Neuroimmune remodelling and secondary inflammatory vulnerability after traumatic brain injury

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Declaration of Authorship

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Abstract

Traumatic brain injury (TBI) represents not merely a mechanical insult but a biologically intricate and evolving disorder characterized by neurovascular disruption, sterile inflammation, leukocyte recruitment, and tissue remodelling, all of which collectively influence neurological outcomes. While the early activation of the innate immune system following TBI is well-documented, the progression of this response into T cell engagement and its potential to leave a lasting immune imprint within the injured brain remains less understood. This thesis explores the hypothesis that controlled cortical impact (CCI) induces a temporally ordered neuroimmune response, wherein early innate immune programs influence subsequent T cell immunity, culminating in a persistent tissue-associated immune state that alters susceptibility to subsequent systemic inflammatory challenges. The study particularly emphasizes the interleukin-1β (IL-1β) axis, innate and T cell immune crosstalk, and the emergence of tissue-resident memory T cell (T_{RM}) populations post-injury.

To investigate this, adult male C57BL/6J mice were subjected to moderate-severe CCI and examined across acute, subacute, and chronic phases post-injury. Brain mononuclear-cell isolation was optimized for downstream immune profiling, using multiparameter spectral flow cytometry, intracellular cytokine staining, and RT-qPCR to delineate immune composition and effector programs over time. Behavioural outcomes were assessed using beam walk, rotarod, Y-maze, novel object recognition, and SNAP scoring. Mechanistic studies targeted distinct innate pathways through anti-Ly6G mediated neutrophil attenuation, MC-21-mediated CCR2-dependent monocyte depletion, and pharmacological NLRP3 inflammasome inhibition with MCC950. Chronic adaptive remodelling was examined using intravascular anti-CD45 labelling to differentiate tissue-associated from blood-circulating leukocytes, while a secondary systemic inflammatory challenge with LPS, with or without CD49d/VLA-4 blockade, was employed to ascertain whether post-traumatic immune amplification relied on continued leukocyte trafficking or resident tissue-associated compartments. The influence of aging on post-traumatic T_{RM} responses was also investigated.

TBI elicited a sequential and compartmentalized neuroimmune response. Early post-injury, inflammatory gene expression was rapidly induced, with $Ly6G^+$ neutrophils and $Ly6C^+$ monocytes emerging as the predominant infiltrating populations and primary cellular sources of IL-1β. As the response progressed, adaptive immune involvement became more

pronounced, with $\gamma\delta^+$ T cells constituting the main interleukin-17 (IL-17) producing population and interferon-γ (IFN-γ) distributed across multiple T cell subsets. Mechanistic studies revealed that targeting individual innate compartments did not simply suppress downstream adaptive immunity, neutrophil attenuation altered inflammatory composition without clear functional benefit, CCR2-dependent monocyte depletion yielded only modest effects, and NLRP3 inhibition with MCC950 selectively reduced microglial activation while leaving broader leukocyte recruitment and T cell responses largely unaffected. Concurrently, T_{RM} cell populations peaked during the subacute phase, and persisted chronically within the injured hemisphere. Aging amplified this response, and secondary systemic inflammation with LPS triggered robust reactivation of T_{RM} cells. CD49d blockade attenuated T cell trafficking; however it did not eliminate the T_{RM} response. This suggests that both continued trafficking and resident tissue-associated immunity contribute to post-traumatic immune amplification.

In conclusion, this thesis elucidates that TBI initiates a prolonged and compartmentalized process of immune remodelling, rather than merely a transient inflammatory response. The findings indicate that post-traumatic pathology is maintained through complex interactions between innate and T cells, persistent tissue-associated immunity, and recruitment-dependent amplification, with aging and secondary challenge further exacerbating vulnerability. These results highlight the IL-1β axis, T_{RM} responses, and trafficking-dependent immune interactions as critical components of post-traumatic neurobiology and as potential therapeutic targets for mitigating chronic inflammation and subsequent vulnerability following TBI.

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Curriculum Vitae

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- 2024, Poster at European congress of Immunology (ECI) entitled "**Temporal analysis of the infiltration dynamics of pro-inflammatory cytokine producing innate and adaptive immune cells following experimental traumatic brain injury in mice"** in Dublin, Ireland
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innate and adaptive immune cells following experimental traumatic brain injury in mice" in Vienna, Austria
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- **Impact of innate immune activation on T cell dynamics and functional recovery following traumatic brain injury** (**Sahil Threja**, Nathan Ryzewski Strogulski, Janeen Laabei, Gloria Vegliante, Carly Douglas, Tizbit Ashine Bogale, Ciara Moynihan, Giusy Di Franco, Matthias Mack[2], Lisa Borkner[3], Béré Diallo[3], Kingston H.G. Mills[3], David J. Loane) **bioRxiv preprint**, 2026 (https://doi.org/10.64898/2026.03.23.713833)
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TEACHING AND RESEARCH SUPERVISION

- Mentored 2 Master's and 2 Bachelor's students in neuroscience and immunology research projects.
- Taught students in teaching laboratories across a range of undergraduate courses

List of Abbreviations

ANOVA	Analysis of variance
APC	Antigen-presenting cell
ARRIVE	Animal Research: Reporting of In Vivo Experiments
ATP	Adenosine triphosphate
AUC	Area under the curve
BBB	Blood-brain barrier
BSA	Bovine serum albumin
CCI	Controlled cortical impact
CCL2	C-C motif chemokine ligand 2
CCR2	C-C chemokine receptor type 2
CD49d	Cluster of differentiation 49d
cDNA	Complementary DNA
CMU	Comparative Medicine Unit
CNS	Central nervous system
cRPMI	Complete Roswell Park Memorial Institute medium
CTE	Chronic traumatic encephalopathy
CXCL1	C-X-C motif chemokine ligand 1
CXCL10	C-X-C motif chemokine ligand 10
CXCR2	C-X-C motif chemokine receptor 2
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DI	Discrimination index
DNA	Deoxyribonucleic acid
dpi	Days post-injury
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FCS	Fetal calf serum
FMO	Fluorescence minus one
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCS	Glasgow Coma Scale
HMGB1	High mobility group box 1
hpi	Hours post-injury
i.p.	Intraperitoneal
i.v.	Intravenous
ICAM-1	Intercellular adhesion molecule 1
ICS	Intracellular cytokine staining
IFN-γ	Interferon gamma
IL-1	Interleukin-1
IL-17	Interleukin-17
IL-1β	Interleukin-1 beta
ITGB2	Integrin subunit beta 2
LPS	Lipopolysaccharide
MCC950	Selective NLRP3 inflammasome inhibitor
MDMs	Monocyte-derived macrophages
MHC-II	Major histocompatibility complex class II
MMP-9	Matrix metalloproteinase 9
MNCs	Mononuclear cells

NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa B
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
NOR	Novel object recognition
NVU	Neurovascular unit
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PMA	Phorbol 12-myristate 13-acetate
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT-qPCR	Real-time quantitative polymerase chain reaction
SEM	Standard error of the mean
SNAP	Simple Neuroassessment of Asymmetric Impairment
TBI	Traumatic brain injury
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th1	T helper 1
Th17	T helper 17
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor alpha
T_{RM}	Tissue-resident memory T cells
UMAP	Uniform manifold approximation and projection
VLA-4	Very late antigen 4

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Chapter 1

General Introduction

1.1 Traumatic brain injury and its systemic complexity

Traumatic brain injury (TBI) is more accurately conceptualized as a syndrome rather than a singular disease entity. It encompasses a broad spectrum of severity, includes both focal and diffuse patterns of tissue damage, and results in outcomes that are only partially elucidated by the initial clinical presentation. Contemporary burden estimates, derived from Global Burden of Disease 1990-2021 data, underscore that the impact at the population level is both substantial and persistent across various countries. Furthermore, the factors influencing incidence differ by region and mechanism of injury (Zhong et al., 2025).

While the Glasgow Coma Scale (GCS) remains a prevalent tool for assessing clinical severity, there is a growing acknowledgment within the field that GCS alone does not adequately account for biological heterogeneity, particularly in patients whose long-term outcomes differ despite similar initial scores. Recent international initiatives, coordinated by NIH-NINDS, have advocated for a modernized approach to classification and nomenclature. This approach emphasizes the importance of multidimensional characterization during the acute phase, with the aim of enhancing stratification and mechanistic interpretation (Manley et al., 2025).

TBI is frequently conceptualized at a biological level through a primary-secondary framework. However, the term "secondary injury" does not refer to a singular cascade; rather, it encompasses a series of interacting processes that manifest over distinct timescales and within specific anatomical compartments **(Fig. 1.1)**. Disruption of the neurovascular unit (NVU), edema, ionic dysregulation, mitochondrial dysfunction, excitotoxicity, programmed cell death, and neuroinflammation concurrently emerge and can exacerbate one another (Hanscom et al., 2021; Kumar & Loane, 2012; Lok et al., 2015; Simon et al., 2017; Unterberg et al., 2004; Xiong et al., 1997; Yi & Hazell, 2006). Recent analyses of post-traumatic neuroinflammation highlight that immune pathways are not merely consequences of tissue damage but are active contributors to secondary pathology and long-term sequelae, contingent upon their magnitude, timing, and cellular origin (Hostiuc & Rusu, 2026). This complexity underscores the necessity of controlled animal models, which facilitate the isolation of biological variables and enable precise, time-specific sampling. Among the experimental paradigms frequently employed, controlled cortical impact (CCI) is particularly prevalent due to its ability to consistently induce graded focal contusion, reproducible neurovascular disruption, perilesional inflammation, and cognitive and behavioural impairments that mirror clinically relevant aspects of human contusional TBI (Osier & Dixon, 2016). In this thesis, CCI serves as a platform to delineate the temporal progression of

neuroimmune responses and to evaluate whether early innate immune nodes influence subsequent adaptive immune programming and establish enduring brain immune "setpoints". The clinical heterogeneity observed following TBI can be attributed to the fact that seemingly similar mechanical insults may diverge biologically. This divergence is influenced by the interplay between the release of damage-associated molecular patterns (DAMPs), complement activation, coagulation signalling, and the extent of microvascular injury. These factors collectively reshape the inflammatory milieu within hours of the trauma (Bouras et al., 2022; Hammad et al., 2018). Endogenous danger signals, including adenosine triphosphate (ATP), high mobility group box 1 (HMGB1), S100 proteins, extracellular matrix fragments, and nucleic acids, activate pattern-recognition and purinergic pathways in glial cells, endothelial cells, and infiltrating myeloid cells. This activation links tissue disruption to a self-propagating sterile inflammatory program (Braun et al., 2017).

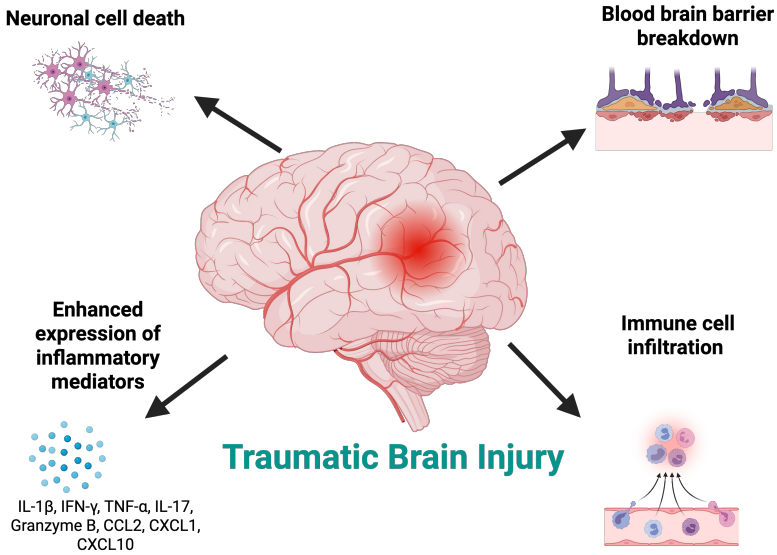

Fig. 1.1: Schematic representation of pathological changes induced by traumatic brain injury

1.2 Blood-Brain Barrier disruption and post-traumatic vulnerability

A primary consequence of mechanical brain injury is the disruption of neurovascular integrity. The blood-brain barrier (BBB) is a specialized interface composed of endothelial cells with tight junctions, including proteins such as claudins and occludin, in conjunction with pericytes, astrocytic end feet, basement membrane components, and regulatory signalling within the neurovascular unit. In the context of TBI, vascular shear and inflammatory signalling converge to enhance permeability, allowing the entry of serum proteins, ions, and circulating immune mediators into central nervous system (CNS) tissue (Bodnar et al., 2021; Chodobski et al., 2011). This process also alters endothelial activation

states, which regulate leukocyte adhesion and migration. A recent review focusing on BBB disruption following TBI underscores that barrier dysfunction serves as both a mechanism of acute pathology and a determinant of recovery biology, influencing edema, oxidative stress, and the exposure of neural tissue to peripheral inflammatory drivers (Bai & Ge, 2024).

Importantly, the disruption of the BBB is not merely an acute event. Persistent or recurrent barrier leakage has been proposed as a mechanism through which an initial injury may evolve into a prolonged inflammatory state, characterized by ongoing microglial activation and altered neurovascular signalling (Abrahamson & Ikonomovic, 2020; Glushakova et al., 2014). Recent research examining TBI in the context of neurodegeneration has highlighted BBB dysfunction as a plausible mediator linking prior trauma to increased susceptibility to later-life pathological trajectories, including chronic traumatic encephalopathy (CTE) and Alzheimer's disease (AD)-like mechanisms in vulnerable contexts (Johnson et al., 2013; Sun et al., 2025). Although not all individuals with TBI develop neurodegenerative diseases, the mechanistic overlap between chronic barrier dysfunction, persistent neuroinflammation, and synaptic vulnerability provides a compelling rationale for detailed investigation of BBB-linked immune processes (Sun et al., 2025). Barrier dysfunction is also directly relevant to immune cell entry. Endothelial activation enhances the expression of adhesion pathways and chemokines that facilitate the rolling, arrest, and transmigration of leukocytes (Man et al., 2007). Post-traumatic vulnerability is influenced by barrier systems beyond the traditional BBB, encompassing the blood-cerebrospinal fluid barrier, meningeal interfaces, and perivascular clearance pathways. These systems regulate the movement of cytokines, metabolites, antigens, and fluids between the brain and the peripheral regions (Bodnar et al., 2021; Mokbel et al., 2024). Experimental and translational research suggests that TBI can compromise glymphatic and meningeal lymphatic drainage, thereby diminishing the clearance of solutes and inflammatory substances to the deep cervical lymph nodes. This impairment may consequently prolong edema and neuroinflammatory signalling (Mokbel et al., 2024; Rasmussen et al., 2018). This thesis specifically examines molecular cues such as intercellular adhesion molecule-1 (Icam1) and integrin-linked pathways, integrin subunit beta 2 (Itgb2)-associated biology, alongside chemokines implicated in lymphocyte recruitment, c-x-c motif chemokine ligand 10 (Cxcl10), as candidate mechanisms enabling sustained adaptive immune accumulation in injured tissue.

1.3 Tissue injury, haemorrhage and antigen-dependent T cell memory after TBI

Mechanical brain trauma results in more than just neuronal loss. At the site of the lesion, rapid tissue deformation can shear axons, disrupt the neurovascular unit, damage the extracellular matrix, rupture microvessels, and cause contusional haemorrhage (Kurland et al., 2012; Logsdon et al., 2015). Haemorrhage holds immunological significance as blood-derived molecules infiltrate a compartment typically shielded from them (Kurland et al., 2012). Erythrocytes, platelets, plasma proteins, and coagulation factors introduce haemoglobin/haem, iron, fibrinogen, thrombin, and complement-active substrates into parenchymal, perivascular, and meningeal spaces (Maegele et al., 2017). These mediators can exacerbate oedema, oxidative stress, endothelial activation, and glial danger signalling, while TBI-associated coagulopathy may further affect haemorrhagic progression and microvascular dysfunction (Nakae et al., 2022). Consequently, the early post-traumatic immune response should be perceived as a reaction not only to damaged neural cells but also to a haemorrhagic, coagulation-active, and barrier-disrupted wound environment (Kurland et al., 2012; Maegele et al., 2017; Nakae et al., 2022).

Naïve T cells are lymphocytes that have not yet encountered antigens and continuously circulate through the bloodstream and secondary lymphoid organs in search of their specific antigen (Sun et al., 2023). A cognate antigen refers to the specific peptide-major histocompatibility complex (peptide-MHC) ligand recognized by a particular T cell receptor (TCR) (Obst, 2015). $CD8^+$ T cells typically recognize peptides presented on MHC class I molecules, whereas $CD4^+$ T cells recognize peptides presented on MHC class II molecules (Obst, 2015). The effective priming of naive T cells generally necessitates three coordinated signals from an antigen-presenting cell. Signal 1 involves the TCR's recognition of the cognate peptide-MHC. Signal 2 is co-stimulation, traditionally mediated through CD28 on the T cell binding to CD80/CD86 on the antigen-presenting cell. Signal 3 comprises inflammatory cytokines that influence the response's quality, such as Interleukin-12 (IL-12) and type I interferons promoting type 1/cytotoxic programs in suitable contexts (Curtsinger & Mescher, 2010; Obst, 2015). Collectively, these signals facilitate clonal expansion, effector differentiation, and the acquisition of migratory receptors, enabling activated T cells to exit the lymph node and infiltrate inflamed tissues (Curtsinger & Mescher, 2010; Obst, 2015; Sun et al., 2023).

The function of cognate antigens evolves as T cells transition from the priming phase to tissue residency (Schenkel et al., 2014; Steinbach et al., 2016). During the priming phase, the recognition of cognate peptide-MHC complexes determines which naive T cell clones undergo expansion (Jenkins & Moon, 2012). In contrast, the formation and maintenance of

tissue-resident memory T cells (T_{RM}) exhibit antigen requirements that are more context-dependent (Schenkel et al., 2014; Steinbach et al., 2016). In certain tissues and disease contexts, T_{RM} populations can persist with minimal ongoing antigen recognition (Curham et al., 2023; Kim & Shin, 2019; Maurice et al., 2021). Within the scope of this thesis, post-traumatic T_{RM} cells may comprise a combination of pre-existing memory cells recruited into an injury-conditioned niche and T cells primed against injury-derived or modified antigens. This framework elucidates why subsequent systemic inflammatory challenges can amplify brain T_{RM} responses even after the original mechanical injury has resolved macroscopically (Ayasoufi et al., 2023). It also provides a conceptual basis for distinguishing between continued trafficking and resident recall in the experimental chapters that follow **(Fig. 1.2)**.

