Molecular determinants of brain-resident CD8+ T cells in health and disease

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Abbreviations

scRNA-seq	Single-cell RNA-sequencing
KLRG1	Killer cell lectin-like receptor subfamily G member 1
IL-7Rα	Interleukin 7 receptor alpha; CD127
CXCR3	C-X-C chemokine receptor type 3
Ly6C	Lymphocyte antigen 6C2
CCR7	C-C chemokine receptor type 7
CX3CR1	CX3C chemokine receptor 1
Trm	Resident memory T cell
IFNγ	Interferon gamma
TNF	Tumor necrosis factor
TCR	T cell receptor
IL-33	Interleukin 33
S1P	Shingosine-1-phosphate
S1PR1	Shingosine-1-phosphate receptor 1
PD-1	Programmed cell death protein 1
LAG3	Lymphocyte activation gene 3
TIM3	T cell immunoglobulin and mucin domain-containing protein 3
Blimp-1	B lymphocyte-induced maturation protein
Hobit	Homolog of Blimp-1 in T cells
KLF2	Krüppfel-like factor 2
Eomes	Eomesodermin
T-bet	T-box transcription factor TBX21
IL-15	Interleukin 15
TCF-1	T cell factor 1
Runx3	Runt-related transcription factor 3
ID3	Inhibitor of DNA Binding 3
TGF-β	Transforming growth factor beta
LAP	Latency associated peptide
TGF-βRII	Transforming growth factor beta receptor II
Bcl-2	B cell lymphoma 2
LCMV	Lymphocytic choriomeningitis virus

gp33	Glycoprotein 33-41
Tpex	Precursor of exhausted T cell
Tex	Terminally exhausted T cell
TOX	Thymocyte selection-associated high mobility group box protein TOX
CCL5	C-C motif chemokine 5
SVZ	Subventricular zone
AD	Alzheimer's disease
NFT	Neurofibrillary tangles
APP	Amyloid precursor protein
PSEN1	Presenilin-1
CXCR6	C-X-C chemokine receptor type 6
NK	Natural killer
IRF4	Interferon regulatory factor 4
GzmB	Granzyme B
Treg	Regulatory T
SPF	Specific-pathogen free
ICB	Immune-checkpoint blockade
RAG	Recombination-activation gene
PMA	Phorbol myristate acetate
i.p.	Intraperitoneally
i.v.	Intravenously
Smad2	Mothers against decapentaplegic homolog 2
Smad3	Mothers against decapentaplegic homolog 3
Smad4	Mothers against decapentaplegic homolog 4
LAP	Latency-associated peptide
KEGG	Kyoto Encyclopedia for Genes and Genomes

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Abstract

T cells populate the brain at steady state, where they contribute to its physiology and protect the host against re-infection. Tissue-resident memory T (Trm) cells residing in various non-lymphoid tissues are known to adopt tissue-specific transcriptional programs shaped by the tissue microenvironment. Whether brain-resident CD8+ T cells similarly acquire a tissue-specific transcriptional landscape, and to what extent this molecular signature is altered in neuropathology, remain to be determined. In addition, the signaling pathways and transcription factors that govern the formation, maintenance, and function of brain CD8+ Trm cells are largely unknown. In this study, I unravel the heterogeneity of brain-resident CD8+ T cells in mice using single-cell RNA-sequencing and high-parameter flow cytometry. Specifically, I profile brain CD8+ Trm cells in naïve young adult mice, and in the contexts of cerebral amyloidosis, systemic acute and chronic viral infection, and aging. From these studies, a framework of a predominantly tissue-specific CD8+ T cell landscape has emerged, largely defined by the expression of the inhibitory receptor PD-1, the surface molecule Ly6C, and the transcription factor TCF-1. Conversely, CD8+ T cells adopted context-specific phenotypic and functional properties in chronic viral infection. Interrogating the molecular determinants of brain CD8+ T cell differentiation and maintenance, I found that TCF-1 promotes the differentiation of brain CD8+ T cells yet negatively regulates the population size upon antigen-specific rechallenge. In addition, PD-1 signaling was important for the differentiation of optimal CD8+ T cell memory in the brain, and was necessary for robust secondary expansion and effector function upon antigen re-encounter. Finally, the cytokine transforming growth factor (TGF)-β was required for the formation of brain-resident CD8+ T cells and for constraining their transition into effector-like cells upon antigenic rechallenge. Taken together, these findings highlight common, tissue-specific as well as context-specific features of brain CD8+ Trm cells, and provide insights into the molecular mechanisms orchestrating their formation and function.

Declaration

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This is to certify that

(i). the thesis comprises only my original work towards the PhD except where indicated in the preface;

(ii). due acknowledgement has been made in the text to all other material used; and

(iii). the thesis is fewer than the 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Bonn, May 7, 2024 Tarek Elmzzahi

Preface

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Chapter 1: Introduction

T cells represent central components of the immune response, playing the roles of orchestrators and effectors in diverse contexts such as infection, autoimmunity, and cancer (Collier et al. 2021). Beyond their crucial role in conferring protection against pathogens, subsets of T cells regulate additional vital developmental and metabolic processes, ranging from promoting microglial cell development and modulating learning/behavior to shaping systemic metabolism (Pasciuto et al. 2020, Ribeiro et al. 2019, Torres et al. 2024). Various conventional and unconventional T cells subsets have been described, based on the dynamics of the T cell response and the composition of the T cell receptor (TCR), i.e. αβ TCR, γδ TCR, or other combinations of invariant and semi-invariant TCR chains. Traditionally described in the context of adaptive immunity, we now know that subsets of T cells, mostly "unconventional" non-αβ T cells, possess innate-like properties. The central theme of this project represents CD8+ T cells, a pillar of the adaptive immune response, in various infectious and non-infectious contexts. Accordingly, the introduction will focus on this subset of αβ T cells.

1.1. Circulating memory CD8+ T cells

1.1.1 Generation of effector and memory CD8+ T cells

The process of V(D)J recombination yields a vast T cell repertoire that allows the host to mount specific T cell responses against an enormous number of antigens. Accordingly, in a naïve mouse, the number of antigen-specific naïve CD8+ T cells recognizing a particular antigenic epitope is only 100-1200 cells (Blattman et al. 2002, Obar et al. 2008). Upon priming or cross-priming of naïve T cells by antigen-presenting cells, the small number of antigen-specific naïve T cells undergo extensive proliferation to generate an exponentially larger (~100,000-fold) progeny of early effector cells (i.e. clonal expansion phase) (Blattman et al. 2002). The classical model of effector/memory T cell differentiation posits that early effector cells predominantly (>90%) differentiate into terminal effector T cells that are chiefly responsible for clearing the pathogen yet are destined to die during the subsequent phase of contraction (Kaech and Cui 2012). Conversely, a small population of memory precursor cells develops in parallel and retains elements of stemness, namely self-renewal, longevity, and pluripotency, i.e. capacity to seed different subsets of mature

memory T cells (Lin et al. 2016, Mueller et al. 2013). Two particular markers that have been classically used to discern terminal effector and memory precursor cells are KLRG1 and the interleukin (IL)-7 receptor α chain (CD127) (Hamann et al. 1997, Kaech et al. 2003). Specifically, terminal effector T cells were found to express KLRG1, whereas effector cells that selectively regain the expression of CD127 acquire a memory precursor state. Compared to a primary immune response by an antigen-specific naïve T cell, memory T cells exhibit a more rapid and more profound effector response upon secondary antigen encounter (Zimmermann et al. 1999, Cho et al. 1999).

1.1.2 Subsets of circulating memory CD8+ T cells

Circulating memory CD8+ T cells have been categorized into two major subsets, namely central memory and effector memory cells, based on their surface phenotype, migration pattern, longevity, and capacity for immediate recall response (Sallusto et al. 1999, Hamann et al. 1997). Both subsets of memory T cells exhibit a surface expression pattern that reflects antigen experience, e.g. the adhesion molecule CD44, the chemokine receptor CXCR3, the surface molecule Ly6C, and the IL-7 receptor α chain (CD127) and IL-15 receptor β chain (CD122) (Wherry et al. 2003b, Graef et al. 2014, Groom and Luster 2011). Indeed, circulating memory CD8+ T cells are known to depend on cytokines, such as IL-7 and IL-15, rather than antigen or tonic TCR signaling, for their long-term survival (Kaech and Cui 2012). On the other hand, central memory T cells express the L-selectin CD62L and the C-C chemokine receptor type 7 (CCR7), which together entail T cell capacity to home to the high endothelial venules of lymph nodes (Sallusto et al. 1999). Accordingly, this subset of memory CD8+ T cells is predominantly found in lymph node as well as in splenic white pulp and blood, and is rarely found in non-lymphoid tissues (Mueller et al. 2013). On the other hand, effector memory T cells do not express CD62L and CCR7, and instead recirculate between blood, splenic red pulp, and non-lymphoid tissues (Jung et al. 2010, Mueller et al. 2013).

Compared to effector memory CD8+ T cells, central memory CD8+ T cells exhibit greater longevity, production of IL-2, and accordingly show a more robust secondary expansion with generation of effector and secondary memory T cells (Graef et al. 2014, Toumi et al. 2022). However, central memory CD8+ T cells possess limited ability to immediately produce effector cytokines and cytolytic molecules upon antigen

reencounter. Instead, effector memory CD8+ T cells are poised for immediate effector function during a secondary immune response (Chen et al. 2018). Therefore, the circulating memory T cell compartment comprises at least two subsets with variable extents of differentiation and longevity to allow for an immediate recall response while simultaneously generate progenies to sustain a long-lived memory T cell pool.

Although the central/effector memory dichotomy has served as a useful model to describe circulating memory T cells, subsequent studies have uncovered additional memory cell populations that do not fit into this simplistic two-subset approach (Jameson and Masopust 2018). For instance, evidence suggests that not all effector-phase KLRG1+ CD8+ T cells die during the contraction phase, with a small subset of "long-lived effector cells" surviving into the memory phase, which combined phenotypic and functional features of canonical effector and memory T cells (Renkema et al. 2020, Olson et al. 2013). Further, another study demonstrated that a subset of KLRG1+ effector cells downregulates KLRG1, acquires CD127 expression, and generates different subsets of memory CD8+ T cells (Herndler-Brandstetter et al. 2018). On the other hand, there is evidence that CX3CR1 functionally demarcates different subsets of memory CD8+ T cells (Bottcher et al. 2015). CX3CR1- memory T cells were chief producers of IL-2 and possessed a greater proliferative potential, whereas CX3CR1+ cells were more terminally differentiated as evidenced by an inferior proliferative potential and yet a greater capacity for cytotoxicity (Bottcher et al. 2015). Moreover, apart from the central memory-like CX3CR1- cells and effector memory-like CX3CR1+ cells, CX3CR1-intermediate memory T cells were also described (Gerlach et al. 2016). Such CX3CR1-intermediate memory T cell population showed a superior proliferative and self-renewal capacity compared to other memory T cells, and were found to be the major memory cell subset recirculating between non-lymphoid tissues and circulation (Gerlach et al. 2016).

1.2. Resident-memory T (Trm) cells

1.2.1 Discovery, tissue distribution, and function of Trm cells

Effector memory T cells have been described to patrol non-lymphoid tissues as part of their re-circulation pattern (Sallusto et al. 1999, Wherry et al. 2003b). However, intense research over the past 15 years has elucidated a new subset of memory T cells in non-lymphoid tissues that are essentially non-recirculating, and hence defined as tissue-resident memory T (Trm) cells (Gebhardt et al. 2009, Masopust et al. 2001). It was

previously shown that systemic infection, e.g. with *Listeria monocytogenes* or vesicular stomatitis virus, results in the formation of stable memory CD8+ and CD4+ T cells in various lymphoid and non-lymphoid tissues, including lung, kidney, and small intestine (Masopust et al. 2001, Reinhardt et al. 2001). Effector T cells seeded non-lymphoid tissues during the expansion phase of the T cell response, and memory-phenotype T cells could still be recovered from non-lymphoid tissues many months post-infection (Masopust et al. 2001). A subsequent study employing parabiosis has shown that memory T cells in many non-lymphoid tissues do not equilibrate to the same extent as T cells in lymphoid tissues (Klonowski et al. 2004). Specifically, highly vascularized organs such as liver and lung exhibited a greater extent of equilibration compared to the brain and small intestine (Klonowski et al. 2004). In addition, transplantation of a skin graft from an immune mouse onto a naïve recipient showed that CD8+ T cells residing in the skin of the donor mouse persisted within the graft and did not migrate to the spleen of the recipient mouse (Gebhardt et al. 2009). A largely similar approach was adopted to show that small intestinal CD8+ Trm cells are also non-recirculating (Masopust et al. 2010). Accordingly, these seminal studies established Trm cells as a sessile, autonomous T cell population that persists long-term in non-lymphoid tissues.

During a secondary immune challenge, CD8+ Trm cells mediate a rapid recall response that includes target cell lysis and secretion of effector cytokines (Gebhardt et al. 2018). In fact, CD8+ Trm cells possess a superior cytolytic capacity compared to their circulating memory CD8+ T cell counterparts (Masopust et al. 2001). In some tissues, Trm cells are found to express elevated amounts of transcripts encoding effector molecules, such as granzyme B, implying that Trm cells are poised for a rapid response upon antigen re-encounter (Gebhardt et al. 2018). Apart from this cardinal feature of memory T cells, an additional facet of CD8+ Trm cell secondary response is the innate-like induction of an “alarm state” in the responding tissue (Schenkel et al. 2014, Schenkel et al. 2013, Ariotti et al. 2014). Upon antigenic rechallenge, Trm cells induce a local state of alert by facilitating the recruitment of natural killer (NK) and myeloid cells, as well as circulating antigen-specific and bystander T cells in an interferon (IFN)γ-dependent manner (Schenkel et al. 2013). Nevertheless, CD8+ Trm cells retain the ability to mediate host protection in a self-sufficient manner, since depletion of circulating T cells prior to rechallenge did not impair Trm cell capacity to mediate viral clearance (Mackay et al. 2015b).

1.2.2. Surface molecules promoting Trm retention and surveillance

As alluded to above, CD8+ Trm cells were definitively shown to be sessile cells using skin or small intestinal graft transplantation and parabiosis experiments (Klonowski et al. 2004, Gebhardt et al. 2009, Masopust et al. 2010). Given the relative technical complexity of such approaches, it was important to identify additional strategies to delineate Trm cells from circulating memory T cells. One strategy is to inject mice with a fluorescently labelled antibody, e.g. anti-CD45, 3 minutes before sacrifice, a total time window of ~8 minutes that allows for labelling circulating immune cells while excluding parenchymal immune cells (Anderson et al. 2014).

In addition, a number of phenotypic markers were uncovered to distinguish Trm cells from their circulatory counterparts (Topham and Reilly 2018). A widely used surface molecule used to identify Trm cells at steady state is CD69. Although it is classically known to be an “early activation marker” following TCR engagement, CD69 is expressed in a stable manner by Trm cells in various tissues independent of ongoing TCR stimulation (Casey et al. 2012, Gebhardt et al. 2009). Multiple signaling pathways have been shown to upregulate CD69 in developing Trm cells, including IL-33 and type I interferon (IFN) (Gebhardt et al. 2018). The sphingolipid sphingosine-1-phosphate (S1P) represents a chemotactic gradient in the lymph that governs T cell egress into the circulation, and CD69 antagonizes S1P receptor 1 (S1PR1) on the T cell surface and promotes its internalization, thereby blocking egress and facilitating the retention of T cells in situ (Matloubian et al. 2004, Skon et al. 2013, Mackay et al. 2015a). Subsequent studies have demonstrated that not all CD8+ Trm cells in a given tissue are necessarily expressing CD69, nor that CD69 is functionally relevant for establishing and/or maintaining residency in a uniform fashion across tissues (Steinert et al. 2015, Walsh et al. 2019). Accordingly, the tissue of residence as well as the pathophysiological context need to be taken into account when interpreting CD69 expression by CD8+ Trm cells.

Apart from CD69, additional molecules widely employed to characterize CD8+ Trm cells include the integrins CD103 and CD49a and the chemokine receptor CXCR6 (Mackay et al. 2013, Casey et al. 2012, Cheuk et al. 2017, Wein et al. 2019). Conversely, Trm cells exhibit a reciprocal downregulation of molecules associated with circulating memory T cells and tissue egress, including CCR7, CD62L, CX3CR1, and CD122 (Behr et al. 2018, Urban et al. 2020). CD103 represents the αE chain of the

αEβ7 integrin that serves as a receptor for E-cadherin, a component of epithelial cell layers (Downer and Speight 1993). In fact, CD103 expression is prominent in CD8+ Trm cells in the skin, small intestine, salivary gland, and lung (Crowl et al. 2022, Casey et al. 2012, Gebhardt et al. 2009, Lee et al. 2011). CD49a is an α1 integrin chain similarly expressed by CD8+ Trm cells in skin, lung, and small intestine (Gebhardt et al. 2009, Zhang and Bevan 2013, Richter and Topham 2007). Together with β1 chain, CD49a serves as a receptor for collagen type I or type IV in the extracellular matrix, and signaling downstream of CD49a has been shown to promote T cell survival (Richter and Topham 2007). Interestingly, CD49a contributes to the capacity of CD8+ Trm cells for local tissue surveillance by controlling its locomotion, while CD103 reciprocally promotes Trm cell anchoring in situ (Reilly et al. 2020). Importantly, it is also known that CD103 and CD49a are not uniformly produced by CD8+ Trm cells across tissues, with local antigen and microenvironmental cues dictating the extent of their expression (Topham and Reilly 2018). In addition, CD49a is expressed by a small fraction of circulating antigen-experienced memory CD8+ T cells, although it is further upregulated by mature Trm cells (Bromley et al. 2020). Accordingly, and similar to CD69, the tissue of residence and the pathological context are important determinants of the expression of CD103 and CD49a by CD8+ Trm cells.

1.2.3 Inhibitory receptors controlling Trm cell function

An efficient immune response aims to balance the rapid clearance of an invading pathogen while minimizing collateral host tissue damage. Inhibitory receptors such as programmed cell death protein 1 (PD-1), lymphocyte activation gene 3 (LAG3), and T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) control the activation state and function of T cells to facilitate such immune balance and maintain self-tolerance (Anderson et al. 2016). Similarly, CD8+ Trm cells in various tissues–including the lung, liver, and pancreas–express one or a combination of such inhibitory receptors, an observation that was made in mouse and human (Le Moine et al. 2023, Pauken et al. 2020, Wang et al. 2019, Weisberg et al. 2019). Conversely, PD-1 expression was not observed in intestinal Trm cells (Casey et al. 2012). PD-1 expression is known to be downstream of TCR signaling, and its expression raises the question of whether Trm cells actively engage their TCR in situ. Interestingly, PD-1+ lung Trm cells were found to partially depend on ongoing TCR engagement for their maintenance, whereas TCR signaling was dispensable for Trm cell persistence in

small intestine and salivary gland (Lee et al. 2011, Wang et al. 2019, Wijeyesinghe et al. 2021).

Independent of its requirement for the TCR, PD-1 expression regulates multiple facets of Trm cell function. On the one hand, PD-1 maintains the homeostasis of CD8+ Trm cells, as its deficiency results in an expanded yet dysfunctional pool of liver CD8+ Trm cells (Charlton et al. 2013, Le Moine et al. 2023). On the other hand, PD-1 was also shown to be critical for robust recall capacity of lung CD8+ Trm cells upon antigen re-encounter (Pauken et al. 2020). Importantly, PD-1 expression by lung Trm cells guards against host tissue damage during a secondary rechallenge, since the absence of PD-1 signaling contributed to a phenotype of lung fibrosis in rechallenged mice (Wang et al. 2019). These findings underscore the important role that inhibitory receptor signaling exerts in CD8+ Trm cells to promote optimal immune memory as well as tissue homeostasis (Hombrink et al. 2016).

1.2.4 Transcription factors governing Trm cell formation

In addition to surface molecules, transcriptional profiling of circulating and resident memory T cells identified transcription factors that either are specifically expressed in Trm cells, or expressed in circulating and resident memory T cells but substantially regulate Trm cell formation and function (Behr et al. 2018). One such transcription factor is homolog of Blimp-1 in T cells (Hobit). Hobit is modestly produced by a minor fraction of effector-stage T cells, yet is highly expressed by the majority of Trm cells in various organs, including skin, small intestine, and kidney (Mackay et al. 2016, Parga-Vidal et al. 2021). Mechanistically, Hobit shapes the acquisition of a mature Trm cell state by suppressing the Krüppfel-like factor 2 (KLF2)-CCR7 axis that drives T cell egress from tissues, thereby promoting tissue retention (Behr et al. 2018). An additional transcription factor implicated in Trm cell formation is Blimp1, a key regulator of effector T cell generation and the recall capacity of circulating memory T cells (Kallies et al. 2009). Although Blimp1 was not necessary for Trm cell differentiation in a self-sufficient manner, it was found to act in synergy with Hobit to promote Trm cell formation, as well as program the capacity of Trm cells to produce granzyme B (Mackay et al. 2016, Kragten et al. 2018).

Runx3 is another transcription factor that was found to be crucial for CD8+ Trm cell formation and maintenance (Milner et al. 2017). Runx3 is known to promote the commitment of double-positive thymocytes to the CD8+ T cell lineage, and to

safeguard the differentiation and function of effector T cells (Shan et al. 2017). Deletion of Runx3 in early effector T cells resulted in a small reduction of splenic effectors yet a significant loss of CD8+ Trm cell precursors in non-lymphoid tissues (Milner et al. 2017). Further, deletion of Runx3 in established memory CD8+ T cells resulted in the preferential attrition of Trm cells, demonstrating a role for Runx3 in the establishment and maintenance of CD8+ Trm cells (Milner et al. 2017).

Apart from transcription factors promoting Trm cell formation, the downregulation of other transcriptional regulators was found to be similarly important for Trm cell establishment and/or maintenance. For example, the majority of CD8+ Trm precursor cells in skin express Eomesodermin (Eomes) and T-bet, with both transcription factors progressively downregulated as CD8+ Trm cells acquire the canonical CD103+ phenotype (Mackay et al. 2015b). Such downregulation was required, since the overexpression of T-bet or Eomes impeded the formation of skin CD8+ Trm cells. Importantly, residual, low-level expression of T-bet was still required in CD8+ Trm cells to preserve CD122 expression and thereby maintain responsiveness to IL-15, a critical determinant of skin CD8+ Trm cell maintenance (discussed below) (Mackay et al. 2013). Another transcription factor that is thought to guard against the development of Trm cells is T cell factor 1 (TCF-1) (Wu et al. 2020). TCF-1, encoded by *Tcf7*, plays an important role in T cell development and is highly expressed in naïve T cells (Escobar et al. 2020). During the peak effector phase, TCF-1 is downregulated in effector CD8+ T cells, yet its expression is maintained in multipotent memory precursor cells (Pais Ferreira et al. 2020). Further, TCF-1 is also crucial for the formation and recall response of central memory CD8+ T cells (Zhou et al. 2010, Jeannet et al. 2010). Conversely, lung CD8+ Trm cells were found to comprise a small proportion of TCF-1+ cells, and TCF-1 also appeared to impede the expression of CD103 by such Trm cells (Wu et al. 2020). Whether TCF-1 plays a similar role in Trm cells in other tissues remains unclear.

1.3. Ontogeny and molecular determinants of memory T cells

A large body of work has characterized the ontogeny of circulating and resident memory T cells, and the extrinsic and intrinsic regulators of their formation and maintenance. Transforming growth factor (TGF)-β is a pleiotropic cytokine that regulates multiple facets of T cell biology, including T cell development, naïve T cell homeostasis, effector T cell generation, and circulating and resident memory T cell formation and maintenance (Sanjabi et al. 2017). Furthermore, multiple molecular signals have been shown to dictate the formation and phenotype of Trm cells in a tissue-specific manner (Gebhardt et al. 2018). In this section, I will provide a brief overview of TGF-β biology and its regulation of effector and circulating memory cells, before giving a detailed account of the molecular regulators shaping Trm cell formation and maintenance, including TGF-β.

1.3.1. TGF-β signaling – a brief overview

TGF-β is a pleiotropic regulatory cytokine with diverse, context-dependent roles in embryogenesis, cancer, and immunity (Vander Ark et al. 2018). Three isoforms of TGF-β have been identified, namely TGF-β1, TGF-β2, and TGF-β3, with TGF-β1 representing the isoform predominantly implicated in immune cell modulation (Li and Flavell 2008). TGF-β is produced in a biologically inactive precursor form, comprising an N-terminal latency associated peptide (LAP) and a C-terminal active TGF-β (Chen 2023). Proteolytic cleavage by means of extracellular matrix proteases (such as matrix metalloproteases) or surface integrins (such as αVβ8) promotes the liberation of the bioactive TGF-β (Li and Flavell 2008). TGF-β signals through a heterotetrameric receptor comprising two chains from each, TGF-β receptor (TGF-βR)I and TGF-βRII (Wrana et al. 1992). Specifically, TGF-β first binds to TGF-βRII, a constitutively active kinase, which subsequently recruits and activates TGF-βRI (Wrana et al. 1992). Upon TGF-β ligation and heteromeric receptor formation, the TGF-βRI kinase domain activates Smad2 and Smad3, signal transducers and transcription factors, allowing them to form a complex with Smad4 (Vander Ark et al. 2018). This trimeric complex subsequently translocates to the nucleus, and–together with additional transcription factors–modulates the expression of TGF-β-responsive genes (Spittau et al. 2020). Downstream signaling of TGF-β receptor complex may also follow the non-canonical Smad-independent pathway in a context-dependent manner (Spittau et al. 2020).

The crucial immunoregulatory role of TGF-β is exemplified by the lethal pro-inflammatory phenotype observed in *Tgfb1* knockout mice (Shull et al., 1992). Subsequent studies limited TGF-β1 or TGF-βRII deficiency to T cells, formally showing that T cells are key effectors of the observed lethal phenotype (Gorelik and Flavell, 2000; Li et al., 2006; Marie et al., 2006). Nevertheless, we now know that TGF-β is not a uniformly immunosuppressive cytokine, and that it modulates T cell activation and differentiation in a context-dependent fashion (Filippi et al. 2008).

1.3.2. Regulation of naïve, effector and circulating memory T cells by TGF-β

TGF-β signaling is known to regulate multiple facets of T cell differentiation and function. In fact, TGF-β regulates the thymic development of various subsets of T cells (Chen, 2023), including the lineage commitment of CD8+ T cells (Ouyang et al. 2013). Specifically, TGF-β promotes IL-7Rα expression by positively selected, intermediate CD4+ CD8-low thymocytes, where IL-7 signaling is an important determinant of CD8 single-positive T cell fate (Ouyang et al., 2013; Park et al., 2010). In naïve T cells, TGF-β signaling enforces cellular quiescence by tuning the threshold for TCR-mediated T cell activation (Tu et al., 2018). In fact, TCR signaling during T cell priming needs to be of sufficient strength to induce the downregulation of TGF-βRI, and hence the abrogation of the TGF-β-mediated cell quiescence (Tu et al., 2018). Moreover, TGF-β regulates the survival of naive T cells by promoting their expression of CD127 and hence their sensitivity to IL-7 signaling-mediated cell survival (Oh and Li, 2013). Therefore, TGF-β maintains naïve T cell longevity by limiting TCR-dependent activation and promoting cytokine-dependent survival.

As concerns effector T cell differentiation, seminal studies have demonstrated a role for TGF-β signaling in limiting the expansion of effector T cells and promoting their apoptosis (Sanjabi et al., 2009). However, early studies addressing the role of TGF-β signaling in T cells have largely relied on one of two models: transgenic dominant negative mutant of TGF-βRII (DNR), which lacks the kinase domain and thereby shows an abrogated signaling capacity; and $Cd4^{Cre}$ $Tgfbr2^{fl/fl}$ mice (Gorelik and Flavell 2000, Li et al. 2006). In both models, abrogation of TGF-β signaling was under control of the *Cd4* promoter, which results in a lack of TGF-β signaling during thymic development of double-positive thymocytes. Subsequent studies have instead employed $dLck^{Cre}$-(distal promoter of Lck)-mediated deletion of *Tgfbr2*, where Cre activity is initiated following positive selection (Zhang and Bevan, 2013). Using $dLck^{Cre}$ $Tgfbr2^{fl/fl}$ mice, it

was shown that TGF-β signaling promotes rather than suppresses the expansion of effector T cells on day 8 post-infection (Ma and Zhang, 2015; Zhang and Bevan, 2013). This discrepancy may be attributed to the altered TGF-β signaling in double-positive thymocytes, and residual TGF-β signaling observed in the DNR model (Ishigame et al., 2013; Zhang and Bevan, 2013).

In addition, TGF-β has been shown to control the phenotype and function of memory CD8+ T cells. Absence of TGF-β at the effector phase did not affect the size but rather the composition of the ensuing memory T cell pool, with a reduced frequency of CD127+ and a reciprocal increase in KLRG1+ cells (Ma and Zhang, 2015). Moreover, when *Tgfbr2* was deleted after memory establishment, similar alterations in the subsets of memory CD8+ T cells were observed, with a reduced frequency of cells expressing CD127, CD62L, and CXCR3 (Ma and Zhang, 2015). Such impaired memory T cell differentiation manifested in a poor recall response of antigen-specific memory CD8+ T cells in the absence of TGF-β signaling (Ma and Zhang, 2015). In summary, TGF-β is important for the formation, maintenance, and function of circulating memory T cells, underscoring the context-specific modulation of T cells by the pleiotropic cytokine.

1.3.3. Trm cell ontogeny

The development of CD8+ Trm cells is initiated during the early stages of the T cell response, with early effector CD8+ T cells seeding non-lymphoid tissues already by day 4.5 to day 7 post-infection (Masopust et al., 2010). In fact, commitment of early effectors to the Trm cell fate is at least partially established during the early stages of tissue infiltration. For instance, effector CD8+ cells seeding non-lymphoid tissues on day 7 post-infection already exhibit >90% of the gene-expression program characterizing mature Trm cells of the same tissue (Milner et al. 2017). In addition, such CD8+ Trm precursor cells show transcriptional divergence from both KLRG1+ CD127- effector cells and KLRG1- CD127+ memory precursor cells in spleen (Milner et al., 2017). Given that KLRG1- effector-stage T cells have been shown to give rise to CD8+ Trm cells (Mackay et al. 2013, Sheridan et al. 2014), the transcriptional distinction between circulating KLRG1- CD127+ cells and tissue-seeding precursors suggests that seeding the tissue imparts a transition of early effectors into the Trm cell fate.

1.3.4. TGF-β and other cytokines controlling Trm cell development

After infiltrating the tissue, Trm precursor cells undergo progressive acquisition of the mature, tissue-specific Trm cell phenotype (Behr et al. 2018). Multiple factors, including cytokines, chemokines, local antigen, and inflammation dictate the eventual phenotype of Trm cells. For instance, IL-15 was found to control the differentiation and/or persistence of CD8+ Trm cells in the salivary gland and skin but not in the small intestine (Mackay et al. 2013, Gebhardt et al. 2018, Adachi et al. 2015). Mechanistically, IL-15 signaling in CD8+ Trm precursors induces the upregulation of Hobit and the anti-apoptotic molecule Bcl-2, which has been shown to be expressed by CD8+ Trm cells in various tissues (Gebhardt et al. 2018, Steinbach et al. 2016, Wakim et al. 2010, Lin et al. 2023). Another interleukin, the alarmin IL-33, was found to promote a CD69+ CD103+ phenotype among Trm cells in the salivary gland, and to upregulate CD69 on T cells in vitro (McLaren et al. 2019, Casey et al. 2012).

Another cytokine that is strongly implicated in the differentiation and maintenance of CD8+ Trm cells is TGF-β. In fact, numerous studies have elucidated a role for TGF-β in the formation and/or persistence of Trm cells in various tissues, including skin (Mackay et al. 2013, Hirai et al. 2021), small intestine (Casey et al. 2012, Zhang and Bevan 2013, Sheridan et al. 2014), lung (Lee et al. 2011, Wakim et al. 2015), salivary gland (Thom et al. 2015), and kidney (Ma et al. 2017). Importantly, TGF-β positively regulates the expression of CD103, CD49a, and, to some extent, CD69 (Zhang and Bevan 2013, Kilshaw and Murant 1990, Bromley et al. 2020). In line with the expression pattern of CD103 among CD8+ Trm cells and its dependence on TGF-β, previous studies have shown that CD103 contributes to CD8+ Trm cell formation and maintenance in a tissue-dependent manner. Specifically, CD103 plays an important role in CD8+ Trm cell establishment in the small intestine and salivary gland and Trm cell maintenance in skin and the lung (Thom et al. 2015, Lee et al. 2011, Mackay et al. 2013, Sheridan et al. 2014). Accordingly, TGF-β regulation of surface integrins represents one potential mechanism whereby TGF-β controls the development of Trm cells.

Another putative mechanism through which TGF-β controls Trm cell differentiation is its reciprocal modulation of Eomes and T-bet expression (Mackay et al. 2015b). On the one hand, TGF-β signaling promoted the downregulation of T-bet and Eomes by day 14 post-infection, which is part of the optimal developmental pathway of skin CD8+

Trm cells. On the other hand, overexpression of Eomes or T-bet resulted in downregulation of TGF-βRII expression on skin CD8+ Trm cell precursors (Mackay et al. 2015b). The ensuing unresponsiveness to TGF-β by Eomes-overexpressing Trm precursor cells was associated with an impaired formation of skin CD8+ Trm cells. Another proposed mechanism for TGF-β-induced Trm cell formation is through its antagonism of TCF-1 binding to the *Itgae* (encoding CD103) locus (Wu et al. 2020). In lung CD8+ Trm cells, TCF-1 was found to block CD103 expression, and TGF-β appeared to act upstream of TCF-1 to de-repress the expression of CD103 (Wu et al. 2020).

At this point, an important question presents itself: does the migration of Trm precursors into non-lymphoid tissue represent a stochastic process, or are such early effector cells endowed with a greater potential to yield Trm cells before seeding the tissue of residence? While both scenarios are not mutually exclusive, recent evidence suggests that CD8+ Trm precursors are–at least partially–imprinted by lymphoid tissue-derived cues to preferentially yield CD8+ Trm cells (Kok et al. 2022). Indeed, apart from the local activity of TGF-β to shape the Trm cell pool described above, there is evidence that TGF-β acts to "pre-condition" naïve CD8+ T cells before their migration to their tissue of residence (Mani et al. 2019). Specifically, a subset of skin migratory dendritic cells was found to facilitate the exposure of naïve CD8+ T cells in draining lymph nodes to TGF-β, with a subsequent propensity to give rise to skin CD8+ Trm cells (Mani et al. 2019). To some extent, this finding is consistent with previous reports showing that distinct early effector CD8+ T cell clones can simultaneously give rise to both, circulating and skin-resident memory T cells (Gaide et al. 2015, Kok et al. 2020). Further studies are required to demonstrate whether this phenomenon is unique to skin CD8+ Trm cells or whether it similarly applies to other tissues.

1.3.5. Local antigen and inflammation as determinants of Trm cells

The vast majority of studies addressing the differentiation and maintenance of CD8+ Trm cells employ either a) adoptive transfer of TCR transgenic T cells followed by infection with a pathogen carrying the cognate antigen, or b) track endogenous antigen-specific T cells following infection using MHC class I tetramers. However, there is also evidence that Trm cells can emerge in the absence of infection, including in the context of sterile inflammation. For example, transfer of naïve T cells into a lymphopenic host, and the subsequent lymphopenia-induced homeostatic

proliferation, results in the formation of Trm cells in the gut (Casey et al. 2012). Furthermore, applying a contact sensitizing agent onto the skin facilitates the establishment of skin CD8+ Trm cells (Mackay et al. 2012). These data suggest that the maturation of Trm cell precursor cells into fully differentiated Trm cells is dependent on local cytokines in the tissue microenvironment. Nevertheless, it is also known that the presence of local antigen, allowing for secondary antigen stimulation by Trm precursor cells in situ, fosters the formation of a substantially larger pool of CD8+ Trm cells (Khan et al. 2016, Wakim et al. 2010, Steinbach et al. 2016). In fact, local presence of antigen in skin served as a "selective pressure" to allow for the retention of CD8+ Trm cell precursors specific for local antigenic epitopes (Muschaweckh et al. 2016). Further, it was shown that antigen-specific CD8+ Trm precursors restimulated in situ preferentially develop into Trm cells–at the expense of bystander cells–under conditions where TGF-β in the microenvironment is limited (Hirai et al. 2021). In summary, although sterile or infection-associated inflammation can promote the formation of Trm cells, the presence of local antigen augments and shapes the composition of the Trm cell pool. The exact mechanisms of why local antigen amplifies Trm cell numbers remain incompletely understood.

1.4. T cell response to chronic antigen exposure

In the context of acute viral or bacterial infection, such as *Listeria monocytogenes*, antigen-specific CD8+ T cells expand and mediate the clearance of the invading pathogen shortly after expansion. After pathogen clearance, the ensuing circulating memory CD8+ T cells persist in a cytokine-dependent but antigen-independent manner (Kaech and Cui 2012). However, certain pathogens–including human immunodeficiency virus (HIV)– when delivered at a high dose, persist and skew the differentiation of CD8+ T cells away from bona fide memory T cells and into a parallel, dysfunctional state, namely T cell exhaustion (Gallimore et al. 1998). Exhausted CD8+ T cells are characterized by a) high and sustained expression of multiple inhibitory receptors, including PD-1 and LAG3; b) distinct transcriptional and epigenetic landscapes compared to memory T cells; and c) impaired functionality manifested by progressive loss of the capacity to produce IL-2, TNF, and IFNγ (McLane et al. 2019, Kallies et al. 2020).

