophages,

mitigating CD8⁺ T cell exhaustion via non-redundant functions of IFN- γ and CD40 [274]. Additionally, several groups have described spatially organized intratumoral T cell-rich immune hubs in immune checkpoint blockade-responsive human tumors, such as mismatch repair-deficient colorectal cancer [275], hepatocellular carcinoma [276], head and neck squamous cell carcinoma (Franken 2024), and non-small cell lung cancer [278]. These intratumoral immune clusters are enriched for putative tumor-reactive CXCL13⁺PD-1⁺CD4⁺ and CXCL13⁺PD-1⁺CD8⁺ T cells and myeloid cells expressing interferon-stimulated gene signatures, including antigen presentation genes and T cell-attracting chemokines. Our data provide insights into how such an immune niche could enhance the function of each cellular immunity compartment and boost antitumor immunity.

Overall, this project defines a crucial peripheral tolerance mechanism operating through the inhibition of Th cell-intrinsic type 1 immunity driven by CD40-expressing myeloid APCs. Our findings provide a deeper understanding of Th cell regulation by cooperative TGF- β and PD-1 signaling, with implications for developing therapeutics targeting Th cells to mitigate autoimmune diseases and enhance protective immunity in chronic infections and cancer. Notably, autoimmunity and cancer/ chronic viral infection, the conditions where CD4⁺ T cells are under chronic antigen stimulation and but arrived at opposite ends of their functional spectrum, are governed by the same fundamental principal and molecular/cellular modules in immune tolerance. Future research should aim to further elucidate these molecular and cellular mechanisms underlying Th cell regulation, while concurrently examining the parallels and distinctions in CD4⁺ T

cell fate decisions and dysfunction across autoimmunity, chronic infection, and cancer. Such investigations could provide critical insights into the remarkably diverse roles of T cells in health and disease and reveal novel therapeutic strategies to rectify immunological disorders.

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