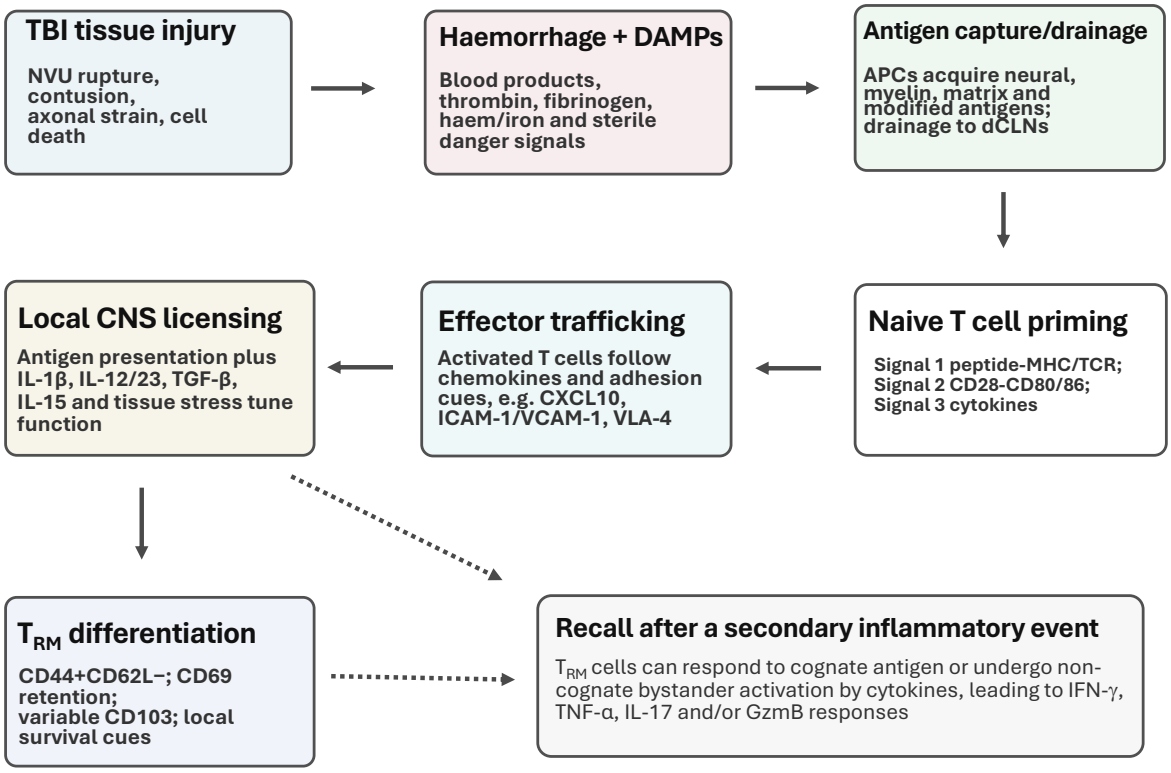

Figure 1.2. Proposed mechanism linking tissue injury, haemorrhage and T cell memory after TBI

1.4 Immune cells after TBI and the emergence of adaptive immune remodelling

Once the integrity of the BBB barrier is compromised, the post-traumatic immune response is influenced not only by brain-resident microglia and infiltrating myeloid cells but also by the progressive involvement of lymphoid compartments at the meninges, cervical lymph nodes, blood, and injured parenchyma (Bolte et al., 2020; Bouras et al., 2022). Mechanical injury also disrupts meningeal lymphatic drainage, and brain-relevant proteins can subsequently be

detected in deep cervical lymph nodes following trauma, indicating that CNS injury is communicated to peripheral immune organs rather than remaining spatially isolated (Bolte et al., 2020; Puhakka et al., 2023). Concurrently, transcriptional profiling of the meninges post-TBI reveals persistent immune remodelling, including inflammatory changes within adaptive immune cell populations, with these alterations being more sustained in aged animals compared to young mice (Bolte et al., 2020). Human studies similarly indicate that T cell biology is altered early after severe brain trauma, including transient reductions in cytotoxic T cell subsets and changes in activation-marker expression on regulatory and Th17-associated CD4 populations (Ditsch et al., 2022; Mrakovcic-Sutic et al., 2010). These observations suggest that T cells should not be regarded as generic late infiltrates, as distinct T cell subsets appear to carry different mechanistic implications post-injury (Abou-El-Hassan et al., 2023; Caplan et al., 2020; Jiang et al., 2024).

For instance, a $CD4^+$ T cell population has recently been shown to exacerbate secondary damage after TBI, and its entry into the injured brain is regulated by brain to cervical-lymph-node signalling, directly linking lymphatic communication to pathogenic T cell recruitment (Jiang et al., 2024). Simultaneously, $\gamma\delta$ T cell subsets are not uniformly deleterious, as Vγ4 cells promote interferon-γ (IFN-γ) and interleukin-17 (IL-17) associated neuroinflammation, whereas Vγ1 cells exert transforming growth factor beta (TGF-β) associated restraining effects on microglial activation and outcome (Abou-El-Hassan et al., 2023). Conversely, enhancement of regulatory T cell programs can modulate both central and peripheral inflammation post-TBI, reinforcing the view that some T cell responses may support repair rather than propagate tissue injury (Caplan et al., 2020). The adaptive consequences of TBI are also not necessarily short-lived, as moderate-to-severe human TBI can be followed by complex autoantibody responses that persist for years and are associated with worse outcomes (Needham et al., 2021). $CD8^+$ T cells are particularly significant in the context of post-traumatic pathology. Experimental TBI has been demonstrated to cause a sustained accumulation of granzyme B (GzmB)-expressing effector/memory $CD8^+$ T cells within the injured brain. Notably, a reduction in this $CD8^+$ response correlates with improved neurological outcomes and diminished myelin pathology (Daglas et al., 2019). Concurrently, acute TBI can provoke systemic dysfunction of both $CD4^+$ and $CD8^+$ T cells through sympathetic activation, with norepinephrine-mediated upregulation of programmed cell death protein 1 (PD-1) impairing T cell function (Yang et al., 2019). This suggests that trauma disrupts $CD8^+$ biology not only within the injured brain but also in the peripheral immune compartment (Yang et al., 2019). Accordingly, the key issue is not merely whether

lymphocytes are present post-TBI, but when specific subsets emerge, which compartments they occupy, and whether they are linked to injury propagation, immune regulation, or longer-term immune imprinting (Bolte et al., 2020; Jiang et al., 2024; Needham et al., 2019; Needham et al., 2021; Yang et al., 2019).

1.5 Neuroimmune remodelling after TBI as a measurement and inference problem

Post-traumatic inflammation is optimally conceptualized as a temporally structured biological response, characterized by shifts in dominant cellular contributors and effector signals across acute, subacute, and chronic phases. Consequently, mechanistic inquiries in neurotrauma often encounter limitations when anchored to a singular sampling day or readout, as these choices may inadvertently capture a transitional state rather than a stable phase. A primary methodological objective of this thesis is to establish an empirically grounded timetable of immune composition and effector programs following CCI, using a flow-cytometry pipeline optimized for viable brain mononuclear cell recovery and multi-parameter phenotyping. The resultant dataset was analyzed using dimensionality reduction and clustering techniques (UMAP and FlowSOM) across multiple timepoints post-injury to ascertain when specific innate and lymphoid programs become detectable at single-cell resolution. This approach enables the planning of future intervention studies to align with biologically determined periods rather than being dictated by convenience. Within this framework, the thesis prioritizes functional immune readouts capable of discerning which lymphoid subsets exhibit key cytokine signatures, as opposed to relying on bulk tissue cytokine measurements. A central example is the identification of IL-17 production as a property highly enriched in $\gamma\delta^+$ T cell compartments in the brain post-injury within the thesis, while IFN-γ signals are distributed across multiple T cell lineages. This subset resolution informs downstream mechanistic experiments in subsequent chapters.

1.6 Selection of mechanistic nodes: testing reprogramming rather than simple suppression

Given the redundancy of inflammatory pathways following TBI, a mere reduction in leukocyte count is insufficient to substantiate meaningful immunological control. Instead, this thesis focuses on the more rigorous inquiry of whether early innate perturbations can influence the subsequent quality of adaptive immune responses and the chronic state of resident myeloid cells. This perspective is consistent with the broader neurotrauma literature, which posits

that effective immune modulation likely necessitates precise targeting of pathways and timing, rather than indiscriminate immunosuppression (Hostiuc & Rusu, 2026).

The interleukin-1 (IL-1) axis serves as a translationally motivated reference point for this mechanistic strategy. In individuals with severe TBI, the recombinant IL-1 receptor antagonist, Anakinra, has been evaluated in a phase II randomized controlled trial with endpoints focused on safety and cerebral micro dialysis-based biomarker profiling, thereby supporting the feasibility and brain exposure as a foundation for biologically informed IL-1-directed studies (Helmy et al., 2014).

To examine how early innate states may affect later immune polarization, this thesis employs three classes of interventions positioned upstream or adjacent to IL-1-linked biology, attenuation of neutrophil-associated early effector pressure, depletion of c-c chemokine receptor type 2 (CCR2)-linked inflammatory monocytes, and pathway-level inflammasome inhibition. These perturbations are explicitly treated as mechanistic probes designed to assess whether early innate modification alters downstream T cell effector profiles and later microglial antigen-presentation-associated phenotypes, rather than merely changing the magnitude of early infiltration.

Given that antibody-based perturbations introduce interpretive constraints, including incomplete depletion, epitope masking, and waning efficacy over time, this thesis also explicitly addresses these limitations as part of causal inference. This is particularly pertinent for anti-Ly6G validation by flow cytometry and for MC-21 dosing windows, where the reliability of "null effects" at later time points may be confounded by technical performance rather than reflecting true biological disengagement.

Inflammasome inhibition is included as a complementary strategy as it targets a post-translational control layer that can influence IL-1 family cytokine maturation. Contemporary reviews of nod-, lrr- and pyrin domain-containing protein 3 (NLRP3) inflammasome biology in CNS disease provide a mechanistic rationale for this approach and support the use of selective inhibitors as tools for pathway-level testing in neuroinflammation contexts (Xu et al., 2025).

1.7 Brain tissue-resident memory T cells as a candidate chronic post-traumatic immune compartment

Tissue-resident memory T cells (T_{RM}) are long-lived, tissue-adapted memory T cells that remain prepared for rapid local recall responses rather than engaging in continuous

recirculation (Korn & Kallies, 2017). In the CNS, their significance is amplified due to the occurrence of local recall responses within a tissue characterized by limited regenerative capacity and a tightly regulated barrier architecture (Korn & Kallies, 2017). Experimental studies indicate that resident T cell pools are detectable in the naïve brain and can expand rapidly following neurological insults, with early expansion partially attributed to local proliferation rather than the delayed arrival of classical antigen-specific infiltrates (Ayasoufi et al., 2023). This makes T_{RM} biology conceptually relevant to TBI, as some of the late lymphoid burden post-injury may reflect persistence and adaptation within the CNS niche rather than mere ongoing recruitment from the bloodstream (Ayasoufi et al., 2023; Wojciechowski et al., 2020). Meningeal lymphatic drainage to the deep cervical lymph nodes provides a pathway through which trauma-derived antigens and inflammatory signals can influence adaptive immune priming, thereby linking local tissue damage to the evolution of tissue-associated memory compartments (Mokbel et al., 2024; Wojciechowski et al., 2020). T_{RM} cells are particularly relevant in secondary-hit paradigms, as reactivation of brain T_{RM} has been demonstrated to drive inflammatory myeloid influx, microglial activation, and BBB disruption even when peripheral T cell trafficking is experimentally constrained (Ayasoufi et al., 2023). Repeated or dysregulated T_{RM} reactivation has also been associated with cumulative microglial activation, proliferation, and prolonged production of neurotoxic mediators, supporting the notion that immune memory in the injured brain may contribute to chronic vulnerability rather than solely host defence (Prasad et al., 2023). Consequently, experimental strategies that combine intravascular labelling with trafficking blockade are particularly informative, as they help distinguish continued leukocyte entry from the recall activity of cells already established within CNS-associated niches (Mueller et al., 2013). This framework also provides the rationale for investigating whether post-traumatic immune amplification following a systemic challenge reflects renewed trafficking, resident recall, or a hybrid of both processes (Ayasoufi et al., 2023).

1.8 Secondary systemic inflammation and resident adaptive immunity

In the realm of clinical neurotrauma care, the management of infections, particularly those affecting the respiratory system, presents significant challenges. Experimental studies substantiate the hypothesis that systemic infections can exacerbate outcomes when superimposed on an already injured brain. This underscores the rationale for employing controlled "secondary insult" paradigms to investigate whether a pre-existing remodelled

neuroimmune environment exhibits disproportionate amplification in response to subsequent systemic inflammatory events (Gandasasmita et al., 2024).

In this thesis, systemic inflammation is induced through the administration of lipopolysaccharide (LPS), a purified endotoxin derived from the outer membrane of Gram-negative bacteria and a recognized agonist of the toll-like receptor 4/myeloid differentiation factor-2 (TLR4/MD-2) receptor complex (Nagai et al., 2002). In mouse models, peripheral administration of LPS is extensively utilized as a controlled model of systemic inflammation due to its ability to elicit a consistent innate immune response and sickness behaviour without the introduction of live infection (Biesmans et al., 2013). Notably, the biological interpretation is dependent upon the dosage and administration schedule, repeated low-dose regimens, such as 0.5 mg/kg administered daily, are used to investigate priming, training, or tolerance, whereas a single acute intraperitoneal (i.p.) challenge within this dosage range is interpreted as a transient systemic inflammatory insult rather than a priming paradigm (Kim et al., 2024). Consequently, LPS is utilized here as a standardized proof-of-concept inflammatory challenge to assess whether a peripheral inflammatory surge can preferentially engage injury-conditioned immune compartments in the brain, rather than serving as a comprehensive surrogate for pneumonia or sepsis.

A central focus of the thesis is the emergence of T_{RM} compartments following CCI and their capacity for rapid reactivation. This focus is grounded in evidence that resident T cells exist in brain tissue, can expand locally after neurological insults, and can exhibit rapid activation features upon challenge (Ayasoufi et al., 2023). These principles are supported by mechanistic studies utilizing intravascular labelling and complementary approaches to differentiate circulating from tissue-localized compartments, as well as by research demonstrating peripherally induced brain T_{RM} with rapid effector reactivation potential (Ayasoufi et al., 2023).

This thesis implements intravascular anti-CD45 labelling prior to harvest to exclude blood-associated leukocytes and strengthen inference about tissue-associated T_{RM} populations, subsequently quantifying resident-phenotype compartments using residency-associated marker combinations. This enables a direct examination of whether systemic inflammation preferentially amplifies activation and proliferation signatures within the injured hemisphere's tissue-associated T cell compartment. To distinguish recruitment-driven effects from intrinsic responsiveness of tissue-associated compartments, this thesis additionally employs α4-integrin (CD49d/VLA-4) blockade during the systemic challenge framework. This choice is supported by evidence that anti-CD49d treatment can improve

survival and attenuate neurocognitive deficits in aged mice after TBI, providing an experimental means to investigate the extent to which the secondary-hit response depends on continued trafficking versus reactivation of cells already positioned within CNS-associated niches (Chen et al., 2024).

1.9 Thesis objectives and hypotheses

The preceding literature inspires a series of interconnected questions that are explored throughout the experimental chapters of this thesis.

Objective 1: To define the temporal dynamics of innate immune and T cell responses after TBI across acute, subacute, and chronic phases.

Hypothesis 1: TBI induces a sequential neuroimmune response, characterized by an initial activation of the innate immune system, followed by the sustained presence of T cells, ultimately leading to a long-term alteration of the immune environment within the injured brain.

Objective 2: To determine whether time-resolved immune profiling identifies therapeutic windows for modulating neuroinflammation and improving recovery after TBI.

Hypothesis 2: The primary early cellular source of IL-1β promotes subsequent effector T cell responses particularly IL-17 and IFN-γ secreting T cells, which subsequently contribute to post-traumatic behavioural dysfunction.

Objective 3: To characterize and identify the role of T_{RM} cells after TBI in both acute and chronic phases and administer LPS post-injury to evaluate whether immune amplification in the brain is driven by peripheral T cell trafficking (CD49d/VLA-4) or resident reactivity.

Hypothesis 3: TBI establishes a persistent brain T_{RM} compartment, which predisposes the injured brain to subsequent systemic inflammatory challenges. The degree to which LPS-induced amplification is mitigated by CD49d/VLA-4 blockade will differentiate between effects dependent on peripheral trafficking and those resulting from resident immune reactivation.