Lymphocytic choriomeningitis virus (LCMV) has been an invaluable tool to study effector and memory T cell generation as well as T cell exhaustion (Abdel-Hakeem 2019). The wildtype variant, LCMV Armstrong, has been used to elucidate the mechanisms of effector and memory T cell differentiation, function, and survival. Conversely, other variants of LCMV, including clone 13 and docile, have been employed to elicit chronic viral infection and thereby model T cell exhaustion (Ahmed et al. 1984, Ahmed et al. 1988). Structurally, the clone 13 strain varies from the Armstrong strain by two coding mutations, with one amino acid substitution in the viral RNA polymerase and another substitution at residue 260 of the viral glycoprotein (gp) (Matloubian et al. 1993). These mutations were found to confer the virus with a greater infectivity of myeloid cell subsets and permit its chronicity in vivo (Matloubian et al. 1993). Importantly, major immunodominant epitopes are conserved across LCMV Armstrong and clone 13, including the H2-Db-restricted gp33–41 epitope (Wherry and Kurachi 2015). This allows for tracking CD8+ T cells with the same antigen specificity and comparing their molecular and functional features in the contexts of a resolved versus. chronic viral infection.

Numerous studies over the past years have led to a more refined understanding of the heterogeneity of exhausted CD8+ T cells (Hudson et al. 2019, Zander et al. 2019, Im et al. 2016, Utzschneider et al. 2016, Tsui et al. 2022). The current model posits that the pool of exhausted CD8+ T cells can be categorized into two major subsets, precursor of exhausted T (Tpex) cells and terminally exhausted T (Tex) cells. Compared to Tex cells, Tpex cells possess a greater capacity for self-renewal, superior proliferative potential, relatively preserved effector function, and the capacity to give rise to Tex cells (Kallies et al. 2020). The transcription factor TCF-1 has been shown to be necessary for the formation and maintenance of Tpex cells (Utzschneider et al. 2016). Accordingly, Tpex cells are defined by the expression of TCF-1 and lack of TIM3 expression, the latter exclusively marking Tex cells (Utzschneider et al. 2020). Moreover, a subpopulation of Tpex cells, expressing CD62L and the transcription factor c-Myb, was found to possess stem-like features of quiescence and multipotency, giving rise to CD62L- Tpex cells and Tex cells (Tsui et al. 2022). On the other hand, Tex cells could be classified into two subsets based on the expression of the chemokine receptor CX3CR1 and the glycoprotein CD101, with the CX3CR1+ CD101- retaining a greater functional capacity compared to the terminally exhausted CX3CR1- CD101+ subset (Hudson et al. 2019).

In line with the heterogeneity of memory CD8+ T cells resident in different tissues, exhausted CD8+ T cells were found to acquire distinct transcriptional programs and effector functions in different tissues (Sandu et al. 2020b). In fact, exhausted T cells in the lung were found to comprise a large frequency of CX3CR1+ cells and exhibit a greater capacity to produce IFNγ and granzyme B compared to other lymphoid or non-lymphoid tissues (Sandu et al. 2020b). This suggests that the tissue microenvironment is actively shaping the differentiation of CD8+ T cells also in the context of chronic viral infection.

1.5. Role of T cells in brain homeostasis

The interaction between the brain and the immune system has long been dominated by the notion of the “immune-privilege” of the brain, and that T cells are only found in the brain in a neuropathological context (Galea et al. 2007). Yet, our understanding of the neuro-immune interface has undergone gradual refinement over the past decade. For instance, lymphatic vessels were discovered to line the dura mater, the outermost of the three layers comprising the meninges (Aspelund et al. 2015, Louveau et al. 2015). In fact, various subsets of αβ and γδ T cells were found to patrol the meninges and produce cytokines that modulate learning and behavior (Alves de Lima et al. 2020, Ribeiro et al. 2019, Kipnis et al. 2012). Apart from the dura, another neuro-immune interface represents the choroid plexus, a structure that produces and filters the cerebrospinal fluid, and whose fenestrated capillaries facilitate immune cell trafficking (Strominger et al. 2018). Of note, both the meninges and choroid plexus host subsets of tissue-resident macrophages that contribute to various aspects of brain physiology (Mrdjen et al. 2018, Kierdorf et al. 2019).

The presence and a role of T cells in the brain parenchyma at steady state has only recently been demonstrated (Mrdjen et al. 2018, Pasciuto et al. 2020). Microglia are tissue-resident macrophages found in the brain parenchyma, comprise 5-10% of total brain cells, and play critical roles in brain development and homeostasis (Frost and Schafer 2016). A recent study has shown that brain-resident CD4+ T cells are required to safeguard microglia maturation (Pasciuto et al. 2020). In addition, CD8+ T cells have also been described in the brain parenchyma of naïve young adult mice (Ayasoufi et al. 2023). This population of CD8+ T cells was found to be distant from the vasculature, yet parabiosis experiments revealed that a fraction of brain CD8+ Trm cells equilibrated between parabionts, implying a requirement for their continuous replenishment from

the periphery (Ayasoufi et al. 2023). Whether brain-resident CD8+ T cells contribute to brain development or homeostasis at steady state remains unclear.

1.6. Brain-resident T cell response to local and systemic infection

Early studies investigating brain CD8+ Trm cells have mostly employed direct brain infection via intracranial or intranasal delivery of pathogen. Brain CD8+ Trm cells were found to undergo homeostatic proliferation, and to persist without replenishment by circulating memory T cells or ongoing antigen encounter (Wakim et al. 2010, Steinbach et al. 2016). The majority of such CD8+ Trm cells expressed CD103, which was found to be necessary to promote T cell retention in the brain (Wakim et al. 2010). Importantly, local recognition of antigen in situ during T cell priming was required for robust CD103 expression, since systemic infection resulted in CD103 expression by only a minor fraction of the ensuing Trm population (Wakim et al. 2010, Steinbach et al. 2016). Similar to what was observed in skin Trm cells (Mackay et al. 2012), brain CD8+ Trm cells robustly mediated pathogen clearance upon antigenic rechallenge independent of circulating T cells (Steinbach et al. 2016).

Intraperitoneal infection with LCMV Armstrong does not manifest in brain infection (Wherry et al. 2003a). Systemic acute infection results in seeding of effector T cells and subsequent CD8+ Trm cell formation in various organs, including organs not extensively infected by the pathogen (Masopust et al. 2001, Casey et al. 2012). Indeed, systemic LCMV Armstrong infection results in a brain CD8+ T cell population that exhibits hallmarks of tissue residency, including surface expression of CD69 and CD49a, and resistance to systemic depletion using anti-CD8α antibody (Urban et al. 2020). Moreover, the frequency of CD103 expressing brain CD8+ Trm cells in this context is negligible, again highlighting the requirement for local infection to induce CD103 expression in brain CD8+ T cells (Wakim et al. 2012, Maru et al. 2017). Further, CD103 deficiency was not found to impair the formation of brain CD8+ Trm cells upon systemic LCMV infection (Urban et al. 2020). Importantly, such “peripherally induced” brain CD8+ Trm cells were sufficient to confer protection against local antigenic rechallenge–in terms of pathogen clearance and mouse survival–independent of the contribution of circulating memory T cells (Maru et al. 2017, Urban et al. 2020). Therefore, systemic viral infection generates bona fide brain-resident CD8+ T cells.

Similar to Trm cells in other tissues, previous studies have addressed the role of inhibitory receptors, primarily PD-1, in the generation of brain CD8+ Trm cells (Shwetank et al. 2019, Scholler et al. 2020, Schachtele et al. 2014, Prasad et al. 2017). These studies yielded contrasting results as to whether PD-1 positively or negatively regulates the formation of brain-resident CD8+ T cells. Although intracranial infection was a common theme among the aforementioned studies, they employed different pathogens with variable extents of persistence and inflammation. Conversely, whether PD-1 contributes to the formation and function of brain CD8+ T cells following a systemic infection is unknown. As alluded to above, TGF-β is critical for the formation and maintenance of CD8+ Trm cells in various tissues. Two previous studies– employing intracranial infection models–have shown a correlation between the amount of Treg-derived TGF-β and the formation of CD103+ CD8+ T cells in the brain (Prasad et al. 2015, Graham et al. 2014). However, it remains unclear whether TGF-β acts on brain CD8+ T cells in a cell-intrinsic manner, or whether TGF-β signaling modulates brain-resident T cells in a setting of systemic infection.

1.7. Ageing-associated brain T cell alterations

Ageing is defined as the gradual loss of tissue integrity and function over time, resulting in an increased susceptibility to a range of pathologies, including cancer and neurodegeneration (reviewed by (Lopez-Otin et al. 2013)). One of the hallmarks of ageing is cellular senescence, where cells lose their proliferative capacity and acquire a pro-inflammatory “secretory phenotype” (Tchkonia et al. 2013). Indeed, senescent cells contribute to generating a chronic, subclinical, sterile inflammation in geriatric patients, or “inflammageing”, culminating in widespread cellular dysfunction (Olmedillas Del Moral et al. 2019).

Both innate and adaptive immune cells are subject to age-dependent alterations. Macrophages, dendritic cells, and neutrophils exhibit impaired phagocytic capacity, production of effector molecules, and cytokine signaling (Nikolich-Zugich 2018). T cells also display age-related alterations, including diminished T cell generation, reduced naïve T cell and increased memory T cell frequencies, and a low-diversity TCR repertoire (Rudd et al. 2011). As concerns CD8+ T cells, a recent study has shown that aged mice exhibit a substantially increased frequency of PD-1+ TOX+ CD8+ T cells across lymphoid and non-lymphoid tissues, which also highly produced the

chemokine CCL5 and granzyme K (Mogilenko et al. 2021). Such CD8+ T cell phenotype represents a terminal differentiation state and develops in a cell-extrinsic fashion, i.e. is dependent on the pro-inflammatory microenvironment in tissues of aged mice (Mogilenko et al. 2021).

Similar to what is observed in the periphery, ageing is associated with a profound alteration of the immune cell landscape in the brain. For instance, a microglia subset that accumulates lipid droplets was identified in aged mice (Marschallinger et al. 2020). This microglia subset exhibited defective phagocytosis and enhanced secretion of proinflammatory cytokines compared to non-lipid-laden microglia (Marschallinger et al. 2020). Ageing is also associated with an increased number of CD8+ T cells in the brain (Gemechu and Bentivoglio 2012). Recent reports have investigated the age-related changes in brain-resident T cells and uncovered additional, brain tissue-specific changes in T cell populations and function (Dulken et al. 2019, Ritzel et al. 2016, Kaya et al. 2022, Groh et al. 2021). For instance, brain CD8+ T cells exhibited a greater capacity to produce effector cytokines compared to their circulating counterparts (Ritzel et al. 2016). In another study, CD8+ T cells were found to infiltrate the subventricular zone (SVZ), one of the few brain regions where neurogenesis persists into adulthood (Dulken et al. 2019). CD8+ T cells were located in close proximity to neural progenitors in the SVZ, and inhibited their proliferation in an IFNγ-dependent manner (Dulken et al. 2019). Further, brain CD8+ T cells were shown to drive ageing-associated axonal demyelination in the retina (Groh et al. 2021). This phenotype was induced in a TCR- and granzyme B-dependent manner, since reconstitution of old *Rag1*$^{-/-}$ mice with bone marrow derived from OT-I or *Gzmb*$^{-/-}$ mice failed to promote the degeneration of optic nerves (Groh et al. 2021). Moreover, Kaya et al demonstrated that CD8+ T cells in the white matter of the aged brain induce oligodendrocyte and microglia states of type I interferon response, an induction that is at least partially mitigated by PD-1 expression by CD8+ T cells (Kaya et al. 2022). Notably, treatment of aged naïve mice with immune checkpoint blockade (ICB) resulted in an increased number of brain CD8+ T cells and an enhanced induction of the interferon-responding subsets of oligodendrocytes and microglia (Kaya et al. 2022). These findings point to a predominantly detrimental impact of brain-resident CD8+ T cells in the aged brain. What remains unclear, however, is whether this adverse effect is promoted by the age-associated increase in total number of CD8+ T cells in the brain, or due to qualitative alterations introduced during ageing in the brain CD8+ T cell compartment.

1.8. Brain CD8+ T cells in the context of cerebral amyloidosis

Alzheimer's disease (AD) is a neurodegenerative disease that is most prevalent among the elderly population. AD manifests with memory and cognitive impairments that progress over time (Breijyeh and Karaman 2020). The histopathological hallmarks of AD are the deposition of amyloid beta (Aβ) aggregates in the brain parenchyma as well as neurofibrillary tangles (NFT) comprised of hyper-phosphorylated tau (Breijyeh and Karaman 2020). Under homeostatic conditions, amyloid precursor protein (APP) is sequentially cleaved by β-secretase and γ-secretase into different Aβ species, which range in size from 38 to 42 amino acids (Haass and Selkoe 2007). Over 90% of the produced Aβ species are shorter than 40 amino acids, whereas only <5% of the product represents the pathogenic, aggregation-prone Aβ42 species at steady state (Li et al. 2016). Mutations in APP or in presenilin 1 (PSEN1; a subunit in an enzymatic complex breaking down APP) favor the accumulation of amyloidogenic Aβ42 species (Haass and Selkoe 2007). Animal models such as APP/PS1 and 5xFAD mice exploit this biochemical pathway to induce amyloid deposition (Oakley et al. 2006, Radde et al. 2006), Specifically, these models are generated by introducing human transgenes encoding mutant APP and PSEN1 that promote the accumulation of pathogenic Aβ42 species (Oakley et al. 2006, Radde et al. 2006). In APP/PS1 mice, amyloid plaques appear at ~1.5 month of age, followed by synaptic dysfunction and cognitive impairment by 7-8 months that further progress up at 15 months (Gengler et al. 2010, Serneels et al. 2009). Using such models of cerebral amyloidosis, numerous studies have explored the myeloid cell contribution and response to amyloidosis aspect of AD pathology. For instance, two studies employing different transcriptional profiling methodologies have identified a CD11c+ microglial subset to cluster around Aβ plaques (Kamphuis et al. 2016, Keren-Shaul et al. 2017). This CD11c+ microglia subpopulation highly expressed genes associated with phagocytosis and was more adept at taking up Aβ, leading authors to label it as a disease-restricting subset (Keren-Shaul et al. 2017).

The role of T cells in the context of cerebral amyloidosis has been subject to several studies, with conflicting results. For instance, upon crossing of APP/PS1 to *Rag2*$^{-/-}$ *Il2rγ*$^{-/-}$ mice (which lack B, T, and NK cells), the immunodeficient APP/PS1 progeny suffered exacerbated Aβ deposition (Marsh et al. 2016). This phenotype was coupled

to enhanced neuroinflammation and defective phagocytic capacity of microglia. Further, bone-marrow reconstitution mitigated Aβ pathology (Marsh et al. 2016). In contrast, depletion of CD8+ T cells in 12 months-old APP/PS1 mice had little to no impact on neuropathological and behavioral outcomes (Unger et al. 2020). A more recent study found that CXCR6-/- 5xFAD mice exhibited an impaired accumulation of brain CD8+ Trm cells, particularly the PD-1+ subset, accompanied by aggravated amyloid plaque pathology and cognitive decline (Su et al. 2023b). One notable variable across these papers is the timepoint–and hence the stage of neuropathology–at which brain CD8+ T cells were manipulated. Importantly, the number of brain CD8+ Trm cells appeared to increase in the 5xFAD mouse brain in an age-dependent manner (Su et al. 2023b). Whether a qualitative difference emerged in brain CD8+ T cells in cerebral amyloidosis models compared to their counterparts in wild-type mice remains unclear.

1.9. Study aims:

The tissue microenvironment plays a significant role in shaping the transcriptome and phenotype of CD8+ Trm cells (Crowl et al. 2022, Christo et al. 2021). A large number of studies have elucidated the molecular profile of CD8+ Trm cells in organs such as skin, small intestine, and salivary glands. In contrast, the transcriptional and phenotypic landscapes of brain CD8+ Trm cells at steady state, and whether they are altered in the context of disease, remain poorly characterized. In addition, there are significant gaps in our knowledge of the signaling pathways and transcription factors that govern the formation, maintenance, and function of brain CD8+ Trm cells. This is perhaps best reflected in the employment of germline *Cd8* KO or systemic depletion of total CD4+ or CD8+ T cells to evaluate their contribution to various neuropathologies (Kaya et al. 2022, Chen et al. 2023). Accordingly, to address these gaps in knowledge, the aims of this study are:

1. Transcriptional and phenotypic characterization of brain-resident CD8+ T cells at steady state, in ageing, and disease
2. Identification of molecular determinants of the differentiation and maintenance of brain-resident CD8+ T cells

Chapter 2: Materials and methods

2.1 Equipment and consumables

Table 1 | Equipment and Instruments

Instrument	Vendor
FACSymphony A5 flow cytometer	BD
FACS Aria III cell sorter	BD
FACSymphony A5 cell sorter	BD
BD High Throughput Sampler	BD
Cytek Aurora 5L spectral cytometer	Cytek Biosciences
NextSeq500	Illumina
NovaSeq6000	Illumina
NextSeq 500/550 High Output Kit v2.5	Illumina
NovaSeq 6000 S1 Reagent Kit (100 cycle)	Illumina
Freedom Evo	Tecan
4200 TapeStation	Agilent Technologies
Qubit fluorometer	Thermo Fisher
BD Rhapsody Express	BD
Heraues Megafuge 40R	Thermo Fisher
Heracell 240i CO2 Incubator	Thermo Fisher
Dissecting instruments	Everhards GmbH
Neubauer counting chamber	Superior Marienfeld
DynaMag-2 Magnet	Thermo Fisher
Vortex Mixer	Velp Scientifica
Thermocycler Biometra Trio Biometra	Biometra
Thermomixer Comfort	Eppendorf GmbH
Orbital Incubator S1500	Stuart
Sartorius Quintix 613	Sartorius
EuroClone Safe-mate 1.8	EuroClone
Spectrophotometer BioMate 3	Thermo Spectronic
Pipette, single or multichannel	Eppendorf GmbH

BD Rhapsody P1200M pipette	BD
BD Rhapsody P1500M pipette	BD
Large magnetic separation stand	V&P Scientific

Table 2 | Disposables

Item	**Vendor**
20 gauge needle	VWR
26 gauge needle	VWR
BD Rhapsody Cartridge Kit	BD
70 µm cell strainer (EASYstrainer)	Greiner Bio-One
Serological pipettes, 5, 10, 25 ml	Sarstedt
50 ml conical tube	Sarstedt
15 ml conical tube	Sarstedt
LIGHTSAFE MICRO CENTRIFUGE TUBES 1.5 ml	Sigma
Inkjekt Luer Solo 5 ml, 10 ml	Braun
OmnicanF (1ml injection syringe)	Braun
P10, P200, P1000 pipette tips	Starlab
5 mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap	Corning
5ml polystyrene round-bottom tube	Corning
SafeSeal reaction tube, 1.5 ml and 2 ml	Sarstedt
DNA LoBind tube, 1.5 ml	Eppendorf
PCR reaction tubes, 0.2 ml	Eppendorf
Reagent reservoirs, 50 ml	Carl Roth
Cell culture plate, 24 well, surface: Standard, flat base	Sarstedt
Cell culture plate, 96 well, surface: Suspension, round base	Sarstedt
Terumo Disposable Syringe without Needle 3 ml	Terumo
Terumo Insulin Syringe - 1ml 27G x 13mm	Terumo
Fisherbrand Low-Nitrogen Weighing Paper 4 x 4	Fisher Scientific
Nunc Cryotube Vials	Thermo Fisher

Inoculation loop, sterile	Westlab
Semi-micro cuvette, 3 ml, (HxW): 45 x 12 mm, PS, transparent	Sarstedt
Alumaseal 384 sealing film	VWR

2.2 Reagents, buffers, and primers

Table 3 | Reagents and chemicals

Reagent	**Vendor**
ACCUCOUNT FLUORESCENT PART 5 ML	Spherotech
BD FACS Accudrop beads	BD
BD Cytometer Setup and Tracking Beads	BD
BD FACS Flow	BD
Brefeldin A	Thermo Fisher
Fetal Bovine Serum	Pan-Biotech
Dulbecco's modified Eagle medium (DMEM)	Gibco
Dulbecco's Phosphate buffered saline (PBS) (1x)	Sigma
Roti-CELL 10x PBS CELLPURE sterile	Carl Roth
Ethanol	Roth
Ethylenediaminetetraacetic acid (EDTA), 0.5 M	Sigma
Brilliant Stain Buffer Plus	BD
eBioscience Foxp3 / Transcription Factor Staining Buffer Set	Thermo Fisher
Percoll	GE Healthcare
HEPES 1 M	Gibco
Ketamine (10%)	Medistar
Non-essential amino-acids (100x)	Thermo Fisher
UltraComp eBeadsCompensationBeads	Life Technologies
Protein Transport Inhibitor (Containing Monensin), BD GolgiStop	BD
eBioscience Cell Stimulation Cocktail (500X)	Thermo Fisher
UltraPure DNase/RNase-Free Distilled Water 10x500ml	Thermo Fisher
Qubit dsDNA HS Assay Kit	Thermo Fisher

4% paraformaldehyde phosphate buffer solution	Novachem
Agencourt Ampure XP beads	Beckman Coulter
Betaine (BioUltra ≥99%)	Sigma-Aldrich
Magnesium chloride (MgCl2; anhydrous)	Sigma-Aldrich
Dithiothreitol (DTT)	Invitrogen
dNTP Set (100 mM)	Thermo Fisher
BD Rhapsody Cartridge Reagent Kit	BD
BD Rhapsody cDNA Kit	BD
BD Rhapsody WTA Amplification Kit	BD

Table 4 | Enzymes

Enzyme	**Vendor**
Collagenase type II	Sigma
DNase type I	Roche
Superscript II reverse transcriptase	Invitrogen
KAPA HiFi HotStart ReadyMix 2x	KAPA Biosystems

Table 5 | Primers

Primer	**Sequence**
Template-switch oligo (TSO)	5′-AAGCAGTGGTATCAACGCAGAGTACATrGrG+G-3′
ISPCR oligo	5′-AAGCAGTGGTATCAACGCAGAGT-3′
Oligo-dT_{30} VN	5′–AAGCAGTGGTATCAACGCAGAGTACT_{30}VN-3′

N, any base; V, either an A, C, or G

Table 6 | Buffers and media

Item	**Ingredients**
80 % ethanol	80 % ethanol (v/v) in nuclease-free water
Fetal bovine serum (FBS)/DMEM	9% FBS (v/v) in DMEM

Digestion buffer (brain)	1 mg/ml collagenase type II 0.02 mg/ml DNase I 10 mM HEPES 9% FBS/DMEM
FACS buffer	3% (v/v) in 1x PBS
T cell medium	IMDM with L-Glutamine 10% FBS 1% GlutaMAX 55 µM β-Mercaptoethanol 0.5 mM sodium pyruvate 1% non-essential amino-acids 5 mM HEPES 100 U/ml penicillin 100 µg/ml streptomycin
PFA 4%	4% (v/v) PFA in 1xPBS
27% Percoll	27% (v/v) Percoll + 9% FBS/DMEM
Red blood cell lysis buffer	Ammonium chloride NH4Cl (8.34g) Sodium hydrogen carbonate (1g) EDTA Tetra Sodium Salt dihydrate (0.037g) Distilled Water up to 1000ml Final pH 7.3 ± 0.2
Smart-seq2 lysis buffer	Guanidine hydrochloride (40 mM) OligodT_{30} VN primer (2.17 µM) dNTP (4.3 mM) Nuclease-free water
Smart-seq2, reverse transcription mix	SuperScript II reverse transcriptase (100 U/µl) RNase inhibitor (10 U/µl) Superscript II first-strand buffer (1x) DTT (5 mM) Betaine (1 M) $MgCl_2$ (6 mM) TSO (1 µM) Nuclease-free water

Table 7 | Antibodies and dyes for flow cytometry

Antigen-Fluorochrome	**Vendor**	**Clone**	**Dilution**
CD44-BUV395	Invitrogen	IM7	1:200
CD4-BUV496	BD	GK1.5	1:400
TCRβ-BUV563	BD	H57-597	1:200
CD49a-BUV661	BD	Ha31/8	1:200
CD69-BUV737	BD	H1.2F3	1:200
CD45.2-BUV737	BD	104	1:200

CD62L-BV650	BD	MEL-14	1:400
PD-1-BV711	BioLegend	29F.1A12	1:200
CD45.1-FITC	Invitrogen	A20	1:200
CD11b-BUV737	BD	M1/70	1:200
KLRG1-BV711	BD	2F1	1:200
CD69-PE-Cy7	Invitrogen	H1.2F3	1:200
Ly6C-eFluor450	Invitrogen	HK1.4	1:200
CX3CR1-BV785	BioLegend	SA011F11	1:200
CD45.2-BUV805	Invitrogen	104	1:200
CD8a-PerCP-Cy5.5	Invitrogen	53-6.7	1:200
CD103-APC-R700	BD	M290	1:200
CD103-eFluor450	Invitrogen	2E7	1:200
CD8a-BV785	BioLegend	53-6.7	1:400
Bcl-2 PE	BD	BCL/10C4	1:200
IFNγ-PE-Cy7	Invitrogen	XMG1.2	1:400
TNFα-APC	Invitrogen	MP6-XT22	1:200
TCF1-Alexa 488	Cell Signaling Technology	C63D9	1:100
TCF1-Alexa 647	Cell Signaling Technology	C63D9	1:100
Ki67-Alexa 488	BD	B56	1:200
Granzyme B-PE	Invitrogen	GB12	1:200
FoxP3-PE eFluor610	Invitrogen	FJK-16S	1:100
CD49a-BUV395	BD	Ha31/8	1:200
CD45-BUV496	BD	30-F11	1:200
CD62L-BUV563	BD	MEL-14	1:200
CD69-BUV737	BD	H1.2F3	1:200
CD8a-BUV805	BD	53-6.7	1:200
PD-1-BV421	BioLegend	29F.1A12	1:200
KLRG1-BV480	BD	2F1	1:200
CD44-BV570	BD	IM7	1:200
CD25-BV650	BD	PC61	1:200
Ly6C-BV711	BioLegend	HK1.4	1:200

CCR2-BV750	BD	475301	1:200
Ki-67-BV785	BD	B56	1:100
TOX-Alexa 488	Cell Signaling Technology	E6G50	1:50
NK1.1-BUV615	BD	PK136	1:200
γδ TCR-BUV661	BD	GL3	1:200
ST2-PerCP7eFluor710	Invitrogen	RMST2-2	1:200
CD3e-PE-Cy5.5	Life Technologies	145-2C11	1:200
CD3-FITC	BioLegend	17A2	1:200
CX3CR1-PE-Cy7	BioLegend	SA011F11	1:200
Bcl2-PE-Cy7	BioLegend	BCL/10C4	1:100
CD11b-APCCy7	BD	M1/70	1:400
CD19-APCCy7	BD	1D3	1:400
Ter119-APCCy7	BioLegend	TER-119	1:400
B220-APCCy7	BioLegend	RA3-6B2	1:400
TruStain FcX (anti-CD16/32)	BioLegend	93	1:200
Live/Dead Fixable Blue Dead Cell Stain	Thermo Fischer		1:1000
Live/Dead Fixable Blue near-IR Cell Stain	Thermo Fischer		1:1000

Table 8 | Antibodies used for scRNA-seq sample multiplexing

Antibody	**Vendor**	**Clone**	**Amount added per sample (µg)**
BD Single-Cell Multiplexing Kit Mouse Immuno Sample Tag 1	BD	30-F11	0.05
BD Single-Cell Multiplexing Kit Mouse Immuno Sample Tag 2	BD	30-F11	0.05
BD Single-Cell Multiplexing Kit Mouse Immuno Sample Tag 3	BD	30-F11	0.05
BD Single-Cell Multiplexing Kit Mouse Immuno Sample Tag 4	BD	30-F11	0.05
BD Single-Cell Multiplexing Kit Mouse Immuno Sample Tag 5	BD	30-F11	0.05

BD Single-Cell Multiplexing Kit Mouse Immuno Sample Tag 6	BD	30-F11	0.05
BD Single-Cell Multiplexing Kit Mouse Immuno Sample Tag 7	BD	30-F11	0.05
BD Single-Cell Multiplexing Kit Mouse Immuno Sample Tag 8	BD	30-F11	0.05
BD Single-Cell Multiplexing Kit Mouse Immuno Sample Tag 9	BD	30-F11	0.05
BD Single-Cell Multiplexing Kit Mouse Immuno Sample Tag 10	BD	30-F11	0.05
BD Single-Cell Multiplexing Kit Mouse Immuno Sample Tag 11	BD	30-F11	0.05
BD Single-Cell Multiplexing Kit Mouse Immuno Sample Tag 12	BD	30-F11	0.05

2.3 Tetramerization

Table 9 | Tetramers and peptides

Item	**Manufacturer**
gp33-41 peptide - KAVYNFATC	JPT Peptide Technologies
MHC class I (H2-Db) / g33-41 monomer	NIH Tetramer Core Facility

Biotinylated MHC class I (H2-Db) monomers were folded in the presence of gp33 peptide to generate a soluble monomeric MHC class I/peptide complex (NIH Tetramer Core Facility). Tetramerization of the MHC-I/g33 monomers was carried out using streptavidin bound to a fluorochrome of interest. Specifically, tetramerization using APC-bound streptavidin (SA-APC) was performed by mixing 1 part of SA-APC to 1.92 part of MHC-I monomer in a step-wise manner. To 50 µg of monomers, increments of SA-APC were added in the following amounts/incubation times at 4 °C:

Step	**Amount of SA-APC (µg)**	**Incubation time (minutes)**
1	6	30
2	6	30
3	5	20
4	5	20
5	4	Until usage

2.4 Mice

Animals were housed in the animal facility of the German Center for Neurodegenerative Diseases, Bonn, Germany, or at the Peter Doherty Institute, the University of Melbourne, with 12 hr light/12 hr dark cycles and unrestricted access to chow and water. Experiments were approved by the Local Animal Care Commission of North Rhine-Westphalia or the Animal Ethics Committee at the University of Melbourne.

Table 10 | Mouse lines

Mouse line	Reference
C57BL/6J wild-type	
B6.SJLPtprcaPep3b/ BoyJ (Ly5.1)	
Hobit^{TomCre}	Kallies Lab (in revision)
Tcf7$^{fl/fl}$	(Steinke et al. 2014)
Tgfbr2$^{fl/fl}$	(Leveen et al. 2002)
Pdcd1$^{fl/fl}$	(Strauss et al. 2020)
Cd8^{Cre}	(Maekawa et al. 2008)
Irf4$^{gfp/gfp}$	Kallies Lab (in revision)
Pdcd1$^{-/-}$	Prof. Daniel Gray (WEHI)
B6.Cg-Tg(Thy1-APPSw,Thy1-PSEN1*L166P)21Jckr (APP/PS1)	Prof. Martin Fuhrmann (DZNE)
Hobit^{TomCre} *Tcf7*$^{fl/fl}$	Kallies Lab
Hobit^{TomCre} *Tgfbr2*$^{fl/fl}$	Kallies Lab
Hobit^{TomCre} *Pdcd1*$^{fl/fl}$	Kallies Lab
Cd8^{Cre} *Tcf7*$^{fl/fl}$	Kallies Lab
Cd8^{Cre} *Tgfbr2*$^{fl/fl}$	Kallies Lab

2.5 Generation of mixed bone-marrow chimeras

Ly5.1 Ly5.2 donor and *Pdcd1* KO Ly5.2 donor mice were sacrificed, and bone marrow was flushed from tibia and femur. Ly5.1 recipient mice were irradiated (2 doses of 550 Rad), and on the same day were reconstituted with bone marrow from Ly5.1 Ly.52 and *Pdcd1* KO Ly5.2 mice, mixed at 1:1 ratio, for a total of 4 million cells via the tail vein.

Mice were monitored and allowed to reconstitute for ~8 weeks. At 8 weeks post-reconstitution, blood was sampled to confirm reconstitution and assess for differences in percentages of cells from each genotype at baseline. Irradiated mice were provided with neomycin supplemented drinking water for the first 4 weeks post-reconstitution.

2.6 Viral and bacterial infections

LCMV Armstrong and clone-13 strains were propagated in baby hamster kidney (BHK) cells and titrated on Vero African green monkey kidney cells. BHK cells were inoculated with virus at a multiplicity of infection of 0.05 and incubated at 37°C and 5% CO2. The flask was shaken every 15 min for the first 1.5 hr of incubation, and then incubation was maintained for a total for 48 hr. At 48 hr, the supernatant–containing viral particles–was collected in 500 µL aliquots in cryotubes and frozen at -80 °C until usage. On the day of infection, frozen stocks were diluted in PBS, and mice were injected with 2 x 10^5 plaque forming units (PFU) intraperitoneally, or with 2 x 10^6 PFU of LCMV-clone-13 via the tail vein.

Listeria monocytogenes expressing the LCMV immunodominant epitope glycoprotein 33-41 (LMgp33) was stored in a glycerol stock at -80 °C. A sterile inoculation loop was used to draw a sample from the glycerol stock of LMgp33, and then to streak a Brain Heart Infusion (BHI) agar plate containing 50 µg/ml streptomycin. Following overnight incubation at 37°C and 5% CO2, a colony of LMgp33 from the agar plate was picked and then inoculated into 20 ml of BHI broth–containing 50 µg/ml of streptomycin–for ~18 hr. An aliquot of the bacterial culture was diluted 1:1000 in a final volume of 25 ml BHI broth, and incubated for 3 hr. Optical density was assessed using a spectrophotometer at 600 nm, and incubation was stopped once the OD value exceeded 0.1, a value that corresponds to ~1 x 10^8 CFU/ml. Bacterial culture was then serially diluted in PBS to an eventual concentration of 5 x 10^5 CFU/ml, and each mouse received 200 µL via the tail vein, corresponding to 1 x 10^5 CFU.

2.7 Labelling of intravascular leukocytes

To label intravascular immune cells, mice received an intravenous injection of 3 µg of CD45 antibody in a final volume of 200 µL PBS. Three minutes later, mice were

euthanized.

2.7 Tissue processing for flow cytometry

Mice were sacrificed using CO_2 asphyxia or an i.p. injection of ketamine (100 mg/Kg bodyweight) and xylazine (10 mg/Kg bodyweight). The head was cut off; fur, skin, and skull cap were removed, followed by isolation of the brain without inclusion of the dura mater. Brain was then immediately put in 2.5 ml of FACS buffer in a 24-well dish on ice. The brain was then drawn and minced in a 50 ml tube containing 4 ml of digestion buffer (1 mg/ml collagenase type II, 0.02 mg/ml DNase I, 10 mM HEPES, 9% FBS/DMEM) and incubated for 30 min 37 °C shaking at 180 rpm. Next, 40 µL of EDTA was added (final concentration of 5 mM) to inhibit the enzymatic activity, together with 10 µL of counting beads, then the tube put on ice. Samples were filtered through a 70-µm strainer into a fresh 50 ml tube, and tissue clumps on the strainer were gently mashed, followed by washing the strainer with 10 ml of 9% FBS/DMEM. Samples were spun down at 300 g 7 minutes 4 °C, followed by aspiration of supernatant using a suction pump. Pellet was resuspended in 10 ml of 27% Percoll, and centrifuged at 600 g for 5 min at 4 °C while setting the brake to 1 (on a scale from 0 to 9). Myelin and debris were aspirated, followed by resuspension in 10 ml of 9% FBS/DMEM. After pelleting and removal of supernatant, the cell pellet was resuspended in 200 µL FACS buffer and transferred to a U-bottom 96-well plate for staining with fluorescent antibodies.

For isolation of lymphocytes from spleen, spleens were mashed over a 70 µm strainer plunged in a 6-well plate filled with 5 ml of PBS. Cell suspension was then transferred to a 50 ml tube, spun down at 400 g 4 °C for 5 min, and supernatant was aspirated using a suction pump. Pellet was resuspended in 1 ml of Red blood cell lysis buffer, incubated at room temperature for 1 min, and the sample was then diluted using 20 ml of PBS. After spinning down and removal of supernatant, cells were resuspended in 2 ml of FACS buffer, and $1/10^{th}$ to $1/20^{th}$ of spleen was transferred to a U-bottom 96-well plate for staining.

2.8 Antibody and tetramer staining

Single-cell suspensions were generated as described in the “tissue processing for flow cytometry” section, and transferred into a U-bottom 96-well plate. After pelleting, cells were resuspended in 50 µL Fc block and fixable Live Dead viability dye–diluted in PBS–for 20 min at 4 °C. Cells were then washed in PBS and incubated with different combinations of fluorescently labelled antibodies targeting surface antigens (Table 7) for 30 min at 4 °C. After washing in PBS, cells were either directly acquired or subject to fixation and intracellular staining.

For experiments involving LCMV infection, CD8+ T cells specific for the LCMV immunodominant epitope gp33-41 were characterized using MHC class I tetramers “presenting” the gp33-41 peptide. After extracellular staining with antibodies, cells were washed once, and resuspended in 50 µL of gp33 tetramers diluted 1:400 in FACS buffer. Cells were incubated with the tetramers for 45 min at 4°C, followed by washing in PBS and fixation for subsequent intracellular staining.

For intracellular staining, cells were fixed using the eBioscience Transcription Factor Staining kit according to the manufacturer instructions. Cells were resuspended in the fixative agent for 1 hr at room temperature, followed by washing twice in 1x perm buffer. This was followed by incubation with antibodies targeting intracellular antigens (Table 7) for 2 hr at room temperature. For experiments involving *Hobit*^{TomCre} mice, cells were first fixed in 4% PFA for 20 min at 4 °C to preserve tdTomato’s fluorescence, followed by washing once in PBS, before proceeding to using the eBioscience kit as stated above.