Chapter 2

Materials and methods

2.1 Materials

2.1.1Media, buffers and solutions

Complete Roswell Park Memorial Institute (cRPMI) medium

RPMI-1640 medium (Sigma-Aldrich)

10% heat inactivated fetal calf serum (FCS; Biosera)

100 mM L-glutamine (Sigma-Aldrich)

100 µg/mL penicillin/streptomycin (Gibco)

50 µM β-mercaptoethanol (Thermo Fisher)

Insulin-Transferrin-Selenium-G Supplement (100X) (Biosciences)

FACS buffer

500 mL 1X PBS (Sigma-Aldrich) + 2% FBS (Biosera)

Phosphate buffered saline (PBS) 10X

400 g Sodium chloride (NaCl; 1.4 M; Sigma-Aldrich)

58 g Sodium hydrogen phosphate (Na2HPO4; 0.08 M)

10 g Potassium di-hydrogen phosphate (KH2PO4; 0.01 M)

10 g Potassium chloride (KCL; 0.03 M)

Stock isotonic Percoll 90%

Percoll (GE Healthcare) 10% + 10X PBS

2.1.2 General reagents:

Reagent	Supplier
Bovine serum albumin (BSA)	Sigma-Aldrich
DMSO	Sigma-Aldrich
EDTA	Sigma-Aldrich
Ethanol	Sigma-Aldrich
Fetal bovine serum (FBS)	Sigma-Aldrich
DNase Type 1	Sigma-Aldrich

NA/LE in vitro antibodies:

Reagent	Clone	Concentration	Application	Supplier
Anti-CD3ε	145-2C11	1 µg/mL	Soluble	BD Pharmigen
Anti-CD28	37.51	2 µg/mL	Soluble	BD Pharmigen

Flow cytometry reagents:

Reagent	Concentration	Product code	Supplier
Brefeldin A	5 µg/mL	B7651	Sigma-Aldrich
Ionomycin	0.5µg/mL	I3909	Sigma-Aldrich
Collagenase-D	1mg/ml	11088866001	Sigma-Aldrich
Deoxyribonuclease I (DNAse I)	20 U/ml	D4263	Sigma-Aldrich
Phorbol 12-myristate 13 Acetate (PMA)	20ng/mL	P1585	Sigma-Aldrich
FoxP3 Transcription Factor Staining Buffer Set		00-5523-00	eBioscience
One Comp eBeads		01-1111-42	eBioscience
ARC TM Amine Reactive Compensation Bead kit		A10346	Invitrogen

FACS antibodies:

Antibody	Fluorochrome	Clone	Product code	Dilution	Supplier
CD11b	APC-eF780/PE-cy7	M1/70	47011282	1:400	eBioscience
CD3ε	BB700	145-2C11	566494	1:200	Biolegend
CD4	BV605/PE-Cy5	RM4-5	15-0041-82	1:200	Biolegend
CD8	APC	53-6.7	17-0081-82	1:200	eBioscience
CD44	PE-Cy5	IM7	103054		Biolegend
CD45	BV785/BV750	30-F11	103149	1:400	Biolegend
CD69	AF647	H1.2F3	104518	1:200	eBioscience
IFN-γ	PE-CF594	XMG1.2	562303	1:200	BD Biosciences
IL-17A	Pacific Blue	TC11-18H10	506918	1:200	Biolegend
NK1.1	AF700	PK136	56-5941-82	1:200	eBioscience
TCR γδ	PerCPeF710	GL3	46-5711-82	1:200	eBioscience
CD62L	PE-Cy5	MEL-14	104410	1:200	Biolegend
Granzyme B	FITC	NGZB	11889882	1:200	eBioscience
CD103	BV711	W19396D	BL-121435	1:200	Biolegend
IL-1β	PE-Cy7	NJTEN3	25-7114-80	1:200	eBioscience

MHC-II	BV785	M5/114.5.2	107645	1:200	Biolegend
CD68	BV421	FA-11	137017	1:200	Biolegend
CD19	BV570	6D5	115535	1:200	Biolegend
Ly6G	BV650	1A8	BL-127641	1:400	Biolegend
Ly6C	BV605	AL-21	563011	1:400	Biolegend
TNF-α	BV711	MP6-XT22	506349	1:200	Biolegend
Fc block		2.4G2	553141	1:200	BD Biosciences
Live/Dead	Aqua		L34957	1:600	eBioscience

Reagents for qPCR:

CXCL1:

forward 5'-CACCCAAACCGAAGTCATAGCC-3',

reverse 5'-AAGCCAGCGTTCACCAGACA-3'

CCL2: forward 5'-GTTGGCTCAGCCAGATGC-3',

reverse 5'-AGCCTACTCATTGGGATCATCTTG-3';

CXCL10: forward 5'-TTTCTGCCTCATCCTGCT-3',

reverse 5'-TCCCTATGGCCCTCATTCT-3';

IL-1β: forward 5'-GAGGACATGAGCACCTTCTTT-3',

reverse 5'-GCCTGTAGTGCAGTTGTCTAA-3';

ICAM-1: forward 5'-AAACCAGACCCTGGAACTGCAC-3',

reverse 5'-GCCTGGCATTTCAGAGTCTGCT-3';

ITGB2: forward 5'-CTTTCCGAGAGCAACATCCAGC-3',

reverse 5'-GTTGCTGGAGTCGTCAGACAGT-3';

GAPDH: forward 5'-TGGTGAAGGTCGGTGTGAAC-3',

reverse 5'-TGAATTTGCCGTGAGTGGAG-3'

βactin: forward 5'-GCC TTC CTT CTT GGG TAT GG -3',

reverse 5'- AGC ACT GTG TTG GCA TAG AG -3'

2.2 Methods

2.2.1 Mice

In vivo studies were conducted using adult male (24-27g) C57BL/6J mice, aged 10–12 weeks, bred by the Comparative Medicine Unit (CMU) at Trinity College Dublin. The mice were housed in groups of five per cage under specific pathogen-free conditions, with a 12-hour light/dark cycle, a temperature of 24 ± 1°C, and 55 ± 5% humidity throughout the experimental period. They had ad libitum access to food and water. The maintenance of the mice adhered to the guidelines and regulations of the Health Products Regulatory Authority (HPRA). All surgical procedures were conducted in accordance with protocols approved by the Trinity College Dublin Animal Research Ethics Committee (Protocol Number: AE19136/P138). The design of all animal experiments in this study followed the Animals in Research: Reporting In vivo Experiments (ARRIVE) guidelines, which emphasize the 3Rs: Refinement, Reduction, and Replacement (Kilkenny et al., 2010). The recently updated ARRIVE guidelines 2.0 include a 21-item checklist that facilitates high-quality reporting of animal research and ensures the reproducibility of experiments (Percie du Sert et al., 2020).

2.2.2 Controlled cortical impact (CCI)

TBI in mice was induced using a CCI device (RWD; 6809II) **(Fig. 2.1)**. C57BL/6J mice were anesthetized in a chamber containing a mixture of 0.75% oxygen flow rate (L/min) and isoflurane (induction at 3% and maintenance at 1.5%). The depth of anaesthesia was assessed prior to the surgical procedure by monitoring respiration rate and pedal withdrawal reflexes. Body temperature was maintained at 37°C. Before securing the mouse's head in a stereotaxic frame, the incision site was shaved, and bupivacaine (0.083%) was administered subcutaneously near the incision site. The researcher achieved sterility with assistance from a non-sterile assistant, utilizing sterile gloves and tools. A sterile sheet was placed over the mouse, and the researcher maintained sterility throughout the procedure. The surgical site was cleaned with ethanol and chlorhexidine, and a 10 mm midline incision was made over the skull. After reflecting the skin and fascia using bulldog clamps (#18050-50; Fine Science Tools), a 5 mm craniotomy was performed on the central aspect of the left parietal bone using a 5 mm drill bit. The stage holding the mouse was then adjusted so that the impactor tip of the injury device, after extending the impactor to its full stroke distance (44 mm), was positioned on the surface of the exposed dura, ensuring it was as flat as possible. The device was then reset to impact the cortical surface. A moderate-severe level injury was induced

using an impactor velocity of 3 m/sec, a deformation depth of 1.5 mm, and a dwell time of 0.18 s. Following CCI, the time of impact and observations on swelling or bleeding were recorded. Bupivacaine (0.083%; 100 µl) was administered at the injury site, the incision was closed with interrupted 5-0 silk sutures, and saline (1 ml) was administered subcutaneously on the dorsal side of the mouse. After withdrawal of anaesthesia, the animal was placed in a heated cage for 60 minutes post-injury to maintain normal body temperature and then returned to a clean home cage. During the 60 minutes, the mouse was monitored, and the righting reflex was noted. Sham animals underwent the same procedure as injured mice, except for craniotomy and cortical impact. The sutures were cleaned daily for three consecutive days with saline and chlorhexidine, and animals were monitored throughout the study using HPRA-approved score sheets. Weight, incision checks or re-suturing requirements, pain assessment scores, whether the mouse was bright, alert, and responsive, and any additional comments were documented on these recovery sheets, as shown in **Fig. 2.2**. If weight loss exceeded 11-19%, 1 ml saline was provided subcutaneously, and the designated veterinarian was contacted.

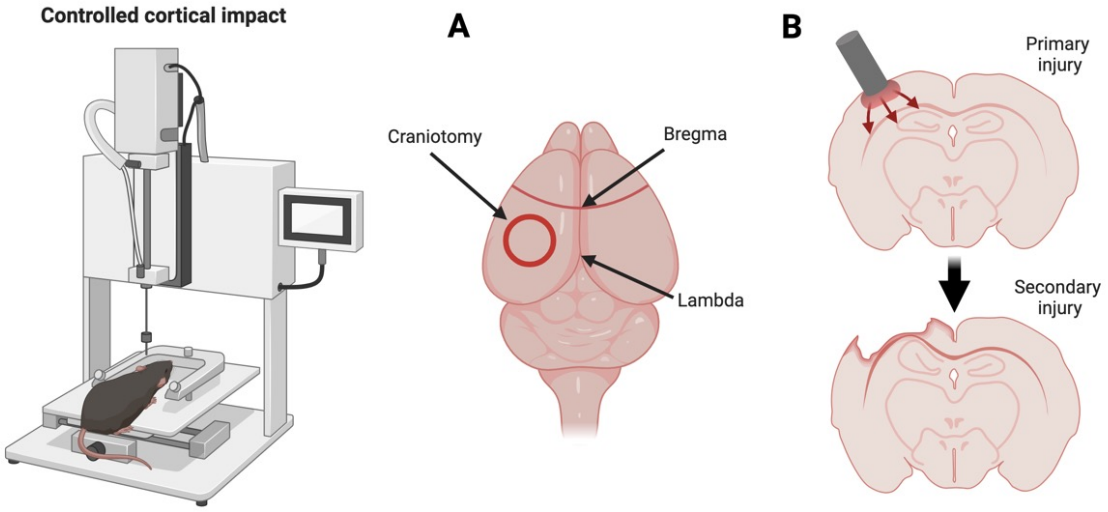

Fig. 2.1: Controlled Cortical Impact (CCI) delivers a focal contusion to the left parietal lobe. This image was created using BioRender

PI: David Loane Researcher: Sahil Threja HPRA #: I641 Species/Strain: C57BL/6J

Procedure date: 30.10.2025 Location: B3. 135 Animal ID: _____

Date	Weight (g)	% Weight	BAR** (Y/N)	Incision Check (√)	PID	PAS*** (0-4)	Re-Suture Required/#	Comments	Initals
2025-10-31 (CCI)					0				
2025-11-01					1				
2025-11-02					2				
2025-11-03					3				

1) Weight Loss: 0-10%, observe animal closely; 11-19%, provide animal fluids and contact DV/ACUO; >20%, humanely euthanize animal & report.
2) BAR (Bright, Alert, Responsive): Yes = no action, No = observe animal closely and contact DV/ACUO.
3) Incision site: check surgical site for signs of swelling/redness/discharge (√ to confirm).
4) Pain Assessment Scoring***:
0 - Normal behavior
1 - Mild behavior and physiologcial changes (slightly depressed behavior, minor guarding of incision site): observe animal closely.
2 - Moderate pain (includes score of 1 plus swelling/redness/discharge at surgical site, reluctance to move, guarding with vocalization or aggression): observe animal closely and contact DV/ACUO for advice.
3 - Severe pain/distress (includes scores of 1 &2 plus immobility, dehiscence of incision, profound dehydration/weight loss): humanely euthanize and report.
4 - Moribund: humanely euthanize and report.

Fig. 2.2: Animal welfare monitoring form for the CCI procedure. Example of recovery sheet for a 3 DPI study. Weight loss, incision site, pain assessment scoring and any additional comments such as hydration level of the animal is all monitored daily until the completion of the study

2.2.3 Antibody and drug treatment

Mice were randomly assigned to the different experimental groups prior to the onset of the study ensuring there was no age, weight or cage effect.

A double antibody-based strategy was used for neutrophil depletion (Boivin et al., 2020). Mice were administered intraperitoneal injections (i.p.) of anti-Ly6G (clone 1A8; 100 µg/mouse; Assay Genie, Cat# IVMB0025) in conjunction with a secondary antibody anti-rat kappa immunoglobulin light chain (clone MAR18.5; 100 µg/mouse; Assay Genie, Cat# IVMB0305). For endpoints at 3 days post-injury (dpi), antibodies were administered on day -1 (pre-injury), and at 1 and 2 dpi. For endpoints at 10 dpi, an additional dose was administered at 3 dpi. Control animals received matched isotype controls for anti-Ly6G and anti-rat kappa immunoglobulin light chain (mouse IgG1, kappa and rat IgG2a, kappa; Assay Genie, Cat# IVMB0198).

For depletion of $CCR2^{+}$ monocytes, mice were administered i.p. anti-CCR2 monoclonal antibody (MC-21; 50 µg/mouse; (Mack et al., 2001) on day -1 and 1 dpi for the 3 dpi endpoint studies, with an additional dose at 3 dpi for the 10 dpi endpoint studies. Control animals received matched isotype rat IgG2b (Assay Genie, Cat# IVMB0200).

For NLRP3 inflammasome inhibition, MCC950 (10 mg/kg; MedChemExpress, Cat# HY-12815A) was administered i.p. beginning 2 h post-injury, followed by repeat doses at 12, 24, and 48 h post-injury for the 3 dpi endpoint studies; dosing was continued every other day beyond 48 h for the 10 dpi endpoint studies. Control animals received vehicle (saline; i.p.).

For blockade of leukocyte trafficking during the systemic challenge paradigm, mice received a CD49d (VLA-4) blocking antibody (αCD49d) (clone PS/2; 200 µg/mouse; Assay Genie, Cat# IVMB0276) or matched isotype mouse IgG2b (Assay Genie, Cat# IVMB0190) in conjunction with systemic LPS (0.5 mg/kg; i.p.), with αCD49d administered at 9 and 13 days post-injury, corresponding to 1 day prior to and 3 days following LPS administration.

2.2.4 Intravascular CD45 labelling

To differentiate leukocytes exposed to blood from non-intravascular cell populations at the time of tissue collection, an intravenous injection (i.v.) of a fluorochrome-conjugated anti-mouse CD45 antibody ([30-F11, biolegend, 103149], [3.5µg] in [200µl] sterile PBS) was administered to mice via the tail vein 10 mins before euthanasia. After 10 mins, the mice were deeply anesthetized, and their brains were harvested for subsequent processing and flow cytometric analysis as detailed below. During analysis, cells that tested positive for the

intravenously administered CD45 were classified as intravascular or blood-exposed, whereas CD45 i.v. negative cells isolated from brain tissue were classified as non-intravascular at the time of collection.

2.2.5 Isolation of mononuclear cells from the CNS

Brain processing using enzymatic digestion:

Mice were humanely euthanized via isoflurane overdose and subsequently perfused through the left ventricle with 30 mL of ice-cold PBS prior to brain extraction. The cerebral hemisphere, specifically the olfactory bulb and cerebellum, was excised from the ipsilateral side for further processing. This tissue was sectioned into 1 mm portions and placed into a tube containing 1 mL of cRPMI solution. DNAse and collagenase were added to a separate tube containing 1 mL RPMI (10 µL each from 1 mg/mL stocks), and the tubes were incubated at 37°C for 30 minutes; on a shaker (Incu-Shaker mini, Benchmark Scientific). The resulting homogenate was filtered through a 70 µm cell strainer to obtain a cellular homogenate. This brain homogenate was re-suspended in 5 mL of 40% isotonic Percoll in cRPMI and layered over 5 mL of 70% Percoll in PBS. The Percoll gradients were centrifuged at 1600 rpm for 20 minutes at room temperature with the centrifuge brake disengaged. The upper myelin layer was removed and discarded, and mononuclear cells were collected from the interface of the Percoll gradient. These cells were washed in cRPMI and either immediately stained or stimulated with PMA, ionomycin, and Brefeldin A for 4 hours at 37°C prior to intracellular cytokine staining.

Brain processing using steel beads:

The ipsilateral hemisphere of the brain was meticulously transferred into a 2 ml centrifuge tube, which had been preloaded with a 50 mm steel bead (Qiagen #69989). Subsequently, the sample was subjected to vibrational agitation using the (QIAGEN Tissue Lyser Retsch MM300 Mixer). The centrifuge tubes were securely positioned within the shaker apparatus, and the shaking protocol was initiated, set for a duration of 5 minutes at a speed of 1250 rpm. Finally, the percoll steps were executed as previously described.

Brain processing using dounce homogenization:

The ipsilateral hemisphere of the brain was meticulously transferred to a 7 ml glass Dounce homogenizer (Merck # D9063) containing 4 ml of cRPMI. The tissue was then homogenized using five strokes with a loose pestle, followed by five strokes with a tight pestle. The resulting

homogenate was subsequently passed through a 70 µm cell strainer and centrifuged at 1350 rpm for 5 minutes. Finally, the Percoll gradient steps were executed as previously described.

Harvesting and processing of meninges:

Mice were humanely euthanized via isoflurane overdose. Where specified, intravascular CD45 labelling was conducted through the intravenous administration of a fluorochrome-conjugated anti-mouse CD45 antibody 10 minutes prior to euthanasia. This procedure was employed to differentiate vascularly accessible leukocytes from non-intravascular cell populations at the time of tissue collection. Post-euthanasia, the scalp was excised, and the skull was exposed. The calvarium was opened using fine scissors and forceps, and the cranial meninges were gently dissected from the inner surface of the skull, with care taken to minimize contamination with adherent brain tissue or bone fragments. The isolated meningeal tissue was then transferred into cRPMI on ice for subsequent processing. DNAse and collagenase were then added to the same tube containing 1 mL RPMI (10 µL each from 1 mg/mL stocks), and the tubes were incubated at 37°C for 30 minutes on a shaker (Incu-Shaker mini, Benchmark Scientific). The meninges were then filtered through a 70 µm cell strainer to obtain a cellular homogenate. The resulting homogenate was then processed for flow cytometric analysis as described below.

2.2.6 Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)

2.2.6.1 RNA extraction:

Hippocampal tissue samples from the ipsilateral cortex of mice were rapidly frozen using CellPathTM Cryospray Freezer Spray and subsequently stored at -80°C until RNA extraction was performed utilizing the RNeasy Mini kit (EasyPure® RNA Kit, Cliniscience). The tissue was thawed, weighed, and placed into a 2 ml RNase-free microcentrifuge tube containing 350 µl of Buffer RLT with β-mercaptoethanol (1:100) and a single stainless steel bead (5 mm; Qiagen). Tissue lysis was conducted using a Tissue Lyser Retsch MM300 Mixer (Qiagen) for 5 minutes at 27.5 Hz, with the adaptor rotated midway through the process. Following homogenization, the bead was removed, and the tissue was centrifuged at maximum speed for 3 minutes. The supernatant was carefully transferred to a new 1.5 ml RNase-free microcentrifuge tube. An equal volume (350 µl) of 70% ethanol was added to the lysates and mixed by pipetting. Up to 700 µl of the sample was then transferred to a spin column placed in a 2 ml collection tube and centrifuged (8,000g for 15 seconds). The flow-through was discarded, and 700 µl of Buffer RW1 was added to the spin column, followed by centrifugation (8,000g for 15 seconds). The flow-through was again discarded, and 500 µl of

Buffer RPE was added to the column and centrifuged (8,000g for 15 seconds). A second addition of 500 µl Buffer RPE was made, followed by centrifugation (8,000g for 2 minutes) to further wash the membrane. The extended centrifugation ensured the complete evaporation of ethanol, preventing its carryover during RNA elution. Once ethanol was fully evaporated, the spin column was placed in a new 1.5 ml collection tube, and the RNA pellet was resuspended in 40 µl of RNase-free H2O. This was centrifuged (8,000g for 1 minute) to elute RNA, a process repeated twice to enhance RNA yield. The RNA concentration in each sample was quantified using NanoDrop software (Applied Biosystems), and the concentrations (ng/µl) were normalized to the sample with the lowest RNA concentration. The purified RNA was stored at -80°C until conversion to complementary DNA (cDNA).