Single-stained UltraComp Compensation Beads were used to set up the compensation matrix (conventional cytometer) or for unmixing (spectral cytometer). For BD FACSSymphony instruments, calibration was performed using CS&T beads for laser delay and 8-peak beads for PMT voltage optimization. For Cytek Aurora, SpectroFlo QC beads were used for calibration.

2.9 Ex vivo stimulation

Cells were isolated as described under “tissue processing for flow cytometry” and subjected to surface staining as described under “antibody and tetramer staining”. For LCMV experiments, cells were restimulated with gp33-41 peptide in T cell medium at a

concentration of 5 μM for 30 min at 37 °C and 5% CO_2. This was followed by addition of brefeldin A (final dilution 1:1000) and incubation for 4 hr at 37 °C and 5% CO_2. For PMA/ionomycin stimulation, cells were stimulated for 4 hr at 37 °C and 5% CO_2 using eBioscience Cell Stimulation Cocktail (500X), and simultaneously adding BD Golgi Plug and Golgi Stop, according to the manufacturer's instructions. Cells were then washed once using PBS, followed by incubation with Live Dead viability dye (1:1000 in PBS) for 15 min, followed by fixation using the eBioscience kit and intracellular staining as described under "Antibody and tetramer staining".

2.10 Cell sorting for scRNA-seq

BD Rhapsody**:** Equivalent amounts (0.05 μg) of a unique hashtag oligo (HTO)-conjugated anti-CD45 antibody (BD Mouse Immune Single-Cell Multiplexing Kit) and fluorescently labelled CD45 antibody were added to each sample during the "Extracellular Staining" step above. Following a 30min incubation at 4°C, cells were washed, pelleted, and resuspended in FACS buffer in preparation for sorting. Drop delay was set semi-automatically using BD AccuDrop beads. Sorting of extravascular (i.e. negative for the intravenously administered antibody) CD11b- CD45+ was carried out using the 4-way purity sorting mode. Cells were collected into BD Sample Buffer in a 1.5 ml tube cooled at 4°C. At the end of the sort, the sample volume was topped up to 615 μL using Sample Buffer as per the manufacturer's recommendations.

Smart-seq2: following extracellular staining and cell washing, cells were resuspended in FACS buffer. An initial round of presorting of non-myeloid cells was carried out using the 4-way purity sort mode, by gating on extravascular CD11b- CD45+ cells. Cells were collected into 9% FBS/DMEM in a 5ml tube. The pre-sorted non-myeloid cells were used to sort CD3+ cells–using the "Single-Cell" sort mode–into 384-well plates containing 2 μL lysis buffer. The plate is then sealed with an aluminum foil, spun down at 600 g 4°C for 2 min, and stored at -80°C until processing.

2.11 BD Rhapsody: cell loading, library preparation, and sequencing

The BD Rhapsody platform is a micro-well-based system that makes use of DNA-barcoded beads, with each "surface" oligonucleotide comprising a PCR handle, cell barcode (common to all oligos per bead), unique molecular identifier (unique to each

oligo), and oligodT primer at the 3' end. Cell and bead loading, cell lysis, and recovery were carried out using the BD Rhapsody Express Single-Cell Analysis System, according to the manufacturer's recommendations. Upon cell lysis, mRNA molecules, as well as oligos bound to the multiplexing antibodies (BD sample tags) will anneal to via their polyA tails to the oligodT primers on the beads. In brief, sorted cells were loaded on a BD Rhapsody cartridge followed by incubation for 15 min at room temperature. Cells that did not settle into the wells were washed away using BD's Sample Buffer. Next, DNA-barcoded beads were loaded on the cartridge and incubated for 3 min, followed by washing of beads that did not settle into wells using Sample Buffer. Next, cell lysis was performed using BD' Lysis Buffer + DTT, and incubated for 2 min at room temperature, followed by recovery of the beads from the cartridge into a 5 ml Eppendorf tube. Beads were washed by placing the 5 ml tube on a magnet, where the beads were pulled towards the magnet and then the supernatant was removed, followed by resuspending the beads in BD's Bead Wash Buffer. Next, reverse transcription of bead-bound mRNA molecules and subsequent exonuclease treatment were performed as per the manufacturer's instructions.

Library preparation was carried out using BD Rhapsody mRNA Whole Transcriptome Analysis and Sample Tag Library Preparation Protocol. In brief, cDNA corresponding to sample tags was denatured from the beads (by heating at 95 °C followed by collection of supernatant), followed by PCR amplification and then Index PCR to ligate sequencing adapters and a library-unique index. As for the transcriptome, second-strand synthesis was carried out using a random-priming strategy annealing to the bead-bound cDNA molecules. This was followed by PCR amplification and Index PCR to ligate sequencing adaptors and a library-unique index.

Quantification of the cDNA libraries was performed using a Qubit Fluorometer with the Qubit dsDNA HS Kit, whereas the size distribution of the libraries was assessed using the Agilent High-Sensitivity D5000 assay on a TapeStation 4200. Paired-end sequencing (2∗75 cycles) was performed on a NextSeq 500 System using NextSeq 500/550 High Output Kit v2.5.

2.12 Smart-seq2: library preparation and sequencing

Single cells sorted into 384-well plates containing Smart-seq2 lysis buffer were processed according to the protocol published by (Picelli et al. 2014) with minor modifications (Table 8). To ensure reproducibility and pipetting accuracy, a Freedom Evo robot handler was used for all steps, namely reverse transcription, PCR amplification, tagmentation, and index PCR. Lysis buffer contained guanidine hydrochloride (40 mM) instead of Triton-X100 and no RNase inhibitor (Table 8). During reverse transcription, a template-switch oligo (TSO) is added to allow for the incorporation of a common 5' barcode at the end of the cDNA molecules. This is followed by PCR amplification using the ISPCR primer, which is complementary to barcodes at both the 5' and 3' ends (Table 8). Full-length cDNA molecules are fragmented and ligated with adapters using the Tn5 transposase enzyme, followed by a final Index PCR step to ligate sequencing adaptors and cell-specific indices.

Size distribution of the libraries was assessed using the Agilent High-Sensitivity D5000 assay on a TapeStation 4200. Single-end sequencing was performed on a NovaSeq6000 System using NovaSeq 6000 S1 Reagent Kit.

2.13 scRNA-seq data preprocessing and analysis

Raw sequencing files (bcl files) were demultiplexed using the Bcl2fastq2 V2.20 tool from Illumina. Sequencing adapters were trimmed and sequencing reads with a PHRED score >20 were filtered using Cutadapt 1.16. Subsequently, STAR aligner (Dobin et al. 2013) was used to align reads against GENCODE vM16 version of mouse reference genome (mm10). Drop-seq tools 2.0.0 were used to generate a unique molecular identifier (UMI)-corrected gene expression count matrix. HTO sequences were added to the reference genome to simultaneously allow for their retrieval during alignment.

Downstream analysis was performed in R (version 4.0.3). The dataset was filtered using the barcodeRanks() function to exclude cells with UMI counts below the inflection point, which represents the sharp transition in UMI counts between cell-containing wells and empty wells (Lun et al. 2019). Downstream analysis was performed using the Seurat R package (version 3.9.9.9032) (Stuart et al. 2019). For Rhapsody datasets, the data was further filtered to exclude cells expressing less than 200 genes, more

than 2000 genes or cells whose mitochondrial reads account for more than 10% of their transcriptomes. For Smart-seq2 datasets, the data was filtered to exclude cells expressing less than 500 genes, more than 4000 genes or cells whose mitochondrial reads account for more than 10% of their transcriptomes.

Normalization and scaling were performed using the SCTransform() function (Hafemeister and Satija 2019). Principal component analysis (PCA) was applied using the RunPCA() function of Seurat, and–based on elbow plot and the inspection of the individual PCs and their contribution to the variance in the data–different number of PCs across datasets were used to run FindNeighbors() funciton. Clustering and non-linear dimensionality reductions were performed using the FindClusters() and RunUMAP() functions, respectively. Seurat's FindSubCluster function was used to segregate CD8 Cluster 2 and NKT cells, which were originally grouped into a single cluster. Differential expression analysis was performed using the FindAllMarkers() function, setting both min.pct and logfc.threshold to 0.2 and using the default Wilcoxon Rank Sum test. Heatmaps were generated by a) computing the average gene expression per cluster using Seurat's AverageExpression function, setting the slot to "scale.data", and b) convert AverageExpression output into a matrix datatype and pass it to pheatmap function, setting the *scale* argument to "row". The "viridis" package was used to set the scale colors in FeaturePlots. TCR signaling and TGFTo compute a TCR signaling or TGF-β signaling score, a gene list corresponding to the respective signaling pathway was imported, converted into a list datatype, and then Seurat's AddModuleScore function was used to compute a gene-expression score. Gene lists of TCR signaling and TGF-β signaling were derived from the KEGG database and Nath et al, respectively (Kanehisa and Goto 2000, Nath et al. 2019)

Trajectory inference was conducted using the monocle3 package (version 0.2.3) (Cao et al. 2019). The cluster_cells function from monocle3 was used while setting k nearest neighbor to 35 and using "Louvain" as the clustering method. Histograms were generated using the dittoSeq package (Bunis et al. 2021).

2.14 Statistics

All statistical analyses, except for scRNA-seq data analysis, were performed using GraphPad Prism 9.5.1. For comparisons between two groups using one variable, unpaired

two-tailed t test was used. For comparison between two groups across two or more variables, two-way Analysis of Variance (ANOVA) with Šídák's multiple comparisons test was used. Statistical differences were considered significant if α was < 0.05. Grubb's test was used to test for statistical outliers by using grubbs.test() function in R. No outliers were left out except for Figure 7, where one *Cd8*^{Cre} *Tcf7*$^{fl/fl}$ mouse was a significant outlier in terms of cell numbers. Data are reported as mean ± standard error of the mean (sem).

Chapter 3: Brain-resident CD8+ T cells exhibit tissue-specific and context-specific features

3.1. Background

Pioneering studies on CD8+ Trm cells established that CD8+ Trm cells in various tissues share a core transcriptional program of tissue residency (Mackay et al. 2013, Kumar et al. 2017). More recent studies employing single-cell RNA-sequencing (scRNA-seq) found that, in addition to a core transcriptional module, the tissue of residence plays a central role in shaping the transcriptional landscape of diverse Trm cells (Crowl et al. 2022). In fact, priming of CD8+ T cells with distinct viral and bacterial pathogens resulted in phenotypically similar CD8+ Trm cells in the same tissue (Urban et al. 2020, Evrard et al. 2023). These findings support the notion of a tissue-specific molecular program imprinted on Trm cells in a given tissue.

In this Chapter, I investigate the transcriptional landscape of resident T cells in the brain of naïve mice using scRNA-seq, and validate these findings using flow cytometry. Next, I apply the same experimental approach to investigate the extent of alteration of the T cell molecular profile in the contexts of ageing and cerebral amyloidosis. Ageing is a strong risk factor for neurodegenerative disease and is associated with an increased number of CD8+ T cells in the brain (Ritzel et al. 2016). However, it remains unclear whether brain CD8+ T cells acquire unique molecular features during ageing, i.e. whether they are qualitatively divergent from their counterparts in young naïve mice. Further, we use mouse models of cerebral amyloidosis to address essentially the same question: to what extent does neuropathology alter the transcriptional makeup of brain-resident T cells. Other contexts in which we evaluate the molecular hallmarks of brain T cells are systemic acute and chronic viral infection using different strains of LCMV. LCMV-Armstrong is cleared ~10 days post-infection and generates robust circulating and resident memory T cells, whereas persistent infection with the clone 13 strain results in T cell exhaustion. Accordingly, the use of a diverse set of animal models allows us to investigate whether the brain T cell landscape is determined in a tissue-specific or in a context/disease-specific manner.

3.2. Results

3.2.1 Brain-resident CD8+ T cells in young adult mice are transcriptionally heterogeneous

Recent studies have reported that T cells populate the CNS at steady state (Mrdjen et al. 2018, Smolders et al. 2018, Pasciuto et al. 2020), but the transcriptional and phenotypic diversity among such cells remains poorly characterized. To uncover the complexity of the brain T cell landscape during homeostasis, we sorted CD11b- CD45+ CD3+ cells from the brains of 5-month-old C57BL/6J mice (Appendix Figure 1A) and examined their transcriptome using single-cell RNA-seq (scRNA-seq). Mice received an intravenous injection of a fluorescently tagged anti-CD45 antibody before euthanasia to exclude circulating leukocytes during sorting (Anderson et al. 2014). Importantly, we confirmed that CD11b is essentially not expressed by brain T cells (Appendix Figure 1B-C). Unbiased clustering of the brain T cell compartment revealed heterogeneous subsets of αβ T cells as well as unconventional T cells (Figure 1A-B). Four main clusters of CD8+ T cells were identified. Cluster 1 exhibited high expression of transcripts encoding granzyme K, chemokines (*Ccl5*, *Ccl4*, *Ccl3*), as well as inhibitory receptors (*Pdcd1*) and transcription factors associated with T cell receptor (TCR) signaling (*Tox*, *Nr4a2*). Cluster 2 was marked by high expression of *Ly6c2*, as well as *Lgals3* and the transcription factor *Hopx*. Cluster 3 showed a pronounced signature of interferon (IFN)-stimulated genes, including IFIT (IFN-induced protein with tetratricopeptide repeats) and IFN-stimulated genes (ISG)s. In addition, we observed a subset of T cells that expressed surface markers and transcription factors associated with naïve, central memory, or stem-like T cells, including *Sell* (encoding CD62L), *Ccr7*, *Tcf7* (encoding TCF-1), *Satb1*, and *Klf2*. This cluster mostly comprised CD8+ T cells but also a fraction of CD4+ T cells. Furthermore, a cluster of CD4+ T cells was identified, marked by expression of *Tnfrsf4* (encoding the costimulatory molecule OX40), *Nrp1* (encoding neuropilin 1), and *Hif1a*. Approximately 25% of CD4+ T cells expressed Foxp3. Apart from conventional T cells, we detected a cluster of γδ T cells characterized by a Th17-like signature, including the expression of *Rora*, *Tmem176a* and *Tmem176b* [encoding intracellular ion channels that are highly expressed in RORγt+ cells (Drujont et al. 2016)], and *Maf* (encoding the transcription factor c-Maf). Finally, a small cluster of NKT cells was detected, as previously described in the steady-state brain (Mrdjen et al. 2018), expressing *Fcer1g* and *Klrb1c* (encoding NK1.1).

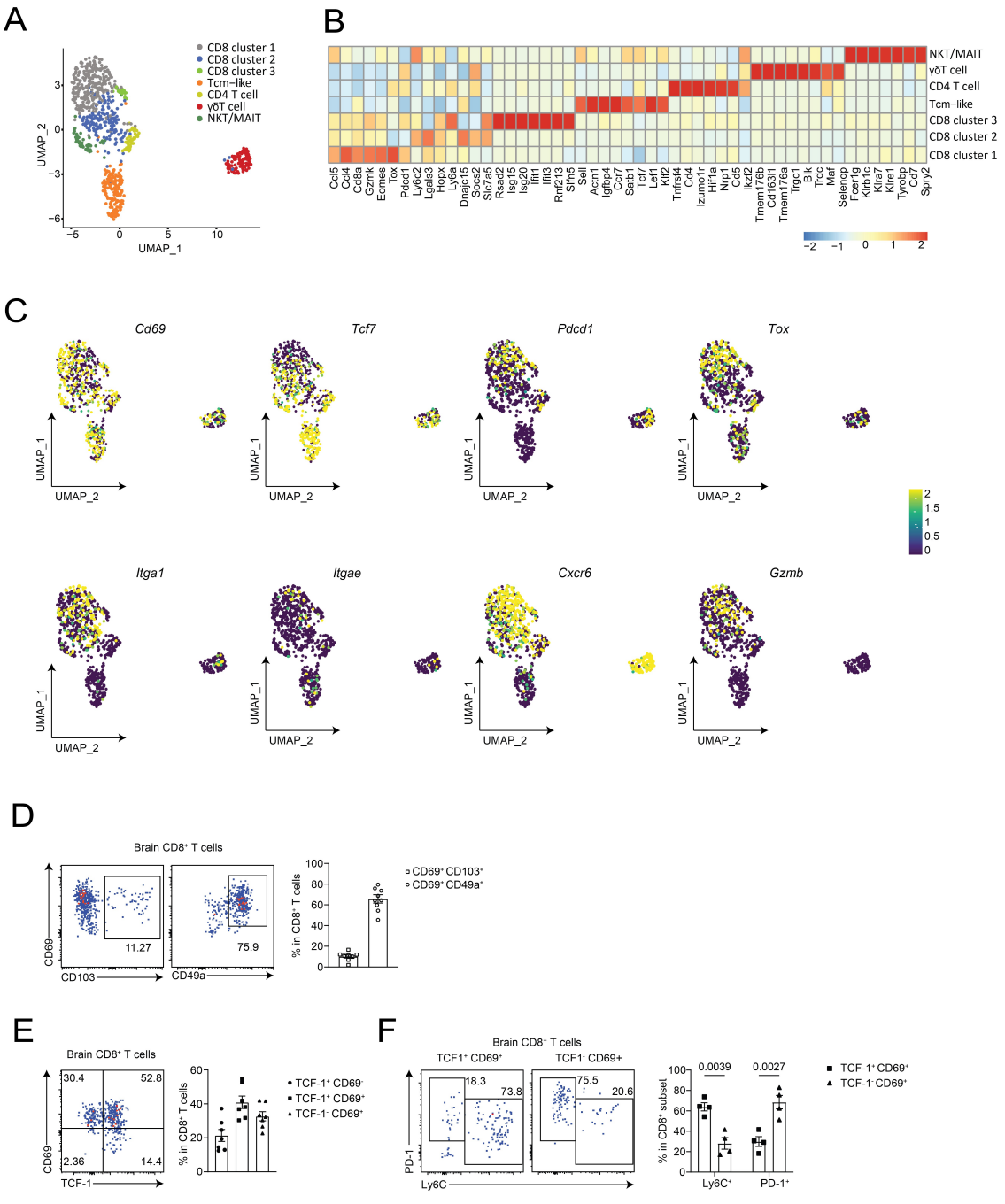

Figure 1 | Transcriptional and phenotypic heterogeneity of the brain T cell compartment at steady state. A, Uniform Manifold Approximation and Projection (UMAP) plot of scRNA-seq analysis of extravascular CD3+ T cells (n = 798 cells) in the brain of 5 month-old mice; n = 6 mice. **B,** Heatmap of the top differentially expressed genes defining each T cells cluster. **C,** FeaturePlots of a select set of genes reported in previous studies to be expressed by subsets of Trm cells. **D,** Flow cytometric analysis and quantification of frequencies of CD69+ CD103+ and CD69+ CD49a+ subsets of brain CD8+ T cells; n = 8 mice. **E,** Frequencies of TCF-1+ CD69-, TCF-1+ CD69+, and TCF-1- CD69+ subsets of brain CD8+ T cells at steady state; n = 7 mice. **F,** Proportions of PD-1+ and Ly6C+ cells among TCF-1+ CD69+ and TCF-1-

CD69+ brain CD8+ T cells at steady state; n = 4 mice. Data are representative (F) or pooled (D-E) from two independent experiments.

Mapping the expression of individual genes with established roles in Trm cells, we found that, as expected, *Cd69* is expressed by the majority of T cells in the brain (Figure 1C). *Cxcr6* was also expressed by CD8+ T cell cluster 1, 2, and 3 (Figure 1C), while *Gzmb* (including granzyme B) was produced by a large fraction of the same clusters of CD8+ T cells. Importantly, *Itgae* (encoding CD103) was expressed by only ~10% of brain-resident CD8+ T cells, whereas *Itga1* (encoding CD49a) was produced by >70% of brain CD8+ Trm cells (Figure 1C-D).

In sum, these findings indicate that brain CD8+ T cells bear transcriptional and phenotypic hallmarks of CD8+ Trm cells described in other tissues, and reinforce the notion that CD103 expression among brain Trm cells requires direct brain infection (Wakim et al. 2010).

3.2.2 TCF-1, CD69, and PD-1 define distinct CD8+ Trm cell subsets with variable proliferative capacity and effector function

TCF-1 is associated with naïve and central memory T cell formation and maintenance (Zhao et al. 2022, Zhou et al. 2010), as well as stem-like T cells in chronic infection and cancer (Utzschneider et al. 2016, Tsui et al. 2022). A previous study has shown that TCF-1 counteracts the formation of CD103+ CD8+ Trm cells in the lungs following influenza infection (Wu et al. 2020). Interestingly, we noticed coexpression of *Cd69* and *Tcf7* that mostly corresponded to CD8 cluster 2, whereas there was little to no co-expression of *Tcf7* and *Pdcd1* or *Tox*, the latter two marking CD8 cluster 1 (Figure 1C). Given the potential discrepancy between mRNA abundance and protein expression (Liu et al. 2016), we assessed CD69 and TCF-1 coexpression in brain CD8+ T cells by flow cytometry, which revealed a discernable CD69+ TCF-1+ CD8+ T cell subset along with a CD69+ TCF-1- subset (Figure 1E). We next aimed to match the key marker genes of brain CD8+ T cell subsets informed by the unbiased scRNA-seq analysis to the identified CD69+ TCF-1+/- brain CD8+ T cell subsets. Accordingly, we examined PD-1 and Ly6C expression by flow cytometry in the respective brain $CD8^+$ $CD69^+$ TCF-1+/- populations. As shown in Figure 1F, CD69+ TCF-1+ $CD8^+$ T cells mostly corresponded to CD8 cluster 2 (i.e. PD-1- Ly6C+), whereas CD69+ TCF-1- CD8+ T cells largely represented CD8+ cluster 1 (PD-1+ Ly6C-).

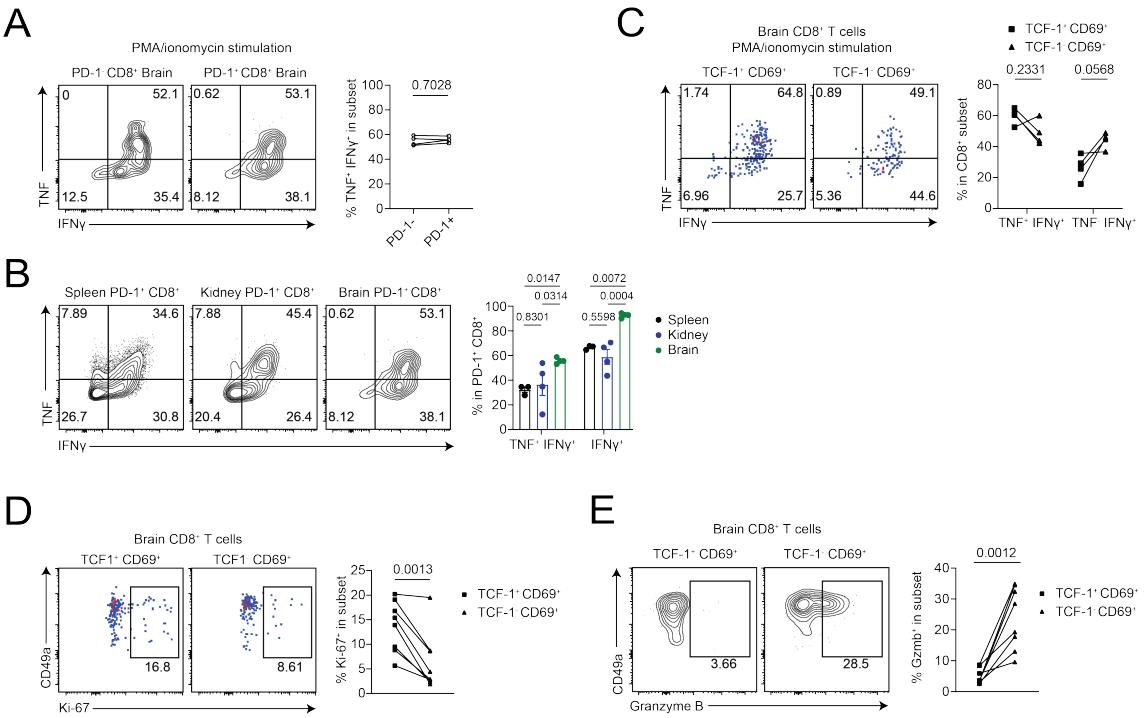

Figure 2 | TCF-1 marks two subsets of brain CD69+ CD8+ Trm cells with distinct proliferative and functional capacities. Data represent brain CD8+ Trm cells in naïve 5 month-old mice. **A,** Frequency of TNF+ IFNγ+ in PD-1- and PD-1+ brain CD8+ Trm cells following ex vivo stimulation with PMA/ionomycin; n = 4 mice. **B,** Proportions of TNF+ IFNγ+ and total IFNγ+ in PD-1+ CD8+ T cells in spleen, kidney, and brain, following ex vivo stimulation with PMA/ionomycin; n = 4 mice. **C,** Percentages of TNF+ IFNγ+ and TNF- IFNγ+ among TCF-1+ CD69+ and TCF-1- CD69+ brain CD8+ T cells following ex vivo stimulation with PMA/ionomycin; n = 4 mice. **D,** Frequency of Ki-67+ among TCF-1+ CD69+ and TCF-1- CD69+ subsets of brain-resident CD8+ T cells; n = 8 mice. **E,** Proportion of GzmB+ in TCF-1+ CD69+ and TCF-1- CD69+ populations of brain CD8+ Trm cells; n = 8 mice. Data are representative (A-C) or pooled (D-E) from two independent experiments. GzmB, granzyme B.

PD-1 is an inhibitory receptor whose elevated and sustained expression is a classical feature of CD8+ T cell exhaustion in chronic viral infection and cancer (Kallies et al. 2020, Jubel et al. 2020). PD-1 is also expressed by tissue-resident CD8+ T cells inthe lung, pancreas, and brain, among other tissues (Wang et al. 2019, Weisberg et al. 2019, Prasad et al. 2017, Scholler et al. 2020). In the lung, antigen-specific PD-1+ CD8+ Trm cells exhibited slightly impaired functionality, as assessed by TNF/IFNγ coexpression, compared to their PD-1- counterparts (Wang et al. 2019). To assess the relative functional capacity of PD-1+ and PD-1- CD8+ T cells in the brain, we stimulated brain leukocytes using PMA/ionomycin and evaluated cytokine expression by CD8+ T cells (Figure 2A). Specifically, we first performed surface staining of PD-1 before PMNA/ionomycin stimulation to more accurately reflect endogenous PD-1 expression

by brain CD8+ T cell subsets. This analysis revealed no difference in the frequency of TNF/ IFNγ double-positive cells between PD-1- and PD-1+ brain CD8+ T cells, with both subsets robustly producing cytokines upon stimulation (Figure 2A). To further probe the functional capacity of brain PD-1+ CD8+ T cells, we compared the cytokine expression by PD-1+ CD8+ T cells in the spleen and kidney, as a representative for lymphoid and non-lymphoid tissues, respectively, relative to their counterparts in the brain (Figure 2B). Interestingly, brain PD-1+ CD8+ T cells comprised a greater fraction of TNF+ IFNγ+ cells, as well as total IFNγ+ cells, compared to their renal and splenic counterparts (Figure 2B). Together, these findings suggest that PD-1 expression represents a feature of brain CD8+ T cell differentiation with no negative modulation of cytokine expression, and indicates that brain CD8+ T cells have a superior capacity to produce effector cytokines compared to peripheral tissue CD8+ T cells.

Having established the capacity for cytokine production at steady state irrespective of PD-1 expression, we then asked whether TCF-1 expression marks two functionally distinct subsets of brain CD8+ T cells. Employing the same experimental setup used in Figure 1F, we found that TCF-1+ CD69+ brain CD8+ T cells comprised a greater fraction of TNF+ IFNγ+ cells compared to their TCF-1- CD69+ counterparts, with a reciprocal increase in the frequency of TNF- IFNγ+ cells among TCF-1- CD69+ CD8+ T cells (Figure 2C). We further explored the expression of cytotoxic effector molecules in the two described CD69+ CD8+ T cell subsets. TCF-1 expression is inversely correlated with granzyme B expression in effector CD8+ T cells (Zhou et al. 2010, Pais Ferreira et al. 2020) as well as in NK cells (Jeevan-Raj et al. 2017). Trm cells in various tissues are known to express granzymes, including granzyme B, as part of their immediate effector-response program (Gebhardt et al. 2018). Consistent with previous reports (Wu et al. 2020), we found that TCF-1- CD69+ CD8+ T cells possessed a greater capacity to produce granzyme B relative to their TCF-1+ CD69+ counterparts in the steady-state brain (Figure 2E). Thus, TCF-1 marks two subsets of CD69+ CD8+ T cells with distinct expression of effector molecules.

In addition to T cell differentiation and function, TCF-1 promotes cell proliferation and self-renewal capacity, by virtue of being a transcription factor downstream of Wnt signaling (Utzschneider et al. 2016, Kratchmarov et al. 2018). Comparing the proliferative potential of TCF-1+ CD69+ and TCF-1- CD69+ brain CD8+ T cells, we could indeed show that TCF-1+ CD69+ brain CD8+ T cells comprised a larger

proportion of Ki-67+ (a nuclear protein expressed in cycling cells (Gerdes et al. 1984)) cells compared to TCF-1- CD69+ cells (Figure 2D).

Taken together, we show that brain T cells are transcriptionally heterogeneous, and identify TCF-1 as marking two subsets of CD69+ CD8+ brain T cells with distinct phenotype, proliferation, and effector function.

3.2.3 Pseudotime analysis infers a developmental trajectory from a TCF-1+ to a PD-1+ state, associated with progressive TCR signaling

The ontogeny of Trm cells is a topic of active investigation. It has been reported that Trm precursors acquire a developmental potential poised towards the Trm cell fate before migrating into the eventual tissue of residence (Kok et al. 2022, Mani et al. 2019). Such Trm precursor cells then undergo a stepwise maturation in situ after recruitment to the tissue of residence (Mackay et al. 2013). To investigate the developmental hierarchy among brain CD8+ T cell subsets, we subsetted the CD8+ T cell clusters that are part of the scRNA-seq dataset described above and performed a pseudotime analysis using monocle3 (Cao et al. 2019) (Figure 3A). This analysis revealed a differentiation trajectory from Tcm-like cells to *Pdcd1*-expressing cells and to an IFN-responding state (Figure 3B). Such trajectory was associated with progressive *Tcf7* downregulation and a reciprocal upregulation of *Pdcd1* and *Tox* (Figure 3C).

The expression of PD-1 and TOX is known to be downstream of TCR signaling (Patsoukis et al. 2020, Maurice et al. 2021). Although circulating memory T cells are maintained in a TCR-independent manner (Kaech and Cui 2012), resident memory T cells in some tissues (e.g. lung) engage and require TCR signaling for their maintenance (Wang et al. 2019). Accordingly, we asked whether PD-1+ and PD-1- CD8+ T cells in the brain are differentially engaging TCR signaling. To this end, we examined their respective expression of IRF4, a transcription factor whose expression is controlled by the magnitude and affinity of TCR signaling (Man et al. 2013). Using an *Irf4*-reporter mouse model (Kallies lab, manuscript in revision), we found that PD-1+ CD8+ T cells in the brain comprised a greater fraction and produced larger amounts of IRF4 compared to their PD-1- counterparts (Figure 3D). We then took an orthogonal approach to examine the extent of TCR signaling by brain CD8+ T cell subsets. We made use of a KEGG TCR signaling module, and computed an average expression score based on this KEGG pathway, and mapped it onto our scRNA-seq data. In agreement with the IRF4 expression data, we observed an increased magnitude of transcripts related to TCR signaling in *Pdcd1*-expressing cluster 1 compared to TCF-1+ or Ly6C+ CD8+ T cells. Interestingly, the progressive increase in TCR signaling largely matched the inferred developmental trajectory of brain CD8+ T cells (Figure

3E). Whether TCR signaling is a determinant of such developmental transition remains to be formally tested

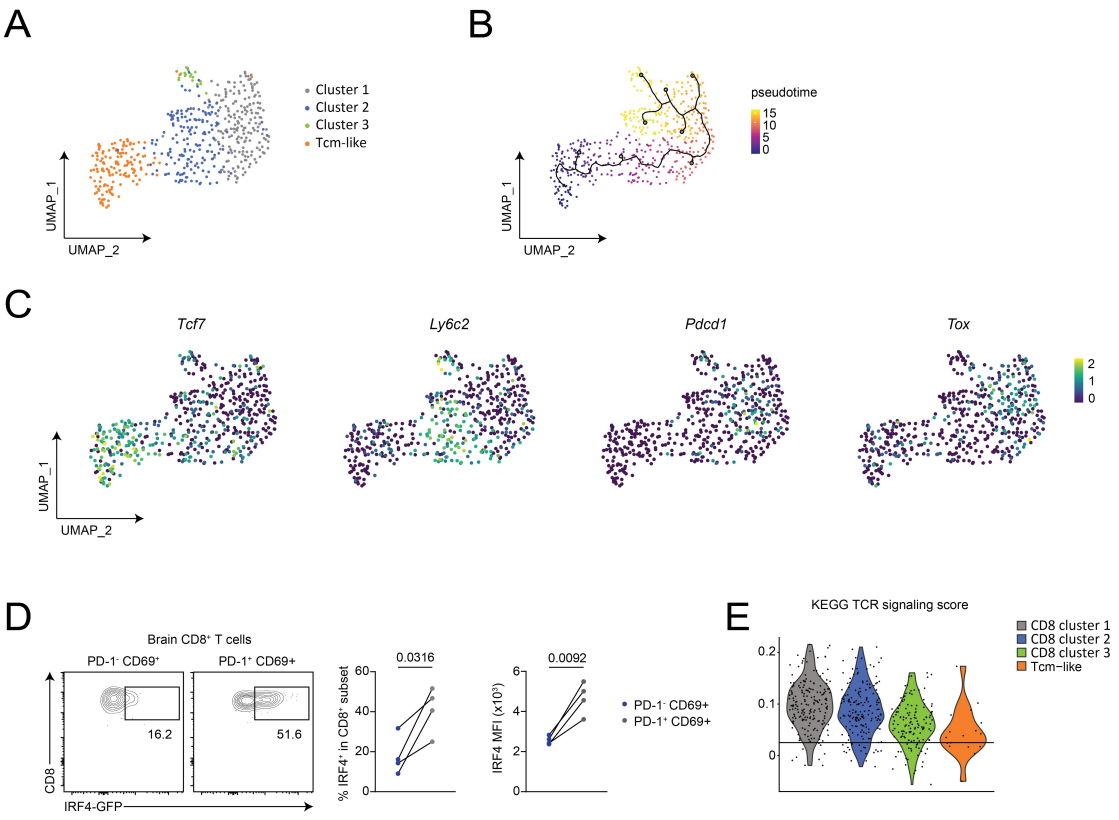

Figure 3 | Developmental trajectory and TCR engagement of brain CD8+ Trm cells. **A**, CD8+ T cell clusters from the scRNA-seq dataset presented in Figure 1 were subsetted and re-analyzed; 527 cells; n = 6 mice. **B**, monocle3 trajectory inferred across the four CD8+ T cell clusters; blue represents least differentiated and yellow represents most differentiated. **C**, FeaturePlots of a subset of genes marking the CD8+ T cell subsets. **D**, Frequency and extent of expression of GFP under control of the IRF4 promoter in PD-1- CD69+ and PD-1+ CD69+ brain CD8+ Trm cells; n = 4 mice, 6-9 month-old. **E**, TCR signaling score by the four CD8+ T cell clusters. GFP, green fluorescent protein; TCR, T cell receptor.

In summary, these data suggest that brain CD8+ T cells undergo a stepwise differentiation from a TCF-1+ state to a TCF-1- PD-1+ state that is correlated with a progressive engagement of TCR signaling.

3.2.4 Ageing is associated with increased numbers of brain-resident CD8+ T cell subsets

Ageing is associated with profound systemic changes in the T cell compartment in lymphoid and non-lymphoid tissues (Mogilenko et al. 2021, Krishnarajah et al. 2022). We next asked how ageing modulates the brain CD8+ T cell compartment observed in young adult mice, i.e. to what extent brain CD8+ T cells adopt a tissue-specific versus context specific molecular profile. Old mice (>20 months of age) exhibited substantially larger numbers of brain CD4+ and CD8+ T cells compared to their young counterparts, with the magnitude of increase being greater for CD8+ T cells (Figure 4A and data not shown).

Next, we assessed whether ageing modulated the TCF-1+/- CD69 subsets of brain CD8+ T cells. Numerically, both TCF-1+ CD69+ and TCF-1- CD69+ subsets increased in the brains of old mice relative to young mice (Figure 4B). TCF-1+ CD69- showed the most profound difference, with a reduced frequency among brain CD8+ T cells in old mice compared to young mice. This was associated with a reciprocal insignificant increase in the frequency of TCF-1- CD69+ CD8+ T cells in the brains of old mice (Figure 4B). PD-1 expression is known to increase on CD8+ T cells in peripheral lymphoid and non-lymphoid tissues of old mice (Mogilenko et al. 2021). However, we noted no differences in the frequency of PD-1 expression between young and old mice (Figure 4C). Collectively, these data show that both TCF-1+ CD69+ and TCF-1- CD69+ brain CD8+ T cells increase in number with age, albeit they show limited qualitative changes in old compared to young mice.