2.2.6.2 cDNA synthesis:

RNA samples were reverse transcribed into complementary DNA (cDNA) utilizing the Applied biosystems cDNA Synthesis Kit, following the manufacturer's instructions. The synthesis of cDNA was conducted using a DNA Engine Dyas Cycler under the conditions specified in table below.

Step 1	Step 2	Step 3	Step 4	Step 5
Temperature	25^0C	37^0C	85^0C	4^0C
Time	10 minutes	120 minutes	5 minutes	Hold

2.2.6.3 Real time qPCR and analysis:

RT-qPCR amplification was conducted using the 7500 Fast Real-Time PCR System with SYBR Green PCR Master Mix (CliniScience, Cat# Q711-03) and the corresponding primers (as mentioned in the reagents section for RT-qPCR). Gene expression levels were normalized to the endogenous control GAPDH/β-actin mRNA level and are presented as fold change relative to the sham group

2.2.7 Flow Cytometry

Mice were humanely euthanized via isoflurane overdose and were perfused through the left ventricle with 30 ml of cold PBS prior to brain extraction. The ipsilateral cortex, excluding the olfactory bulb and cerebellum, was sectioned and collected into 1 ml of cRPMI. DNase and Collagenase (1mg/ml) were added to the tubes and incubated at 37°C for 1 h on a shaker (Incu-Shaker mini, Benchmark Scientific). The homogenate was passed through a 70 µm cell

strainer, supplemented with cRPMI, centrifuged, and resuspended in 40% Percoll before being layered over 70% Percoll and centrifuged without the brake (600 x g; 20 min, 4°C). The upper myelin layer was discarded, and the isolated mononuclear cells were collected from the interface of the Percoll gradient. Cells were washed with cRPMI and plated on a 96-well V-plate, then immediately stained for flow cytometry. Following incubation with Brefeldin A (5 µg/ml), PMA (20 ng/ml) and Ionomycin (0.5 µg/ml) for 3h at 37°C, cells were centrifuged and stained with Live/Dead (10min; dark, room temperature). Cells were washed with FACS buffer and resuspended in a master mix of mouse Fc Block and surface fluor-conjugated antibodies as mentioned in the reagents section. Antibodies used in chapter- 3 & 4 are listed here (CD19-BV570, Ly6C-BV605, Ly6G-BV650, CD45-BV750 or BV785, MHC-II-BV785, CD3e-BB700, TCR$\gamma\delta$-PerCP-ef710, CD4-PE-Cy-5, CD8-APC, NK1.1-AF700, and CD11b-PE-Cy7 or APCeF780), antibodies used in chapter 5 are (CD19-BV570, CD4-BV605, MHC-II-BV785, CD103-BV711, CD3e-BB700, TCR$\gamma\delta$-PerCP-ef710, CD45-PE, CD44-AF594, CD62L-PE-Cy5,

CD11b-PE-Cy7, CD69-AF647, and CD8-APCeF780) Following a wash step, cells underwent intracellular staining by adding Fixation/Permeabilization working solution and incubated for 20 min at room temperature. Cells were washed twice with 1X Permeabilization buffer and centrifuged (1350 RPM; 5 min, 4°C). The pelleted cells were resuspended in the intracellular antibody master mix, containing IL-17A-Pacific blue, TNF-α-BV711 or BV650, Granzyme-B-FITC, IFN-γ-PE-CF594 or APC, and IL-1β-PE-Cy7 in 1X Permeabilization buffer and incubated overnight at 4°C (in dark). Cells were washed twice in 1X Permeabilization buffer and finally washed in FACS buffer for analysis. All flow cytometric data were acquired using Cytek Aurora Spectral flow cytometry with Spectro Flo software. Compensation was calculated using single-stained cells. Cell-specific fluorescence minus one (FMO) controls were employed for gating strategies, and flow cytometric analysis was performed using FloJo software (TreeStar Inc.), where UMAP (Uniform Manifold Approximation and Projection) was applied to concatenated, down sampled live singlet events using the FlowJo UMAP plugin and a marker set restricted to phenotypic channels of interest (excluding FSC/SSC, time, and viability channels). UMAP settings (e.g., number of nearest neighbours and minimum distance) were held constant across samples within an analysis to enable direct comparison of embeddings. Cell populations were identified using the gating strategy shown below in **Fig 2.3 (Chapter 3 & 4)** and **Fig 2.4 (Chapter 5)**. The absolute number of cells was calculated based on the frequency of the total parent population.

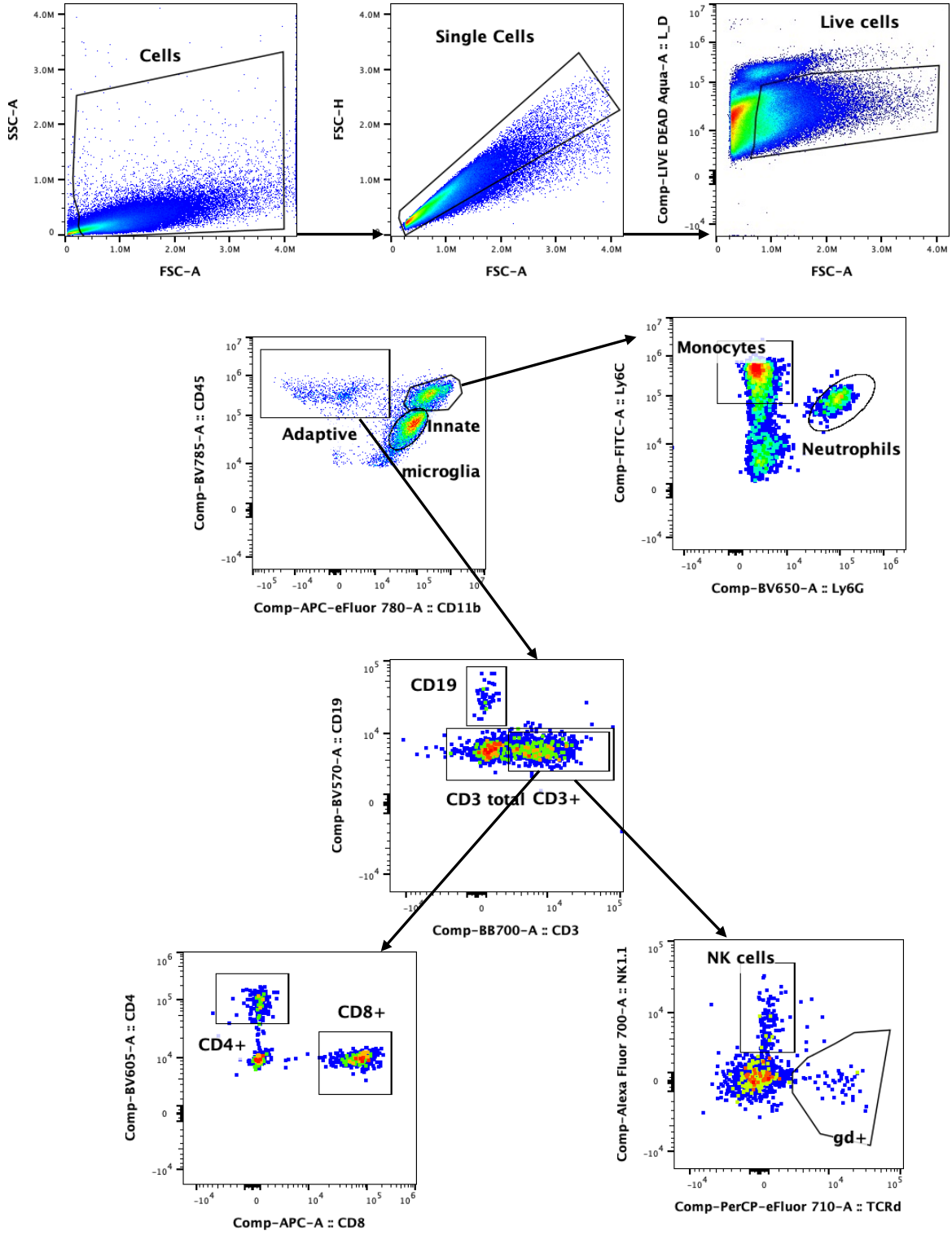

Fig. 2.3: Gating strategy for infiltrating innate and adaptive immune cells in the brain following TBI

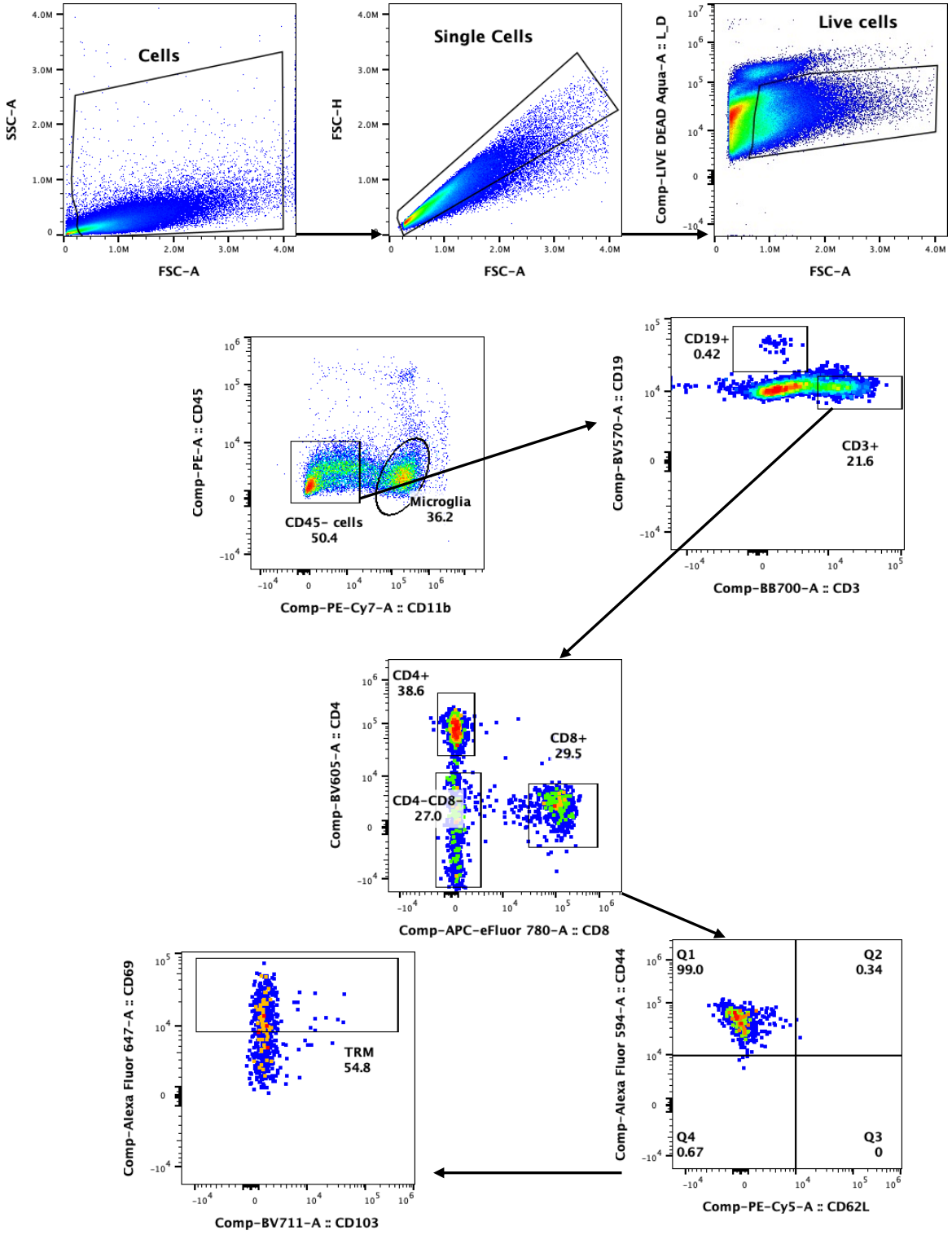

Fig. 2.4: Gating strategy for T_{RM} cells in the brain following TBI

2.2.8 Neuro-behavioural testing

Researchers conducted a handling protocol with mice for 5 minutes per day over a period of 14 days prior to the commencement of experiments. This procedure was implemented to mitigate stress and anxiety by familiarizing the mice with handling. Before initiating all tests, the mice were allowed an acclimatization period of 30 to 60 minutes in the behaviour room. The cage lids were replaced with ones equipped with a water bottle to ensure the mice had access to water during extended testing and habituation periods. A separate holding cage, containing food and water, was designated for each experimental cage to prevent the reintroduction of tested mice to untested ones, as the former may experience stress that could affect the latter. All apparatus were sanitized with water and 70% ethanol prior to testing, between animals, and after testing. Appropriate lighting was maintained at 35 lux, as measured by a luminometer (Extech LT300 Light Meter, Amazon), to ensure uniform illumination in the room and to facilitate clear video recordings when necessary. A white noise machine was also activated. By maintaining consistent lighting and noise levels (45 dB, measured using a decibel meter, Extech 407730 Digital Sound Level Meter) throughout all tests and for all animals, these variables were controlled to prevent interference with the analysis. Mice were video-tracked during all behavioural tests using ANY-maze software (Stoelting Company, Europe). The neurobehavioral tests included the Beam Walk, 2-trial Y-maze, Novel Object Recognition (NOR), Rotarod, and Simple Neuroassessment of Asymmetric Impairment (SNAP). For precise behavioural outlines according to the experiment, refer to the individual chapters.

2.2.8.1 Beam walk:

The Beam walk test was employed to assess the fine motor coordination and balance of mice, as described by (Zhao et al., 2012). The apparatus consisted of a narrow beam, measuring 5-6 mm in width and 1300 mm in length, suspended 30-40 cm above foam padding, with an escape house positioned at the beam's end. The protocol required three consecutive training days prior to testing. On day -3, each mouse was placed on the beam near the escape house and guided towards it. The mouse was permitted to remain in the house for a few minutes to recognize it as a safe location. Gradually, the mouse was positioned further away to learn to traverse the beam towards the house. Ultimately, the mouse began at the opposite end of the beam and learned to navigate from the start of the beam to the house, as illustrated in **Fig. 2.5**. At this stage, the number of foot-faults in a total of 50 steps was recorded. A foot-fault was defined as an instance where the mouse's right

hind limb slipped off the beam or when the lower body was not elevated, resulting in the mouse dragging itself to the house. This indicated an inability to utilize the right hind limb, thus constituting a foot-fault. By day -1, all experimental mice exhibited fewer than 10 foot-faults; those that did not meet this criterion were excluded from the study. All tests were recorded for subsequent analysis by an independent researcher blinded to the treatment groups. The image below illustrates a representative example from a mouse demonstrating both a "hit" and a "miss" score on the beam walk test. The red circle highlights the right hind limb either successfully stepping on the beam, as depicted in the upper panel, or slipping off the beam, as shown in the lower panel **Fig. 2.6**. To summarize the post-injury performance throughout the recovery period, the area-under-the-curve (AUC) was computed for each animal from day 1 onwards using the trapezoidal rule

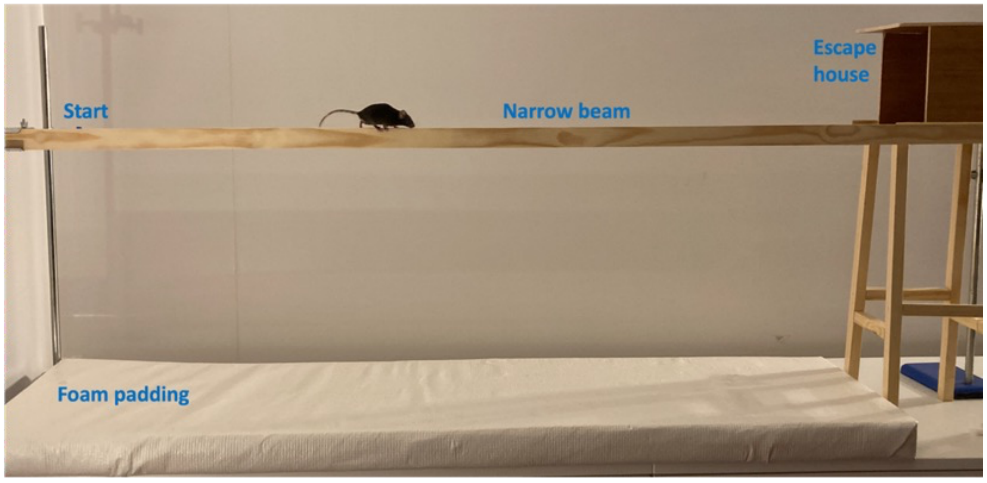

Fig. 2.5: The beam walk apparatus comprised of a narrow beam with an escape house at the end, suspended over foam padding which assessed fine motor coordination longitudinally

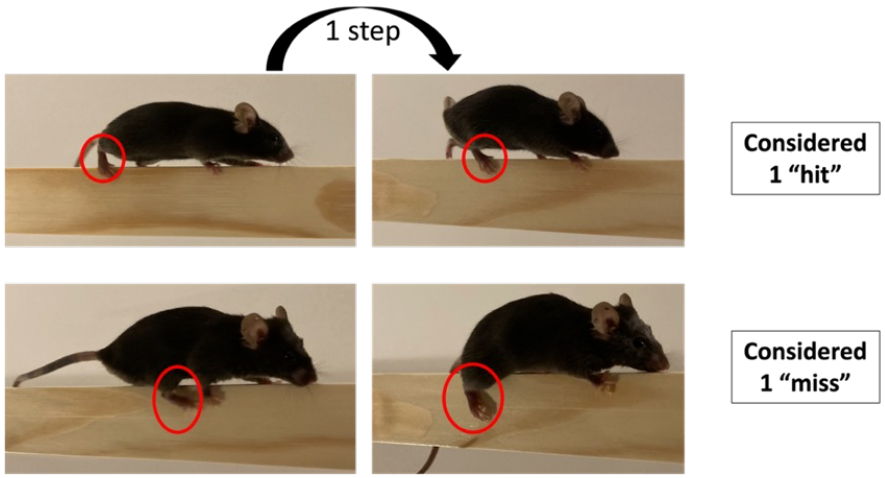

Fig. 2.6: Visual representation of a "hit" and "miss" score on the beam walk

2.2.8.2 2 Trail Y maze:

The two-trial Y-maze test was employed to assess spatial memory, as described by (Di Sapia et al., 2021). The experimental setup consisted of a Y-maze arena with dimensions of 5 cm in width, 36 cm in length, and 10 cm in height, positioned on a folding table. A transparent Plexiglas cover was used to prevent the mice from escaping the arena during the test. Visual cues, in the form of distinct shapes, were strategically placed within the Y-maze to enable the mice to distinguish between the arms. Bedding was laid on the floor of the maze to mitigate stress and enhance contrast for tracking video analysis. The test comprised two trials. In the initial trial, one arm, selected at random, was obstructed with a guillotine. The mouse was introduced into a randomly chosen starting arm, oriented towards the wall. Both the blocked and starting arms were randomized across all mice prior to the commencement of the experiment. The mouse was permitted to explore the maze freely for a duration of 5 minutes. The conclusion time of the test was documented, and the mouse was subsequently placed in a holding cage for an inter-trial interval of 1 hour before the initiation of the second trial. In the subsequent trial, the guillotine was removed from the previously blocked arm, and the mouse was placed in the same starting arm as in the first trial, allowing it to explore all three arms freely for 5 minutes, as illustrated in **Fig 2.7**. For the ANY-maze analysis, the start arm was excluded, and arm occupancy during trial 2 was quantified as the percentage of time spent in the previously blocked (novel) arm compared to the other (old/familiar) arm. A score significantly greater than 50% preference/occupancy, which represents the chance level for selecting the unfamiliar arm, was indicative of spatial working memory.