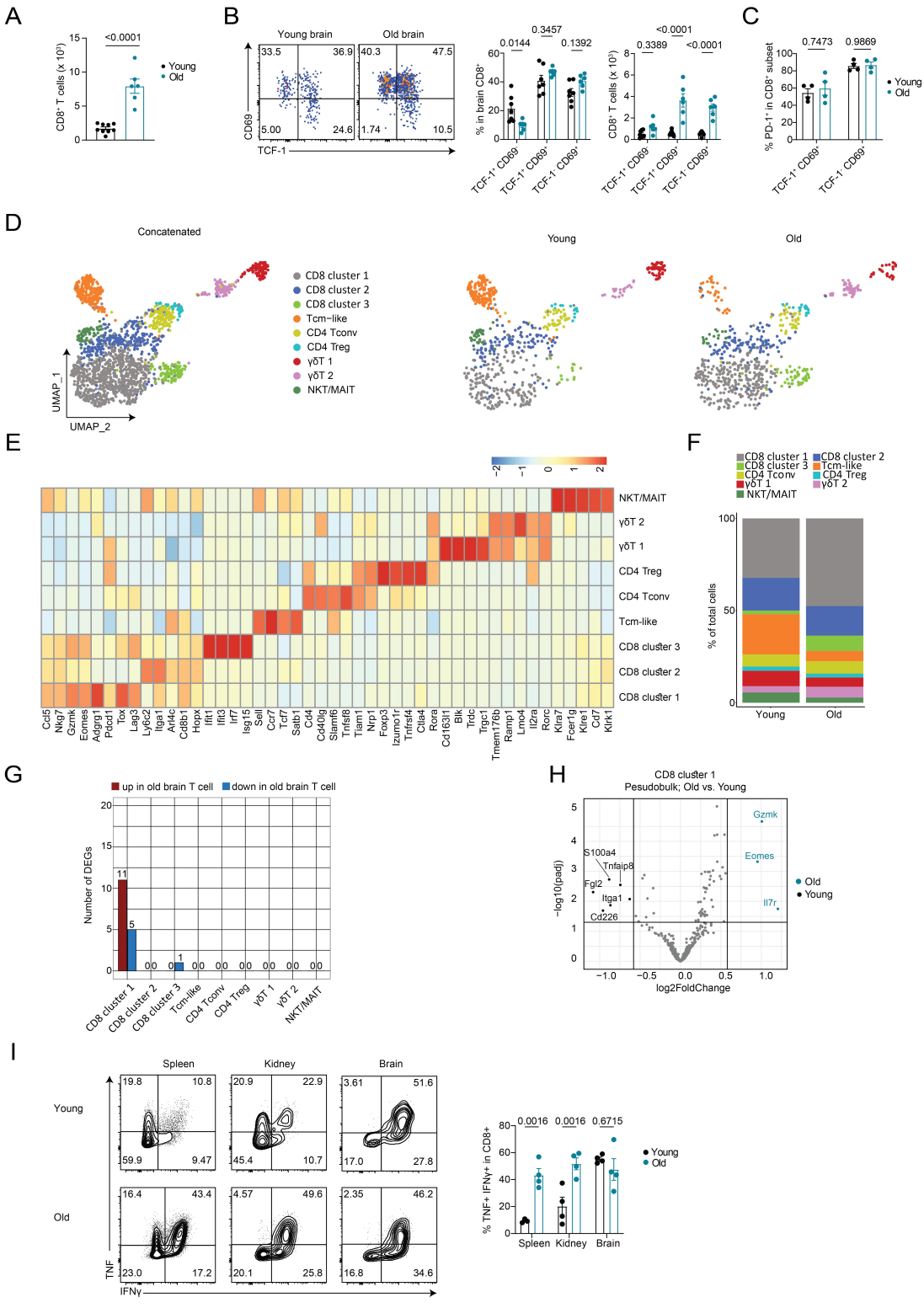

Figure 4 | Ageing-induced alteration of the brain CD8+ T cell compartment is primarily quantitative rather than qualitative. A, Quantification of brain CD8+ Trm cells in young (5-6 months) and old (20-24 months)-old naïve, sex-matched mice; n = 6-9 mice. **B,** Frequency and number of TCF-1+ CD69-, TCF-1+ CD69+, and TCF-1- CD69+ subsets of brain CD8+ T cells in young and old mice; n = 6-9 mice. **C,**

Percentage of PD-1+ among TCF-1+ CD69+ and TCF-1- CD69+ subsets of brain CD8+ Trm cells; n = 4 mice. **D,** Uniform Manifold Approximation and Projection (UMAP) plot of scRNA-seq analysis of extravascular CD3+ T cells (n = 1870 cells) in the brain of 5 month- and 20 month-old naïve mice; n = 5 mice per age group. **E,** Heatmap showing the top differentially expressed (DE) genes defining each T cell cluster. **F,** Frequencies of the T cell clusters in young and old mice. **G,** Barplot showing the number of DE genes between young and old per T cell cluster. **H,** Volcano plot depicting the DE genes between cells corresponding to CD8 cluster 1 in young vs. old mouse brain. **I,** Frequency of TNF+ IFNγ+ CD8+ T cells in the spleen, kidney, and brain of young (6 month) and old (24 month) mice following ex vivo stimulation using PMA/ionomycin; n = 4 mice. Data are representative (C, I) or pooled (A, B) from at least two independent experiments.

3.2.5 Ageing alters the representation of brain CD8+ Trm cell subsets with limited qualitative alteration of their transcriptional composition or effector function

To gain deeper insights into potential age-induced alteration of the brain T cell landscape, we sorted CD11b- CD3+ cells from young and old mouse brains and performed scRNA-seq. Remarkably, the global architecture of the T cell compartment in aged mice was similar to young adult mice (Figure 4D-E). We again observed four main subsets of CD8+ T cells, the first being defined by the expression of *Pdcd1*, *Tox*, *Lag3*, as well as *Ccl5* and *Gzmk*; the second being marked by high *Ly6c2* expression; a third cluster whose transcriptome was dominated by type I IFN-responsive genes; and a fourth cluster with a central memory-like phenotype. Due to a larger number of cells included in this dataset compared to the one reported in Figure 1, we could reliably resolve the CD4+ T cell compartment into *Foxp3*- CD4+ conventional T cells (Tconv) and *Foxp3*+ regulatory T (Treg) cells. CD4+ Tconv cells expressed the costimulatory molecules *Cd40l* (encoding CD40 ligand) and *Tnfsf8* (encoding CD153), whereas Treg cells expressed *Ctla4*, as well as the costimulatory molecule *Tnfrsf4* (encoding OX40). Finally, two γδ T cell subsets could be identified, distinguished by the expression of *Cd163l1*, *Blk*, and *Lmo4* (Tan et al. 2019, Laird et al. 2010).

Comparing the frequencies of the identified T cell clusters across young and old mice, we found the most substantial differences in percentages of CD8+ T cell subsets (Figure 4D and 4F). Specifically, we observed an increased in frequency of *Pdcd1*-expressing cluster 1 and a reciprocal decrease in the fraction of central memory-like CD8+ T cells in old mice. Moreover, type I IFN-responding CD8 cluster 3 was enriched in the brains of old mice relative to young mice, which is in agreement with a previous

study (Baruch et al. 2014). Thus, ageing induces a marked alteration of the frequencies and numbers of CD8+ T cell subsets in the brain. However, such changes in cell frequencies do not necessarily imply that T cells in the brains of young and old mice exhibit an intrinsically different transcriptome. To address whether ageing induces a qualitative alteration in the gene expression program of brain T cells, we performed differential gene expression (DE) analysis per each brain T cell cluster across young and old mice (Figure 4G). Remarkably, the majority of brain T cell clusters exhibited no differentially expressed genes between young and old mice, with the exception of CD8 cluster 1, which showed a relatively small total number of 16 DE genes (Figure 4G).

The analysis shown in figure 4G employed a computational tool that considers each cell to be an independent sample. Given the sparsity of scRNA-seq data, and to conduct a more robust DE analysis, we made use of a pseudobulk approach that aggregates transcript counts of cells of the same cluster before running DE analysis (as detailed in the Methods section). Using this approach, we could confirm that the number of DE genes for a given T cell cluster between young and old mice was remarkably small, i.e. a total of 9 DE genes for cluster 1 (Figure 4H). Accordingly, we conclude that brain CD8+ T cells show negligible qualitative alterations in the context of ageing, arguing for a tissue-specific rather than a context-specific transcriptional signature of brain CD8+ T cells.

To further interrogate potential ageing-induced alteration of the brain CD8+ T cell compartment, we assessed the capacity of CD8+ T cells of young and old mouse brains to produce cytokines upon ex vivo stimulation with PMA/ionomycin. Consistent with previous studies (Mogilenko et al. 2021), splenic and renal CD8+ T cells exhibited an increased frequency of TNF+ IFNγ+ CD8+ T cells in old compared to young mice (Figure 4I). In contrast, brain CD8+ Trm cells displayed similar capacities to co-express TNF and IFNγ in young and old mice (Figure 3I). This suggests that the increased capacity to produce effector cytokines upon stimulation of CD8+ T cells in ageing is not a feature of brain CD8+ T cells.

Collectively, these data demonstrate that the brains of old mice are infiltrated by large numbers of CD8+ T cells that exhibit transcriptional, phenotypic and functional

overlaps with their counterparts in young mice, suggestive of a tissue-specific imprinting of the molecular features of brain-resident CD8+ T cells.

3.2.6 Cerebral amyloidosis precipitates an accumulation of brain CD8+ Trm cells in an age-dependent manner

After investigating the impact of ageing on the brain T cell compartment, we then evaluated how cerebral amyloidosis affects the composition and transcriptional profile of the brain T cell populations. To this end, we made use of the APP/PS1 model. APP/PS1 mice carry two human transgenes encoding mutants of amyloid precursor protein (APP) and PSEN1 (Radde et al. 2006). PSEN1 is a subunit of the γ-secretase complex that, in homeostasis, cleaves APP into short (< 40 amino acids) amyloid β (Aβ) peptides (O'Brien and Wong 2011). In APP/PS1 mice, APP and PSEN1 mutants favor the production and accumulation of pathogenic 42-amino acid Aβ (Aβ2) species at the expense of shorter Aβ peptides (O'Brien and Wong 2011, Radde et al. 2006). Amyloid plaques appear at 6 weeks of age (Radde et al. 2006), followed by synaptic loss at 3-4 months of age (Bittner et al. 2012) and cognitive impairment at 7-8 months of age (Radde et al. 2006, Serneels et al. 2009).

We studied APP/PS1 mouse brains at different age groups to disentangle the contributions of ageing vs. amyloid deposition to any potential alteration in T cell phenotype. A hallmark of the innate immune response to cerebral amyloidosis is the differentiation of a subset of microglia referred to as disease-associated microglia (DAM). DAM cells are characterized by the expression of a number of phagocytic receptors, including dectin-1 and CD11c (Kamphuis et al. 2016, Keren-Shaul et al. 2017). Indeed, the fraction of CD11c+ microglia increased proportionately as mice aged and pathology progressed (data not shown). In parallel, we observed a gradual increase in T cell numbers, particularly that of CD8+ T cells, as APP/PS1 mice grew from 4 months to 10 months of age (Figure 5A). There was no difference in T cell numbers in the brain of 4-month-old APP/PS1 compared to age- and sex-matched control mice. On the other hand, non-significant increases in T cells in APP/PS1 mouse brains were noted at 10 months of age when compared to control non-transgenic mice (Figure 5A-B). Conversely, the number of CD8+ T cells sharply increased in the brains of APP/PS1 mice at 15 months of age (Figure 5G), suggesting that ageing represents a greater determinant of T cell accumulation in the brain compared to neuropathology per se.

We then examined whether the increase in brain CD8+ T cells in APP/PS1 mice at 10 months of age was differentially reflected in the TCF-1+/- CD69+ CD8+ T cell subsets.

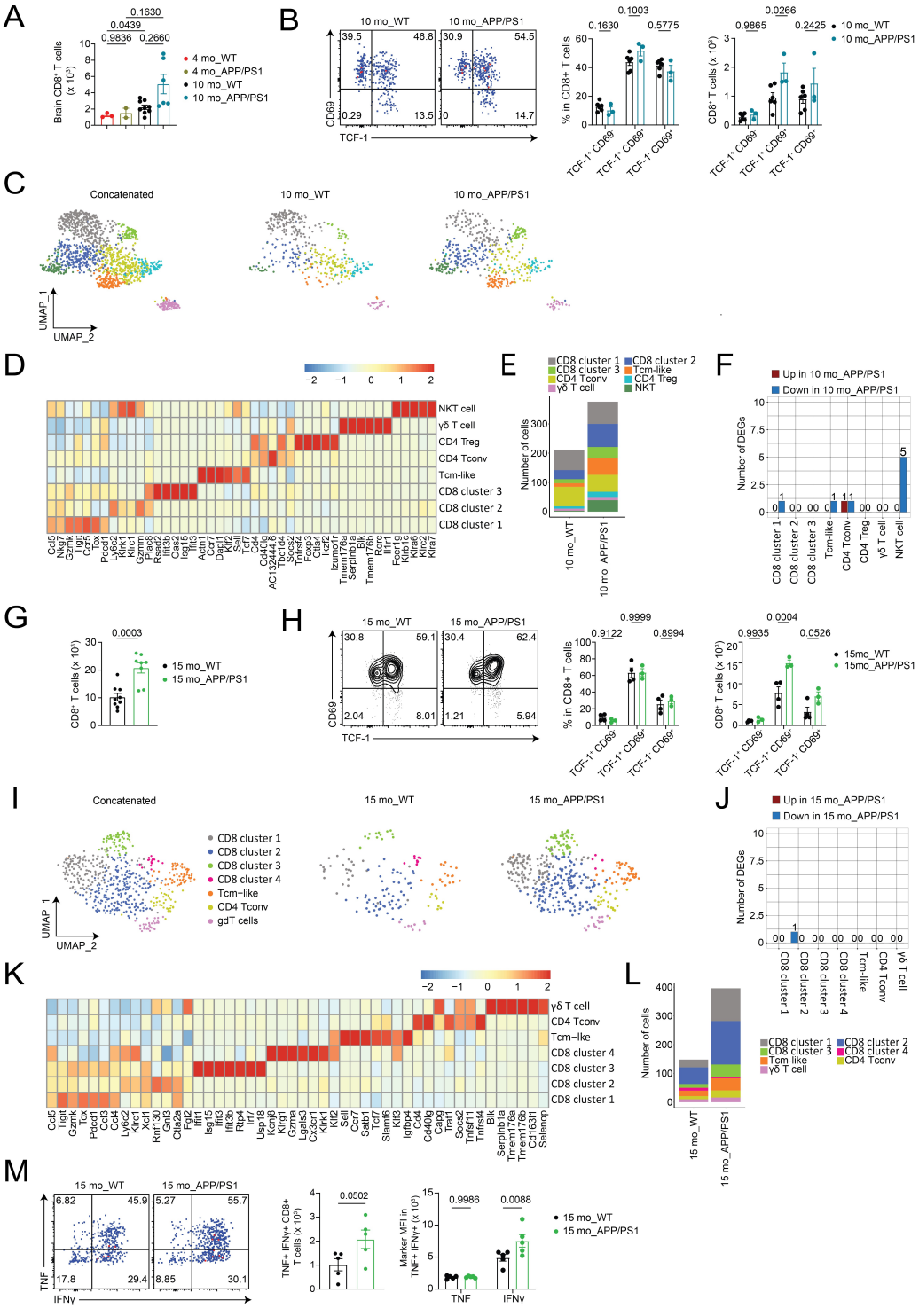

Figure 5 | Age-dependent modulation of number but not quality of brain CD8+ T cells in cerebral amyloidosis. APP/PS1 transgenic mice and sex- and age-matched mice were used for transcriptional and phenotypic profiling of brain-resident T cells. **A,** Enumeration of extravascular CD8+ T cells in 4- and 10 moth-old WT and APP/PS1

by flow cytometry. **B,** Frequency and number of TCF-1+ CD69-, TCF-1+ CD69+, and TCF-1- CD69+ CD8+ T cells in the brain of 10 month-old WT and APP/PS1 mice; n= 3-6 mice. **C,** scRNA-seq of extravascular brain CD3+ T cells from 10 month-old WT and APP/PS1 mice; n = 3-4 mice per genotype. **D,** Heatmap of top differentially expressed genes defining each cluster. **E,** Number of cells per T cell cluster in 10 month-old WT vs. APP/PS1 mice. **F,** DE genes between WT and APP/PS1 for each T cell cluster. **G,** Quantification of brain-resident CD8+ T cells in 15 month-old WT and APP/PS; n = 8-9 mice. **H,** Proportions and numbers of TCF-1+ CD69-, TCF-1+ CD69+, and TCF-1- CD69+ CD8+ T cell subsets in WT and APP/PS1 mouse brain; n = 3-4 mice. **I,** scRNA-seq analysis of brain extravascular CD3+ T cells in 15 month-old WT and APP/PS1 mice; n = 3-4 mice. **J,** Barplot showing the number of DE genes per cluster between age-matched WT and APP/PS1 mice. **K,** Heatmap of the top DE genes of the T cell clusters identified in **I. L,** Number of cells corresponding to the different scRNA-seq T cell clusters identified in **I**. **M,** Number of TNF+ IFNγ+ CD8+ T cells in the brain of 15 month-old WT and APP/PS1 mice, and MFI of TNF and IFNγ in TNF+ IFNγ+ CD8+ T cells. Data are representative (B, H, M) or pooled (A, G) from two independent experiments. DE, differentially expressed; MFI; median fluorescence intensity; WT, wild type.

We observed no substantial alterations in the frequencies of TCF-1+ or TCF-1- CD8+ T cell subsets in the brains of APP/PS1 mice compared to wild-type controls (Figure 5B). Conversely, the TCF-1+ CD69+ subset of brain CD8+ T cells was found to be specifically increased in 10-month-old APP/PS1 mice compared to age-matched control mice (Figure 5B). Similarly, at 15 months of age, we noted a substantial increase in the TCF-1+ CD69+ subset of brain CD8+ Trm cells in APP/PS1 mice compared to age- and sex-matched controls (Figure 5H).

Taken together, these data demonstrate that ageing is a stronger driver of T cell accumulation in the brain compared to early-onset neurodegeneration. They also highlight the TCF-1+ CD69+ subset of CD8+ Trm cells to be most prominently increased in the brains of ageing APP/PS1. Further studies are needed to clarify whether the accumulation of this particular subset of CD8+T cells represents an adaptation to the ongoing neuropathology in APP/PS1 mice.

3.2.7 Tissue-specific rather than disease-specific imprinting of the brain T cell landscape in cerebral amyloidosis

In the context of ageing, we observed that the accumulating CD8+ T cells in the brain displayed minimal qualitative differences–transcriptional or functional–compared to their counterparts in the brains of young mice. We asked if the progressive increase in the number of brain CD8+ T cells in 10 month-old APP/PS1 was associated with qualitative alterations of the transcriptome of T cell subsets. Accordingly, we used scRNA-seq to profile the transcriptional landscape of T cells in the brains of 10 month-old APP/PS1 and wild-type controls. Consistent with what we observed at steady state and in ageing, we identified 4 clusters of CD8+ T cells, defined largely by the expression of PD-1, Ly6C, type I IFN signaling, and a central memory phenotype; in addition to CD4 Tconv and Treg cells, and unconventional γδ and NKT cells. In line with the numerical increase of TCF-1+ CD69+ CD8+ T cells in APP/PS1 (Figure 5B), we noted that CD8 cluster 2–which largely corresponds to the TCF-1+ CD69+ subset identified by flow cytometry (Figure 1E-F)–was also increased in APP/PS1 mouse brain (Figure 5E). This was accompanied by an increase in type I-responding CD8 T cells in the brains of APP/PS1 mice (Figure 5E). However, when we tested for DE genes across wild-type and APP/PS1 mice for each cluster, there was essentially no difference in the transcriptional makeup of the different T cell subsets between APP/PS1 and non-transgenic control mice (Figure 5F). Thus, these findings lend further support to the notion that CD8+ T cells in the brain are instructed to acquire a tissue-specific transcriptional signature.

APP/PS1 mice at 15 months of age had a two-fold increase in the number of brain CD8+ Trm cells. To examine if brain T cells adopted disease-specific transcriptional features, we employed the same experimental strategy as above and assessed the transcriptional profile of brain T cells in 15 month-old APP/PS1 mice and age- and sex-matched control mice. In agreement with our previous findings, we essentially observed the same cellular landscape as in 10 month-old mice, with the exception of a very small (15 cells) cluster that was marked by *Klrg1* and *Cx3cr1* expression (Figure 5I and 5K). Given that these cells mostly emanated from the wild-type control and not APP/PS1 mice (Figure 5I), we hypothesize that these cells are likely effector memory T cells that were not reliably excluded by CD45 intravascular labelling. In line with the sharp increase of TCF-1+ CD69+ CD8+ T cells in 15 moth-old APP/PS1 mice (Figure 5H), we similarly noted a substantially larger number of cluster-2 cells in the brain of

APP/PS1 mice, as well as IFN-responsive CD8+ T cells (Figure 5L). Finally, we could not identify any transcripts that were differentially regulated between APP/PS1 and wild-type control mice per each cluster, consistent with the comparison of 10 month-old animals (Figure 5J).

Finally, we examined the ability of brain CD8+ T cells in 15 month-old APP/PS1 and wild-type control mice to produce cytokines upon ex vivo stimulation with PMA/ionomycin. In line with the larger number of total CD8+ T cells in APP/PS1 mice (Figure 5G), we observed a two-fold increase in the number of CD8+ T cells co-producing TNF and IFNγ (Figure 5M). Notably, the amount of IFNγ synthesized by TNF+ IFNγ+ CD8+ T cells in APP/PS1 mice was moderately higher than non-transgenic control mice (Figure 5M). This may point to post-transcriptional mechanisms favoring the production of a greater amount of IFNγ in this context.

Overall, these data add further evidence to the conclusion that, in cerebral amyloidosis, the brain tissue residency transcriptional signature drives CD8+ T cell identity in a disease-independent manner. Thus, these results align with previous findings that ageing is a stronger driver of T cell infiltration into the brain compared to early-onset neurodegeneration (Unger et al. 2020)

3.2.8 Brain CD8+ T cells exhibit phenotypic and functional features of exhaustion in the context of chronic viral infection

The inhibitory receptor PD-1 is expressed by a subset of brain CD8+ T cells. Previous work studying brain CD8+ T cells in the contexts of ageing or cerebral amyloidosis have characterized such PD-1+ cells as being exhausted-like (Chen et al. 2023, Kaya et al. 2022). Conversely, our PMA/ionomycin stimulation data suggest that these cells possess similar capacity to produce cytokines as their PD-1- counterparts.

PD-1 is also expressed by effector CD8+ T cells, being induced downstream of TCR signaling (Ahn et al. 2018). Circulating memory CD8+ T cells exhibit low to a lack of PD-1 expression, while T cell exhaustion is marked by an elevated and persistent PD-1 expression, in addition to other inhibitory receptors including TIM-3 (Utzschneider et al. 2020). In order to address whether brain-resident PD-1+ CD8+ T cells are exhausted, we compared the brain-resident CD8+ T cell landscape in steady state mice to that of mice with chronic LCMV clone-13 infection. The clone-13 variant, which differs in structure from the wild-type Armstrong variant by only two amino acids, represents a well-established model for inducing CD8+ T cell exhaustion (Bergthaler et al. 2010, Ahmed et al. 1988). As a control, we investigated the brain CD8+ T cell compartment upon systemic infection with LCMV Armstrong, a robust model for generating memory CD8+ T cells that has been invaluable for studying Trm cells in various organs (Casey et al. 2012). Accordingly, we ask how a systemic acute vs. chronic viral infection shapes brain-resident CD8+ T cells in comparison to the non-infection settings described above (Figure 6A).

In terms of cell numbers, chronic LCMV clone 13 infection resulted in a substantially larger number of CD8+ T cells compared to naïve or LCMV Armstrong-infected mice (Figure 6B). This increase in numbers applied to both, polyclonal CD8+ and CD8+ T cell specific for the LCMV immunodominant epitope glycoprotein (gp)33-41 (hereafter referred to as gp33), which is conserved across both, LCMV Armstrong and clone-13 strains (Figure 6B). Phenotypically, brain-resident CD8+ T cells in steady state and Armstrong-immune mice showed negligible PD-1 and TIM-3 co-expression, while clone 13-infected mice exhibited a substantial PD-1+ TIM-3+ CD8+ T cell population in the brain (Figure 6C). This also applied to both, polyclonal CD8+ and gp33-specific CD8+ T cells (Figure 6C). Importantly, the amount of PD-1 expressed per CD8+ T cell was

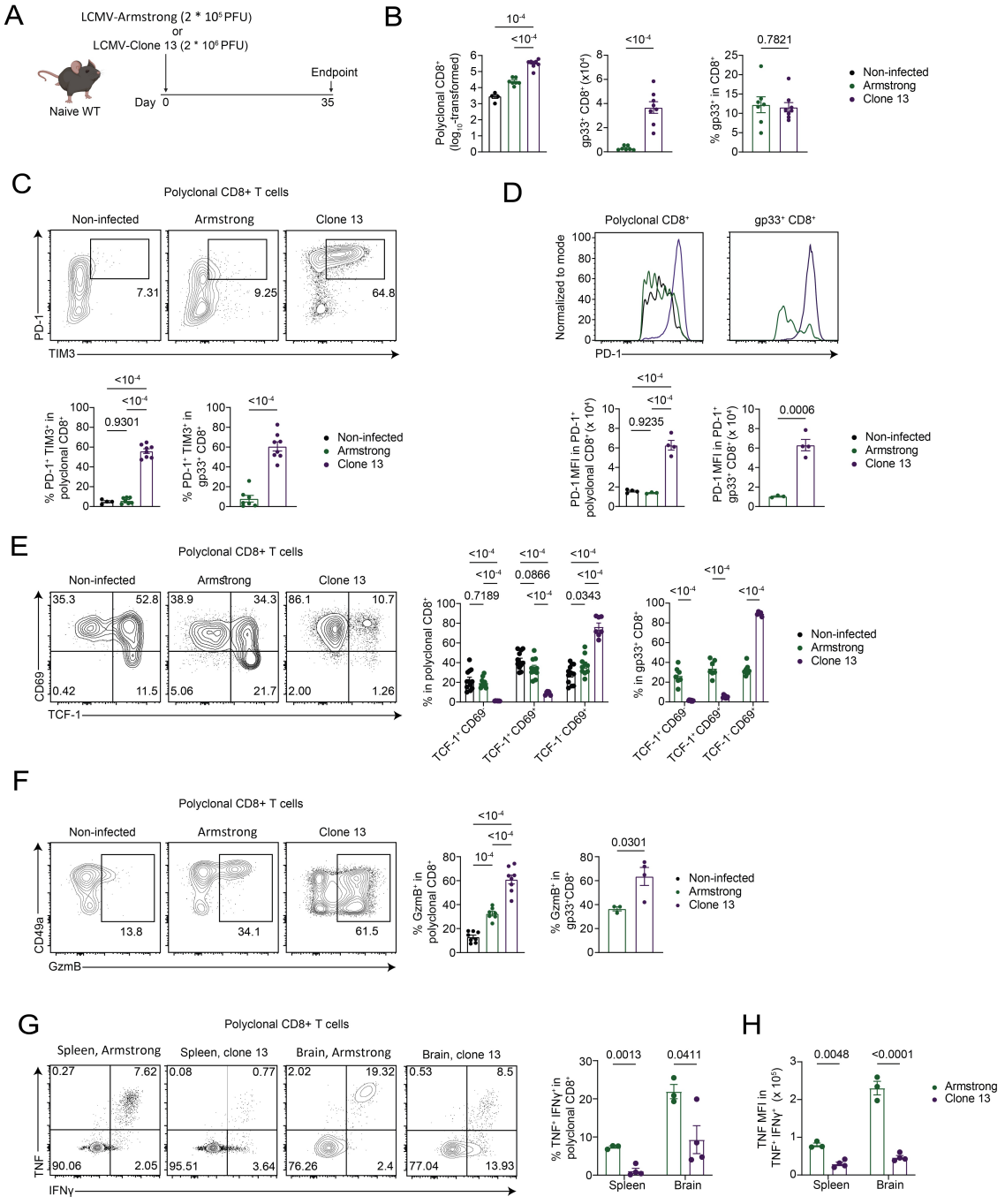

Figure 6 | Chronic, but not acute, viral infection profoundly alters the brain CD8+ T cell compartment. A, Five month-old female mice were either infected with 2 x 10^5 PFU of LCMV-Armstrong (Arm) or 2 x 10^6 PFU of LCMV-Clone13 (C13) as indicated, and naïve mice were used as controls. **B,** Quantification of polyclonal and gp33-specific CD8+ T cells in the brains of naïve, Arm, or C13-infected mice; n = 4-8. **C,** Co-expression of PD-1 and TIM3 in brain CD8+ Trm cells in naïve, Arm- or C13-infected mice, shown for polyclonal and gp33-specific cells; n = 4-8 mice. **D,** PD-1 MFI in PD-1+ polyclonal and gp33-specific brain CD8+ T cells; n = 4. **E,** Frequency of TCF-1+ CD69-, TCF-1+ CD69+, and TCF-1- CD69+ in polyclonal and gp33-specific CD8+ T cells in the brains of naïve, Arm-, and C13-infected mice; n = 7-8 mice. **F,** Proportion of GzmB+ in brain CD8+ Trm cells, shown for polyclonal and gp33-specific CD8+ T

cells; n = 7-8 mice. **G,** Frequency of TNF+ IFNγ+, and TNF MFI in TNF+ IFNγ+, in splenic and brain CD8+ T cells upon ex vivo stimulation with gp33 peptide for 4 hr in the presence of brefeldin A; n = 3-4. GzmB, granzyme B; LCMV, lymphocytic choriomeningitis virus; MFI, median fluorescence intensity.

substantially higher in the brain of clone 13-infected mice compared to non-infected or Armstrong-infected mice (Figure 6D). Therefore, brain CD8+ T cells in the context of a systemic chronic viral infection displayed phenotypic features of T cell exhaustion, which were absent in mice with a resolved acute infection or non-infected mice.

A cardinal feature of T cell exhaustion is the progressive loss of the capacity to produce cytokines in a stepwise manner. Exhausted CD8+ T cells first lose the ability to produce IL-2, followed by TNF, with IFNγ being most resistant to functional deterioration (Mackerness et al. 2010, Wherry et al. 2003a). To assess the functional potential of antigen-specific brain CD8+ T cells in acute versus chronic infection, we restimulated CD8+ T cells from the brains and spleens of Armstrong- or clone 13-infected mice ex vivo using gp33 peptide. As expected, spleen CD8+ T cells displayed a loss of TNF IFNγ co-expression in the context of chronic viral infection (Figure 6G). Similarly, brain CD8+ T cells exhibited a marked reduction in the capacity to co-produce TNF IFNγ in clone 13-infected compared to Armstrong-infected mice (Figure 6G). This deficit was not attributed to a difference in the frequency of gp33 antigen-specific CD8+ T cells in the brain across the two infection contexts (Figure 6B). Moreover, the amount of TNF made by brain CD8+ T cells in chronically infected mice was drastically smaller than their counterparts in Armstrong-infected mice (Figure 6H). In summary, CD8+ T cells in the brain of chronically infected mice exhibited functional hallmarks of T cell exhaustion.

We next asked how acute or chronic viral infection affected the TCF-1+/- CD69+ subsets of brain CD8+ Trm cells. In the context of acute Armstrong infection, we observed a small, non-significant reduction in the frequency of the TCF-1+ CD69+ subset and a reciprocal increase in TCF-1- CD69+ CD8+ T cells compared to non-infected mice (Figure 6E). Conversely, chronic viral infection was associated with a sharp loss of the TCF-1+ subset of brain CD8+ T cells, with 76% of cells bearing by a TCF-1- CD69+ phenotype. This was observed for both, polyclonal CD8+ T cells as well as gp33 antigen-specific cells (Figure 6E). Consistent with this loss of TCF-1+ CD8+

T cells cells, chronically infected mice showed a significantly larger frequency of granzyme B-producing CD8+ T cells in the brain compared to non-infected or Armstrong-infected mice (Figure 6F). Thus, whereas acute viral infection induced subtle alterations to the brain CD8+ T cell pool, chronic viral infection significantly altered the differentiation of CD8+ T cells in the brain.

Taken together, these findings demonstrate that brain CD8+ T cells display phenotypic and functional features of exhaustion in the context of chronic viral infection. This represents an example of a disease-specific alteration of the tissue-specific signature of brain CD8+ T cells established in non-infection contexts. Conversely, resolved infection was not associated with a marked alteration of the phenotype of brain CD8+ Trm cells relative to steady state. Further, these findings support the notion that the PD-1 expression in ageing and amyloidosis is not reflective of classical T cell exhaustion.

3.3. Discussion

3.3.1. Tissue-instructed vs. disease-specific features of brain CD8+ Trm cells

Tissue-resident memory CD8+ T cells seed most non-lymphoid tissues following a systemic acute infection or sterile inflammation (Casey et al. 2012, Wijeyesinghe et al. 2021). Brain CD8+ Trm cells have been described in mouse and human (Smolders et al. 2018, Urban et al. 2020); however, the molecular landscape of brain-resident memory CD8+ T cells remain ill-defined. The key finding of this chapter is that T cells residing in the brain appeared to adopt a primarily tissue-instructed rather than context-defined molecular profile. Using scRNA-seq and flow cytometry, our analysis consistently uncovered the same subsets of CD8+ T cells in young and aged naïve mice, in cerebral amyloidosis, and following an acute infection. Specifically, brain CD8+ T cells could be classified into distinct subsets based on the combinatorial expression of diverse molecules, including PD-1, Ly6C, CD69, and TCF-1.

The notion of tissue-specific imprinting of CD8+ Trm cells has been reported in peripheral non-lymphoid tissues. For instance, high-dimensional analysis (scRNA-seq or flow cytometry) of CD8+ Trm cells isolated from various tissues revealed that T cells segregated based on their tissue of origin, arguing that the tissue microenvironment was the predominant factor in shaping Trm cell transcriptome and phenotype (Christo et al. 2021, Crowl et al. 2022). Similarly, priming of CD8+ T cells with diverse viral and bacterial pathogens resulted in phenotypically similar CD8+ Trm cells in the same tissue, particularly in the small intestine and salivary glands (Evrard et al. 2023). This is of particular interest, since distinct infectious agents are known to promote qualitatively different effector as well as circulating memory T cell responses (Martin et al. 2015, Obar et al. 2011). For example, *Listeria monocytogenes* and vesicular stomatitis virus elicit different magnitudes and qualities of early effector/terminally differentiated effector CD8+ T cells, which were associated with variable secondary recall responses (Obar et al. 2011). This suggests that, despite the discrepancies in antigen presentation and effector T cell responses to different pathogens, the process of Trm cell maturation in situ appears to “negate” qualitative differences emanating from distinct priming events.

In agreement with what is reported in peripheral tissues, the phenotype of brain CD8+ Trm cells appears to be primarily dependent on the tissue of residence. Indeed, systemic acute infection using different pathogens (i.e. LCMV Armstrong or *Listeria*

monocytogenes) established brain CD8+ Trm cells with similar phenotype as well as long-term stability (Urban et al. 2020). Similarly, and consistent with our findings, Groh et al reported that CD8+ T cells in the aged brain displayed minimal differentially expressed genes compared to the same cell types in young adult mice (Groh et al. 2021). Moreover, bulk transcriptomic profiling of brain CD8+ T cells of APP/PS1 mice and age-matched wild-type mice revealed a substantial transcriptional overlap among transgenic and non-transgenic mice (Altendorfer et al. 2022). However, given the greater depth of transcript coverage achieved by bulk RNA-seq, a larger number of DE genes were detected between wild-type and APP/PS1 brain CD8+ T cells compared to what we observed (Altendorfer et al. 2022). Notably, the notion of a tissue-specific transcriptional profile in the brain extends beyond T cells. For instance, oligodendrocytes responded in a largely context-independent manner to various neuropathological insults (Kenigsbuch et al. 2022). Specifically, across pathologies ranging from cerebral amyloidosis and tauopathy to autoimmunity, a subset of oligodendrocytes adopted a transcriptional module that was conserved across these divergent disease states. However, the described disease-associated state of oligodendrocytes was largely absent in steady-state mice, suggestive of context and tissue-dependent features of oligodendrocytes. Although parallels seem to exist in terms of tissue adaptation between T cells and oligodendrocytes, microglia displayed context-specific adaptations (Mrdjen et al. 2018).

Whereas the data presented above support the notion that brain T cell residency instructs a common molecular program, it has long been known that acute intracranial infection generates brain CD8+ Trm cells comprising a large fraction of CD103+ cells (Wakim et al. 2010, Wakim et al. 2012, Steinbach et al. 2016, Rosen et al. 2022). This context is characterized by a high local antigen burden, achieved through the delivery of pathogen directly in situ, resulting in a qualitatively distinct CD8+ T cell response in the brain. As alluded to above, brain CD8+ Trm cells generated by a systemic acute infection or through sterile inflammation consist of a small CD103+ population (Altendorfer et al. 2022, Smolders et al. 2018, Urban et al. 2020, Su et al. 2023b, Ritzel et al. 2016). In contrast, we noticed that another context of elevated antigen burden, i.e. $2*10^6$ PFU of LCMV clone 13 infection, induced a state of exhaustion in brain CD8+ T cells, akin to the extensively described phenotype in peripheral lymphoid and non-lymphoid tissues (Wherry et al. 2003a, Kallies et al. 2020, Sandu et al. 2020b). In this study, chronic viral infection represented one example where the underlying

pathophysiology constituted a stronger determinant of the brain CD8+ T cell phenotype than the tissue residency per se. Importantly, the fraction of CD103+ CD8 brain Trm cells remained small in chronically infected mice. It is likely that the mode of localized, intracranial brain infection, resulting in a high antigen burden but also tissue injury and potential subsequent release of alarmins such as IL-33, is required for the differentiation of a large CD103-expressing CD8+ Trm population. Indeed, in the context of tumors, CD8+ Trm-like cells deficient in ST2, the IL-33 receptor, fail to upregulate CD103 to the same extent as ST2-sufficient CD8+ T cells (Chen et al. 2020). Further studies are needed to delineate the precise requirements for generating the CD103+ subset of brain CD8+ Trm cells (beyond TGF-β signaling, as described in chapter 4).