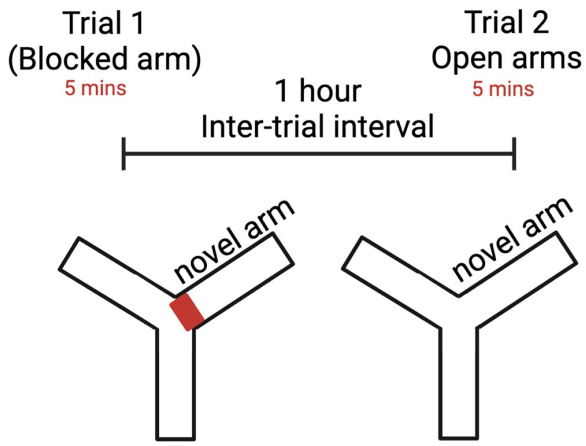

Fig. 2.7: 2-trials Y-Maze consisted of blocked arm trial and 1h later, unblocked trial and assessed spatial memory. This image was created using BioRender

2.2.8.3 Novel Object Recognition (NOR):

NOR test was employed to assess recognition memory, as described by (Hanscom et al., 2021). The experimental setup consisted of a square, open-top black plexiglass box serving as the testing arena, along with a selection of objects, including Rubik's cubes, heart/star shapes, and intertwined circles, examples of which are depicted in **Fig 2.8**. The objects were selected based on the criterion that naive mice exhibit no inherent preference between objects during baseline testing. On the initial day of testing, referred to as habituation, each mouse was placed in the empty arena, oriented towards the wall opposite the future object placement, for a duration of 10 minutes. The researcher exited the room to minimize disturbances. This day also facilitated an Open Field test to evaluate locomotor activity, measured by distance moved, and anxiety-like behaviour, assessed by proximity to the arena's border, known as thigmotaxis. Mice, being inherently curious, tend to explore new environments; thus, proximity to the border indicated heightened anxiety-like behaviour. The second day involved a similar 10-minute habituation period. On the third day, the familiarization test was conducted, wherein two identical objects, such as the intertwined circles shown in **Fig 2.8**, were placed in the arena, and mice were allowed to explore the objects freely for 5 minutes. The duration of exploration was recorded manually using timers. Behaviours such as sniffing, direct interaction, or close proximity to the object were classified as "exploring the object," whereas climbing on, walking past, or sitting adjacent to the object without interaction were not included in the recorded time. Post-familiarization, exploration times were graphed to ensure no side preference within the arena. Mice with a cumulative exploration time of less than 6 seconds for both objects were excluded from the study. The fourth day involved the novel object test, where one object was replaced with a novel item, such as a Rubik's cube depicted in **Fig 2.8**, and mice were allowed to explore for 5 minutes. The time spent with both the familiar and novel objects was recorded. The novel object was evenly distributed across experimental groups, and its placement was randomized and balanced to prevent object and side bias. The primary outcome of the NOR test was the discrimination index (DI), calculated as (time exploring novel – time exploring familiar) / (total time novel + familiar object). Mice with a DI greater than 0.2 were considered to have intact cognitive function, with higher DI values indicating superior performance.

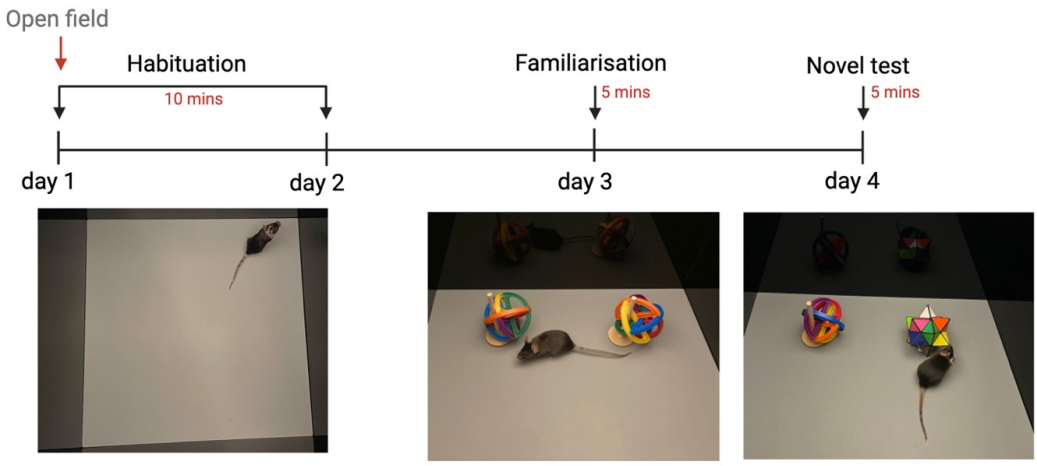

Fig. 2.8: The Novel Object Recognition was a 4-day test which evaluated recognition memory. The first 2 days allowed the mouse to freely explore and habituate the empty arena. On day 3 familiarisation, two identical objects were placed in the arena and the time the mice spent interacting with the objects was recorded. On day 4, the novel test consisted of one object being replaced by a novel object and the interaction time was recorded.

2.2.8.4 Accelerating rotarod:

The accelerating rotarod was employed to assess gross motor function in mice, as described by (Doran et al., 2019). The apparatus comprised an accelerating rotarod device (Ugo Basile; Italy), which initiated at a speed of 4 RPM and increased to a final speed of 60 RPM over a duration of 180 seconds, as illustrated by the "ramp" setup in **Fig 2.9**. This assessment requires three consecutive days of training to establish a baseline measurement prior to the CCI. Post-injury, the mice were evaluated daily. The mice were positioned on the rod facing the wall, away from the researcher, and three mice were tested simultaneously to ensure precision. Once all mice were positioned on the rod, the "run" button was activated, and the rod commenced movement at a constant speed of 4 RPM. If any mouse attempted to turn around on the rod, it was corrected to maintain its orientation towards the wall. Upon ensuring all mice faced the wall, the "start" button was pressed, initiating the speed increase. The latency, measured in seconds, for the mice to remain on the rod was recorded. Each mouse underwent three trials per day, with a 5-10 minute rest period, during which they had access to food and water, between each trial. The scores from the three trials were averaged to yield a single score per mouse per day. If a mouse fell off or clung to the rod for two consecutive complete rotations, the timing was halted.

Fig. 2.9: The accelerating rotarod measured gross motor function. This image was created using BioRender

2.2.8.5 Simple Neuroassessment of Asymmetric Impairment (SNAP):

SNAP test was employed to evaluate neurological deficits in mice following CCI (Moro et al., 2022). SNAP consists of a series of eight neurological assessments that evaluate vision, coordination and proprioception, motor strength, and posture, as illustrated in the schematic below **Fig 2.10**. Each of the eight assessments was scored on a scale from 0 (neurologically intact) to 5 (severely impaired). The scores from each assessment were aggregated to yield a final overall score, with higher scores indicating greater neurological impairment. The tests and scoring guidelines were conducted in accordance with the SNAP testing protocol (Moro et al., 2022).

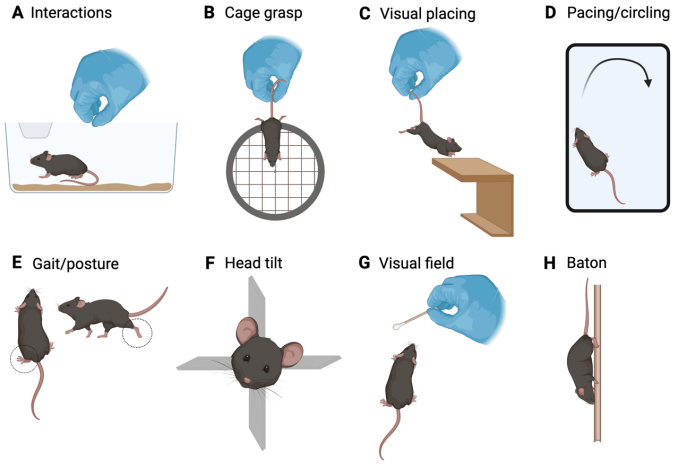

Fig. 2.10: SNAP measured neurological deficits. This image was created using BioRender

2.2.9 Statistical Analysis:

Power analysis calculations were conducted based on prior data from the Loane laboratory to predict effect size and variability. Statistical analyses and graph generation were executed using Prism v10.2.1 software (GraphPad Software, San Diego, CA, USA; RRID:SCR_002798). Normality testing was conducted, and parametric statistical analysis was applied to data sets that passed the Shapiro-Wilk test for normality. Outliers were identified using Grubb's test and were excluded prior to analysis. Where animals were excluded from an experiment, the number and percentage of excluded mice were recorded and reported for transparency. Across the studies included in this thesis, fewer than 5% of animals were excluded. Data from these experiments were analyzed using an unpaired Student's t-test for two groups, and a one-way ANOVA for more than two groups, with post hoc Dunnett's test for comparisons to a single group of interest or Tukey's test for comparisons among all groups. For analyses involving more than two groups and two factors, a two-way ANOVA, with or without repeated measures, was performed using the Tukey's test to correct for multiple comparisons. For analyses involving more than two groups and three factors, a three-way ANOVA, without repeated measures, was performed using Benjamini, Kreiger and Yekutieli test to correct for multiple comparisons. All quantitative data are presented as mean ± standard error of the mean (SEM). p-values less than 0.05 were considered statistically significant.

Chapter 3

Temporal dynamics of innate immune and T cell infiltration post-injury

Hypothesis: TBI induces a sequential neuroimmune response, characterized by an initial activation of the innate immune system, followed by the sustained presence of T cells, ultimately leading to a long-term alteration of the immune environment within the injured brain.

3.1 Introduction

3.1.1 Immune response dynamics in TBI

TBI triggers a highly dynamic and context-dependent immune response that involves both CNS-resident immune cells and peripherally derived leukocytes. This immune activity is temporally categorized into acute, subacute, and chronic phases, each characterized by distinct patterns of cellular infiltration, activation, and resolution. The immune response to TBI is not merely a secondary effect of tissue damage; rather, it plays a crucial role in determining neurological outcomes by influencing lesion expansion, tissue repair, and long-term neuropathology. The acute and subacute phases are of particular interest as they encompass the initial activation of innate immunity and the subsequent recruitment of adaptive immune effectors. This section examines the role of innate and adaptive immune cells following TBI, focusing on infiltration kinetics, phenotypic diversity, functional polarization, and methodological considerations for their identification.

3.1.2 Extraction and phenotyping immune cells in the brain

Isolating viable mononuclear immune cells (MNCs) from the brain following TBI necessitates balancing four competing priorities: (i) yield, (ii) viability, (iii) preservation of surface epitopes for accurate immunophenotyping, and (iv) minimization of artefactual activation induced by dissociation. Brain tissue presents unique technical challenges, including high lipid and myelin content, necrotic debris, haemorrhagic components, and post-injury extracellular matrix remodelling that can trap cells and increase clumping. These challenges are exacerbated when analysing early time points post-injury, when debris burden is highest and infiltrating populations may be relatively sparse compared to resident myeloid cells. Transcardial perfusion with ice-cold buffered saline is a widely employed technique to minimize contamination from intravascular leukocytes, which can otherwise artificially elevate the apparent numbers of "brain immune" cells, particularly neutrophils and monocytes that accumulate within injured microvasculature. The anatomical delineation of the sampled region is equally critical. Numerous studies concentrate on the ipsilateral hemisphere, peri-contusional cortex, or a defined lesion-adjacent block to enhance sensitivity to injury-induced changes. However, this focus must align with the specific biological question, as immune responses vary between the lesion core, perilesional penumbra, and distal connected regions. Consistent dissection planes and weights enhance comparability across cohorts and time points. A highly reproducible protocol for isolating

brain MNCs from the ipsilateral hemisphere of mice subjected to CCI has been described previously (Ritzel et al., 2023). The protocol involves mechanical homogenisation of perfused tissue followed by enzymatic digestion using papain and collagenase/dispase, yielding single-cell suspensions that maintain delicate surface epitopes necessary for flow cytometry. Following digestion, the tissue is passed through a 70 µm strainer and resuspended in RPMI medium containing DNase I to reduce clumping and preserve cell integrity. A 70%/30% Percoll gradient is commonly used to separate MNCs from debris and myelin. The yield of viable MNCs from each brain hemisphere is typically around 1.5-2 × 10^5 cells, suitable for multicolour flow cytometry. The optimization of such isolation methods is particularly crucial for investigating immune cell infiltration at specific time points post-injury, notably during the acute (0-3 days) and subacute (3-14 days) phases, when immune responses are dynamic and cell populations undergo rapid shifts. A thorough analysis of both innate and adaptive immune cells during these phases offers valuable insights into the evolution of immune responses and their impact on injury progression or resolution.

Following isolation, the cellular composition of the brain's immune landscape can be analyzed using multiparameter flow cytometry. For TBI studies, following key markers are selected to differentiate between resident and infiltrating populations:

- **CD45 (low on microglia, high on infiltrates)**
- **CD11b (myeloid lineage)**
- **Ly6C and Ly6G (monocytes and neutrophils)**
- **CD3, CD4, CD8 (T lymphocytes)**
- **MHC-II, CD80/86 (APC activation)**

3.1.3 Innate and adaptive immune cell dynamics and interactions in TBI

The acute immune response following TBI is predominantly characterized by the infiltration of innate immune cells into the parenchyma, induced by the disruption of the BBB and chemokine gradients (Theus, 2024). Neutrophils appear as early as 4-6 hours post-injury, peaking between 24-72 hours (Soares et al., 1995). Their infiltration is mediated largely by c-x-c motif chemokine receptor 2 – c-x-c motif chemokine ligand 1(CXCR2-CXCL1/2) signalling (Semple et al., 2010). Neutrophils contribute to debris clearance but can exacerbate secondary injury through the release of matrix metalloproteases 9 (MMP-9), ROS, and neutrophil extracellular traps (NETs) (Semple et al., 2015). Monocyte-derived macrophages (MDMs), which commence differentiation into macrophages by 3 dpi (Szmydynger-

Chodobska et al., 2012). These cells are recruited through CCL2-CCR2 signalling, and CCR2 deficiency significantly reduces their accumulation and attenuates lesion volume (Hsieh et al., 2014; Morganti et al., 2015). Once in the parenchyma, MDMs undergo polarisation influenced by local cytokines, adopting phenotypes along a pro-inflammatory to reparative spectrum (Hsieh et al., 2013; Kumar et al., 2016). The early innate response is characterized by elevated levels of TNF-α, IL-6, and CXCL10, which contribute to bystander tissue injury and astrocyte activation (Kumar & Loane, 2012). While microglia exhibit a stereotypical reactive morphology (amoeboid shape, CD68 upregulation), infiltrating monocytes express higher levels of Ly6C and CD11c, distinguishing them both functionally and phenotypically (Braun et al., 2017).

Following the initial innate immune response, the subacute phase is characterized by the emergence and proliferation of adaptive immune cells, particularly T lymphocytes. In a murine model of controlled cortical contusion injury, $CD4^+$ and $CD8^+$ T cells were increased by 7 dpi, with continued accumulation observed through 10 dpi (Clausen et al., 2007). The presence of T cells was observed in both the perivascular and parenchymal areas, suggesting true infiltration into the CNS rather than just being located around the blood vessels. It has previously been shown that the adaptive immune response encompasses both effector and regulatory T cells. At this stage, $CD4^+$ T cells exhibit elevated expression of CD69 and CD44, with some producing IFN-γ and IL-17, indicative of Th1/Th17 polarization (Braun et al., 2017; Daglas et al., 2019). Conversely, $CD8^+$ T cells express GzmB and perforin, suggesting potential cytotoxic interactions with neurons or glial cells (Daglas et al., 2019).

In the context of TBI, there is significant interaction between innate and adaptive immune cells. Microglia and macrophages play a pivotal role in influencing T cell recruitment through cytokine production and in determining their functional polarization via antigen presentation and costimulatory signals (Becher et al., 2006; Braun et al., 2017). Conversely, T cells possess the ability to modulate the function of innate immune cells. For example, IFN-γ produced by Th1 cells facilitates the pro-inflammatory activation of microglia and macrophages, whereas IL-4 from Th2 cells directs these cells towards a reparative phenotype (Klebe et al., 2015). This bidirectional communication underscores the critical importance of maintaining temporal balance, while initial pro-inflammatory activity is crucial for pathogen defence and debris clearance, its prolonged activation can lead to chronic neuroinflammation and subsequent neurodegeneration (Ayerra et al., 2025; Donat et al., 2017). In this context, persistently reactive microglia can perpetuate chemokine and cytokine signalling, which recruits and activates T cells (Abou-El-Hassan et al., 2023; Krämer et al.,

2019). In turn, these activated T cells can further enhance the inflammatory state of microglia, thereby establishing a feed-forward loop that sustains neuroinflammation over time.

3.2 Results

3.2.1 Optimization of the mononuclear cell preparation from the brain

Various methodologies have been employed by different research groups to isolate mononuclear cells from the brain. In the present study, three commonly used dissociation strategies were compared in naïve brain tissue, manual mechanical disruption using a dounce homogenizer, mechanical dissociation by magnetic bead agitation, and an enzymatic dissociation protocol. Each one described in the methods section. The dounce and magnetic bead approaches rely primarily on physical disruption of tissue, whereas the enzymatic method additionally facilitate the breakdown of extracellular matrix components that can retain cells within the brain parenchyma. In the enzymatic protocol, collagenase was included to digest matrix proteins and thereby improve the release of mononuclear cells, while DNase was used to degrade extracellular DNA released from damaged cells during processing, reducing viscosity and cell clumping and improving the quality of the single-cell suspension for flow-cytometric analysis (Srakočić et al., 2022). Mononuclear cells isolated using each protocol were stained with antibodies against CD45 and CD11b, and representative flow cytometry plots for the three methods are shown in **(Fig. 3.2.1 A)**. Microglia, the predominant innate immune cell population in the naïve brain, were successfully identified and isolated with all three methods. However, the dissociation protocol had a marked effect on cell recovery. Enzymatic digestion yielded the highest number of cells, significantly exceeding both Dounce homogenization and magnetic bead dissociation **(Fig. 3.2.1 B)**. Mean ± SEM cell numbers were 21,167 ± 1,922 for Dounce homogenization, 36,500 ± 764 for magnetic bead dissociation, and 63,617 ± 2,648 for enzymatic digestion. Thus, although mechanical dissociation methods are often favored where preservation of the native cellular state is a priority, the enzymatic approach provided superior recovery under the present conditions. Cell recovery was quantified after exclusion of non-viable events during flow-cytometric analysis, ensuring that differences between protocols reflected viable cell yield rather than total acquired events.

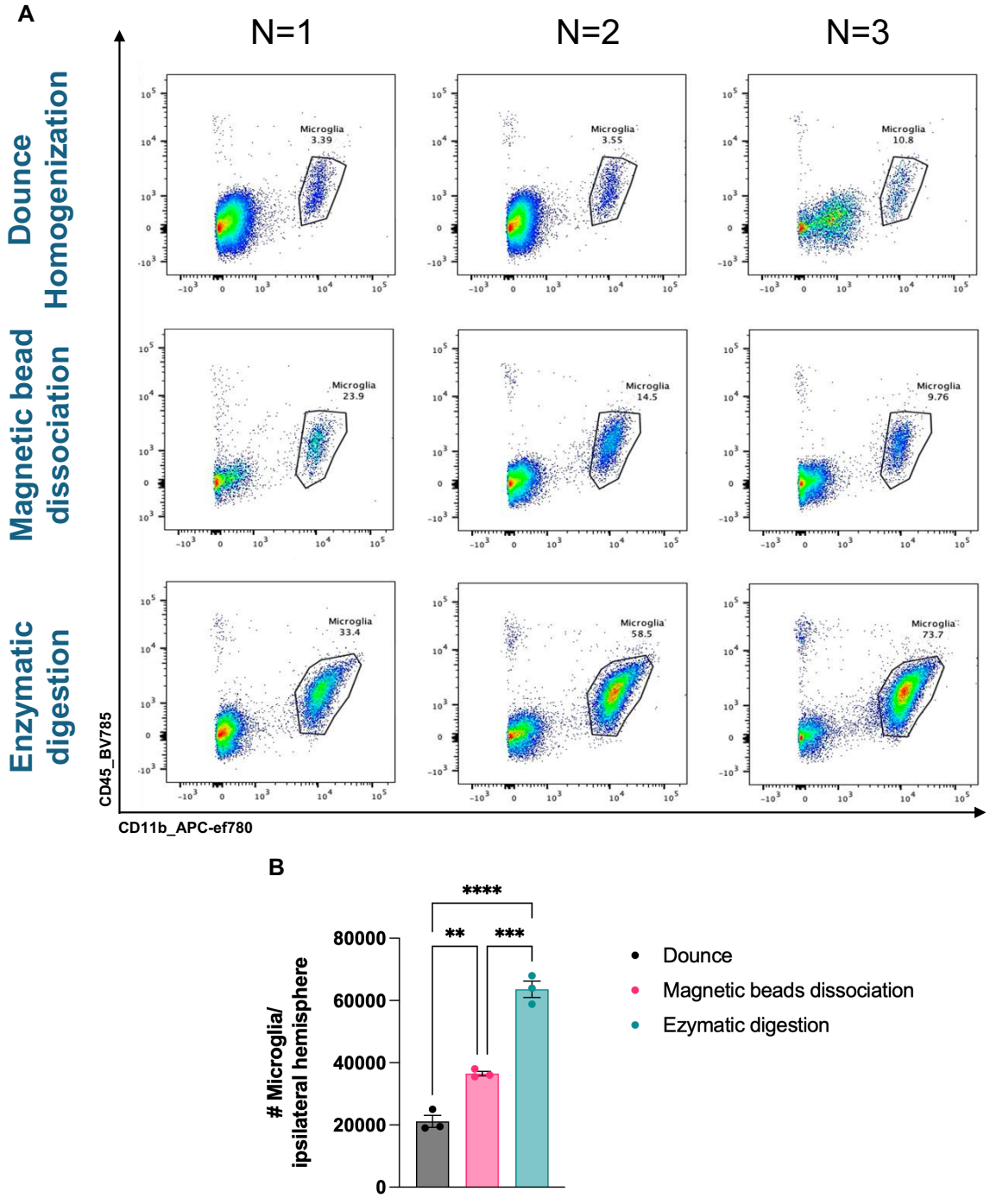

Fig. 3.2.1: Isolation of mononuclear cells from the naïve mouse brain. Flow cytometry was employed to quantify microglia in order to determine the most effective method [(a) dounce homogenization, (b) magnetic bead dissociation, (c) enzymatic digestion] for isolating mononuclear cells from the brain. (A) The dot plot represents the microglia ($CD11b^+/CD45int$) cells in the naïve brain, (B) The bar graph represents the absolute number of microglia ($CD11b^+/CD45int$) cells obtained from each mononuclear isolation method. Data are mean ± SEM (n=3 per method). *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA.

3.2.2 Optimization and assessment of cytokine production by infiltrating T cells within the cerebral environment

In order to gain a deeper understanding of the functional dynamics of the post-traumatic T cell response, this research focused on analyzing the identity of T cell subsets along with their capacity to produce essential effector cytokines. Brains were collected from adult male C57BL/6J mice subjected to CCI at 10 dpi, and infiltrating mononuclear cells were isolated using the enzymatic digestion protocol optimized in **section 3.2.1**. Preliminary investigations indicated that T cell infiltration peaks at 10 dpi (data not shown), thus this timepoint was selected to evaluate intracellular cytokine production, specifically IL-17 and IFN-γ, across T cell subsets. To determine the most appropriate stimulation protocol for subsequent experiments, isolated mononuclear cells were subjected to three distinct stimulation conditions varying in strength and mode of activation. Condition 1 involved phorbol 12-myristate 13-acetate (PMA; 20 ng/ml) plus ionomycin (0.5 µg/ml), whereas Condition 2 employed a lower-dose PMA/ionomycin combination (5 ng/ml and 0.25 µg/ml, respectively). PMA and ionomycin are widely recognized as potent non-specific stimulants for intracellular cytokine staining as they bypass proximal T cell receptor signalling, with PMA activating protein kinase C and ionomycin increasing intracellular calcium, collectively driving rapid cytokine production (Mandala et al., 2021). The inclusion of both a higher-dose and lower-dose PMA/ionomycin condition facilitated comparison between a stronger pharmacological stimulus and a milder stimulus that might better preserve viability while still inducing detectable cytokine expression. Condition 3 comprised anti-CD3 (1 µg/ml) plus anti-CD28 (1 µg/ml), serving as a more physiological mode of activation because anti-CD3 engages the T cell receptor complex, while anti-CD28 provides the co-stimulatory signal necessary for effective T cell activation and cytokine induction (Trickett & Kwan, 2003).

For Conditions 1 and 2, cells were stimulated for three hours in the presence of brefeldin A, whereas Condition 3 was conducted overnight with brefeldin A added after 4 hours. Brefeldin A was included to block intracellular protein transport and retain newly synthesized cytokines within the cell, thereby enabling their detection by intracellular flow cytometry. The production of IL-17 and IFN-γ by $CD4^+$, $CD8^+$, and $\gamma\delta^+$ T cells was subsequently assessed. Representative flow-cytometric plots of cytokine-producing T cells under the three stimulation conditions are depicted in **Fig. 3.2.2 A**. There was no significant IFN-γ production by $CD4^+$ or $\gamma\delta^+$ T cells across the conditions **(Fig. 3.2.2 C,G)**. In contrast, $CD8^+$ T cells exhibited

significantly greater IFN-γ production in Conditions 1 and 3 than in Condition 2 **(Fig. 3.2.2 E)**, indicating that the lower-dose PMA/ionomycin stimulus was less effective at inducing a robust IFN-γ response. IL-17 production by $CD4^+$ and $CD8^+$ T cells was minimal under all conditions **(Fig. 3.2.2 B,D)**. Notably, $\gamma\delta^+$ T cells were the principal IL-17 producing population, and IL-17 expression in these cells was detected under all three stimulation conditions, with no significant differences between them **(Fig. 3.2.2 F)**. Thus, all three conditions were sufficient to induce measurable cytokine production in at least some T cell subsets, but Condition 1 was selected for the subsequent time-course experiment because it provided a strong and rapid non-specific stimulus that reliably elicited intracellular cytokine accumulation within a short incubation period.

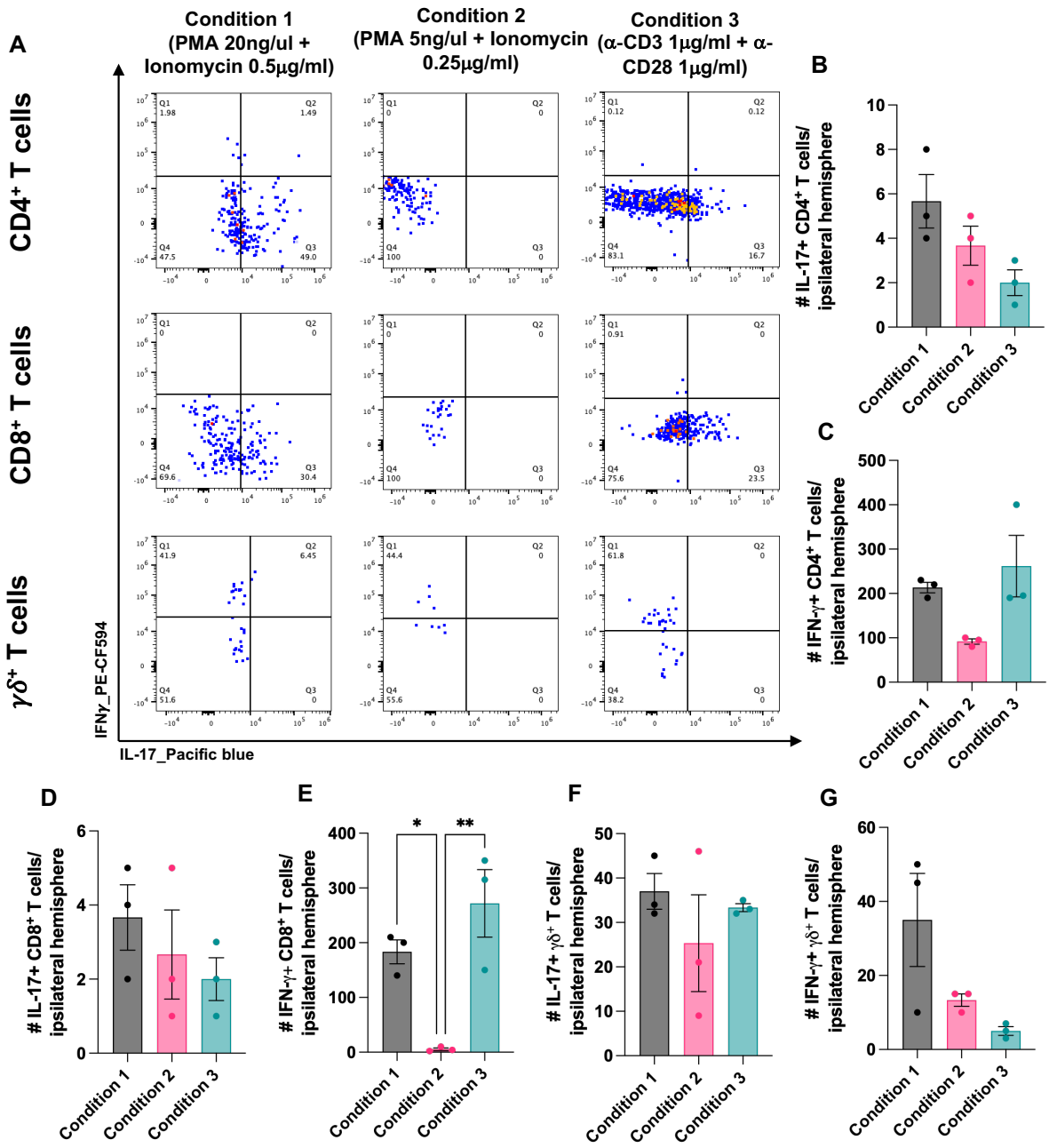

Fig. 3.2.2: Evaluation of cytokine production (IL-17, IFN-γ) by T cells under different stimulating conditions. Cells were isolated and stimulated under three different conditions which were Condition 1 [PMA (20ng/ml) + Ionomycin (0.5µg/ml)]; Condition 2 [PMA (5ng/ml) + Ionomycin (0.25µg/ml)]; Condition 3 [α-CD3(1µg/ml)+ α-CD28 (1µg/ml)]. (A) Representative flow cytometry plots of cytokine producing (IL-17, IFN-γ) T cells, (B-G) The bar graphs show the absolute numbers of IL-17 producing CD4⁺ T cells (B), IFN-γ producing CD4⁺ T cells (C), IL-17 producing CD8⁺ T cells (D), IFN-γ producing CD8⁺ T cells (E), IL-17 producing γδ⁺ T cells (F), IFN-γ producing γδ⁺ T cells (G). Data are mean ± SEM (n=3 per method). *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA.

3.2.3 TBI produces a rapid IL-1β production in infiltrating myeloid cells

Following the establishment of the methodological framework, the subsequent aim was to characterize the initial innate immune response to TBI. Accordingly, the expression of inflammatory genes and the populations of infiltrating leukocytes were analyzed within the first 24 hours post-injury (hpi). Adult male C57BL/6J mice were subjected to moderate CCI or sham surgery, with tissue samples collected at 3, 6, 12, and 24 hpi **(Fig. 3.2.3 A)**. Hippocampal punches were utilized for qPCR analysis of inflammatory gene expression, while the remaining ipsilateral tissue, excluding the olfactory bulb and cerebellum, was processed for multiparameter flow cytometric analysis of infiltrating innate immune cells. The qPCR analysis revealed a rapid and dynamic induction of inflammatory genes within the ipsilateral hippocampus. *Cxcl1* expression was markedly elevated early post-injury, peaking at 3 hpi relative to sham animals, before declining towards baseline at subsequent timepoints **(Fig. 3.2.3 B)**. Conversely, *Ccl2* expression exhibited a more delayed profile, with minimal change at earlier timepoints and a significant increase by 24 hpi **(Fig. 3.2.3 C)**. *Il1b* expression also rose rapidly post-injury, with the strongest induction observed at 6 hpi, followed by a decline at 12 and 24 hpi **(Fig. 3.2.3 D)**.

To ascertain the relationship between these transcriptional changes and leukocyte recruitment, infiltrating innate immune cells were quantified via flow cytometry using the gating strategy described in the **Fig 2.4** of the methods section. To detect intracellular IL-1β, isolated brain mononuclear cells were ex vivo stimulated with PMA and ionomycin in the presence of Brefeldin A prior to staining. This procedure ensures that IL-1β$^+$ populations indicate the inducible cytokine-producing capacity under standardized assay conditions. Representative gating plots are presented in **(Fig. 3.2.3 E,F)**. Ly6G$^+$ neutrophils were the earliest infiltrating population, with a pronounced increase evident at 6 hpi compared to sham controls, followed by a decline at later timepoints, although numbers remained elevated above baseline **(Fig. 3.2.3 G)**. In contrast, Ly6C$^+$ monocytes accumulated more gradually, reaching peak abundance at 12 hpi **(Fig. 3.2.3 H)**. CD11c$^+$ cells were relatively scarce at earlier timepoints but increased at later stages, particularly at 12 and 24 hpi **(Fig. 3.2.3 I)**. The cellular sources of IL-1β were subsequently examined using intracellular staining and a back-gating strategy **(Fig. 3.2.3 F)**. Quantification of IL-1β$^+$ infiltrating cells revealed that Ly6G$^+$ neutrophils and Ly6C$^+$ monocytes were the principal IL-1β producing populations during the acute post-injury period. IL-1β$^+$ Ly6G$^+$ cells were most prominent at early timepoints, consistent with the rapid influx of neutrophils **(Fig. 3.2.3 J)**, whereas IL-1β$^+$ Ly6C$^+$

cells accumulated later, peaking alongside the expansion of the Ly6C$^+$ population **(Fig. 3.2.3 K)**. Thus, the temporal pattern of IL-1β production closely mirrored the recruitment kinetics of these infiltrating innate immune subsets. Overall, these data demonstrate that moderate CCI induces a rapid inflammatory program in the injured hemisphere, characterized by early *Cxcl1* and *Il1b* induction, followed by delayed *Ccl2* expression, and accompanied by temporally distinct recruitment of Ly6G$^+$ neutrophils, Ly6C$^+$ monocytes, and CD11c$^+$ cells. Neutrophils and monocytes emerged as the dominant IL-1β producing infiltrating populations in the acute phase post-injury.