3.3.2 Identification of novel as well as established markers of tissue residency expressed by CD8+ Trm cells

A major aim behind the described experiments was to investigate the existence of a conserved molecular signature of brain CD8+ T cells across diverse physiological and pathophysiological contexts. Our unbiased scRNA-seq and confirmatory flow cytometry data identified molecules that are well established to mark CD8+ Trm cells in various tissues, such as CD49a, CD69, and PD-1 and other molecules that are either not well studied in Trm cells in general or in brain Trm cells in particular, such as TCF-1 and Ly6C.

A canonical marker of Trm cells in various tissues is CD69 (Topham and Reilly 2018). Consistent with revious reports, we found that CD69 marked >80% of extravascular CD8+ T cells. CD69 contributes to the retention of Trm cells by antagonizing the sphingosine-1-phospate (S1P) receptor on Trm precursors and mature Trm cells, thereby guarding against T cell egress along S1P gradients in blood and lymph (Mackay et al. 2015a). However, subsequent studies have shown that in many non-lymphoid tissues, a fraction of Trm cells, as defined by parabiosis and imaging studies, does not express CD69 (Steinert et al. 2015, Li et al. 2022). Further, Trm cells in various non-lymphoid tissues do not require CD69 for their formation (Walsh et al. 2019). As concerns the brain, previous reports show that >80% of extravascular brain CD8+ T cells express CD69 (Urban et al. 2020, Kaya et al. 2022, Ayasoufi et al. 2023). Whether CD69 actively contributes to brain CD8+ Trm cell maintenance remains to be clarified. Another canonical marker of Trm cells in skin, gut, salivary glands, and lungs

is the TGF-β-dependent integrin CD103 (Mackay et al. 2013, Casey et al. 2012, Wakim et al. 2015). As alluded to above, brain CD8+ Trm cells generated in the absence of local infection consist of a small fraction of CD103-expressing cells. Similarly, we observed a minor CD103+ subset among extravascular brain CD8+ T cells in all contexts studied. Conversely, another TGF-β-controlled integrin, CD49a, was found to be produced in >60% of brain CD8+ Trm cells, in agreement with a previous study (Urban et al. 2020).

TCF-1 is a transcription factor that plays well-established and critical roles in T cell development, as well as in naïve and central memory T cell maintenance (Zhao et al. 2022). However, few studies have addressed its expression pattern and function in Trm cells, where the authors concluded that TCF-1 downregulation was required for proper Trm formation (Dave et al. 2021, Liao et al. 2021, Wu et al. 2020). Conversely we observed a substantial fraction of TCF-1+ cells among brain CD8+ Trm cells across various models. The TCF-1+ subset of CD8+ T cells exhibited features that are in agreement with past studies. For instance, TCF-1+ CD69+ cells showed a higher proliferation rate compared to their TCF-1- counterparts. This was consistent with a TCF-1+ subset among CD8+ T cells resident in the cervicovaginal tissue (Dave et al. 2021), as well as the greater proliferative potential among TCF-1+ central memory or Tpex CD8+ T cells in acute and chronic infection, respectively (Utzschneider et al. 2016, Kratchmarov et al. 2018, Lin et al. 2016). Further, pseudotime analysis inferred a developmental trajectory that progresses along a gradient of TCF-1+ to a TCF-1- state. This is consistent with the notion that TCF-1 marks precursors of memory and exhausted T cells in acute and chronic viral infection, respectively (Pais Ferreira et al. 2020, Tsui et al. 2022, Utzschneider et al. 2016). Moreover, we noted that, upon ex vivo stimulation, TCF-1+ CD69+ brain CD8+ Trm cells comprised a higher proportion of TNF+ IFNγ+ cells compared to TCF-1- cells. Likewise, *Tcf7*-deficient circulating memory CD8+ T cells exhibited a reduced capacity to produce TNF, suggestive of at least a correlation between TCF-1 and TNF expression (Zhou and Xue 2012).

Further, previous studies have characterized a TCF-1+ subset among brain CD8+ Trm cells in a model of autoimmune neuroinflammation (Vincenti et al. 2022, Page et al. 2021, Page et al. 2018). The authors found that such TCF-1+ cells expressed fewer transcripts encoding effector molecules (granzymes and perforin), and that the transition into TCF-1- CD8+ T cells was required for neuropathology to manifest in mice. They also found that brain CD8+ Trm cell differentiation into a TCF-1- state was

at least partially dependent on CD4+ T cell help (Vincenti et al. 2022). The enhanced expression of granzymes by brain TCF-1- CD8+ T cells is in agreement with our data. Further, the notion of TCF-1+ cells among brain CD8+ T cells acting as precursors for a more terminally differentiated state is consistent with our findings. Conversely, the fraction of TCF-1+ cells in brain CD8+ Trm cells reported (at 5-20%) was substantially smaller than what we observed across diverse infection and non-infection models. This might be attributed to the context of autoimmunity employed by the authors, since inflammation is known to downregulate TCF-1 expression in T cells (Danilo et al. 2018). Moreover, the authors made use of TCR transgenic P14 cells in their experimental setup (Page et al. 2021, Vincenti et al. 2022): preliminary experiments using *Hobit*^{Cre} P14 cells to study brain CD8+ Trm cells revealed a substantially reduced fraction of TCF-1+ compared to polyclonal CD8+ T cells (see section 4.2.2 and data not shown).

PD-1 is an inhibitory receptor that is expressed downstream of TCR signaling, and whose interplay with TCR signaling controls T cell differentiation and activation (Mizuno et al. 2019, Ahn et al. 2018). PD-1 is perhaps best studied in the context of CD8+ T cell exhaustion in the contexts of chronic viral infection and cancer. Whereas effector T cells transiently upregulate PD-1, exhausted CD8+ T cells show an elevated and sustained expression of PD-1. Originally thought to promote T cell exhaustion, studies have shown that PD-1 mitigates the development of a terminally exhausted phenotype and sustains long-term T cell response by maintaining the Tpex subset (Odorizzi et al. 2015, Tsui et al. 2022, Chen et al. 2019). Unlike circulating memory CD8+ T cells, Trm subsets in various non-lymphoid tissues stably express PD-1 (Smith and Snyder 2021, Kumar et al. 2017, Scholler et al. 2020, Wang et al. 2019, Chen et al. 2023).

Given its prominent expression by exhausted T cells, PD-1+ subsets of Trm cells, including in the brain, are occasionally labelled as “exhausted” or “exhausted-like” without the authors providing sufficient evidence of functional exhaustion in the context in question (Chen et al. 2023, Kaya et al. 2022). In another context, namely lung Trm cells specific for different influenza epitopes, a subset of lung Trm cells was found to highly express PD-1, exhibit reduced cytokine production compared to their PD-1- counterparts, yet retain a robust recall response characteristic of memory T cells (Wang et al. 2019). Accordingly, we assessed the capacity of PD-1+ and PD-1- CD8+ T cells in the brain to produce cytokines following PMA/ionomycin stimulation, and found no evidence for a reduced functionality in PD-1+ cells. Conversely, in the setting

of chronic LCMV clone-13 infection, we observed–as expected–a marked reduction in TNF IFNγ co-production compared to LCMV Armstrong infected mice. Interestingly, we noted that the frequency of TNF+ IFNγ+ CD8+ T cells, as well as TNF MFI, was markedly higher among brain compared to splenic CD8+ T cells. This is in line with evidence pointing to heterogeneity in exhausted CD8+ T cells across lymphoid and non-lymphoid tissues in terms of terminal differentiation and effector function (Sandu et al. 2020b). Similarly, PMA/ionomycin stimulation of brain CD8+ T cells in naïve mice revealed a greater frequency of TNF+ IFNγ+ compared to their counterparts in spleen or kidneys. This suggests that brain CD8+ Trm cells are poised for a heightened cytokine production upon restimulation, although the exact molecular mechanisms underpinning this propensity remain unclear.

Notably, TCR signaling is known to be impaired in exhausted CD8+ T cells in a PD-1 dependent manner (Sandu et al. 2020a). This raises the question of whether PMA/ionomycin stimulation, which bypasses proximal TCR signaling and instead acts on distal TCR signaling components protein kinase C and calcineurin, would reflect potential deficits in cytokine production. Yet, Zajac et al. have previously demonstrated that the impaired cytokine production characteristic of exhausted antigen-specific T cells was recapitulated upon PMA stimulation (Zajac et al. 1998). Therefore, in contexts where a specific antigen(s) is not defined, we made use of PMA/ionomycin for ex vivo stimulation.

Another question pertaining to PD-1 expression by Trm cells is whether it reflects persistent TCR signaling in situ, or if it represents part of the differentiation program of Trm subsets independent of ongoing antigen recognition. Different T cell subsets variably depend on TCR signaling to promote their survival. Naïve T cells require tonic TCR signaling for their longevity (Eggert and Au-Yeung 2021). Circulating memory T cells typically do not depend on TCR signaling, but instead on cytokines such as IL-7 and IL-15, for their maintenance (Kaech and Cui 2012). The engagement and requirement of TCR signaling for Trm cell formation and maintenance varies across tissues. For instance, gut CD8+ Trm cells do not actively engage their TCR in situ, as evidenced by a lack of Nur77 expression (Casey et al. 2012). Conversely, lung Trm cells show evidence for local TCR engagement, as well as brain CD8+ Trm cells in the context of intracranial infection (Wang et al. 2019, Scholler et al. 2020). As a corollary, TCR signaling is dispensable for long-term Trm maintenance in the gut and salivary gland (Wijeyesinghe et al. 2021), yet is important for the longevity and phenotype of

the PD-1+ subset of lung Trm cells (Uddback et al. 2021, Wang et al. 2019). Intriguingly, a previous study has shown that the TCRs of brain Trm cells–in the context of intracranial infection–exhibit a significantly higher affinity to antigen compared to their counterparts in spleen (Frost et al. 2015). However, the authors did not show whether such increased affinity was associated with actual TCR signaling. In the brain of naïve mice, we observed that PD1+ CD8+ Trm cells express higher amounts of IRF4 compared to PD-1- cells, suggestive of TCR signaling in situ. However, an elegant study by Shwetank et al has shown that, in a local infection setting, PD-1 expression among brain CD8+ Trm cells is independent of cognate antigen recognition or inflammation, but rather dependent on the *Pdcd1* promoter being set in a stably demethylated state (Shwetank et al. 2017). These findings leave open a couple of possibilities: a) brain CD8+ Trm cells engage their TCR in situ but PD-1 expression is independent of such engagement; b) PD-1 expression is cognate antigen-independent but, in other settings, PD-1 is maintained by cross-reactive TCR engagement of self-antigens. Accordingly, conditional deletion of the TCR in established memory CD8+ T cells is needed to formally assess a requirement for TCR signaling for the maintenance of brain CD8+ Trm cells and their expression of PD-1.

A cluster of CD8+ T cells that was consistently observed in our scRNA-seq datasets comprised cells with a prominent type I IFN signature. Type I IFN response is known to be prominent in the aged brain, primarily driven by an alteration in the choroid plexus and cerebrospinal fluid composition in old mice (Baruch et al. 2014). In fact, a heightened type I IFN response has been demonstrated both in ageing and in neurodegenerative diseases (Sanford and McEwan 2022). This is thought to be a consequence of protein misfolding and aggregate formation, which acts on various cell types in the brain and triggers the release of type I interferon (IFN)s such IFNα and IFNβ, with subsequent autocrine or paracrine IFN signaling (reviewed by (Sanford and McEwan 2022)). Accordingly, we also observed an increased frequency and numbers of type I IFN-responsive CD8+ T cells in the brains of old naïve and APP/PS1 mice. Interestingly, such CD8+ T cell subset was also resolved in the brain of naïve young mice, consistent with the description of similar CD4+ and CD8+ T cell states in other tissues at steady state (Le Moine et al. 2023, Zemmour et al. 2020). This raises the question if indeed protein misfolding and aggregate formation are necessary for inducing this T cell state in the brain or if other factors are instead causative. Notably, our pseudotime analysis inferred that such type I IFN-responding CD8+ T cells

differentiated from PD-1+ CD8+ T cells. This suggests that a subset of PD-1+ CD8+ T cells respond to type I IFN and acquire a distinct transcriptional state. Nevertheless, the precise ontogeny and function of type I IFN-responding CD8+ T cells in the brain warrant further investigation.

Ly6C consistently marked a subset of CD8+ T cells in the brain during homeostasis and disease. Ly6C is known to be expressed by naïve and memory CD8+ T cells, particularly CD8+ Tcm cells (Walunas et al. 1995, DeLong et al. 2018). Notably, evidence points to a role for Ly6C in mediating the adhesion of T cells to the vascular endothelium via clustering with CD11a (Jaakkola et al. 2003). However, a recent study found that deletion of *Ly6c1* and *Ly6c2* genes resulted in virtually no impact on T cell distribution in spleen and lymph nodes. Nevertheless, a subsequent study found that administration of anti-Gr1 antibody specifically depletes a Ly6C+ subset of Trm cells in the liver, kidney, and salivary glands (Evrard et al. 2023). Our data suggest that Ly6C-expressing brain CD8+ T cells mostly correspond to TCF-1+ CD69+ cells, and pseudotime inference shows that Ly6C+ CD8+ T cells represent an intermediate stage of differentiation towards a TCF-1- PD-1+ state. Adoptive cell transfer using Ly6C-deficient cells are needed to formally address the developmental potential of Ly6C+ CD8+ T cells, and whether Ly6C mediates trans-endothelial migration of Trm cell precursors into the brain.

Granzyme B is known to be expressed by subsets of Trm cells in various tissues as part of a poised state for immediate effector function (Gebhardt et al. 2018). The discrepancy in granzyme B expression by brain Trm cells at steady state vs. LCMV Armstrong infection was of interest. A previous study has found that liver CD8+ Trm cells in naïve mice exhibited small frequencies (<10%) of granzyme B expression (Le Moine et al. 2023). Conversely, Trm cells lodged in the gut by virtue of lymphopenia-induced proliferation and activation, i.e. in the absence of infection, appeared to highly express granzyme B (Casey et al. 2012). However, it is known that at a “memory” phase following T cell transfer into a lymphopenic host, such T cells acquire a gene expression program that is largely similar to that observed in memory T cells generated following an acute infection (Goldrath et al. 2004). This indicates that the high granzyme B expression by intestinal Trm cells observed by Casey et al. may not be recapitulated in an immunocompetent, steady-state mouse. Instead, existing evidence points to inflammation during memory T cell generation as the likely factor for the enhanced granzyme B expression generated in the context of acute infection. Indeed,

it has been show that the extent of granzyme B expression was more dependent on systemic inflammation during priming rather than TCR signal strength (Zehn et al. 2014, Kohlmeier et al. 2010). Similarly, in the cervicovaginal tissue, localized infection resulted in an ensuing persistent inflammation and generation of local CD8+ Trm cells with a greater frequency of GzmB+ cells, compared to systemic infection without persistent local inflammation (Dave et al. 2021). As for chronic LCMV clone-13 infection, the frequency of granzyme B expression among brain CD8+ T cells was markedly higher compared to LCMV-Armstrong immune mice, consistent with past observations in other tissues (Wherry et al. 2007). It is known that the elevated expression of Blimp-1 in Tex cells promotes their expression of granzyme B, similar to what was previously reported in effector T cells (Gong and Malek 2007, Shin et al. 2009, Kallies et al. 2009). The persistent systemic inflammation associated with chronic LCMV infection (Baazim et al. 2019) may offer an additional potential mechanism for the enhanced granzyme B expression by exhausted CD8+ T cells compared to bona fide memory T cells (Wherry et al. 2007, Casey et al. 2012). In summary, transient and persistent inflammation in resolving and chronic infection, respectively, may contribute to differential expression of granzyme B by CD8+ T cells in the brain, among other tissues.

3.3.3 CD8+ Trm cells in the aged brain: quantitative vs. qualitative alterations

Protective and detrimental roles have been ascribed to CD8+ T cells in the aged brain (Groh et al. 2021, Ritzel et al. 2016). Whether such roles are mediated by the increased numbers of CD8+ T cells in the aged brain, or by ageing-induced qualitative alteration of brain CD8+ T cells, remains unclear. We found that, in ageing, the transcriptional and functional qualities of brain CD8+ Trm cells exhibited remarkable similarities to their counterparts in young mice, arguing against the suggestion that CD8+ T cells acquire additional cytotoxic features in the aged brain.

In a study by Ritzel et al., the number of CD8+ T cells in the aged brain was positively correlated with the number and phagocytic capacity of microglia, yet inversely correlated with microglial size and TNF production (Ritzel et al. 2016). However, these findings do not rule out the likelihood of microglial alteration being the primary driver of the enhanced CD8+ T cell recruitment to the aged brain. Furthermore, administration of brefeldin A before sacrifice of aged mice (to capture the endogenous expression of

TNF and IFNγ) revealed an enhanced expression of the aforementioned cytokines in brain compared to blood CD8+ T cells (Ritzel et al. 2016). Yet, the authors did not perform such comparison between brain CD8+ T cells in young and old mice. In our hands, we observed no difference in TNF IFNγ co-production by brain CD8+ Trm cells across young and old mice, further supporting the notion of the acclimatization of brain CD8+ T cells to their tissue of residence in ageing.

In another study, Dulken et al investigated CD8+ T cell infiltration and function in the subventricular zone (SVZ) of the aged brain, one of the few brain regions where adult neurogenesis occurs (Dulken et al. 2019). SVZ CD8+ T cells were found to be producing IFNγ, located in close proximity to neural progenitors and inhibiting their proliferation (Dulken et al. 2019). However, the authors did not explicitly show that this phenomenon is attributed to an inherent age-related alteration of CD8+ T cells in the aged brain. In fact, the same detrimental impact of CD8+ T cell on neural precursors was observed in young mice through their immunization with myelin oligodendrocytes glycoprotein to promote T cell infiltration into the brain (Dulken et al. 2019). Together, these findings suggest that the enhanced IFNγ responsiveness by neural progenitors in the old brain is likely due to the concurrent increase in the number of IFNγ-producing CD8+ T cells, and not due to unique characteristics of CD8+ T cells resident in old brains.

Apart from the SVZ, Groh et al examined how CD8+ T cells in the aged brain affects the axonal demyelination and degeneration observed in the retina (Groh et al. 2021). They found that aged *Rag1*$^{-/-}$ mice were largely protected from retinal neurodegeneration compared to age-matched wild-type mice. Further, the authors showed that reconstitution of *Rag1*$^{-/-}$ mice with wild-type, and not *Cd8*$^{-/-}$, bone marrow rescued the neurodegenerative phenotype observed in old mice. In addition, reconstitution of *Rag1*$^{-/-}$ old mice with bone marrow derived from *Gzmb*$^{-/-}$ mice failed to rescue the age-associated degeneration of optic nerves (Groh et al. 2021). However, the authors did not compare whether reconstitution with bone marrow derived from young vs. old donor mice resulted in variable outcomes. This precludes the conclusion that the degenerative phenotype in the retina is caused by ageing-associated qualitative alteration of brain CD8+ T cells. Instead, these data, together with our findings, suggest that neurodegeneration is driven by the ageing-associated accumulation of CD8+ T cells expressing largely similar amounts of effector molecules compared to young adult mice.

As alluded to above, we observed elevated frequencies and numbers of CD8+ T cells showing a type I IFN response in ageing. Focusing on CD8+ T cell-glial cell interaction, a study by Kaya et al demonstrated that CD8+ T cells in the white matter of the aged brain induce oligodendrocyte and microglia states of type I IFN response (Kaya et al. 2022). Notably, treatment of aged naïve mice with immune checkpoint blockade (ICB) resulted in an increased number of brain CD8+ T cells and an enhanced induction of the type I IFN-responding subsets of oligodendrocytes and microglia (Kaya et al. 2022). Yet, the authors did not demonstrate whether the ICB-induced increase of IFN-responsive oligodendrocytes was promoted by the increased total number of CD8+ T cells per se, or through a heightened frequency of cytokine-producing CD8+ T cells. In other words, it was not formally shown whether such CD8+ T cell alteration of brain homeostasis was down to an altered brain CD8+ T cell transcriptome or phenotype, or a consequence of the increased total number of CD8+ T cells in the aged brain.

In summary, we argue that the data reported in previous studies, coupled with our findings, point to the age-associated increase of brain CD8+ T cell numbers posing a detrimental impact to brain homeostasis, rather than ageing driving a substantial alteration of the quality of brain-resident CD8+ T cells.

3.3.4 CD8+ Trm cells in cerebral amyloidosis and other models of neurodegeneration

In the context of cerebral amyloidosis, we observed that the number of CD8+ T cells in the brain increases as a function of ageing, with a slight increase noted at 10 months of age and a sharp increase at 15 months of age. This is in agreement with two recent reports. On the one hand, Unger et al also showed a progressive increase of CD8+ T cells in the hippocampi of APP/PS1 mice starting at 10 months and peaking at 18 months of age (Unger et al. 2020). Similarly, 5xFAD mice–which exhibit a more severe neurodegenerative phenotype relative to APP/PS1 mice (Oakley et al. 2006)–showed an increase in brain CD8+ T cell numbers at 9 months but not 6 months of age (Su et al. 2023b). These data suggest that ageing and neuropathology act in synergy to facilitate T cell infiltration into the brain. Notably, it is known that microglia in 5xFAD mice exhibit a disease-associated phenotype in <2 month-old mice (Keren-Shaul et al. 2017), indicating that an innate immune response to neuropathology is prominent independent of ageing.

A number of studies have addressed the contribution of CD8+ T cells to disease progression in mouse models of dementia (i.e. cerebral amyloidosis and tauopathy). Laurent et al used the THY-Tau22 model of tauopathy, characterized by transgenic expression of a mutant human *MAPT* transgene (encoding tau), and where mice exhibit a heightened T cell infiltration at 7-12 month of age (Laurent et al. 2017). Systemic depletion of total T cells at 4-9 months of age rescued the memory impairment otherwise observed in T cell-sufficient THY-Tau22 mice (Laurent et al. 2017). In another tauopathy model expressing transgenic human mutant tau and mutant APOE4, depletion of CD4+ and CD8+ T cells resulted in microglia adopting a homeostatic rather than disease-associated state, in addition to a reversal of hippocampal atrophy and improved behavioral performance (Chen et al. 2023). Conversely, 5xFAD mice deficient in total T/B cells, αβ T cells, or CD8+ T cells exhibited enhanced amyloid plaque formation and worsened cognitive impairment (Marsh et al. 2016, Su et al. 2023b). These studies reflect differential modulation by T cells of the pathological processes of tauopathy and amyloidosis. Yet, and as critically, these studies employed crude models of depleting the entire pool of T cells, overlooking the heterogeneity of brain-resident T cells. In the study by Chen et al, scRNA-seq of brain CD8+ T cells revealed a modest decrease in the fraction of the PD-1+ subset and a reciprocal increase in the fraction of interferon-responsive cells, consistent with our observation (Chen et al. 2023). Further studies are warranted to identify and selectively manipulate functionally pathogenic and “regulatory” subsets of brain-resident T cells.

More recently, an elegant study by Su et al characterized the role of the CXCR6+ subset of brain CD8+ Trm cells in 5xFAD mice. CXCR6-/- 5xFAD mice showed an impaired differentiation and accumulation of CD8+ Trm cells, particularly the PD-1+ subset, accompanied by aggravated amyloid plaque pathology and cognitive decline. Further, brain CD8+ T cells in CXCR6-/- 5xFAD mice showed an impaired capacity to co-localize with amyloid-associated mciroglia, which resulted in an enhanced microglial production of pro-inflammatory cytokines (Su et al. 2023b). Although this study employed a more targeted manipulation of T cells in 5xFAD mice, CXCR6 is also known to be expressed by subsets of CD4+ T cells and NKT cells, which both are found in the 5xFAD brain and do express CXCR6 (Su et al. 2023b). However, given that a CXCL16-CXCR6 ligand-receptor interaction was inferred specifically between microglia and CD8+ T cells, it is likely that the disease alterations observed in CXCR6-

/- 5xFAD mice was mediated by CD8+ T cells in a cell-intrinsic manner, without ruling out a contribution from CXCR6+ CD4 and/or NKT cells. Finally, these data also suggest that early manipulation of T cells in neurodegeneration is required, since total T cell depletion in 12 month-old APP/PS1 mice did not mitigate cognitive decline (Unger et al. 2020).

In our studies, we noticed a selective increase of the TCF-1+ CD69+ subset of brain CD8+ Trm cells both in 10 month-old and 15 month-old APP/PS1 mice, which corresponded to an increase of CD8 cluster 2 in our scRNA-seq data. To some extent, this finding is consistent with a post-mortem immunohistochemical assessment of CD8+ T cells in the AD brain, whereby granzyme B was not detected (Merlini et al. 2018). Thus, we speculate that the preferential accumulation of TCF-1+ CD69+ CD8+ T cells (which barely express granzyme B) may reflect their tissue adaptation in the mouse brain with cerebral amyloidosis to limit neuropathology. Selective manipulation of TCF-1 expression in brain CD8+ Trm cells in APP/PS1 mice (e.g. using *Hobit*^{Cre} *Tcf7*$^{f/fl}$, described below), and assessment of the neuropathological and behavioral consequences of such modulation, is an area of potential future investigation.

Chapter 4: TCF-1, PD-1, and TGFβ signaling jointly regulate brain CD8+ T cell formation and function

4.1. Background

After establishing the molecular profile of brain-resident CD8+ T cells across diverse contexts, we next aimed to understand the role these molecules play in shaping the formation and maintenance of the brain CD8+ T cell pool. In particular, we addressed the contribution of the transcription factor TCF-1, the inhibitory receptor PD-1, and the TGF-β signaling pathway to the differentiation of brain CD8+ Trm cells.

The expression pattern of TCF-1, namely its co-expression with CD69 and other residency markers, was unexpected, given that previous reports have associated TCF-1 downregulation with the formation of CD103+ Trm cells in the brain and lung (Wu et al. 2020, Wakim et al. 2012). Given that pseudotime analysis inferred a trajectory starting from a TCF-1+ to a TCF-1- state, we hypothesized that deletion of *Tcf7* in CD8+ T cells would impede the differentiation of CD8+ Trm cells in the brain. Yet, the establishment and maintenance phases of Trm cell formation in a given tissue do not necessarily rely on the same molecular pathways (Gebhardt et al. 2018). Therefore, we also aimed to investigate the regulation of CD8+ Trm cells by TCF-1 during the maintenance phase.

PD-1 signaling has been shown to be important for the differentiation and recall response of CD8+ Trm cells in the lung (Wang et al. 2019, Pauken et al. 2020). In addition, previous studies have investigated the regulation of brain CD8+ Trm cells by PD-1 (Phares et al. 2009, Prasad et al. 2017, Scholler et al. 2020, Shwetank et al. 2019). These studies employed different viral strains with variable extents of viral persistence in the brain, which led to inconsistent results as to whether PD-1 promotes or inhibits brain Trm differentiation. In addition, the aforementioned studies employed constitutive PD-1 KO or PD-L1 KO mice, which do not allow for studying the consequences of PD-1 ablation in a CD8+ T cell-intrinsic manner. Accordingly, further investigations are warranted to clarify the role of PD-1 in regulating the differentiation and maintenance of brain CD8+ Trm cells.

As alluded to in Chapter 1, TGF-β signaling is critical for the differentiation and maintenance of Trm cells in various tissues (Casey et al. 2012, Mackay et al. 2013). Two previous studies have provided indirect evidence on the implication of TGF-β in

the formation of CD103+ CD8+ Trm cells in the brain following intracranial infection (Graham et al. 2014, Prasad et al. 2015). Graham et al have shown that depleting Treg cells resulted in a smaller number of polyclonal and antigen-specific CD103+ CD8+ T cells in the brains of West Nile virus-infected mice. Indeed, the amount of TGF-β in the brain was found to be higher in Treg cell-sufficient compared to Treg-deficient mice, with Treg cells also shown to robustly produce TGF-β in this setting. (Graham et al. 2014). In another study, Prasad et al employed a mouse model of localized MCMV brain infection, and depleted Treg cells during the early effector stage of infection. This led to the generation of a CD8+ Trm cell pool with a smaller CD103+ fraction, larger frequency of KLRG1+ cells, higher proliferation rate, and greater production of cytokines (Prasad et al. 2015). Notably, the notion of tissue Treg cells locally providing TGF-β to Trm cell precursors for their subsequent maturation has also been described in other tissues, including the gut (Ferreira et al. 2020). However, these findings do not formally show that TGF-β acts on CD8+ Trm precursor cells in a cell-intrinsic manner, nor rule out an (additional) TGF-β-independent, Treg-mediated regulation of CD8+ Trm cells.

As described in Chapter 3, LCMV-Armstrong infection generated a brain CD8+ Trm cell pool that exhibited subtle to no phenotypic differences compared to its counterpart in naïve mice. Yet, systemic acute infection resulted in a substantially larger number of CD8+ Trm cells in the brain, an important consideration for a robust analysis of brain-resident CD8+ T cells. Accordingly, to investigate the contribution of TCF-1, PD-1, and TGF-β signaling to the differentiation of brain CD8+ T cells, we made use of various genetic models that allow for targeted deletion of the respective molecules/receptors, infected such mice with LCMV Armstrong, and analyzed the mice at the memory phase post-infection. In these experiments, we address two main questions: how does TCF-1, PD-1, and TGF-β contribute to the differentiation and homeostasis of polyclonal and antigen-specific CD8+ T cells in the brain; and whether such molecules of interest regulated the magnitude of the recall response of brain CD8+ T cells upon antigenic rechallenge.

4.2. Results

4.2.1. CD8+ T cell-specific deletion of TCF-1 impairs the differentiation of brain Trm cells

To begin to investigate the role of TCF-1 in the regulation of brain-resident CD8+ T cells, we crossed *Tcf7*$^{fl/fl}$ mice to *Cd8*^{Cre} mice to generate mice with a CD8+ T cell-specific deletion of TCF-1. In this model, *Cd8*^{Cre} is expressed under control of the E8I enhancer as well as the *Cd8a* promoter (Ellmeier et al. 1997, Hostert et al. 1997), allowing for Cre recombinase expression in mature peripheral CD8+ T cells but not in CD4+ CD8+ double-positive thymocytes, thereby avoiding the deletion of *Tcf7* in the CD4+ T cell lineage (Maekawa et al. 2008). We then infected *Tcf7*$^{fl/fl}$ *Cd8*^{Cre} and *Tcf7*$^{fl/fl}$ control mice with LCMV Armstrong and assessed the splenic and brain CD8+ T cell compartments on day 44 post-acute LCMV infection (Figure 7A). We first confirmed the efficiency of *Cd8*^{Cre}-mediated deletion of *Tcf7* by intracellular staining of TCF-1 in spleen and brain CD8+ T cells (Figure 7B). TCF-1 deficiency was associated with a decreased number of brain-resident CD8+ T cells, a reduction that was not observed in the spleen (Figure 7C). Thus, deletion of TCF-1 in the CD8+ T cell compartment resulted in a smaller population size of brain CD8+ T cells.

TCF-1 is crucial for the formation and maintenance of central memory CD8+ T cells (Jeannet et al. 2010, Zhou et al. 2010). *Tcf7*$^{fl/fl}$ *Cd8*^{Cre} mice exhibited a systemic reduction in the frequencies and absolute numbers of gp33-specific central memory CD8+ T cells compared to *Tcf7*$^{fl/fl}$ mice (Figure 7D). We then examined whether the reduced number of brain CD8+ T cells in *Tcf7*$^{fl/fl}$ *Cd8*^{Cre} mice was associated with an altered cellular phenotype. As we have shown earlier (Figure 1E), CD69+ TCF-1- cells chiefly represent the PD-1+ subset of brain CD8+ T cells. In the absence of TCF-1 in CD8+ T cells, brain CD8+ T cells contained a substantially reduced fraction of PD-1+ cells compared to TCF-1-sufficient cells (Figure 7E). Moreover, CD8+ T cell-specific deletion of TCF-1 resulted in an increased frequency of granzyme B-expressing brain CD8+ T cells (Figure 7F).

In addition to CD8+ T cells, the *Cd8a* locus is transcriptionally active in conventional dendritic cells 1 (cDC1s)(Guilliams et al. 2016). The brain contains a population of cDC1s(Mrdjen et al. 2018), raising the possibility that the *Tcf7*$^{fl/fl}$ *Cd8*^{Cre} model additionally manipulates cDC1s. However, TCF-1 is not expressed by cDC1s (Appendix Figure 2). Accordingly, TCF-1 modulates the formation of brain CD8+ Trm

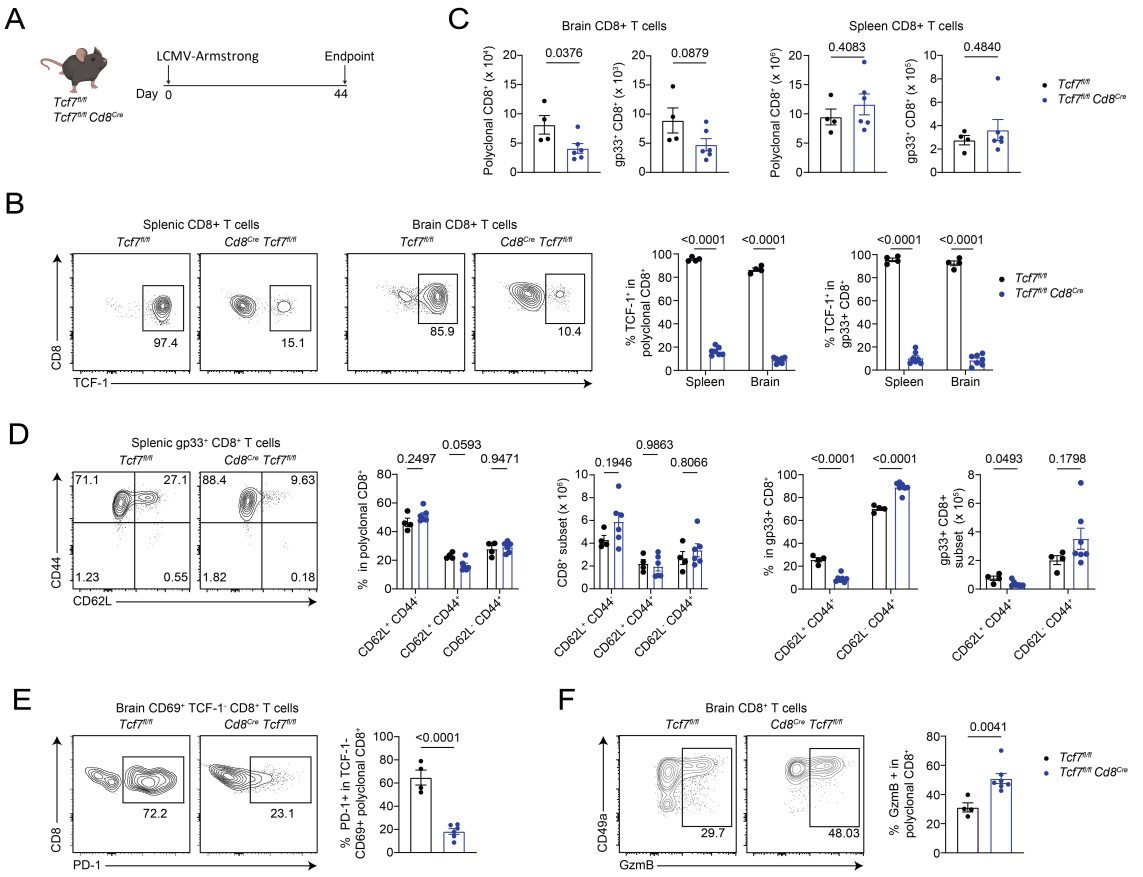

Figure 7 | Cell-intrinsic TCF-1 expression is important for the formation of brain CD8+ Trm cells. **A,** Sex- and age-matched *Cd8*^{Cre} *Tcf7*$^{f/fll}$ and *Tcf7*$^{f/fll}$ control mice were infected with LCMV-Armstrong (2 x 10^5 PFU i.p.) and sacrificed on day 44 post-infection. **B,** Frequency of TCF-1+ in polyclonal and gp33-specific CD8+ T cells in spleen and brain. **C,** Flow cytometric quantification of polyclonal and gp33-specific CD8+ T cells in the spleen and brain of *Cd8*^{Cre} *Tcf7*$^{f/fll}$ and *Tcf7*$^{f/fll}$ mice. **D,** Frequencies and numbers of naïve (CD62L+ CD44-), central memory (CD62L+ CD44+), and effector memory (CD62L- CD44+) CD8+ T cells in spleen. **E,** Proportion of PD-1+ in TCF-1- CD69+ brain CD8+ Trm cells. **F,** Percentage of GzmB+ in brain CD8+ Trm cells. Data are representative of one out of two independent experiments, n = 4-6 mice per genotype. One outlier in the *Cd8*^{Cre} *Tcf7*$^{f/fll}$ group was left out based on Grubb's test. i.p., intraperitoneally; LCMV, lymphocytic choriomeningitis virus, PFU, plaque-forming unit.

cells in a CD8+ T cell-intrinsic manner.

Taken together, these data show that TCF-1 expression in CD8+ T cells contributes to the optimal differentiation of brain-resident CD8+ T cells.

4.2.2 Hobit, a transcription factor specific for tissue-resident lymphoyctes, is expressed by a subset of brain CD8+ T cells

The *Tcf7*$^{fl/fl}$ *Cd8*^{Cre} model revealed a role for TCF-1 in regulating brain CD8+ T cells. However, this model inevitably results in non-specific modulation of the CD8+ T cell compartment in peripheral lymphoid tissues. To manipulate TCF-1 expression specifically in Trm cells, a Cre driver line selectively expressed in Trm cells is required.

Transcriptional profiling of Trm cells in various tissues revealed that they exhibit a distinct gene-expression programs compared to circulating memory T cells (Milner et al. 2020, Mackay et al. 2013, Crowl et al. 2022). Homolog of Blimp1 in T cells (Hobit) represents a transcriptional regulator differentially expressed by circulating and resident memory T cells, being downregulated in T cells at the effector stage and then stably expressed by Trm cells (Mackay et al. 2016). Indeed, Hobit is required for the differentiation and functional fitness of CD8+ Trm cells in various tissues, including the skin, gut, and kidney (Kragten et al. 2018, Mackay et al. 2016). We first assessed the expression pattern of Hobit by brain CD8+ T cells at steady state. To this end, we made use of a Hobit reporter mouse whereby a tdTomato cassette and a Cre transgene are inserted into the *Zfp683* (encoding Hobit) locus (Kallies lab, manuscript in revision). Indeed, we could observe that a substantial fraction of brain-resident CD8+ T cells express Hobit, co-expressed with the residency markers CD49a and CD69 (Figure 8A-B and data not shown).

4.2.3. TCF-1 maintains the homeostasis and limits the secondary expansion of brain-resident CD8+ T cells

To investigate the role of TCF-1 in brain CD8+ T cells in a Trm cell-specific manner, we crossed *Tcf7*$^{fl/fl}$ to *Hobit*^{Cre} mice to allow for *Tcf7* deletion specifically in *Hobit*-expressing cells (Figure 8C). We infected *Hobit*^{Cre}*Tcf7*$^{fl/fl}$ and *Hobit*^{Cre} control mice with LCMV Armstrong, and assessed brain and spleen T cells on day 60 post-infection. The splenic CD8+ T cell compartment in *Hobit*^{Cre} *Tcf7*$^{fl/fl}$ mice showed no significant reduction of TCF-1+ cells and was largely unaltered, with similar frequencies and absolute cell numbers of naïve and central/effector memory CD8+ T cells subsets compared to *Hobit*^{Cre} mice (Figure 8E, 8G). In contrast, in the brain, there was a marked decrease in the frequency of TCF-1+ CD8+ T cells in *Hobit*^{Cre} *Tcf7*$^{fl/fl}$ compared to *Hobit*^{Cre} cells (Figure 8F). Thus, *Hobit*^{Cre}*Tcf7*$^{fl/fl}$ allows for the manipulation of TCF-1

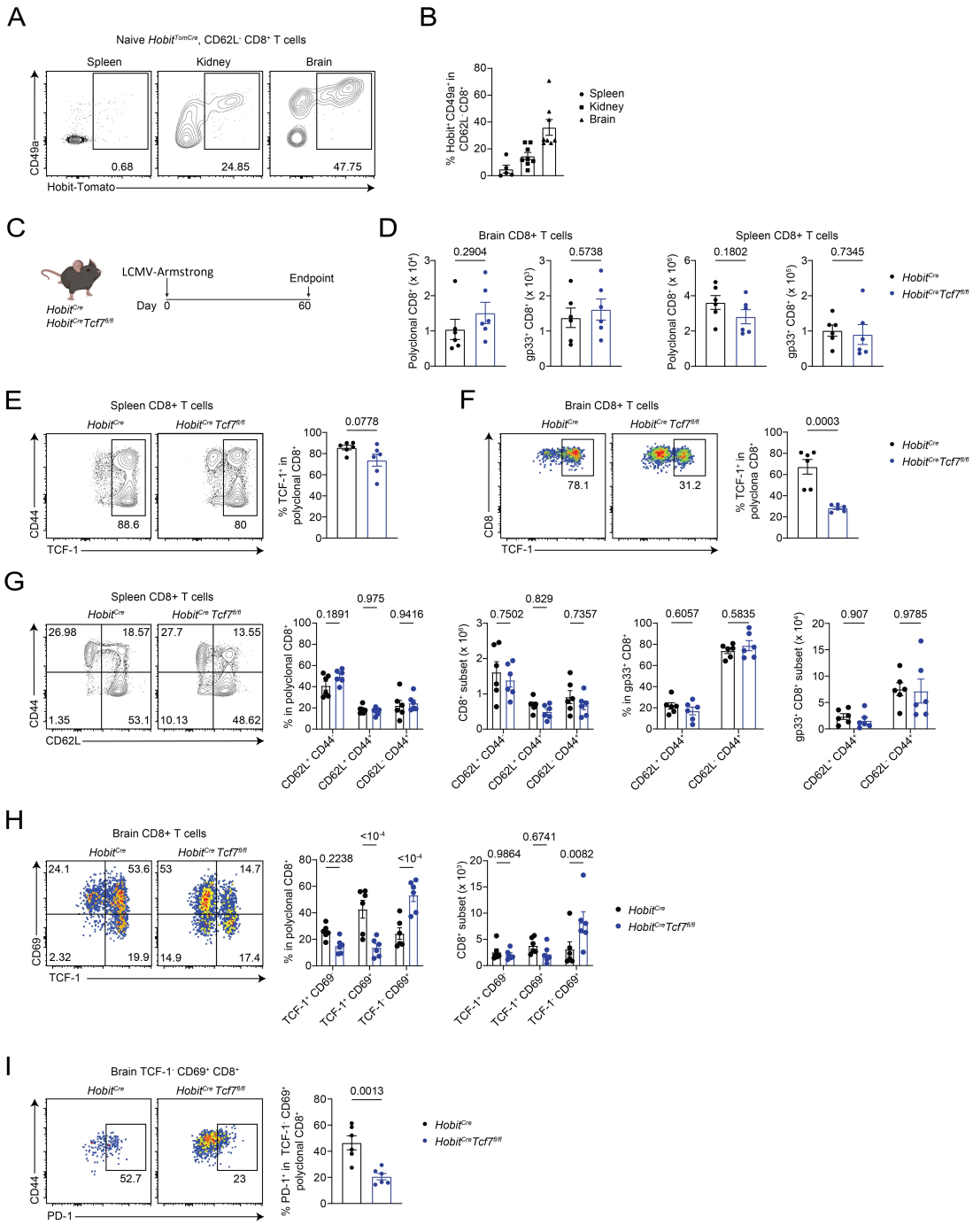

Figure 8 | TCF-1 maintains brain CD8+ T cell homeostasis in a Trm cell-specific manner. **A-B,** Frequency of Hobit+ in splenic CD62L- CD8+ and kidney and brain extravascular CD62L- CD8+ Trm cells in naïve *Hobit*^{Cre} mice, which carries a Cre transgene and tdTomato reporter downstream of *Hobit* promoter (n = 5-8 mice). **C,** Sex- and age-matched *Hobit*^{Cre} *Tcf7*$^{fl/fl}$ and *Hobit*^{Cre} mice were infected with LCMV-Armstrong (2 x 10^5 PFU i.p.) and analyzed 60 days post-infection. **D,** Flow cytometric enumeration of polyclonal and gp33-specific CD8+ T cells in spleen and brain (n = 6 mice per genotype). **E-F,** Frequency of TCF-1+ in spleen CD8+ (**E**) and brain-resident CD8+ (**F**) T cells (n = 6 mice per genotype). **G,** Frequency and numbers of naïve (CD62L+ CD44-), central memory (CD62L+ CD44+), and effector memory (CD62L-

CD44+) CD8+ T cells in spleen (n = 6 mice). **H,** Proportions and numbers of TCF-1+ CD69-, TCF-1+ CD69+, and TCF-1- CD69+ brain CD8+ Trm cells (n = 6 mice). **I,** Percentage of PD-1+ in TCF-1- CD69+ brain CD8+ Trm cells (n = 6 mice). Data are pooled from three (A-B) or two (D-I) independent experiments. i.p., intraperitoneally; LCMV, lymphocytic choriomeningitis virus, PFU, plaque-forming unit.

expression in brain CD8+ T cells while leaving TCF-1 expression intact in splenic CD8+ T cells.

Next, we assessed numbers and phenotype of brain-resident CD8+ T cells. There was no difference in numbers of polyclonal or gp33 antigen-specific CD8+ T cells in the brains of *Hobit*^{Cre}*Tcf7*$^{fl/fl}$ compared to *Hobit*^{Cre} mice (Figure 8D). Yet, TCF-1- CD69+ CD8+ T cells selectively and substantially exhibited an increase in cell numbers in *Hobit*^{Cre}*Tcf7*$^{fl/fl}$ compared to *Hobit*^{Cre} mice (Figure 8H). Moreover, TCF-1- CD69+ CD8+ T cells in *Hobit*^{Cre}*Tcf7*$^{fl/fl}$ mice showed reduced frequencies of PD-1+ cells (Figure 8I), in agreement with the phenotype observed in *Tcf7*$^{fl/fl}$ *Cd8*^{Cre} cells. In sum, these data show that TCF-1 expression in brain CD8+ T cells limits the population size and promotes PD-1 expression in brain-resident CD8+ T cells.

A key feature of Trm cells is the capacity to mount an immediate effector response upon cognate antigen re-encounter. Thus, we asked whether *Hobit*^{Cre}*Tcf7*$^{fl/fl}$ CD8+ T cells would mount an altered recall response in the brain upon antigenic rechallenge. Accordingly, we rechallenged LCMV Armstrong-immune *Hobit*^{Cre}*Tcf7*$^{fl/fl}$ and *Hobit*^{Cre} mice with *Listeria monocytogenes* (*L. monocytogenes*) expressing the LCMV immunodominant epitope gp33 (hereafter referred to as LMgp33; Figure 9A). This experimental setup allowed for assessing the antigen-specific recall response of CD8+ T cells while mitigating the contribution of LCMV-specific neutralizing antibodies to pathogen clearance. We examined the splenic and brain CD8+ T cell response 7 days post-rechallenge at peak effector response to *L. monocytogenes*. Whereas we noted no difference in the magnitude of the CD8+ T cell response in the spleen, *Hobit*^{Cre}*Tcf7*$^{fl/fl}$ mice showed a stronger increase in brain CD8+ T cell numbers compared *Hobit*^{Cre} mice (Figure 9B). Specifically, the increase in brain CD8+ T cell numbers in *Hobit*^{Cre}*Tcf7*$^{fl/fl}$ mice was selectively attributed to the increase of the TCF-1- CD69+ CD8+ T cell subset (Figure 9C).

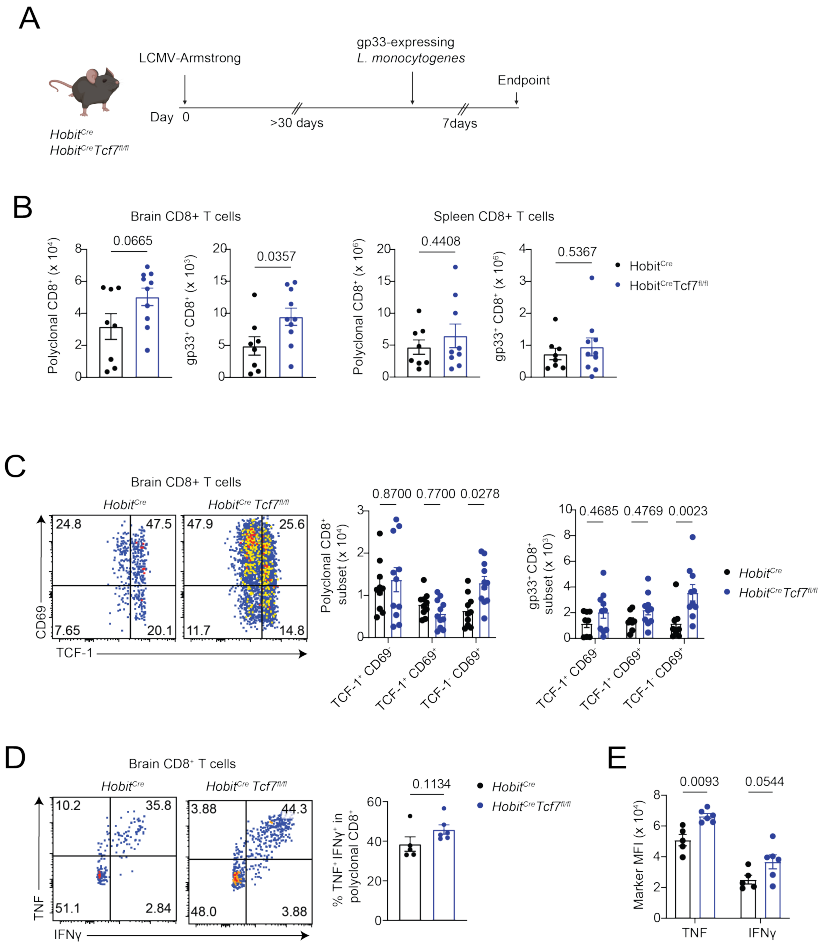

Figure 9 | TCF-1 limits the recall capacity of brain CD8+ Trm cells upon antigenic rechallenge. A, Sex- and age-matched *Hobit*^{Cre} *Tcf7*$^{fl/fl}$ and *Hobit*^{Cre} mice were infected with LCMV-Armstrong (2 x 10^5 PFU i.p.); >30 days post-infection, mice were rechallenged with *Listeria monocytogenes* expressing the LCMV immunodominant epitope gp33-41 (LMgp33; 1 x 10^5 CFU i.v.), and analyzed 7 days post-rechallenge. **B,** Flow cytometric enumeration of polyclonal and gp33-specific CD8+ T cells in spleen and brain (n = 8-10 mice per genotype). **C,** Quantification of polyclonal and gp33-specific TCF-1+ CD69-, TCF-1+ CD69+, and TCF-1- CD69+ subsets of brain CD8+ Trm cells (n = 9-11 mice per genotype). **D,** Frequency of TNF+ IFNγ+ in brain CD8+ Trm cells (n = 5-6 mice per genotype). **E,** TNF and IFNγ MFI in TNF+ IFNγ+ CD8+ brain Trm cells following a 4 hr ex vivo stimulation with gp33 (n = 5-6 mice per genotype). Data are representative (D-E) or pooled (B-C) from 2-3 independent experiments. CFU, colony-forming unit; i.p., intraperitoneally; i.v., intravenously; IFNγ; interferon gamma; LCMV, lymphocytic choriomeningitis virus; MFI, median fluorescence intensity; PFU, plaque-forming unit; TNF, tumor necrosis factor.

To further examine the impact of TCF-1 deletion on the recall response of brain CD8+ Trm cells, we restimulated CD8+ T cells isolated from the brains of *Hobit*^{Cre}*Tcf7*$^{fl/fl}$ and *Hobit*^{Cre} mice with the cognate peptide gp33 ex vivo. Whereas no significant difference was observed in the frequency of polyfunctional TNF+ IFNγ+ CD8+ T cells (Figure 9D),

Hobit^{Cre}*Tcf7*$^{fl/fl}$ cells produced larger amounts of TNF compared to *Hobit*^{Cre} cells (Figure 9E). This supports the notion that TCF-1 acts a negative regulator of brain CD8+ Trm cell capacity to expand upon antigen-specific recall response.

Collectively, these findings demonstrate that TCF-1 expression in brain CD8+ Trm cells limits the population size at steady state and upon antigenic rechallenge.

4.2.4. PD-1 signaling safeguards the differentiation of brain CD8+ T cells

PD-1 signaling has been implicated in brain CD8+ Trm formation in the context of localized brain infection, a setting that favors the development of CD103+ CD8+ Trm cells (Shwetank et al. 2019, Scholler et al. 2020, Prasad et al. 2017, Phares et al. 2009). These studies reported contrasting results on whether PD-1 promotes or hinders the development of CD103+ Trm cells in the brain, albeit different pathogens were used to elicit brain Trm cell formation. Accordingly, we aimed to shed further light on the question of how PD-1 signaling controls the differentiation and maintenance of brain CD8+ Trm cells. In particular, we addressed three key questions left unanswered by previous studies: a) how PD-1 regulates brain-resident CD8+ T cells in the context of a systemic, rather than localized, acute infection; b) how PD-1 signaling shapes the different subsets of brain CD8+ T cells identified in this study (e.g., TCF-1+ CD69+ cells); and c) whether PD-1 modulates brain CD8+ T cells in a cell-intrinsic manner, since past studies employed infection models of mice with constitutive PD-1 or PD-L1 deficiency, which may impact the differentiation of additional T cell subsets such as Treg cells (Tan et al. 2021). Finally, given the impaired PD-1 expression in brain CD8+ Trm cells in the absence of TCF-1 (Figure 6E and Figure 7I), we aimed to disentangle the contribution of a primary PD-1 deficiency to the observed phenotype upon conditional *Tcf7* deletion.

To this end, we generated mixed bone-marrow chimeras of *Pdcd1* KO and congenically marked WT cells at a ratio of 1:1, followed by LCMV Armstrong infection. Sampling mouse blood one day before infection, *Pdcd1* KO CD8+ T cells comprised a slight majority in the circulation compared to WT cells (data not shown); such increased frequency of PD-1 KO cells at baseline was adjusted for in subsequent analyses involving infection of chimeric mice.

We first examined the relative expansion of PD-1 KO and PD-1-sufficient CD8+ T cells at the peak of effector response (Figure 10A). Consistent with a previous report (Pauken et al. 2020), PD-1-deficient virus-specific CD8+ T cells expanded to a substantially greater extent compared to WT cells in the spleen on day 8 post-infection (Figure 10B). Similarly, antigen-specific effector CD8+ T cells infiltrating the brain consisted of a ~3-fold greater percentage of PD-1 KO compared to WT cells (Figure 10B). Conversely, PD-1 deficiency did not alter the representation of KLRG1- TCF-1+ memory precursor cells in the spleen or brain (Figure 10C). Thus, PD-1 signaling limits

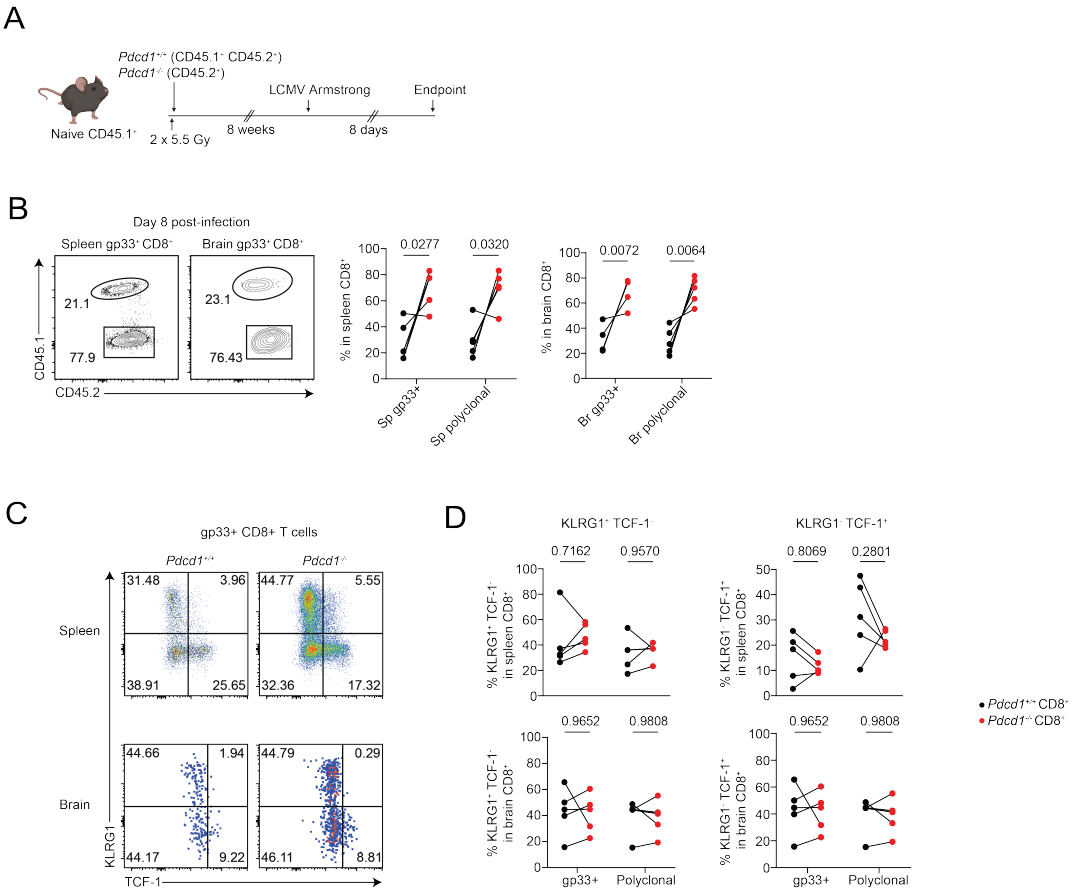

Figure 10 | PD-1 signaling restricts the expansion of effector CD8+ T cells in the brain and lymphoid tissue. A, Ly5.1 (CD45.1) recipient mice were irradiated followed by bone-marrow reconstitution using hematopoietic stem cells (HSCs) from CD45.1+ CD45.2+ and *Pdcd1* KO CD45.2 mice; a total of 4 million HSCs, mixed at a 1:1 ratio from each donor mouse, were administered i.v. to irradiated CD45.1 mice; chimeric mice were infected with LCMV-Armstrong (2 x 10^5 PFU i.p.) and analyzed on day 8 post-infection. **B,** Representation of WT and *Pdcd1* KO in CD8+ T cells, polyclonal and gp33-specific, in spleen and brain of chimera mice (n = 5 mice). **C-D,** Frequencies of KLRG1+ TCF-1- and KLRG1- TCF-1+ in polyclonal and gp33-specific CD8+ T cells in spleen and brain (n = 5 mice). Data are representative of one experiment. i.p., intraperitoneally; i.v., intravenously; KO, knockout; LCMV, lymphocytic choriomeningitis virus, PFU, plaque-forming unit; WT, wild type.

the expansion of effector CD8+ T cells but does not appear to modulate their propensity to give rise to terminal effector or memory precursor cells.

To examine whether this augmented effector response of PD-1 KO cells eventually translated into an altered T cell memory, we employed the same experimental strategy of *Pdcd1* KO and WT mixed bone-marrow chimeras and assessed the chimera mice on day 45-47 post-infection (Figure 11A). Polyclonal CD8+ T cells lacking PD-1 signaling outnumbered PD-1-sufficient cells both in the spleen and brain (Figure 11B).

Notably, the fold-change of KO over WT CD8+ T cells in the brain was superior to that in the spleen (Figure 11B), indicating that PD-1 signaling restrains memory CD8+ T cell formation in the brain. In spite of the increase of *Pdcd1* KO polyclonal CD8+ T cells, the frequency of gp33-specific CD8+ T cells was decreased among PD-1-deficient CD8+ T cells compared to their WT counterparts in the brain but not in the spleen (Figure 11B, right). This indicates that PD-1 signaling contributes to the formation of antigen-specific memory CD8+ T cells systemically and in the brain.

We next aimed to understand how PD-1 deficiency shaped the differentiation of CD8+ T cell subsets systemically and in the brain during the memory phase following acute infection. In the spleen, PD-1 KO polyclonal CD8+ T cells showed a greater proportion of CD62L- CD44+ effector memory cells and a reciprocal reduction in CD62L+ CD44- naïve cells, compared to WT cells (Figure 11C). Conversely, no difference was observed in the frequencies of effector memory and CD62L+ CD44+ central memory CD8+ T cell subsets among antigen-specific CD8+ T cells in the spleen (data not shown). In the brain, PD-1-deficient CD8+ T cells comprised fewer TCF-1+ cells, with concomitant increase in TCF-1- CD69+ cells, compared to WT CD8+ T cells (Figure 11D). Of note, the magnitude of reduction of TCF-1+ CD8+ T cells was not reflected among splenic CD8+ T cells (Figure 11E). As another readout for the differentiation of brain Trm cells, we examined their expression of CD49a, an α1 integrin that serves as a collagen receptor and that is expressed by Trm cells in various tissues (Gebhardt and Mackay 2012, Zhang and Bevan 2013, Cheuk et al. 2017, Reilly et al. 2020), including the brain (Urban et al. 2020). Brain CD8+ T cells lacking PD-1 signaling consisted of a smaller fraction of CD49a+ cells relative to their WT counterparts (Figure 11F). In the absence of PD-1 signaling, brain CD8+ Trm cells also exhibited a reduced fraction of granzyme B-producing cells relative to WT cells (Figure 11G). Notably, no difference in the frequencies of granzyme B+ cells was observed in the spleen (Figure 11G). Taken together, these data demonstrate that PD-1 signaling limits the number of CD8+ Trm cells and safeguards their differentiation.

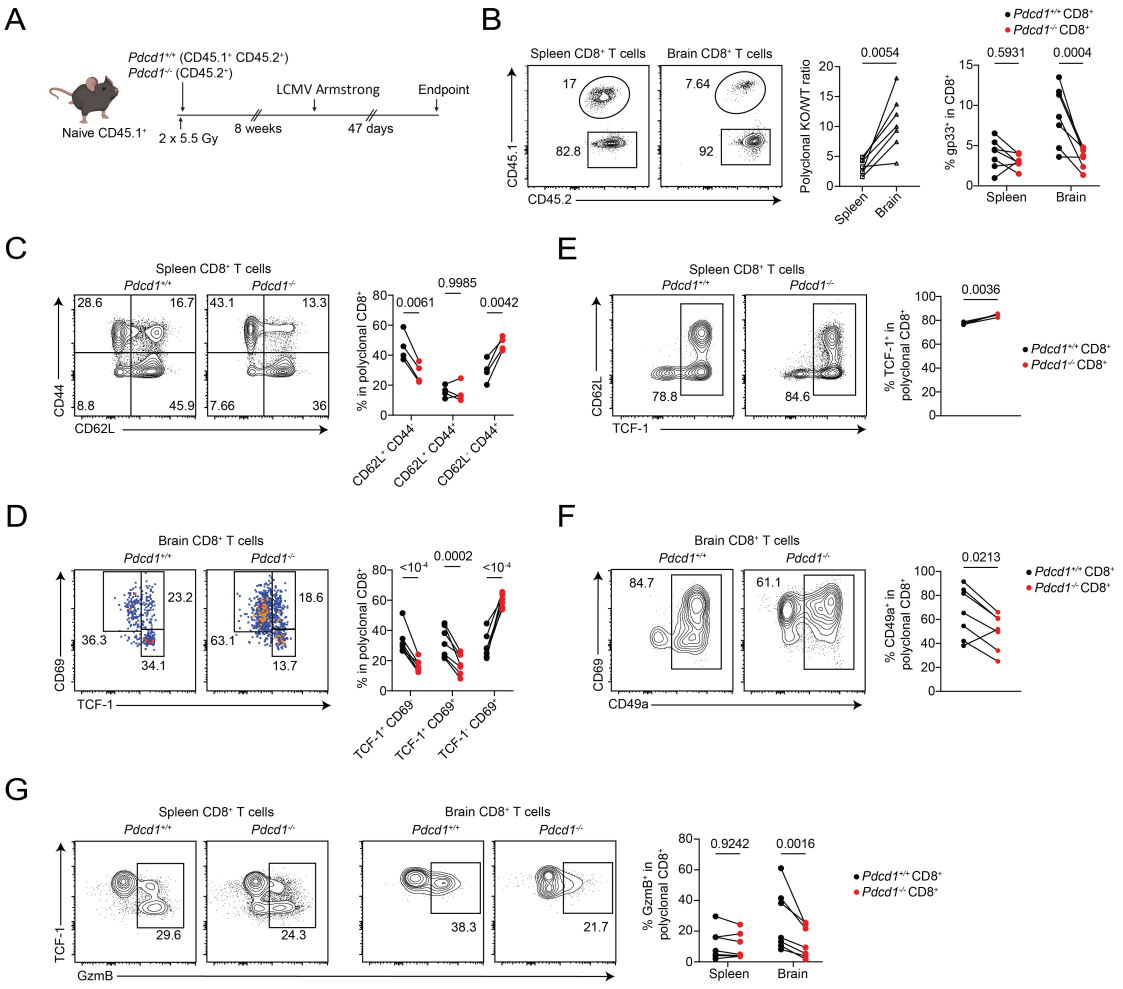

Figure 11 | PD-1 signaling is important for the maintenance of brain CD8+ Trm cells. A, Ly5.1 (CD45.1) recipient mice were irradiated followed by bone-marrow reconstitution using hematopoietic stem cells (HSCs) from CD45.1+ CD45.2+ and *Pdcd1* KO CD45.2 mice; a total of 4 million HSCs, mixed at a 1:1 ratio from each donor mouse, were administered i.v. to irradiated CD45.1 mice; chimeric mice were infected with LCMV-Armstrong (2 x 10^5 PFU i.p.) and analyzed on day 47 post-infection. **B,** Ratio of *Pdcd1* KO to WT CD8+ T cells in spleen and brain (left); frequencies of gp33-specific CD8+ T cells per genotype in spleen and brain (right; n = 7 mice). **C,** Frequencies of naïve (CD62L+ CD44-), central memory (CD62L+ CD44+), and effector memory (CD62L- CD44+) CD8+ T cells in spleen (n = 7 mice). **D,** Proportions of TCF-1+ CD69-, TCF-1+ CD69+, and TCF-1- CD69+ subsets in brain CD8+ T cells (n = 7 mice). **E,** Frequency of TCF-1+ in spleen CD8+ T cells (n = 3 mice). **F,** Frequency of CD49a+ in brain CD8+ Trm cells (n = 7 mice). **F,** Proportion of GzmB+ in spleen and brain CD8+ T cells (n = 7 mice). Data are representative (E) or pooled (B-C; D-G) of two independent experiments. GzmB, granzyme B; i.p., intraperitoneally; KO, knockout; LCMV, lymphocytic choriomeningitis virus; PFU, plaque-forming unit; WT, wild type.

4.2.5. PD-1 is important for optimal memory recall of brain-resident CD8+ T cells

Next, we asked how PD-1 impacted the recall response of brain Trm cells upon cognate antigen re-encounter. To answer this question, we again employed LCMV Armstrong infection of PD-1 KO mixed bone marrow chimeras, followed by anti-Gr1 treatment at the memory phase to deplete circulating memory CD8+ T cells (Matsuzaki et al. 2003), and then rechallenged the mice one week later with L. *monocytogenes* (LM) expressing gp33 (LMgp33) (Figure 12A). Given that LM readily infects the brain (Cassidy et al. 2020, Ghosh and Higgins 2018), and coupled with the depletion of circulating memory via anti-Gr1 treatment, this experimental setup allows for examining Trm cell response to antigenic rechallenge with minimal to no contribution from circulating memory T cells (Evrard et al. 2023). Mice were sacrificed one week post-rechallenge, a time-point corresponding to peak effector response to LM (Porter and Harty 2006, Toumi et al. 2022).

Similar to the memory phase post-LCMV Armstrong infection, PD-1 KO cells dominated the CD8+ T cell pool upon LMgp33 rechallenge relative to WT cells, both in spleen and brain (Figure 11B). However, whereas the ratio of KO to WT CD8+ T cells in the spleen remained largely the same in memory and rechallenged mice, it dropped by 59% in the brain upon rechallenge compared to memory mice (Figure 11B). Indeed, PD-1-KO and WT gp33-specific CD8+ T cells in the spleen expanded similarly upon LMgp33 rechallenge (Figure 12C), whereas WT gp33-specific CD8+ T cells in the brain expanded much better than PD-1 KO cells (Figure 12D).

We next assessed the expression of granzyme B, TNF and IFNγ during the recall of memory cells. In contrast to the memory phase, no difference in granzyme B expression was observed between PD-1 deficient and PD-1 sufficient antigen-specific CD8+ T cells upon LMgp33 rechallenge, in spleen or brain (Figure 12E). Conversely, brain-resident CD8+ T cells showed a profound loss of capacity to co-produce TNF and IFNγ relative to their WT counterparts (Figure 12F). Although splenic CD8+ T cells lacking PD-1 signaling showed a small, non-significant reduction in TNF/IFNγ coproduction, the magnitude of the impairment of cytokine production upon rechallenge was more pronounced among PD-1 deficient brain CD8+ Trm cells (Figure 12F, right). Collectively, these data demonstrate that PD-1 is required for optimal recall response of brain CD8+ Trm cells upon antigenic rechallenge.

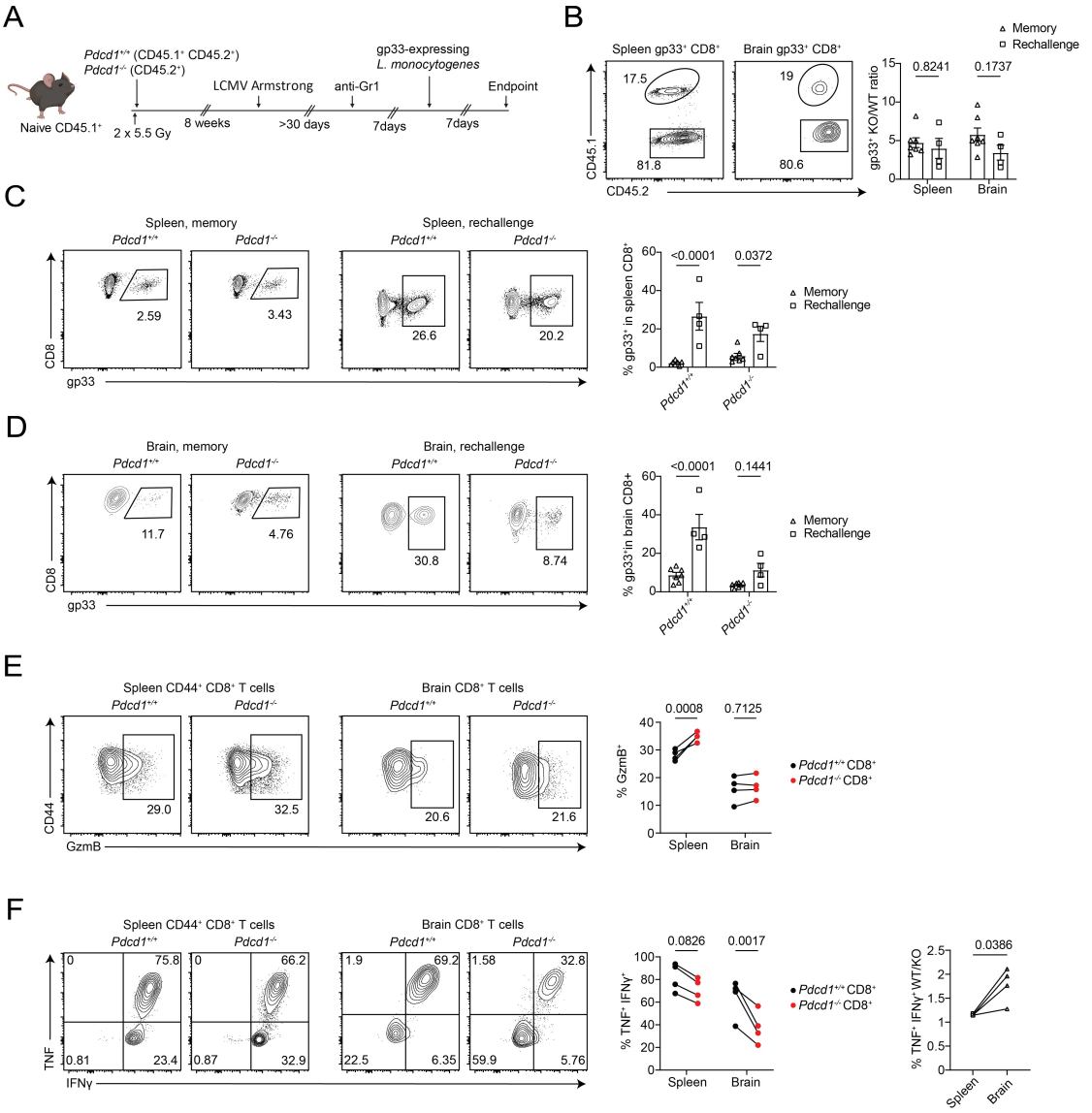

Figure 12 | PD-1 is important for optimal recall response by brain CD8+ Trm cells. **A,** Ly5.1 (CD45.1) recipient mice were irradiated followed by bone-marrow reconstitution using hematopoietic stem cells (HSCs) from CD45.1+ CD45.2+ and *Pdcd1* KO CD45.2 mice; a total of 4 million HSCs, mixed at a 1:1 ratio from each donor mouse, were administered i.v. to irradiated CD45.1 mice; chimeric mice were infected with LCMV-Armstrong (2 x 10^5 PFU i.p.); >30 days later, mice received 200 µg of anti-Gr1 i.p., and one week later mice were rechallenged with 1 x 10^5 CFU of LMgp33 i.v., and sacrificed 7 days post-rechallenge. **b-c,** frequency of gp33-specific cells among WT and *Pdcd1* KO CD8+ T cells during memory and following rechallenge in spleen (b) and brain (c); n = 4-7 mice. **d,** frequency of TNF+ IFNγ+ in spleen and brain CD8+ T cells (left); and ratio of the TNF+ IFNγ+ frequency in WT over *Pdcd1* KO cells in spleen and brain (right); n = 4 mice, following a 4 hr ex vivo stimulation with gp33. Data are representative of one out of two independent experiments. GzmB, granzyme B; i.p., intraperitoneally; i.v., intravenously; KO, knockout; LCMV, lymphocytic choriomeningitis virus; LMgp33, Listeria monocytogenes expressing gp33-41; PFU, plaque-forming unit; WT, wild type.