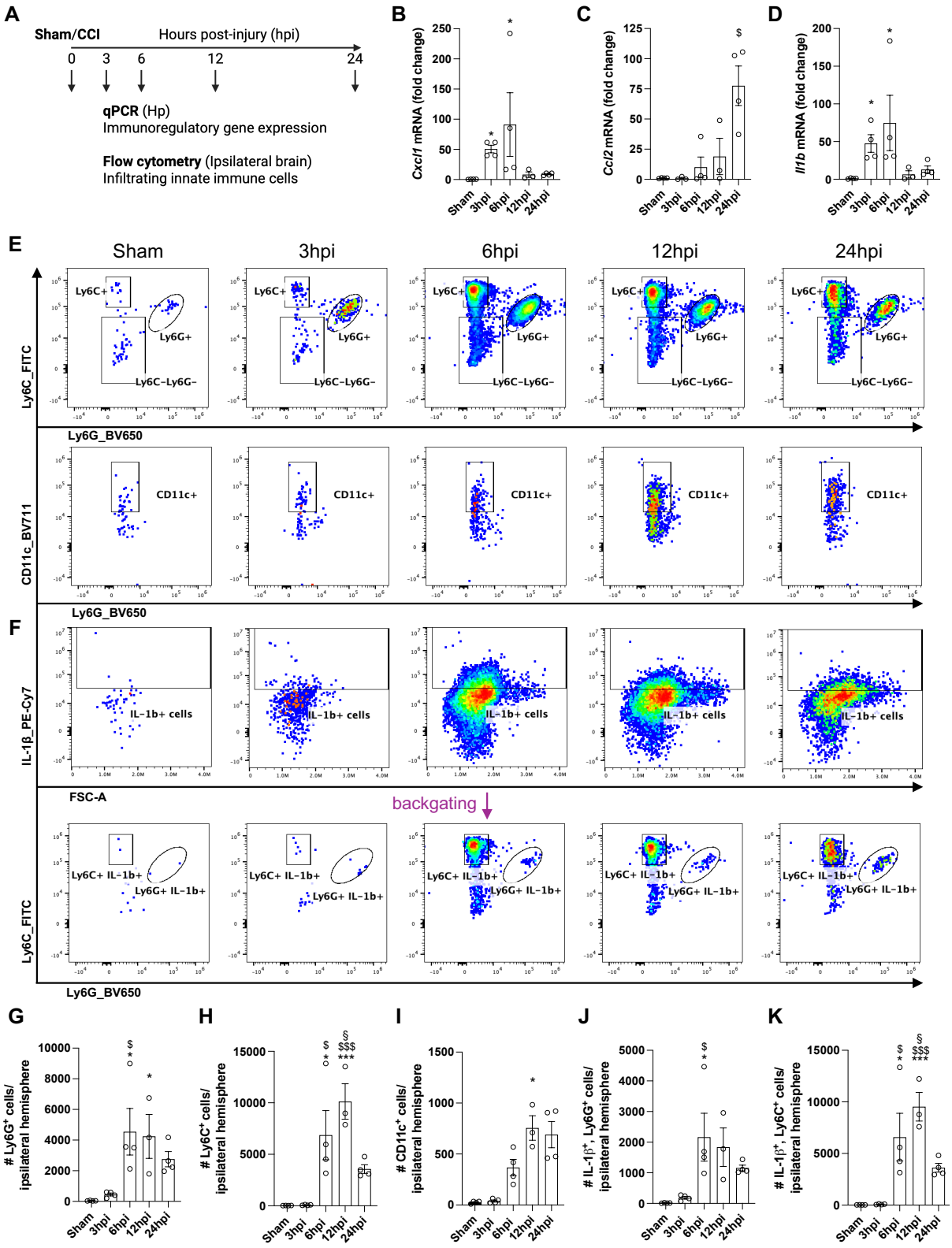

Fig. 3.2.3. Early infiltration of IL-1β producing innate immune cells into the injured brain. (A) Experimental design, (B-D) Relative fold-change hippocampal gene expression of *Cxcl1*, *Ccl2*, *Il1b*. (E) Representative flow cytometry plots for Ly6C$^+$ monocytes, Ly6G$^+$ neutrophils and CD11c$^+$ dendritic cells. (F) Back-gating strategy to identify IL-1β producing innate immune cells. (G-K) Bar graphs illustrate the absolute numbers of Ly6G$^+$ neutrophils (G), Ly6C$^+$ monocytes (H), CD11c$^+$ dendritic cells (I), IL-1β producing neutrophils (J), IL-1β producing monocytes (K). Data = Mean ± SEM (n=3-4 per time point); differences vs. Sham *p<0.05, **p<0.01, ***p<0.001; differences vs. 3hpi $^{\$}$p<0.05, $^{\$\$}$p<0.01, $^{\$\$\$}$p<0.001; differences vs. 6hpi $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001; differences vs. 12hpi §p<0.05, §§p<0.01, §§§p<0.001 by one-way ANOVA with Tukey's multiple comparisons test.

3.2.4 UMAP visualization of innate immune cell infiltration into the brain following injury

UMAP plots of concatenated samples from the acute time course experiment were used to visualize the high-dimensional flow cytometry data, allowing for the identification and tracking of diverse brain cell populations and their cytokine signatures post-injury. The clusters of each point on the UMAP represents a single cell, positioned according to its overall marker expression profile, identified using their marker expression using cluster explorer on FlowJo. Cells with similar marker expression are grouped together, resulting in visually distinct clusters that correspond to different cell types, as depicted in **Fig. 3.2.4 A**. Comparison of UMAP plots across acute time points post-injury shows clear changes in cluster size and density, reflecting the expansion or reduction of innate immune cell populations **(Fig. 3.2.4 B)**. Notably, the highest expression of IL-1β was predominantly observed in the clusters of infiltrating monocytes and neutrophils throughout this time course study, suggesting the capacity of these cells to produce IL-1β at these acute time points, as depicted in **(Fig. 3.2.4 C)**. In contrast, microglia persisted as a prominent resident immune cluster throughout the acute phase, without any obvious shift indicating their dominance as the IL-1β high population. Instead, although some IL-1β signal was detectable within the microglial compartment, the highest expression was predominantly associated with infiltrating monocyte and neutrophil clusters. This suggests that these infiltrating innate populations are likely the primary contributors to acute IL-1β production following injury **(Fig. 3.2.4 C)**.

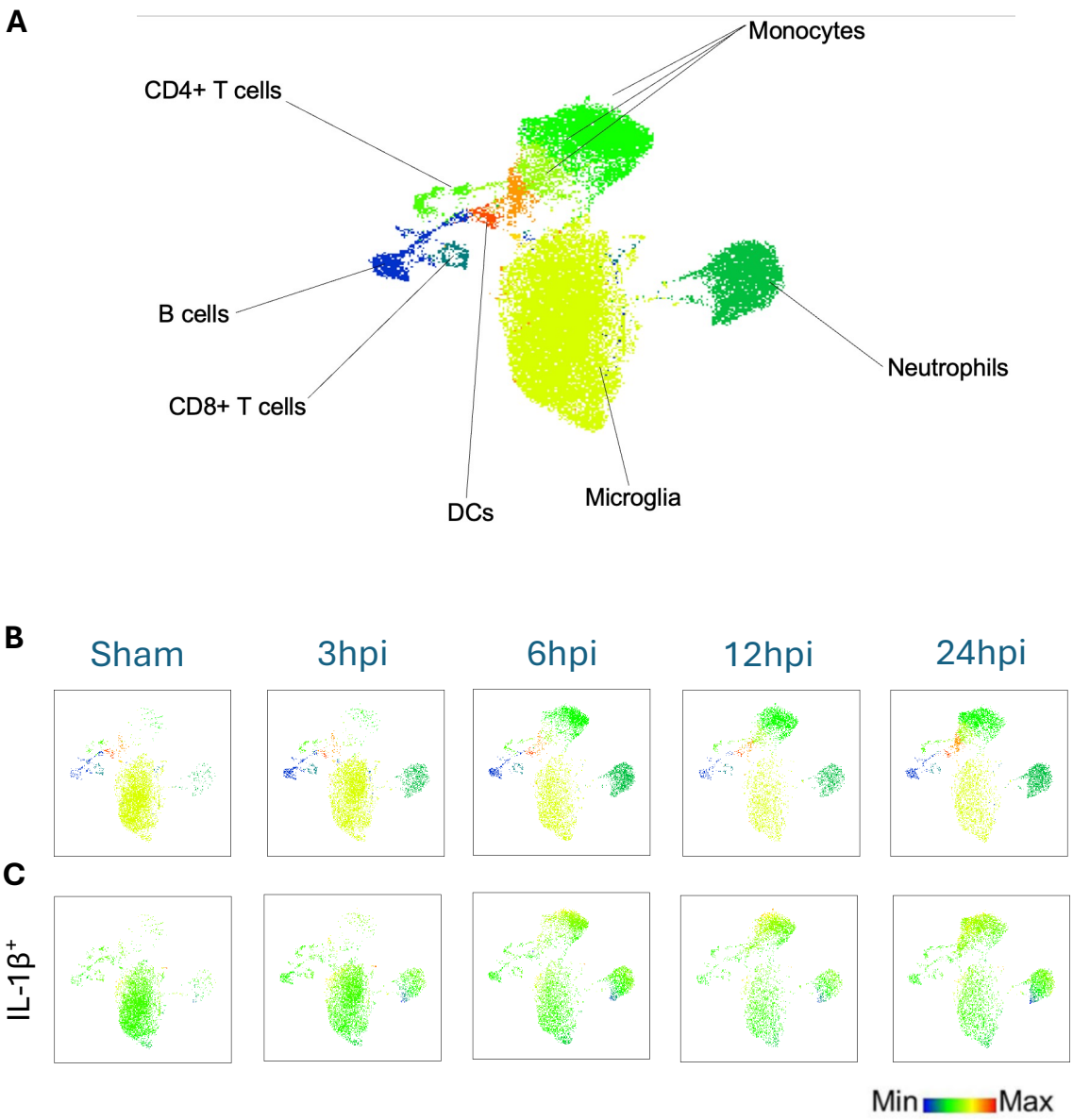

Fig. 3.2.4: Temporal evolution of innate immune cell populations post-injury. UMAP projection of flow cytometry data from the brain collected at (3, 6, 12, 24 hours post-injury) and the clustering was performed using FlowSOM. (A) Each point represents a single cell, immune cell type identified by marker expression (CD45, CD11b, Ly6G, Ly6C, CD11c, CD3, CD19, CD4, CD8, IL-1β), (B) Plot highlighting temporal shifts in the infiltrating immune cell composition over the acute course, (C) Plot highlighting the IL-1β dynamics over the acute time course.

3.2.5 TBI drives pro-inflammatory lymphocyte recruitment during the subacute phase post-injury

To delineate the temporal dynamics of adaptive immune cell recruitment following TBI, an extended temporal analysis was conducted, adult male C57BL/6J mice underwent either CCI or sham surgery, with tissue collection occurring at 1, 3, 10, and 28 dpi **(Fig. 3.2.5 A)**. Hippocampal punches were utilized for qPCR analysis to assess immunoregulatory gene expression, while the remaining ipsilateral tissue, excluding the olfactory bulb and cerebellum, was processed for multiparameter flow cytometry to quantify infiltrating adaptive immune populations and their cytokine production. The qPCR analysis revealed a subacute induction of genes associated with leukocyte recruitment and cellular adhesion within the injured hippocampus. *Cxcl10* expression was significantly elevated post-injury, peaking at 3 dpi before declining at subsequent time points **(Fig. 3.2.5 B)**. *Icam1* expression also showed a significant increase, with a strong induction at 3 dpi that remained elevated at 10 dpi before returning towards baseline by 28 dpi **(Fig. 3.2.5 C)**. A similar expression pattern was observed for *Itgb2*, which was significantly upregulated at 3 and 10 dpi, subsequently declining by 28 dpi **(Fig. 3.2.5 D)**.

Flow cytometry was employed to assess adaptive immune cell infiltration using the gating strategy described in the **Fig 2.4** of the methods section. Representative gating plots for $CD4^+$ T cells, $CD8^+$ T cells, $\gamma\delta^+$ T cells, and NK-T cells are depicted in **Fig. 3.2.5 E**. Quantitative analysis indicated that $CD4^+$ T cell numbers progressively increased post-injury, peaking at 10 dpi relative to sham controls, before declining by 28 dpi **(Fig. 3.2.5 G)**. $CD8^+$ T cells exhibited a similar pattern, with maximal accumulation also observed at 10 dpi **(Fig. 3.2.5 H)**. NK-T cells demonstrated the most pronounced expansion, with a marked increase at 10 dpi that remained elevated, albeit reduced, at 28 dpi **(Fig. 3.2.5 I)**. In contrast, $\gamma\delta^+$ T cells accumulated earlier, with their highest numbers observed at 3 dpi, followed by a decline at 10 and 28 dpi **(Fig. 3.2.5 J)**. To elucidate the functional characteristics of these infiltrating populations, intracellular cytokine staining was conducted for IFN-γ and IL-17,where isolated brain mononuclear cells were ex vivo stimulated with PMA and ionomycin in the presence of Brefeldin A prior to staining, with representative plots depicted in **(Fig. 3.2.5 F)**. The quantity of IFN-γ^+ $CD4^+$ T cells exhibited an increase over time, peaking at 10 dpi and remaining elevated at 28 dpi **(Fig. 3.2.5 K)**. Similarly, IFN-γ^+ $CD8^+$ T cells showed an increase following injury, with the most substantial response observed at later time points, particularly at 28 dpi **(Fig. 3.2.5 L)**. NK-T cells emerged as a significant source of IFN-γ, displaying a pronounced

peak at 10 dpi, followed by a reduction by 28 dpi, although levels remained above those of sham controls **(Fig. 3.2.5 M)**. In contrast to these IFN-γ dominant populations, γδ$^+$ T cells constituted the primary IL-17 producing lymphocyte subset. IL-17$^+$ γδ$^+$ T cells were elevated as early as 1 dpi, reaching their peak at 3 dpi before subsequently declining **(Fig. 3.2.5 N)**. Additionally, γδ$^+$ T cells produced IFN-γ, but this response exhibited a delayed profile, peaking at 10 dpi rather than 3 dpi **(Fig. 3.2.5 O)**. Collectively, these findings indicate that TBI induces a temporally ordered adaptive immune response within the injured hemisphere. This response is characterized by increased expression of *Cxcl10*, *Icam1*, and *Itgb2*, followed by the progressive accumulation of CD4$^+$ and CD8$^+$ T cells and NK-T cells, predominantly associated with IFN-γ production. Concurrently, γδ$^+$ T cells are recruited earlier and represent the major IL-17 producing population, suggesting that distinct lymphocyte subsets contribute to different inflammatory programs at various stages post-injury.

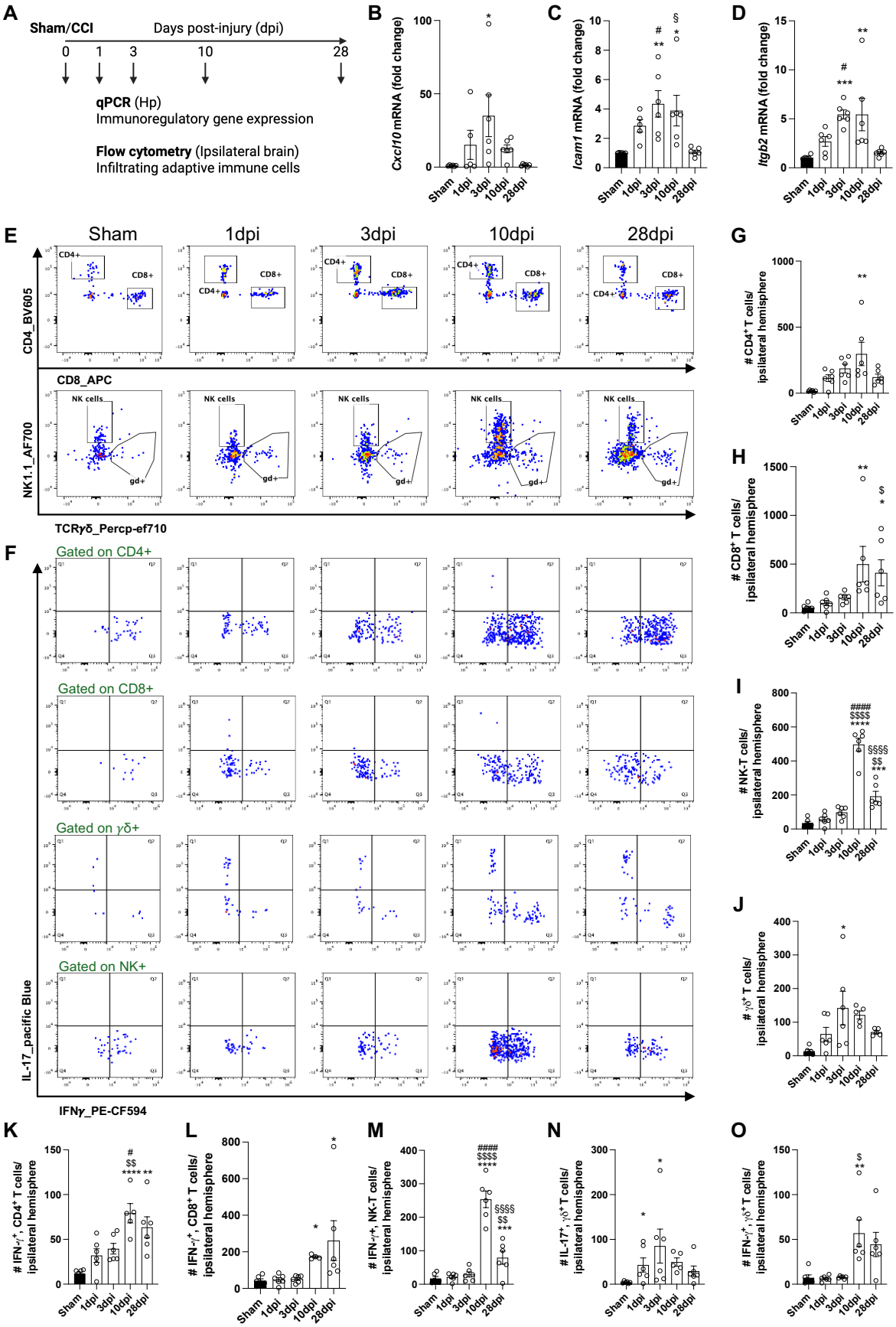

Fig. 3.2.5: Sustained infiltration of adaptive immune cells and pro-inflammatory cytokine production in the subacute phase after TBI. (A) Experimental design. (B-D) Relative fold-change expression levels of *Cxcl10*, *Icam1*, *Itgb2*. (E) Flow

cytometry plots of $CD4^+$, $CD8^+$, $\gamma\delta^+$, and NK^+ T cells. (F) Representative flow cytometry plots for IL-17 and IFNγ production by different T cell subsets. (G-O) Bar graphs illustrate the absolute numbers of $CD4^+$ T cells (G), $CD8^+$ T cells (H), NK-T cells (I), $\gamma\delta^+$ T cells (J), IFN-γ producing $CD4^+$ T cells (K), IFN-γ producing $CD8^+$ T cells (L), IFN-γ producing NK-T cells (M), IL-17 producing $\gamma\delta^+$ T cells (N), and IFN-γ producing $\gamma\delta^+$ T cells (O). Data = Mean ± SEM (n=6 per time point); differences vs. Sham *p<0.05, **p<0.01, ***p<0.001; differences vs. 1dpi $^{\$}$p<0.05, $^{\$\$}$p<0.01, $^{\$\$\$}$p<0.001; differences vs. 3dpi $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001; differences vs. 10dpi §p<0.05, §§p<0.01, §§§p<0.001 by one-way ANOVA with Tukey's multiple comparisons.