4.2.6. PD-1 maintains the homeostasis and function of brain memory CD8+ T cells in a Trm cell-specific manner

The data presented above demonstrate a role for PD-1 signaling in regulating brain CD8+ T cell differentiation and function in a CD8+ T cell-intrinsic manner. The establishment and maintenance phases of Trm cell formation, however, may not necessarily rely on the same molecular mechanisms. To directly test if PD-1 signaling controls the maintenance of brain CD8+ T cells, we crossed *Hobit*^{Cre} mice to *Pdcd1*$^{fl/fl}$ to generate mice with Trm cell-specific deletion of PD-1. Given that Hobit expression in non-lymphoid tissue marks CD8+ T cells acquiring tissue residency, we reasoned that PD-1 deletion downstream of Hobit expression would allow for the assessment of the role of PD-1 in the maintenance of brain CD8+ Trm cells.

Using the same experimental setup of systemic acute viral infection, we infected *Hobit*^{Cre} *Pdcd1*$^{fl/fl}$ and *Hobit*^{Cre} control mice and sacrificed them 40 days post-infection (Figure 13A). Confirming the efficiency of *Pdcd1* deletion downstream of Hobit, we noted a 75% reduction in the fraction of PD-1-expressing brain CD8+ T cells in *Hobit*^{Cre} *Pdcd1*$^{fl/fl}$ compared to *Hobit*^{Cre} mice, while PD-1 expression in the spleen was unimpaired (data not shown) as were frequencies and numbers of naïve, central memory or effector memory (polyclonal or gp33-specific) CD8+ T cells (Figure 13C). Notably, we also found no significant differences in numbers of total or gp33 antigen-specific CD8+ T cells in the brains of *Hobit*^{Cre}*Pdcd1*$^{fl/fl}$ and *Hobit*^{Cre} control mice (Figure 13B). *Hobit*^{Cre}-mediated deletion of PD-1, however, resulted in an altered brain CD8+ T cell composition, with an increased frequency and absolute number of the TCF-1-CD69+ subset (Figure 13D). This finding was consistent with the increase in TCF-1-CD69+ T cells among CD8+ cells with a constitutive deletion of PD-1.

To further investigate the impact of PD-1 deletion downstream of Hobit on brain CD8+ T cells, we examined their expression of the residency marker CD49a. In agreement with the PD-1 KO mixed bone marrow chimera model, we observed a reduced fraction of CD49a-expessing cells among *Hobit*^{Cre} *Pdcd1*$^{fl/fl}$ brain CD8+ Trm cells, although this difference did not reach statistical significance (Figure 13E). Furthermore, brain CD8+ Trm cells lacking PD-1 exhibited a reduced proportion of granzyme B expressing cells (Figure 13F), consistent with what was observed in brain CD8+ T cells with *Pdcd1*

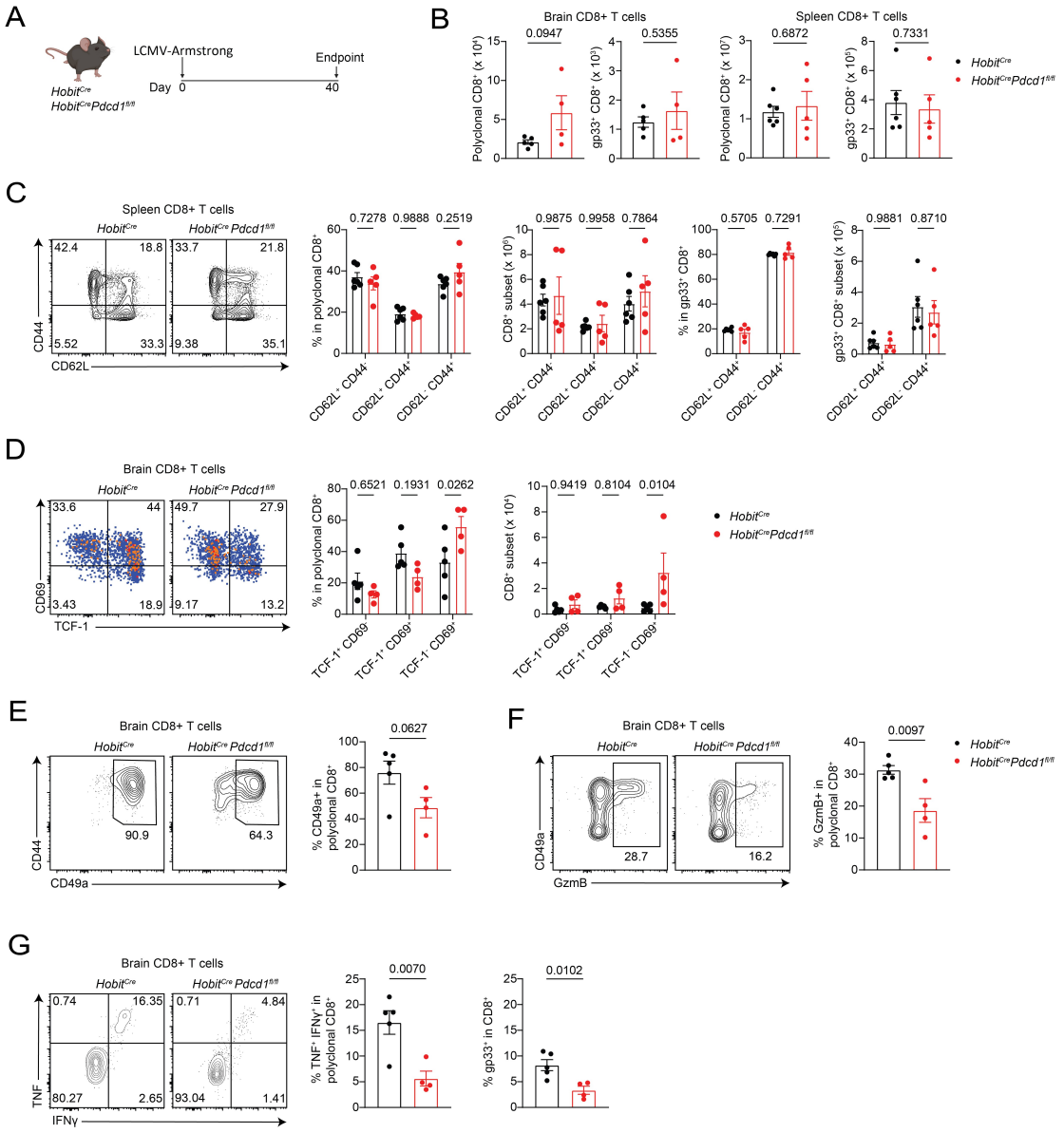

Figure 13 | PD-1 maintains brain CD8+ T cell phenotype and effector function in a Trm cell-specific manner. A, Sex- and age-matched *Hobit*^{Cre} *Pdcd1*$^{fl/fl}$ and *Hobit*^{Cre} mice were infected with LCMV-Armstrong (2 x 10^5 PFU i.p.) and analyzed 40 days post-infection. **B,** Flow cytometric enumeration of polyclonal and gp33-specific CD8+ T cells in spleen and brain (n = 4-6 mice per genotype). **C,** Frequencies and numbers of naïve (CD62L+ CD44-), central memory (CD62L+ CD44+), and effector memory (CD62L- CD44+) CD8+ T cells in spleen (n = 4-6 mice). **D,** Proportions and numbers of TCF-1+ CD69-, TCF-1+ CD69+, and TCF-1- CD69+ brain CD8+ Trm cells (n = 4-5 mice). **E,** Proportion of CD49a+ among brain CD8+ Trm cells (n = 4-5 mice per genotype). **F,** Frequency of GzmB+ among brain-resident CD8+ T cells (n = 4-5 mice). **G,** Percentage of TNF+ IFNγ+ in brain CD8+ Trm cells (left) and frequency of gp33-specfici cells (right; n = 4-5 mice) following a 4 hr ex vivo stimulation with gp33. Data are pooled from two independent experiments. GzmB, granzyme B; i.p.,

intraperitoneally; IFNγ, interferon gamma; LCMV, lymphocytic choriomeningitis virus, PFU, plaque-forming unit; TNF, tumor necrosis factor.

germline deletion. Finally, we assessed cytokine production through the ex vivo stimulation of CD8+ T cells from the brains of LCMV immune mice with the cognate antigen gp33. Brain CD8+ T cells with conditional deletion of PD-1 exhibited a reduced capacity to co-produce TNF and IFNγ following ex vivo stimulation (Figure 13G). This was in line with a reduced frequency of gp33-specific brain CD8+ T cells in *Hobit*^{Cre} *Pdcd1*$^{fl/fl}$ mice compared to *Hobit*^{Cre} mice (Figure 13G), albeit the magnitude of decrease in TNF IFNγ co-production was larger. Collectively, these findings suggest that PD-1 contributes in a Trm cell-specific manner to the maintenance of brain CD8+ cell homeostasis, and their acquisition of the capacity for immediate effector response.

We next assessed the capacity of CD8+ Trm cells lacking PD-1 signaling to mount an effective recall response. We administered anti-Gr1 to LCMV-immune *Hobit*^{Cre} *Pdcd1*$^{fl/fl}$ and *Hobit*^{Cre} to deplete circulating memory CD8+ T cells, followed by heterologous rechallenge using LMgp33 (Figure 14A). Brain CD8+ T cells with conditional deletion of PD-1 exhibited a marked reduction in their capacity to expand upon antigenic rechallenge (Figure 14B), a phenomenon that was not observed in polyclonal CD8+ T cells. This was largely in agreement with the phenotype of brain CD8+ T cells with constitutive *Pdcd1* deficiency upon LMgp33 rechallenge.

To further evaluate the functional alterations of brain CD8+ T cells in *Hobit*^{Cre} *Pdcd1*$^{fl/fl}$ mice, we examined their differentiation after rechallenge. We observed no difference in the frequencies of KLRG1+ CX3CR1+ or total KLRG1+ among responding gp33-specific CD8+ T cells in the brains of *Hobit*^{Cre} *Pdcd1*$^{fl/fl}$ mice compared *Hobit*^{Cre} mice (Figure 14C). We then asked whether brain Trm cells lacking PD-1 expression would display a reduced capacity to produce cytokines following rechallenge. CD8+ T cells isolated from the brains *Hobit*^{Cre} *Pdcd1*$^{fl/fl}$ mice–and restimulated in vitro with gp33 peptide for 4 hours–displayed a dramatic reduction in their ability to produce TNF and IFNγ compared *Hobit*^{Cre} CD8+ T cells (Figure 14D). Collectively, these data provide evidence that PD-1 promotes the capacity of brain CD8+ Trm cells to mount a robust recall response in a Trm cell-specific manner.

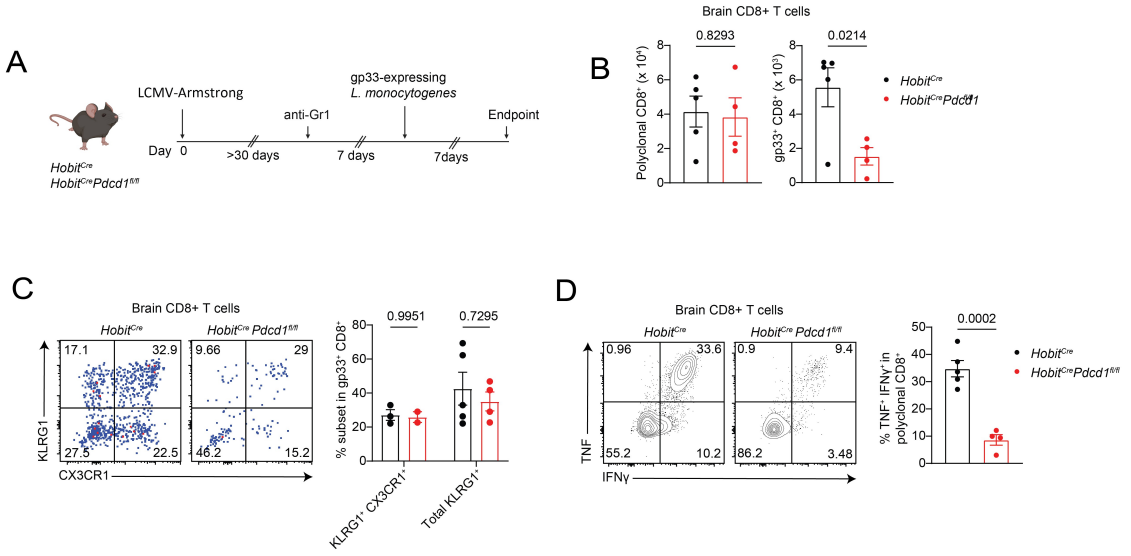

Figure 14 | PD-1 is required for brain CD8+ Trm cell expansion and effector function upon antigenic rechallenge. **A,** Sex- and age-matched *Hobit*^{Cre} *Pdcd1*$^{fl/fl}$ and *Hobit*^{Cre} mice were infected with LCMV-Armstrong (2 x 10^5 PFU i.p.); >30 days later, mice received 200 µg of anti-Gr1 i.p., and one week later mice were rechallenged with 1 x 10^5 CFU of LMgp33 i.v., and sacrificed 7 days post-rechallenge. **B,** Flow cytometric quantification of polyclonal and gp33-specific CD8+ T cells in spleen and brain (n = 4-5 mice per genotype). **C,** Frequency of CX3CR1+ KLRG1+ and total KLRG1+ in brain CD8+ T cells (n = 4-5 mice per genotype). **D,** Percentage of TNF+ IFNγ+ in brain CD8+ Trm cells (n = 4-5 mice) following a 4 hr ex vivo stimulation with gp33. Data are pooled from two independent experiments. CFU, colony-forming unit; i.p., intraperitoneally; i.v., intravenously; IFNγ; interferon gamma; LCMV, lymphocytic choriomeningitis virus; PFU, plaque-forming unit; TNF, tumor necrosis factor.

In summary, PD-1 signaling is important for the differentiation of CD8+ Trm cells with optimal phenotypic and functional profile, as well as for their secondary expansion and effector function upon antigen re-encounter.

4.2.7. TGF-β promotes the formation of brain-resident CD8+ T cells

TGF-β shapes the T cell landscape at various stages of a T cell's lifetime, from thymic development, during the naïve state and during effector and memory T cell generation (Oh and Li 2013). Numerous studies have shown that TGF-β is necessary for the establishment and/or maintenance of Trm cells in the skin (Christo et al. 2021, Mackay et al. 2013, Hirai et al. 2021), small intestine (Casey et al. 2012, Crowl et al. 2022, Zhang and Bevan 2013), as well as the kidneys (Ma et al. 2017) and lungs (Wang et al. 2019, Lee et al. 2011). Whether brain Trm cells depends on TGF-β signaling for their differentiation remains unclear

To begin to investigate a potential role for TGF-β in the regulation of brain CD8+ T cells, we first confirmed TGF-βRII expression on brain CD8+ T cells using our scRNA-seq dataset (Figure 15). In addition, we made use of a transcriptional signature of TGF-β signaling generated following stimulation of CD8+ T cells with TGF-β in vitro (Nath et al. 2019). We computed an enrichment score of the average expression of this gene-set (using Seurat's AddModuleScore function) and plotted it onto the UMAP of our steady-state scRNA-seq dataset. Interestingly, TGF-β signaling appeared to be enriched in CD8 clusters 1, 2 and 3, whereas Tcm-like T cells displayed a smaller score of TGF-β signaling (Figure 15). As alluded to above (Figure 1), we noted that the majority of brain CD8+ T cells expressed CD49a, a T cell residency-associated α1 integrin whose expression is regulated by TGF-β, in addition to IL-12 (Bromley et al. 2020).

Next, to address whether TGF-β controls brain CD8+ T cells in a cell-intrinsic fashion, we crossed *Cd8*^{Cre} mice to *Tgfbr2*$^{fl/fl}$ mice to generate *Tgfbr2*$^{fl/fl}$ *Cd8*^{Cre} mice, resulting in the CD8+ T cell-specific deletion of TGF-βRII. We infected *Tgfbr2*$^{fl/fl}$ *Cd8*^{Cre} mice and *Tgfbr2*$^{fl/fl}$ control mice with LCMV Armstrong, and assessed brain and splenic T cells on day 60 post-infection (Figure 16A). In the spleen, we observed no significant differences in the numbers of antigen-specific or polyclonal TGF-βRII deficient CD8+ T cells compared to TGF-βRII sufficient CD8+ T cells (Figure 16B), in agreement with previous studies (Ma and Zhang 2015, Zhang and Bevan 2013). Instead, the composition of such memory T cells was altered, with increased frequencies and numbers of CD44+ CD62L- memory but not central memory CD8+ T cells (Figure 16C). This was coupled to reduced frequencies and numbers of naïve CD62L+ CD44- T cells (Figure 16C),

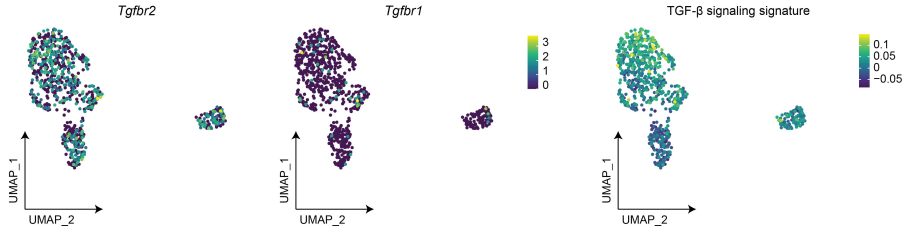

Figure 15 | TGF-βRII expression and TGF-β signaling in brain Trm cells. scRNA-seq of brain T cells, related to Figure 1. FeaturePlots depicting the expression of *Tgfbr2*, *Tgfbr1*, and gene-expression score of TGF-β signaling, derived from Nath et al., 2019.

which is consistent with the reported role of TGF-β in maintaining the quiescence of naïve CD8+ T cells (Filippi et al. 2008, Tu et al. 2018).

Despite the systemic increase in numbers of effector memory CD8+ T cells in the absence of intrinsic TGF-β signaling, polyclonal and gp33-specific CD8+ Trm cells were significantly decreased in the brains of *Tgfbr2*$^{fl/fl}$ *Cd8*^{Cre} mice (Figure 16B). This suggests that TGF-β signaling contributes to the acquisition and/or maintenance of tissue residency in the brain. Phenotypically, brain CD8+ T cells exhibited a reduced frequency of CD49a, as expected, whereas the frequency of total CD69+ CD8+ T cells remained unchanged (Figure 16D). Consistent with the reduction in total CD8+ T cell numbers, both CD49a+ and CD69+ CD8+ T cells were numerically decreased (Figure 16D). On the other hand, the lack of TGF-β signaling did not influence the frequencies of TCF-1+ CD69+ and TCF-1- CD69+ subsets among brain CD8+ T cells, although there was a trend toward a reduction of both subsets, as well as of TCF-1+ CD69- cells, in *Tgfbr2*$^{fl/fl}$ *Cd8*^{Cre} mice (Figure 16E).

Further investigating the impact of loss of TGF-β signaling on brain CD8+ T cells, we found a modest increase in frequency of KLRG1+ cells among TGF-βRII-deficient cells compared to *Tgfbr2*$^{fl/fl}$ CD8+ T cells (Figure 16F). In addition, brain CD8+ T cells in *Tgfbr2*$^{fl/fl}$ *Cd8*^{Cre} mice exhibited an increased frequency of ganzyme B+ cells (Figure 16G). These two findings point to a skewed differentiation of a subset of brain CD8+ T cells towards an effector-like phenotype in the absence of TGF-β signaling. Conversely, we noted no difference in the frequency of TNF+ IFNγ co-producing brain CD8+ Trm cells in *Tgfbr2*$^{fl/fl}$ *Cd8*^{Cre} compared to *Tgfbr2*$^{fl/fl}$ mice (Figure 16H).

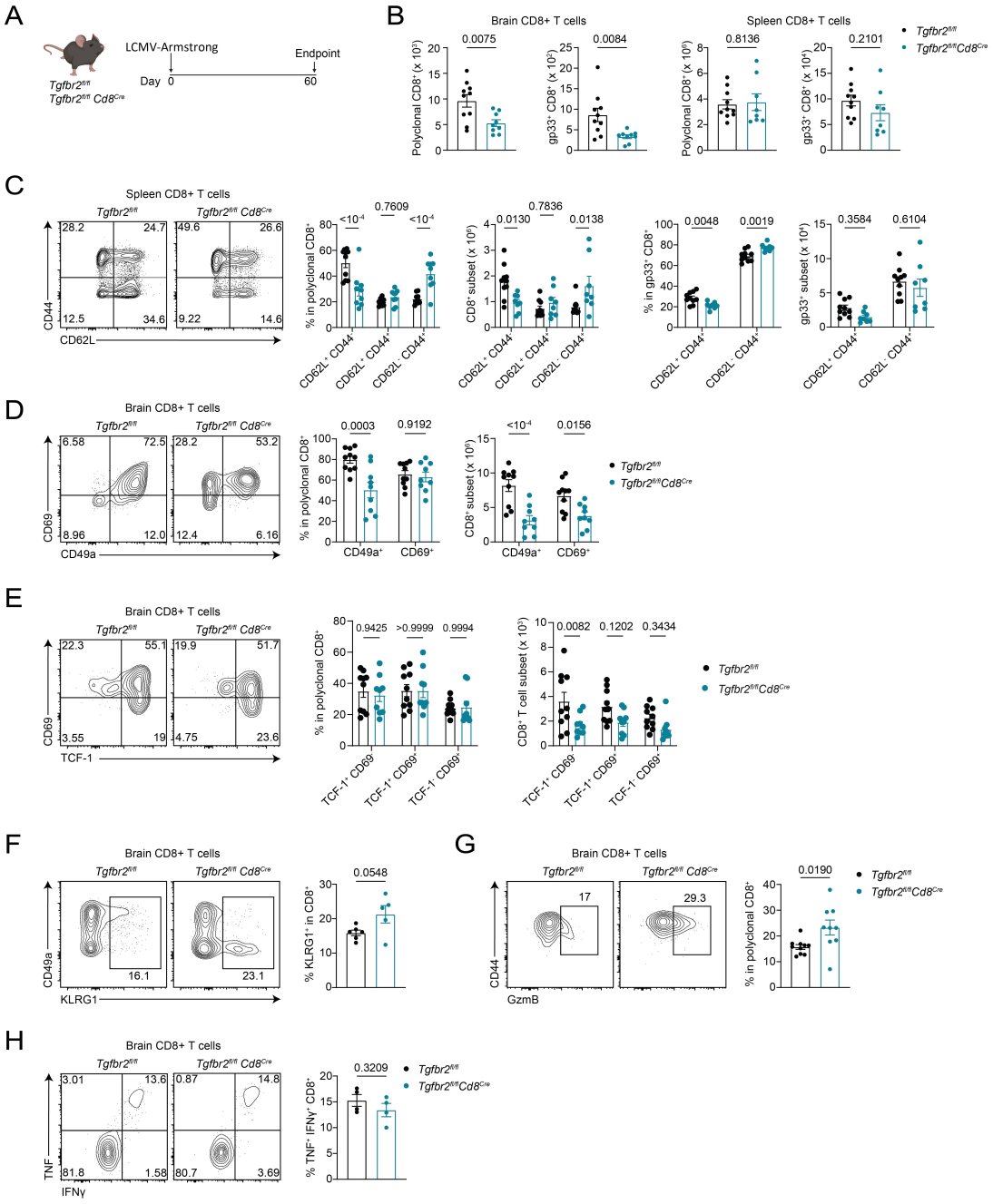

Figure 16 | TGF-β signaling is important for the formation of brain-resident CD8+ T cells. A, Sex- and age-matched *Cd8*^{Cre} *Tgfbr2*$^{f/fl}$ and *Tgfbr2*$^{f/fl}$ control mice were infected with LCMV-Armstrong (2 x 10^5 PFU i.p.) and sacrificed on day 60 post-infection. **B,** Flow cytometric quantification of polyclonal and gp33-specific CD8+ T cells in the spleen and brain (n = 9-10 mice per group). **C,** Frequencies and numbers of naïve (CD62L+ CD44-), central memory (CD62L+ CD44+), and effector memory (CD62L- CD44+) CD8+ T cells in spleen (n = 8-10 mice per group). **D,** Percentages and numbers of CD49a+ and CD69+ subsets of brain CD8+ Trm cells (n = 9-10 mice). **E,** Frequencies and numbers of TCF-1+ CD69-, TCF-1+ CD69+, and TCF-1- CD69+ subsets of brain CD8+ Trm cells (n = 9-10 mice per genotype). **F,** Frequency of

KLRG1+ in brain CD8+ Trm cells (n = 5-6 mice per group). **G,** Proportion of GzmB+ in brain CD8+ Trm cells (n = 9-10 mice). **H,** Frequency of TNF+ IFNγ+ in brain CD8+ T cells following a 4 hr ex vivo stimulation with gp33 (n = 4 per genotype). Data are representative (F, H) or pooled (B-E, G) from two independent experiments. GzmB, granzyme B; i.p., intraperitoneally; IFNγ, interferon gamma; LCMV, lymphocytic choriomeningitis virus, PFU, plaque-forming unit; TNF, tumor necrosis factor.

Taken together, these data demonstrate that cell-intrinsic TGF-β signaling shapes the formation and proper differentiation of brain-resident CD8+ T cells.

4.2.8. TGF-β contributes to the establishment phase of brain-resident CD8+ T cells

TGFβ is required for both the establishment and maintenance phases of CD8+ Trm cell formation in the skin (Mackay et al. 2013, Hirai et al. 2021) and small intestine (Casey et al. 2012, Zhang and Bevan 2013, Crowl et al. 2022). The data presented in the previous section does not disentangle whether the contribution of TGF-β signaling to brain Trm cell formation involves Trm cell differentiation, maintenance, or both.

Trm cell precursors are known to seed the non-lymphoid tissue of residence during the effector phase of infection, and progressively mature into the Trm cell phenotype in situ (Kok et al. 2022, Mackay et al. 2013). To address the role of TGF-β in the establishment phase of brain CD8+ Trm cells, we again made use of *Tgfbr2*$^{fl/fl}$ *Cd8*^{Cre} and *Tgfbr2*$^{fl/fl}$ mice. Following LCMV Armstrong infection, we assessed the splenic and brain CD8+ T cell compartments at the peak of the CD8+ T cell response on day 8 (Figure 17A; (Butz and Bevan 1998, Buchmeier et al. 1980)). Consistent with previous studies (Ma and Zhang 2015, Zhang and Bevan 2013), the expansion of gp33 antigen-specific CD8+ T cells in the spleen was impaired in the absence of TGF-β signaling (Figure 17B). Conversely, no difference in the expansion of gp33-specific CD8+ T cells in the brain, or of polyclonal CD8+ T cells in brain or spleen, was observed between *Tgfbr2*$^{fl/fl}$ *Cd8*^{Cre} and *Tgfbr2*$^{fl/fl}$ mice (Figure 17B).

Previous studies suggest that the bifurcation of early effector T cells into terminal effectors and memory precursors occurs by the third cell division following antigen presentation (Pais Ferreira et al. 2020, Lin et al. 2016, Mercado et al. 2000). To assess the divergence of CD8+ T cell fates in the presence or absence of TGF-β in spleen and brain, we examined their cellular co-expression of KLRG1 and TCF-1 on day 8 post-infection. In the spleen, we observed no significant differences in the percentages of KLRG1+ TCF-1- and KLRG1- TCF-1+ cell subsets between *Tgfbr2*$^{fl/fl}$ *Cd8*^{Cre} and *Tgfbr2*$^{fl/fl}$ mice (Figure 17 C-D, top panels). This applied to both, gp33 antigen-specific and polyclonal CD8+ T cells. Conversely, TGF-βRII deficient brain CD8+ T cells comprised a reduced fraction of KLRG1- TCF-1+ cells and a modestly higher frequency of KLRG1+ CD8+ T cells (Figure 17 C-D, bottom panels). Thus, TGF-β signaling did not impact the numbers of antigen-specific or polyclonal effector CD8+

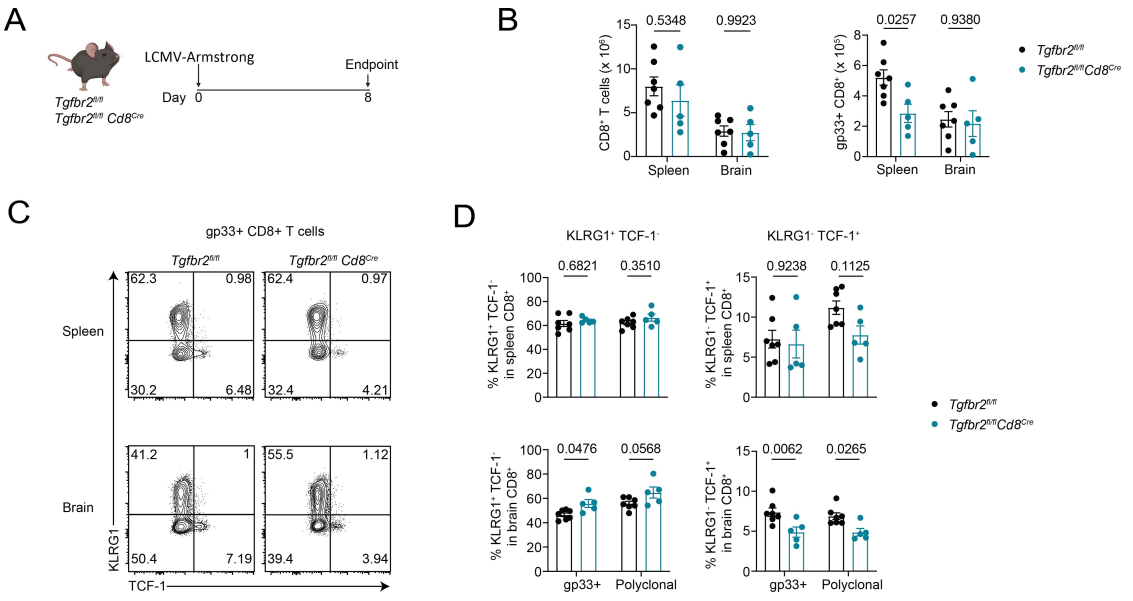

Figure 17 | TGF-β signaling contributes to the establishment of the pool of brain CD8+ Trm cells. A, Sex- and age-matched *Cd8*^{Cre} *Tgfbr2*$^{f/fll}$ and *Tgfbr2*$^{f/fll}$ control mice were infected with LCMV-Armstrong (2 x 10^5 PFU i.p.) and sacrificed on day 8 post-infection. **B,** Flow cytometric quantification of polyclonal and gp33-specific CD8+ T cells in the spleen and brain (n = 5-7 mice per group). **C-D,** Frequencies of KLRG1+ TCF-1- and KLRG1- TCF-1+ in polyclonal and gp33-specific CD8+ T cells in spleen and brain (n = 5-7 mice). Data are representative pooled from two independent experiments. i.p., intraperitoneally; LCMV, lymphocytic choriomeningitis virus, PFU, plaque-forming unit.

T cells seeding the brain, but rather modulated their propensity to differentiate into memory precursor cells. These findings, combined with the reduced numbers of brain CD8+ Trm cells in the absence of TGF-β (Figure 16), suggest that TGF-β signaling represents one determinant of the establishment of brain-resident CD8+ T cells.

4.2.9. TGF-β represses the acquisition of an effector-like state by brain CD8+ T cells in a Trm cell-specific manner

Having shown a role for TGF-β signaling in regulating the establishment of brain Trm cells, we then asked whether TGF-β is required to maintain brain Trm cells. Accordingly, to delete *Tgfbr2* in T cells that have initiated the acquisition of the tissue residency program, we crossed *Hobit*^{Cre} mice to *Tgfbr2*$^{fl/fl}$ to generate mice with Trm cell-specific deletion of TGF-βRII. We infected *Hobit*^{Cre} *Tgfbr2*$^{fl/fl}$ mice and *Hobit*^{Cre} control mice with LCMV Armstrong and assessed brain and spleen CD8+ T cells on day 45 post-infection (Figure 18A). As expected, there was no qualitative or quantitative alterations in CD8+ T cells in the spleen: the frequencies and absolute numbers of naïve, central memory, and effector memory CD8+ T cells were similar across *Hobit*^{Cre} *Tgfbr2*$^{fl/fl}$ and *Hobit*^{Cre} mice (Figure 18B-C). Notably, there was also no difference in the numbers of polyclonal or gp33 antigen-specific CD8+ T cells in the brain upon conditional deletion of TGF-βRII (Figure 18B).

We then assessed whether the abrogation of TGF-β signaling downstream of Hobit expression altered the phenotype of brain CD8+ Trm cells. Strikingly, the frequency of total CD49a+ brain CD8+ T cells was reduced in *Hobit*^{Cre} *Tgfbr2*$^{fl/fl}$ mice (Figure 18D), albeit there was no difference in absolute numbers of total CD49a+ or CD69+ brain CD8+ T cells (Figure 18D). Similarly, we observed no alteration in the frequencies or total count of TCF-1+ CD69+ and TCF-1- CD69+ CD8+ T cell subsets in the brains of *Hobit*^{Cre} *Tgfbr2*$^{fl/fl}$ compared to *Hobit*^{Cre} mice (Figure 18E).

Hobit^{Cre} *Tgfbr2*$^{fl/fl}$ CD8+ Trm cells, however, upregulated CD69 expression compared to their *Hobit*^{Cre} counterparts (Figure 18F). Furthermore, Hobit-dependent TGF-βRII deletion resulted in an increased frequency of KLRG1+ cells among brain CD8+ T cells compared to TGF-βRII-sufficient cells (Figure 18G), which is in agreement with our observations in *Tgfbr2*$^{fl/fl}$ *Cd8*^{Cre} mice. In addition, *Hobit*^{Cre} *Tgfbr2*$^{fl/fl}$ CD8+ T cells displayed a greater proportion of granzyme B expression in the brain compared to *Hobit*^{Cre} CD8+ cells (Figure 18H). Moreover, Bcl-2, an anti-apoptotic molecule that is known to be expressed in CD8+ Trm cells in various tissues (Lin et al. 2023, Steinbach et al. 2016), was downregulated in the absence of TGF-β signaling in *Hobit*^{Cre} *Tgfbr2*$^{fl/fl}$ brain CD8+ T cells (Figure 18I). Finally, we assessed cytokine expression by *Hobit*^{Cre} *Tgfbr2*$^{fl/fl}$ and *Hobit*^{Cre} brain CD8+ Trm cells following gp33 restimulation ex vivo and found no difference in their capacity to co-produce TNF and IFNγ (Figure 18J).

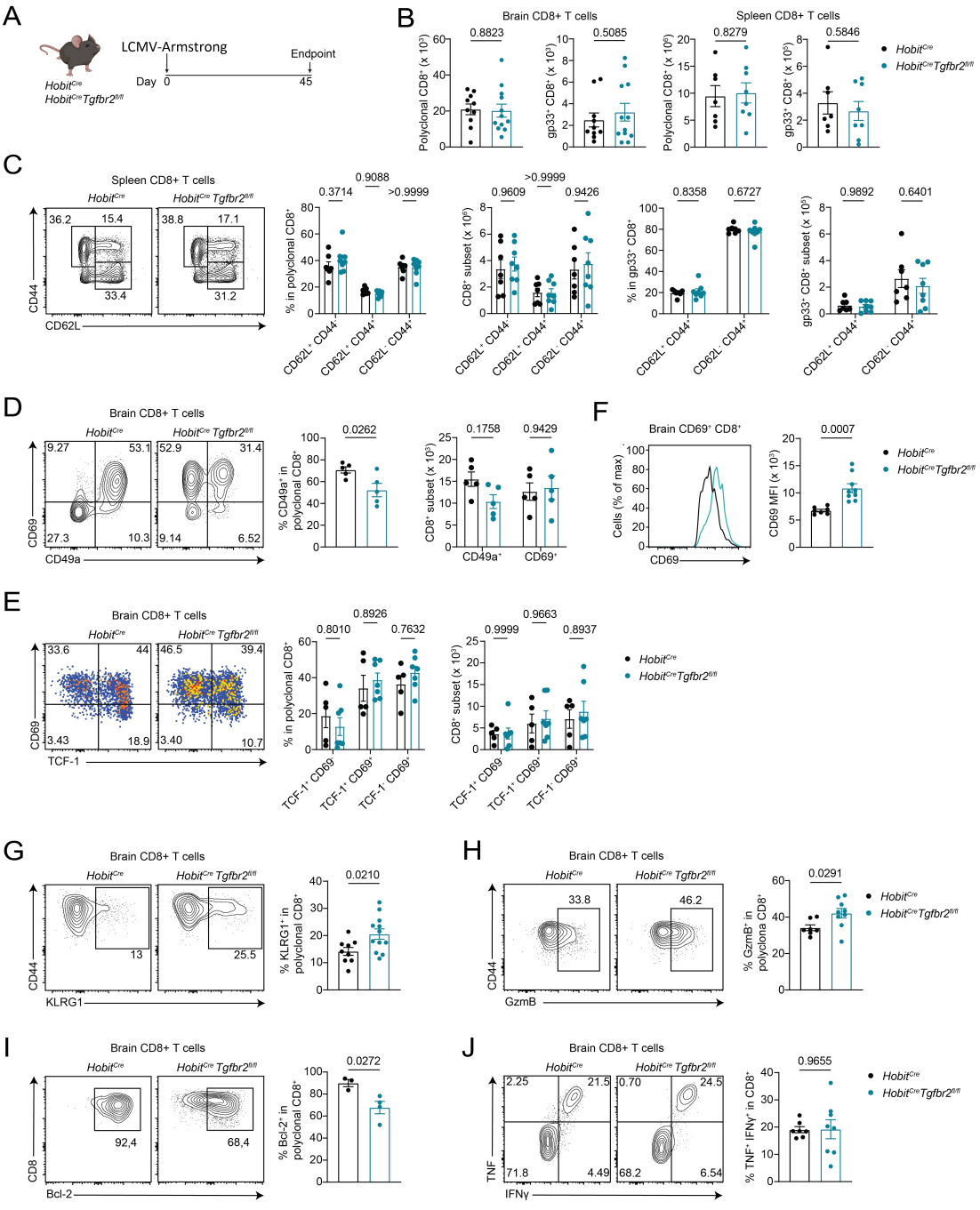

Figure 18 | TGF-β maintains the phenotypic identity of brain CD8+ T cells in a Trm cell-specific manner. A, Sex- and age-matched *Hobit*^{Cre} *Tgfbr2*$^{f/fll}$ and *Hobit*^{Cre} control mice were infected with LCMV-Armstrong (2 x 10^5 PFU i.p.) and sacrificed on day 45 post-infection. **B,** Flow cytometric quantification of polyclonal and gp33-specific CD8+ T cells in the spleen (n = 7-8 mice per group) and brain (n = 9-12 mice per group). **C,** Frequencies and numbers of naïve (CD62L+ CD44-), central memory (CD62L+ CD44+), and effector memory (CD62L- CD44+) CD8+ T cells in spleen (n = 7-8 mice per group). **D,** Percentages and numbers of CD49a+ and CD69+ subsets of brain CD8+ Trm cells (n = 5 mice per genotype). **E,** Frequencies and numbers of TCF-

1+ CD69-, TCF-1+ CD69+, and TCF-1- CD69+ subsets of brain CD8+ Trm cells (n = 5-7 mice per genotype). **F,** CD69 MFI in CD69+ brain CD8+ Trm cells (n = 7-9 mice). **G-I,** Proportion of KLRG1+ (**G**; n = 9-12 mice), GzmB+ (**H**; n = 7-9 mice), and Bcl-2+ (**I**; n = 3-4 mice) in brain CD8+ Trm cells. **J,** Frequency of TNF+ IFNγ+ in brain CD8+ T cells following a 4 hr ex vivo stimulation with gp33 (n = 7-8 mice per genotype). Data are representative (D, I) or pooled (B-C, E-H, J) from 2-3 independent experiments. GzmB, granzyme B; i.p., intraperitoneally; IFNγ, interferon gamma; LCMV, lymphocytic choriomeningitis virus, PFU, plaque-forming unit; TNF, tumor necrosis factor.

In summary, these data show that TGF-β plays a role in shaping brain CD8+ Trm cell identity and guards against their acquisition of an effector-like state. More experiments, however, are needed to decipher whether TGF-β affects the long-term survival of brain CD8+ Trm cells.

4.2.10. TGF-β constrains the transition of brain CD8+ Trm cells into effector cells upon antigen-specific rechallenge

A previous study has demonstrated that the ability of circulating memory CD8+ T cells to mount a recall response is drastically impaired in the absence of TGF-β signaling (Ma and Zhang 2015). Brain CD8+ Trm cells generated in *Hobit*^{Cre} *Tgfbr2*$^{fl/fl}$ mice displayed an enhanced effector state following antigen clearance. To assess the capacity of TGF-βRII deficient CD8+ Trm cells for secondary expansion, we generated *Hobit*^{Cre} *Tgfbr2*$^{fl/fl}$ and *Hobit*^{Cre} immune mice, depleted them of circulating memory cells via anti-Gr1 treatment, and rechallenged the mice using LMgp33. Brain and spleen CD8+ T cells were assessed 7 days post-rechallenge (Figure 19A).

In line with what we observed at the memory time point, *Hobit*^{Cre} *Tgfbr2*$^{fl/fl}$ CD8+ T cells in brain or spleen did not exhibit a change in number upon rechallenge compared to *Hobit*^{Cre} mice (Figure 19B). However, the composition of the responding antigen-specific CD8+ T cells was altered. Specifically, the lack of TGF-β signaling facilitated the transition of antigen-specific CD8+ Trm cells into KLRG1+ CX3CR1+ (as well as total KLRG1+) effector-like cells (Figure 19C). Moreover, TGF-βRII-deficient brain CD8+ cells showed an enhanced production of granzyme B compared to TGF-βRII sufficient cells (Figure 19D). Finally, CD8+ T cells from the brains of *Hobit*^{Cre} *Tgfbr2*$^{fl/fl}$ mice displayed a non-significant increased frequency of TNF+ IFNγ+ cells following gp33 restimulation in vitro, compared to *Hobit*^{Cre} CD8+ T cells (Figure 19E). Taken together, TGF-β signaling negatively regulates the differentiation of brain Trm cells into effector-like cells upon antigen-specific rechallenge, albeit in a qualitative but not quantitative manner.

In summary, TGF-β plays a critical role in the establishment of brain-resident memory CD8+ T cells, the maintenance of CD8+ Trm cell identity, and in constraining their transition into effector-like cells upon antigen re-encounter. These findings underscore the complex, content-dependent nature of TGF-β regulation of CD8+ T cells in general and brain CD8+ Trm cells in particular.

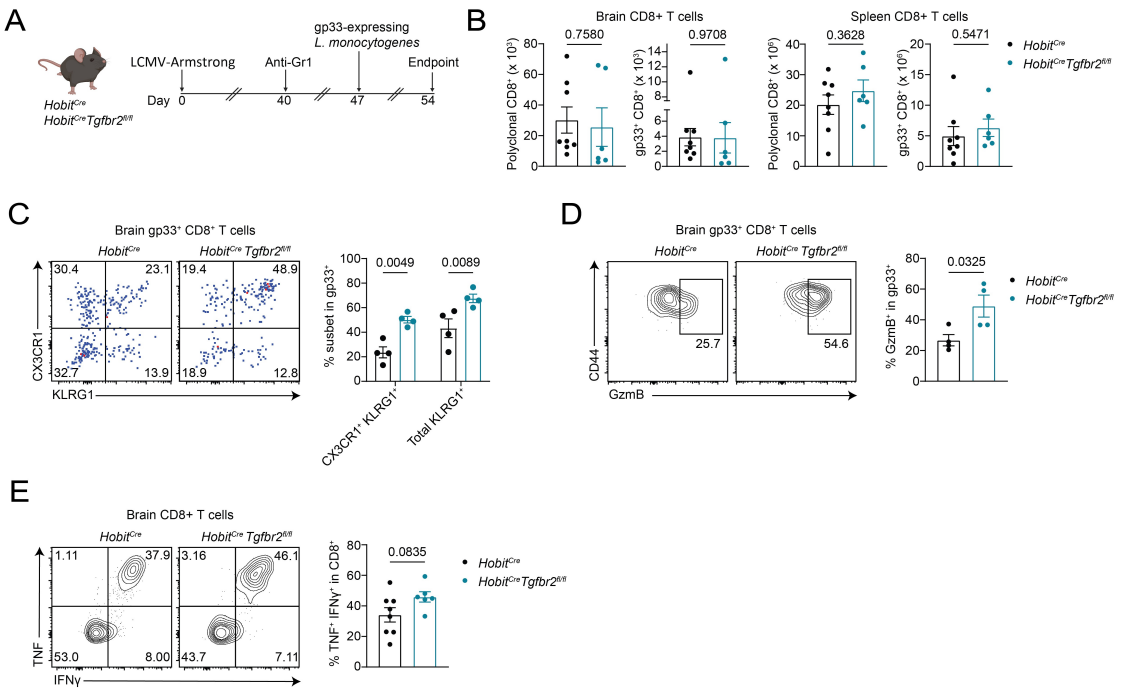

Figure 19 | TGF-β restrains the acquisition of an effector state by brain CD8+ Trm cells upon rechallenge. A, Sex- and age-matched *Hobit*^{Cre} *Tgfbr2*$^{f/fl}$ and *Hobit*^{Cre} control mice were infected with LCMV-Armstrong (2 x 10^5 PFU i.p.); >30 days later, mice received 200 µg of anti-Gr1 i.p., and one week later mice were rechallenged with 1 x 10^5 CFU of LMgp33 i.v., and sacrificed 7 days post-rechallenge. **B,** Flow cytometric quantification of polyclonal and gp33-specific CD8+ T cells in the spleen and brain (n = 6-8 mice per group). **C,** Frequencies of KLRG1+ CX3CR1+ and total KLRG1+ among brain gp33-specific CD8+ Trm cells (n = 4 mice per genotype). **D,** Percentage of GzmB+ in gp33-specific CD8+ Trm cells (n = 4 mice per genotype). **E,** Frequency of TNF+ IFNγ+ in brain CD8+ T cells following a 4 hr ex vivo stimulation with gp33 (n = 6-8 mice). Data are representative (C-D) or pooled (B, E) from two independent experiments. CFU, colony-forming unit; i.p., intraperitoneally; i.v., intravenously; IFNγ; interferon gamma; LCMV, lymphocytic choriomeningitis virus; PFU, plaque-forming unit; TNF, tumor necrosis factor.

4.3. Discussion

4.3.1 TCF-1 as a regulator of brain CD8+ T cell homeostasis and function

A large body of evidence has clarified the requirement of TCF-1 for the differentiation and secondary expansion of circulating memory CD8+ T cells, particularly central memory T cells (Jeannet et al. 2010, Lin et al. 2016, Zhou and Xue 2012, Zhou et al. 2010). TCF-1 was also found to be produced by subsets of CD8+ Trm cells in the kidney, cervicovaginal tissue (CVT), and lung (Wu et al. 2020, Dave et al. 2021, Liao et al. 2021). Its role in Trm cells, however, remains poorly characterized. In this work, we describe an important role for TCF-1 in regulating the homeostasis and antigen-specific recall capacity of brain CD8+ Trm cells.

Hobit is a transcription factor that is necessary for the development of Trm cells in various organs (Mackay et al. 2016). We could show that Hobit was expressed by a subset of brain CD8+ Trm cells, a fraction that was larger than in kidneys and liver CD8+ Trm cells, organs where Hobit expression by Trm cells was first reported (Mackay et al. 2016). Accordingly, to manipulate TCF-1 expression specifically in Trm cells, we crossed *Tcf7*$^{fl/fl}$ mice to *Hobit*^{Cre} mice. Notably, a study by Parga-Vidal et al. has shown that a small population of Hobit-expressing CD8+ T cells in the spleen marks cells with a higher propensity to differentiate into Trm cells (Parga-Vidal et al. 2021). Such low extent of Hobit expression in the spleen potentially explains the mild reduction observed in TCF-1 expression among splenic CD8+ T cells in *Hobit*^{Cre} *Tcf7*$^{fl/fl}$ mice (Figure 8). However, the same study showed substantial Hobit upregulation by established Trm cells compared to the putative precursors in the spleen (Parga-Vidal et al. 2021). Importantly, we observed no difference in the frequency or numbers of naïve or memory CD8+ T cells in *Hobit*^{Cre} *Tcf7*$^{fl/fl}$ compared to *Hobit*^{Cre} controls. This was in contrast to the marked decrease in TCF-1 expression and the increased number of TCF-1- CD69+ CD8+ T cells in the brain. Together, this underscores the utility of the *Hobit*^{Cre} model to investigate the role of *Tcf7* (or other genes) in Trm cells without perturbation of circulating memory T cells.

In one of the earliest studies to report *Tcf7* expression by Trm cells, Wakim et al noted that brain CD8+ Trm cells made small amounts of *Tcf7* transcripts compared to circulating memory T cells (Wakim et al. 2012). This was in contrast to the robust TCF-1 expression we observed at the mRNA and protein levels in brain CD8+ Trm cells in various models. A number of biological and technical variations across the paper in

question and our work may explain the discrepancy in *Tcf7* detection: a) employment of an intracranial infection model, driving the formation a predominantly CD103+ CD8+ Trm cell population; b) brain Trm generation using adoptive transfer of TCR-transgenic T cells, which potentially inherently comprises a smaller fraction of TCF-1+ cells; and c) bulk transcriptional profiling of brain CD8+ Trm cells, which precluded the identification of a T cell subset highly expressing TCF-1.

An elegant study by Wu et al has characterized the regulation of lung CD8+ Trm cells by TCF-1 (Wu et al. 2020). In the absence of TCF-1, lung CD8+ Trm cells increased in numbers and consisted of a greater frequency of CD103+ and CXCR6+ cells as well as TNF+ IFNγ+ cells. Conversely, upon secondary antigenic re-encounter, *Tcf7*-deficient CD8+ Trm cells in the lung did not exhibit a greater magnitude of recall response compared to *Tcf7*-sufficient cells (Wu et al. 2020). These data have commonalities and discrepancies compared to the regulation of brain CD8+ T cells by TCF-1. In terms of commonalities, we also observed that TCF-1 expression appeared to restrict CD8+ T cell population size at the memory phase in the brain. As for the discrepancies, TCF-1 expression in the brain was found to promote, rather than inhibit, the phenotypic maturation of brain CD8+ Trm cells, as evidenced by their downregulation of PD-1 in the absence of TCF-1. In addition, we found that TCF-1 deletion resulted in a more pronounced secondary expansion upon antigenic rechallenge by brain CD8+ T cells. Together, TCF-1 appears to regulate the homeostasis and recall response of brain and lung CD8+ Trm cells in a tissue-specific manner. However, there are other experimental variables across the two studies that could potentially explain the differential contribution of TCF-1 to lung and brain Trm cell dynamics, including the route of infection, usage of TCR transgenic T cells, and the pathogen used.

As described earlier, we found that TCF-1 marks two populations of CD69+ CD8+ Trm cells with distinct proliferation and effector function. Moreover, pseudotime analysis inferred a developmental trajectory where brain CD8+ T cells progressively lose TCF-1 expression as they terminally differentiate (Figure 3). Similarly, the notion of the existence of discrete Trm subsets with variable extents of differentiation, effector function, and longevity, has been previously reported in the small intestine (Milner et al. 2020). In this study, two populations of intestinal Trm cells were delineated based on the reciprocal expression of Blimp1 and ID3 (Milner et al. 2020). Notably, ID3 and TCF-1 show near-total co-expression in circulating memory CD8+ T cells following

acute infection (Utzschneider et al. 2020). Transcriptional profiling of intestinal CD8+ Trm cells revealed that ID3+ cells displayed elements of central memory T cell transcriptional signature, whereas Blimp1+ cells showed an effector-like transcriptome suggestive of terminal differentiation (Milner et al. 2020). The ID3+ subset of intestinal CD8 Trm cells comprised ~5% of the Trm precursors seeding the intestine on day 7 post-LCMV Armstrong infection, and steadily increased to represent ~40% of mature intestinal Trm cells post-day 30. This was in agreement with the appearance of a small subset of TCF-1+ CD8+ T cells in the brain during the expansion phase of the T cell response, and the eventual increase of the TCF-1+ cell frequency at the memory phase. Moreover, adoptive transfer experiments of intestinal CD8+ Trm cells showed that ID3+ cells were multipotent, possessing the capacity to generate a larger number of CD8+ Trm cells in the intestine (Milner et al. 2020). While we did not perform cell re-transfer experiments of TCF-1+ and TCF-1- brain Trm cells, these data align well with the inferred developmental potential of TCF-1+ cells in the brain serving as a putative precursor population. Finally, ID3+ CD8+ Trm cells in the intestine were found to possess a greater ability to produce cytokines upon ex vivo restimulation (Milner et al. 2020). This was also consistent with the larger fraction of TNF IFNγ co-producing cells among TCF-1+ CD69+ brain CD8+ T cells, as well as the greater cytokine production by memory precursors and Tpex cells reported in previous studies (Tsui et al. 2022, Joshi et al. 2007, Gerlach et al. 2016).

Finally, our data point to a role for PD-1 signaling in controlling the size of the TCF-1+ population among brain CD8+ Trm cells. Using germline *Pdcd1* KO or *Hobit*-mediated deletion of *Pdcd1*, we could show that PD-1 signaling positively regulates the TCF-1+ CD69+ subset of brain CD8+ Trm cells. A previous study has shown that, in the context of chronic infection, PD-1 contributed to the maintenance of the TCF-1+ Tpex cell population and guarded against their differentiation into a TCF-1- terminally differentiated subset (Chen et al. 2019). Further, Tsui et al have shown that lack of PD-1 signaling resulted in a reduced frequency of the CD62L+ Myb+ subset of Tpex cells and a concomitant increase in number of TCF-1- Tex cells (Tsui et al. 2022). In another setting, naïve PD-1 KO mice exhibit an expansion of liver CD8+ Trm cells that drastically downregulate TCF-1 (Le Moine et al. 2023). Thus, I speculate that PD-1 expression in TCF-1+ CD69+ brain CD8+ Trm cells is required to sustain this subset against TCR-driven conversion into a TCF-1- CD69+ subset. Whether there exists a

transcriptional module that acts downstream of PD-1 signaling to maintain TCF-1 expression in Trm cells remains to be clarified.

4.3.2 Profound impact of PD-1 signaling on brain CD8+ T cell memory

The inhibitory receptor PD-1 has been extensively studied in the context of chronic infection and cancer (McLane et al. 2019, Kallies et al. 2020). Persistent, elevated expression of PD-1 on CD8+ T cells in such contexts represents one of the key features of T cell exhaustion. On the other hand, PD-1 is transiently expressed by effector CD8+ T cells during the early stages of acute infection, followed by its downregulation by circulating memory cells (Jubel et al. 2020). As concerns Trm cells, PD-1 was found to be part of a core transcriptional module shared by CD8+ Trm cells in diverse tissues in mouse and human (Smith and Snyder 2021, Kumar et al. 2017). However, the regulation of Trm cells by PD-1 signaling remains poorly defined. Our data show that PD-1 signaling is important for safeguarding the differentiation of CD8+ Trm cells in the brain, guarding against an increase in population size while promoting/maintaining the acquisition of the mature Trm phenotype, including TCF-1, CD49a and granzyme B. Importantly, lack of PD-1 signaling also resulted in a severely impaired recall response of brain CD8+ Trm cells upon antigenic rechallenge.

Previous studies have examined the consequences of ablating PD-1:PD-L1 signaling on the formation of brain Trm cells. These studies employed localized intracranial infection models, which generate a sizeable CD103+ subset of CD8+ Trm cells (Scholler et al. 2020, Prasad et al. 2017, Shwetank et al. 2019). In the context of acute mouse cytomegalovirus (MCMV) infection, which precipitates an ensuing state of viral latency and chronic neuroinflammation, lack of PD-1 signaling yielded fewer brain CD8+ Trm cells, particularly the CD69+ CD103+ subset (Prasad et al. 2017, Schachtele et al. 2014). Importantly, the frequency of CD127+ CD8+ Trm cells (which putatively correspond to TCF-1+ cells) was substantially smaller among Trm cells lacking PD-1 (Prasad et al. 2017). Conversely, infection of PD-L1 KO mice with polyomavirus, which persists at a low-level antigen burden, generated a slightly larger frequency and number of CD103+ brain Trm cells compared to infected WT mice (Shwetank et al. 2019). In another study, PD-1 KO mice infected with an adenoviral vector expressing LCMV glycoproteins exhibited a greater fraction CD127+ and CD103+ brain CD8+ Trm cells compared to infected PD-1- sufficient mice (Scholler et

al. 2020). The discrepancies across these papers, namely the impact of PD-1 signaling on the formation of CD127+ and CD103+ brain CD8+ Trm subsets, may reflect variable extents of viral persistence of the different pathogens used. Nevertheless, the common theme in the aforementioned papers, intracranial infection of long-persisting viruses, differs from our experimental setup where LCMV Armstrong does not persist in the brain (Wherry et al. 2003a, Urban et al. 2020). In addition, the majority of the experiments reported above were carried out in constitutive PD-1 KO (or PD-L1 KO) mice. Given that PD-1 is expressed by additional cell types, including Treg cells, more evidence is needed to identify how PD-1 regulates brain CD8+ T cells in a cell-intrinsic manner.

Accordingly, we examined the contribution of PD-1 signaling to brain CD8+ Trm formation and recall response in the setting of acute systemic infection. We first made use of a mixed bone marrow chimera model of PD-1 KO and WT cells to assess the role of PD-1 in CD8+ T cells in a cell-intrinsic manner, followed by employing the *Hobit*^{Cre} *Pdcd1*$^{f/fl}$ model to investigate PD-1 in a Trm cell-specific fashion. As alluded to above, we could demonstrate a critical role for PD-1 in shaping brain CD8+ Trm cell differentiation and recall capacity.

The role of PD-1 signaling in shaping CD8+ Trm cells has also been addressed in the lung in the context of influenza infection (Pauken et al. 2020, Wang et al. 2019). At the peak of the effector phase, loss of PD-1 signaling coincided with an enhanced expansion of antigen-specific effector CD8+ cells and a more efficient viral clearance (Pauken et al. 2020). However, PD-L1/PD-L2 double-deficient or PD-1 deficient mice exhibited a more profound contraction of antigen-specific CD8+ T cells in the lung compared to WT mice, which extended to a reduced number of antigen-specific memory cells on day 60 post-infection (Pauken et al. 2020). These data mirror our findings on brain CD8+ T cells, in the sense that PD-1 KO cells expanded to a greater extent, which was followed by an impaired memory formation on day 45-47. The discrepancy between lung and brain CD8+ T cells manifests in that both antigen-specific and polyclonal memory cells in the brain outnumbered their WT counterparts, even though the frequency of antigen-specific cells was smaller. This may reflect a greater reliance by brain CD8+ T cells on PD-1 signaling to promote cellular quiescence, in addition to the requirement of PD-1 for robust antigen-specific memory development. Furthermore, Paulken et al. showed that suboptimal differentiation of lung CD8+ Trm cells in the absence of PD-1 resulted in an impaired recall response

upon an influenza heterologous rechallenge. This manifested in an abrogated CD8+ T cell secondary expansion and cytokine production upon rechallenge and a slower viral clearance. In an orthogonal approach, Wang et al described a subset of lung CD8+ Trm cells that highly expresses PD-1 and which exhibited a greater secondary expansion upon antigen re-encounter compared to their PD-1-low counterparts (Wang et al. 2019). These findings align well with our observation of an important role of PD-1 in shaping the recall capacity of brain CD8+ Trm cells, where PD-1-deficient CD8+ T cells exhibited an impaired secondary expansion and cytokine production upon antigen-specific rechallenge.

The notion of PD1 acting as a negative regulator of Trm cell expansion has also been recently demonstrated in liver CD8+ Trm cells (Le Moine et al. 2023, Charlton et al. 2013). Expanded PD1 KO cells comprised a greater proportion of CD69+ (as well as CXCR6+) cells and exhibited an exhaustion-like state, characterized by upregulation of inhibitory receptors (Tim3 and Tigit) and the transcription factor TOX, and diminished cytokine production (Le Moine et al. 2023). Such reduced functionality is in line with the impaired capacity of brain CD8+ T cells for cytokine expression observed during recall response in both, constitutive *Pdcd1* KO and Hobit-dependent *Pdcd1* deletion. This suggests that PD-1 plays a largely similar role in maintaining the homeostasis and effector function of CD8 Trm cells in a number of non-lymphoid tissues including brain and liver.

4.3.3 TGF-β regulates different facets of brain Trm formation and function

A large number of studies have illuminated the pivotal roles that TGF-β play in regulating naïve, effector, and circulating memory T cells (Oh and Li 2013, Li and Flavell 2008). In addition, it is now well established that TGF-β shapes the differentiation, maintenance, and/or function of resident-memory T cells in a number of non-lymphoid tissues, particularly skin and small intestine (Mackay et al. 2013, Hirai et al. 2021, Zhang and Bevan 2013). Apart from T cells, TGF-β is also known to be an active player in brain development and homeostasis. In fact, TGF-β is locally produced in the brain by various cell types, mainly by astrocytes, and has been shown to regulate neuronal survival, synapse formation, and cognition (Kapoor and Chinnathambi 2023, Su et al. 2023a). In the absence of TGF-β signaling, developing microglia exhibit impaired maturation, an altered transcriptional profile, and compromised long-term survival, whereas adult microglia require TGF-β to maintain their morphology and homeostasis (reviewed by (Spittau et al. 2020). Given the important roles exerted by TGF-β in peripheral T cells, coupled with its activity in the brain, we hypothesized that TGF-β signaling may also regulate the formation of brain-resident CD8+ T cells.

Our data uncover a novel and critical role for TGF-β in shaping the differentiation of bona fide CD8+ Trm cells in the brain. In the absence of TGF-β signaling, effector CD8+ T cells comprised a smaller fraction of memory precursor cells in the brain, which eventually led to a smaller number of mature brain CD8+ Trm cells at the memory phase. Further, ongoing TGF-β signaling appeared to mitigate the acquisition of an effector state by brain CD8+ Trm cells at the steady state, as well as restraining their capacity to fully differentiae into effector-like cells upon antigenic rechallenge. These data serve as an additional example of the remarkable context-dependent regulation of CD8+ T cells by TGF-β. In fact, it has long been known that the status of T cell activation dictates the outcome of TGF-β signaling, partially because TGF-βRI is known to be downregulated upon TCR signaling (Chen 2023, Tu et al. 2018, Filippi et al. 2008). Furthermore, our data argue that TGF-β signaling safeguards the homeostasis and promotes optimal function of CD8+ Trm cells. Specifically, TGF-β acts to promote the population size and phenotypic maturation of CD8+ Trm cells, yet subsequently curbs CD8+ Trm cell effector function upon rechallenge. This presumably serves to ensure the formation of a Trm cell pool poised for antigen re-encounter, yet its response is kept in check to guard against collateral tissue damage.

Initial studies showing the necessary role of TGF-β for the establishment of Trm cells in the skin employed direct intradermal transfer of *Tgfbr2*-deficient activated T cells (Mackay et al. 2013). Given that we were interested in understanding the molecular determinants of brain CD8+ Trm cells in acute systemic infection without local tissue damage, we instead employed the *Cd8*^{Cre} *Tgfbr2*$^{fl/fl}$ model. In this model, TGF-βRII is deleted in mature CD8+ T cells and not in CD4+ CD8+ double-positive thymocytes. Deletion of *Tgfbr2* downstream of *Cd8*^{Cre} rather than *Hobit*^{Cre} would then allow for investigating the contribution of TGF-β signaling to the early events governing the acquisition of tissue residency, independent of Hobit expression. A key feature of TGF-β signaling is that it regulates T cells in a context-dependent manner, e.g. depending on the cellular activation state (Filippi et al. 2008). Indeed, we observed that *Tgfbr2* deletion led to loss of naïve CD8+ T cell homeostasis and an increased number of effector memory CD8+ T cells in the spleen at the memory phase post-LCMV Armstrong infection (Ma and Zhang 2015), yet resulted in a decreased number of CD8+ Trm cells in the brain. Coupled with the impaired differentiation of the putative memory precursors (TCF-1+ KLRG1-) during the effector phase of infection, these findings suggest that TGF-β signaling is important for the establishment of CD8+ Trm cells in the brain. We noted that the magnitude of the contribution of TGF- β to brain CD8+ Trm formation was closer to that observed in the skin rather than in the small intestine (Mackay et al. 2013, Zhang and Bevan 2013). Specifically, co-transfer of WT and *Tgfbr2* KO antigen-specific cells resulted in WT cells outnumbering *Tgfbr2* KO cells by ~3 folds in the skin yet >45 folds in the gut, whereas we observed a ~2-fold reduction in number of brain CD8+ Trm cells lacking TGF-β signaling.

As concerns Trm cell maintenance, previous studies have uncovered a prominent role for TGF-β in preserving Trm cell numbers long after pathogen clearance in the skin, gut, and salivary glands (Hirai et al. 2021, Crowl et al. 2022). Moreover, it was also shown that TGF-β-dependent skin Trm cells exhibited a restrained effector program compared to Trm cells that do not engage TGF-β signaling (Christo et al. 2021). On the other hand, *Tgfbr2*-deficient circulating memory CD8+ T cells exhibit an impaired recall response upon antigen re-encounter (Ma and Zhang 2015). Using the *Hobit*^{Cre} *Tgfbr2*$^{fl/fl}$ model, we could show that TGF-β does not impact the brain CD8+ Trm population size in the maintenance phase but serves to impede their acquisition of an effector state, particularly upon antigen-specific rechallenge. This indicates that TGF-β qualitatively but not quantitatively modulates the brain CD8+ T cell Trm pool after its

establishment, arguing for a relatively limited contribution to the maintenance of such Trm population.

Bcl2 is anti-apoptotic molecule that balances cell death and cell survival in tandem with pro-apoptotic molecules such as Bim (Grayson et al. 2000). In fact, Bcl-2 is expressed in naïve CD8+ T cells, downregulated by short-lived effector T cells during the expansion phase, and then its expression is regained and further upregulated (compared to naïve cells) in memory CD8+ T cells (Grayson et al. 2000). Further, Bcl-2 has been shown to promote the survival of circulating memory T cells (Wojciechowski et al. 2007) as well as of resident memory CD8+ T cells in the small intestine (Lin et al. 2023). In the brain, *Hobit*^{Cre} *Tgfbr2*$^{fl/fl}$ CD8+ Trm cells showed a reduced fraction of Bcl2+ cells at the memory phase following LCMV infection, suggestive of a reduced capacity for long-term survival. In contrast, we did not observe a reduced number of *Hobit*^{Cre} *Tgfbr2*$^{fl/fl}$ CD8+ Trm cells on day 45-60 post-infection. Whether reduced expression of Bcl-2 in this model would result in poor long-term maintenance (e.g. 4-6 months post-infection) remains to be tested.

In both sets of experiments, involving *Hobit*^{Cre} *Tgfbr2*$^{fl/fl}$ or *Cd8*^{Cre} *Tgfbr2*$^{fl/fl}$, we assessed CD49a expression as a positive control for the lack of TGF-β signaling. Whereas the majority of studies on TGF-β regulation of skin and gut Trm cells focused on CD103 expression as a readout for TGF-β signaling, the small and inconsistent frequency of CD103+ among brain CD8+ Trm cells rendered CD103 a largely non-reliable marker in this setting. Instead, CD49a, another TGF-β-dependent integrin, exhibited robust expression in brain CD8+ Trm cells in all contexts studied, including acute infection. Yet, the fraction of CD49a+ CD8+ Trm cells in *Cd8*^{Cre} *Tgfbr2*$^{fl/fl}$ mice was reduced by only ~2 folds compared to WT, comprising about 40% of brain CD8+ Trm cells lacking TGF-β signaling. This may be attributed to the fact that CD49a is initially upregulated by a fraction of effector cells before tissue entry in an IL-12- and TGF-β-dependent manner, and further upregulated during Trm cell maturation (Bromley et al. 2020). Apart from CD103 and CD49a, another indicator for TGF-β signaling in T cells is the extent of expression of KLRG1, since it is known that TGF-β suppresses KLRG1 expression (Schwartzkopff et al. 2015). Indeed, we observed that the frequency of KLRG1-producing brain CD8+ Trm cells–at memory time points post-infection–was elevated in both *Cd8*^{Cre} *Tgfbr2*$^{fl/fl}$ and *Hobit*^{Cre} *Tgfbr2*$^{fl/fl}$ cells.

Finally, our findings further confirm the notion that TGF-β promotes rather than inhibit the expansion of effector CD8+ T cells at the peak of T cell response. Seminal studies using T cells carrying a dominant negative mutant of TGF-βRII (with abrogated signaling capacity) showed that effector CD8+ T cells expanded three-folds more than their wild-type counterparts (Gorelik and Flavell 2000, Sanjabi et al. 2009). Caveats associated with this model included the manipulation of TGF-β signaling in double-positive thymocytes, incomplete blockade of TGF-β signaling, and additional signaling "artifacts" introduced by the dominant negative TGF-βRII mutant that are independent of TGF-β signaling (Ishigame et al. 2013, Zhang and Bevan 2013). Conversely, subsequent studies employed *Lck*^{Cre}-mediated deletion of *Tgfbr2*, and found that KLRG1+ effector T cell expansion was inhibited in the absence of TGF-β signaling (Ma and Zhang 2015, Zhang and Bevan 2013). Therefore, these findings further underscore the context-dependent activity of TGF-B in regulating different subsets of CD8+ T cells.

Chapter 5: Concluding remarks and future directions

In this study, we demonstrate that brain CD8+ T cells are heterogeneous and adopt a molecular landscape that is strongly driven by the tissue microenvironment but also shaped by the disease in some contexts. We also provide evidence on the important contributions of TCF-1, PD-1, and TGF-β to the formation and function of brain CD8+ Trm cells. Accordingly, our study provides a detailed molecular map of brain CD8+ T cells as well as tools that allow for selectively manipulating them. This lays the foundation for future work that identify and manipulate pathogenic and/or protective subsets of brain CD8+ T cells in diverse disease settings. An example would be to investigate the role of the TCF-1+ CD69+ subset of brain CD8+ Trm cells in cerebral amyloidosis, since it was selectively increased in the brains of aged APP/PS1 mice. This subset produces less GzmB yet more TNF compared to their TCF-1- CD69+ counterparts. Does this T cell state represent brain tissue adaptation to limit further tissue damage, or is this subset actively promoting neuroinflammation? Crossing *Hobit*^{Cre} *Tcf7*$^{fl/fl}$ to APP/PS1 mice, and evaluating neuropathology and cognitive function in such mice, would be one experimental setup that makes use of the Trm cell-specific manipulation presented in this study.

Recall response upon antigenic re-encounter is one of the cardinal feature of memory T cells. Our main readout for the function of CD8+ Trm cells lacking TCF-1, PD-1, or TGF-β signaling has been to assess their secondary expansion and production of cytokines. Future experiments are required to identify whether the enhanced recall response in *Hobit*^{Cre} *Tcf7*$^{fl/fl}$ mice or impaired response in *Hobit*^{Cre} *Pdcd1*$^{fl/fl}$ translates into a faster/slower bacterial clearance upon rechallenge, including intracranial rechallenge.

Finally, more work is needed to decipher the differentiation trajectory that CD8+ Trm precursors undertake to eventually adopt a mature brain Trm phenotype. Our trajectory inference data suggest an association between a progressive engagement of TCR signaling and brain CD8+ T cells differentiation into TCF-1- CD69+ PD-1+ cells. Does TCR signaling contribute to this maturation? Conditional deletion of the TCR on mature or precursors of Trm cells would address this notion. Further, Ly6C is expressed by a large fraction of circulating memory T cells (DeLong et al. 2018), and was found to be gradually lost in our trajectory inference data. More experimental data is needed to assess if Ly6C+ CD69+ CD8+ T cells in the brain represent early emigrants, and

whether Ly6C per se is important for the extrvasation/infiltration of CD8+ T cells into the brain parenchyma.

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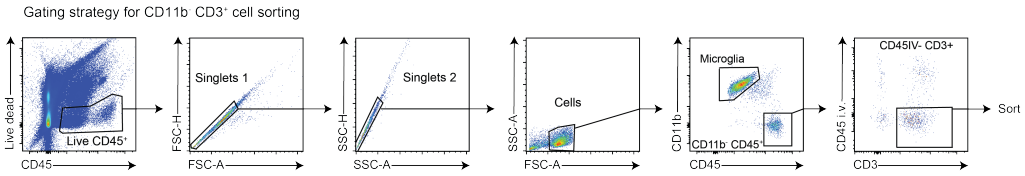
Chapter 6: Appendix

A



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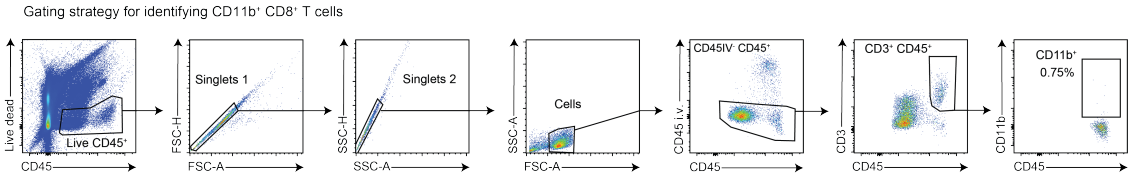

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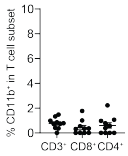
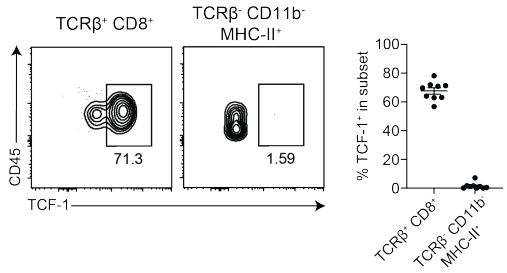


Appendix Figure 20 | Gating strategy and assessment of CD11b expression by brain T cells. A, gating strategy used to sort CD3+ T cells for downstream single-cell RNA-sequencing. **B-C**, gating strategy and quantification of the frequency of CD11b+ T cell subsets.



Appendix Figure 21 | TCF-1 is not expressed by cDC1 cells in the brain. CD8+ T cells (defined as TCRβ+ CD8+) and conventional dendritic cells 1 (cDC1s; defined as TCRβ- CD11b- MHC-II+) were assessed for TCF-1 expression. Cells were pre-gated on live single extravascular CD45+ cells.