3.2.6 UMAP visualization of adaptive immune cell infiltration into the brain post-injury

UMAP plots of concatenated samples from the sub-acute and chronic time course experiment were used to visualize the high-dimensional flow cytometry data, as shown previously for the innate immune cell infiltration, allowing for the identification and tracking of diverse brain cell populations and their cytokine signatures post-injury. The clusters of each point on the UMAP represents a single cell, positioned according to its overall marker expression profile, identified using their marker expression using cluster explorer on FlowJo. Cells with similar marker expression are grouped together, resulting in visually distinct clusters that correspond to different cell types, as depicted in **Fig. 3.2.6 A**. Comparison of UMAP plots across sub-acute and chronic time points post-injury shows clear changes in cluster size and density, reflecting the expansion or reduction of adaptive immune cell (CD4, CD8, γδ, NK-T) populations **(Fig. 3.2.6 B)**. Notably, the highest expression of IL-17 was predominantly observed in the clusters of infiltrating $\gamma\delta^+$ T cells, whereas all the other adaptive immune subsets (CD4, CD8, NK-T) showed higher expression of IFN-γ **(Fig. 3.2.6 C,D)**.

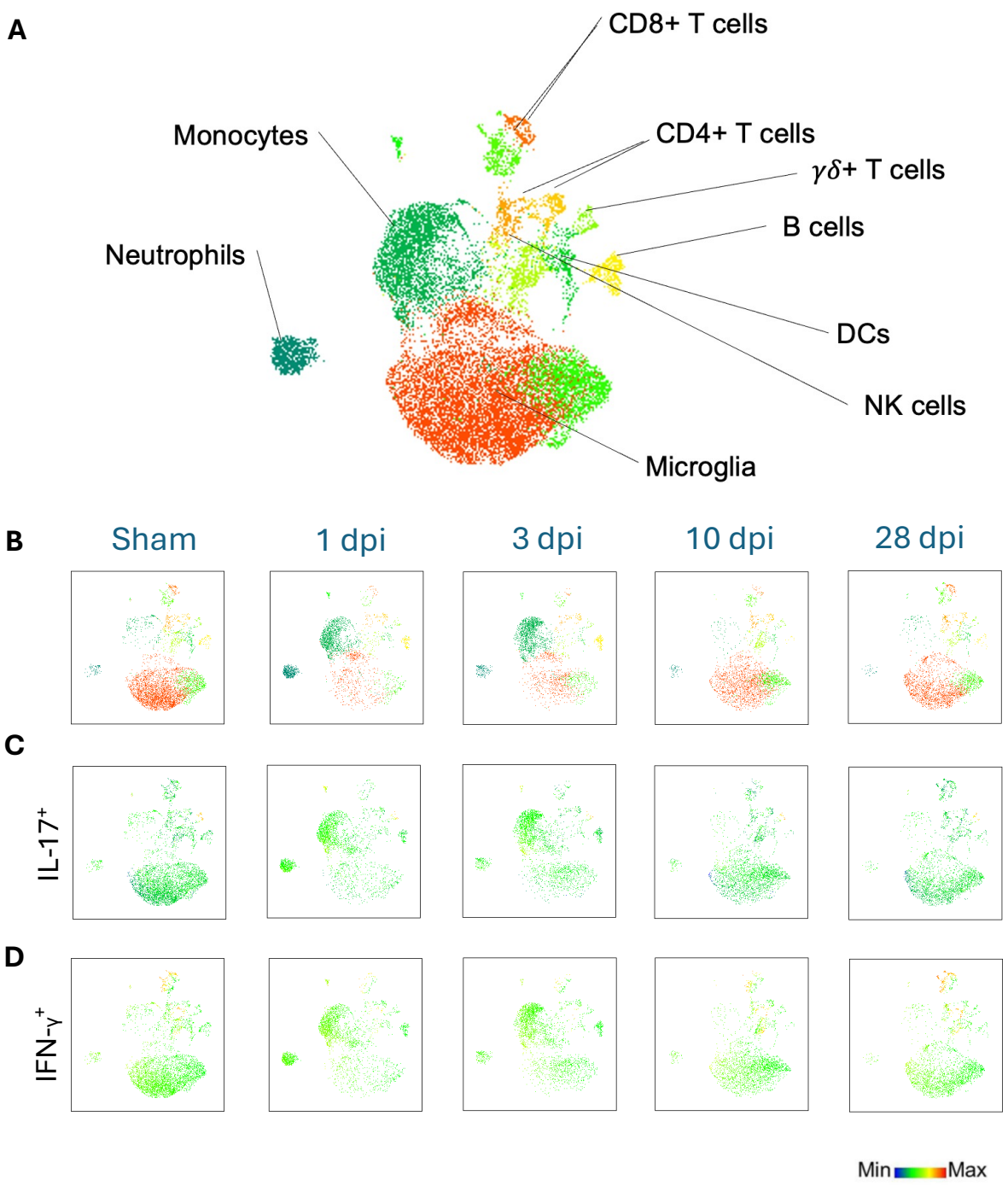

Fig. 3.2.6: Temporal evolution of adaptive immune cell populations post-injury. UMAP projection of flow cytometry data from the brain collected at (1, 3, 10, 28 days post-injury) and the clustering was performed using Flow SOM. (A) Each point represents a single cell, immune cell type identified by marker expression (CD45, CD11b, Ly6G, Ly6C, CD11c, CD3, CD19, CD4, CD8, TCRγδ, NK1.1, IL-17, IFN-γ), (B) Plot highlighting temporal shifts in the infiltrating immune cell composition over the sub-acute and chronic time course, (C) Plot highlining the IL-17 dynamics over the acute time course, (D) Plot highlining the IFN-γ dynamics over the subacute/chronic time course.

3.3 Discussion

This chapter delineates an experimental pipeline designed to quantify post-traumatic immune responses in the brain via flow cytometry, subsequently employing this pipeline to construct a time-resolved "immune atlas" that encompasses early innate activation and the subsequent emergence of effector lymphocyte programs. The practical significance of this work is two-fold. Firstly, it offers a technically optimized framework for the recovery of viable brain immune cells with sufficient yield and marker fidelity to facilitate multi-parameter phenotyping. Secondly, it identifies the temporal onset of specific inflammatory cytokine programs at the single-cell level, thereby enabling downstream hypothesis-driven intervention studies to be aligned with biologically meaningful windows rather than arbitrary post-injury time points.

A key methodological outcome was the empirical comparison of brain mononuclear cell isolation strategies. Enzymatic digestion consistently yielded the highest microglial recovery compared to dounce homogenization or magnetic bead-based approaches, endorsing its selection as the default method for subsequent time-course experiments. This finding is consistent with broader methodological literature indicating that mechanical dissociation alone may inadequately recover tightly embedded parenchymal immune cells and may leave substantial debris and myelin that complicate downstream cytometry, whereas carefully optimized enzymatic dissociation enhances the liberation of microglia and infiltrating leukocytes for gradient purification and phenotyping (Srakočić et al., 2022). Another consideration is that the cell isolation procedure itself can influence downstream immune readouts. Enzymatic digestion was necessary in this study as it enhanced leukocyte recovery from brain tissue sufficiently to allow for multiparameter flow cytometry and intracellular cytokine staining (ICS) on infiltrating populations. However, recent research has demonstrated that enzymatic dissociation of brain tissue can induce ex vivo activation signatures, particularly in myeloid cells (Mattei et al., 2020). This does not invalidate the current findings, but it does imply that the strength of the dataset lies in the use of a consistent isolation workflow across groups, along with viability gating and matched handling, rather than in asserting that the isolated cells are a completely unperturbed reflection of their in vivo state (Srakočić et al., 2022).

This chapter addresses a critical methodological issue concerning the interpretation of ICS in leukocytes derived from brain tissue. ICS does not offer a passive representation of all cytokines produced by a cell in vivo prior to isolation. Instead, it identifies cytokines that are

actively synthesized and retained during the ex vivo capture period. It suggests that, once cells are extracted from the injured brain, many cease translating sufficient cytokine to accumulate a detectable intracellular pool during the assay period. This distinction is crucial for interpretation, as the results should be understood as indicative of inducible cytokine competence rather than direct evidence of ongoing spontaneous cytokine secretion at the time of harvest. This distinction is particularly pertinent in brain tissue, where low cell numbers, myelin contamination, and dissociation stress can all diminish assay sensitivity. Within this framework, the stimulation comparison conducted in this chapter was essential rather than merely technical. The objective was not only to optimize signal but also to identify a condition that could consistently capture cytokines from scarce post-traumatic brain leukocytes while maintaining acceptable viability. PMA (20ng/ml) + Ionomycin (0.5μg/ml) was ultimately deemed most suitable for the downstream time course, as it facilitated robust cytokine accumulation within a short incubation period, thereby reducing reliance on prolonged culture and the preservation of accessory cell interactions post-tissue isolation and is supported by the studies in neurotrauma and ischemia-associated neuroinflammation (Daglas et al., 2019; Shichita et al., 2009). In contrast, receptor-mediated stimulation is biologically appealing due to its closer resemblance to physiological activation, but in dissociated brain preparations, it is more susceptible to variability in cell fitness, receptor integrity, and the loss of in vivo contextual signals. Consequently, the assay employed here should be regarded as a functional standardization step that enabled the comparison of effector programs longitudinally across injury time points (Mandala et al., 2021). Practically, the protocol established in this chapter represents a compromise between maximal recovery and minimal artifact, which is often unavoidable in neuroimmunology studies of low-frequency brain-infiltrating leukocytes.

The biological framework that emerges aligns closely with contemporary TBI literature. Initial tissue disruption results in the release of DAMPs, that rapidly trigger chemokine expression and the recruitment of peripheral leukocytes (Corps et al., 2015). Neutrophils are among the earliest cells to infiltrate following experimental TBI, with the choroid plexus identified as a significant pathway and source of neutrophil-attracting chemokines, such as CXCL1, following CCI (Chodobski et al., 2003; Szmydynger-Chodobska et al., 2009). This is important as neutrophils are now recognized as more than mere early indicators of TBI. Collectively, these observations substantiate the interpretation that the early neutrophil wave identified in this chapter is likely to contribute directly to secondary injury cascades (Alam et al., 2020).

The subsequent phase involving monocytes and dendritic cells is of equal significance, as it offers a plausible connection between the acute sensing of innate injury and the subsequent recruitment of lymphocytes. The production of CCL2 associated with TBI by the choroid plexus has been demonstrated to facilitate monocyte trafficking across the blood-cerebrospinal fluid (CSF) barrier (Szmydynger-Chodobska et al., 2012). Furthermore, age exacerbates this CCR2/CCR5-associated monocyte response, indicating that monocyte recruitment is not merely a by-product of injury but a regulated axis that may affect vulnerability and recovery (Morganti et al., 2016). The role of dendritic cells (DCs) in TBI is less well-defined compared to neutrophils or monocytes; however, existing research suggests that dendritic cells do appear in the injured cortex, are influenced by chemokine networks including CCR2 and CXCL10, and may engage in antigen presentation and lymphocyte organization (Israelsson et al., 2014). Concurrently, systemic studies in TBI patients and related acute brain injury cohorts have reported both quantitative and functional defects in dendritic cells (Fridh et al., 2025) , supporting the notion that dendritic cell biology following TBI is dual in nature, potentially pro-inflammatory within the injured CNS, yet systemically associated with post-traumatic immune dysfunction (Israelsson et al., 2014; Roquilly et al., 2013).

The subacute study primarily focused on the infiltration dynamics of T cells post-injury and it aligns well with the broader field of TBI research. The observed upregulation of Icam1, Itgb2, and Cxcl10 indicates that the injured brain establishes a vascular and chemotactic milieu conducive to lymphocyte infiltration and retention. This interpretation is corroborated by studies demonstrating that Icam1 facilitates leukocyte adhesion and transmigration following TBI (Bhowmick et al., 2021). Similarly, clusters of CXCL10-expressing inflammatory cells emerge rapidly following TBI, with subsequent research linking CXCL10 signalling to Th1-type T cell infiltration (Israelsson et al., 2010). The predominance of IFN-γ producing $CD4^+$, $CD8^+$, and NK-T lymphocytes in this chapter is thus biologically plausible and consistent with an inflammatory type 1 program. This is particularly pertinent for $CD8^+$ T cells, as persistent effector or memory $GzmB^+$ $CD8^+$ T cells have been shown to accumulate post-injury, contributing to chronic neurological impairment and myelin pathology (Daglas et al., 2019). The observation that $\gamma\delta^+$ T cells constitute the predominant IL-17 producing subset, and that this program manifests prior to the more pronounced IFN-γ-associated phase, substantiates the hypothesis that $\gamma\delta^+$ T cells serve as an initial link between innate and adaptive immunity following TBI. Recent research specific to TBI reinforces this interpretation by demonstrating that $\gamma\delta^+$ T cells influence pathology from the acute phase

onward, with distinct $\gamma\delta^+$ T cell subsets exhibiting opposing roles (Abou-El-Hassan et al., 2023). In that study, Vγ4 $\gamma\delta^+$ T cells infiltrated the injured brain and secreted IL-17 and IFN-γ, thereby promoting microglial activation and neuroinflammation, whereas Vγ1 $\gamma\delta^+$ T cells produced TGF-β and were comparatively protective (Abou-El-Hassan et al., 2023). IL-17 has been implicated in the pathogenesis of multiple sclerosis (MS), with Th17 lymphocytes demonstrating effective transmigration across the BBB and exhibiting cytolytic GzmB (Kebir et al., 2007). Recent preclinical studies have further indicated that an increase in Th17 cells is followed by a rise in cytotoxic $CD8^+$ T cells, suggesting a potential role for Th17 cells in mediating cytotoxicity and neuroinflammation (Daglas et al., 2019). Notably, it was observed that $\gamma\delta^+$ T cells commenced IL-17 production prior to IFN-γ. Previous research has shown that IL-17 producing $\gamma\delta^+$ T cells are antigen-naïve, whereas IFN- γ producing $\gamma\delta^+$ T cells are antigen-experienced. These findings imply that $\gamma\delta^+$ T cells may acquire antigen specificity at later stages (Jensen et al., 2008).

The findings presented in this chapter indicate that the innate immune system is the initial responder following TBI, followed by the activation of T cells. The temporal analysis conducted using flow cytometry is essential for establishing intervention studies aimed at mitigating neuroinflammation.

Chapter 4

From innate activation to T cell engagement post-TBI

Hypothesis: The primary early cellular source of IL-1β promotes subsequent effector T cell responses particularly IL-17 and IFN-γ secreting T cells, which subsequently contribute to post-traumatic behavioural dysfunction.

4.1 Introduction

4.1.1 From descriptive immune profiling to mechanistic testing

The preceding chapter delineated the temporal framework of post-traumatic neuroimmune activity. The subsequent logical progression involves transitioning from mere description to an exploration of causality by examining whether key innate immune effector pathways actively influence downstream disease mechanisms and the development of pathogenic adaptive immunity. This chapter, therefore, investigates the role of the innate immune system as a catalyst in secondary injury biology, with a particular emphasis on the IL-1β axis as a potential "licensing" signal that connects early innate activation to subsequent T cell effector programs. This rationale is consistent with the broader neurotrauma literature, which underscores that neuroinflammation significantly contributes to secondary injury and chronic sequelae, while effective therapy likely necessitates targeted immunomodulation rather than broad suppression (Simon et al., 2017).

4.1.2 Sterile danger sensing and amplification loops upstream of IL-1β

A characteristic feature of TBI is the occurrence of sterile inflammation, which is initiated by DAMPs. Molecules such as HMGB1 can activate innate signalling pathways through receptors such as TLR4 and the receptor for advanced glycation end-products (RAGE). This activation promotes nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-driven cytokine programs, including IL-1 family mediators, which can perpetuate neurovascular dysfunction and cognitive impairment (Ahmad et al., 2013; Bodnar et al., 2021; Du et al., 2024). These DAMP-triggered pathways provide a rationale for targeting downstream innate effector nodes to interrupt amplification loops once injury signalling is established (Paudel et al., 2018).

IL-1β is a critical inflammatory cytokine in the context of experimental neurotrauma, as it plays a pivotal role in initiating extensive transcriptional inflammatory pathways and influences vascular integrity, glial activation, and neuronal function (Bodnar et al., 2021; Lu et al., 2005; Ozen et al., 2020). In murine models of TBI, the neutralization of IL-1β has been shown to reduce lesion burden and tissue loss, as well as enhance cognitive outcomes, thereby supporting its direct role rather than being merely an epiphenomenon (Clausen et al., 2009). The IL-1 pathway presents a viable target for clinical intervention. A phase II randomized controlled trial investigating the use of recombinant human IL-1 receptor

antagonist (Anakinra) in cases of severe TBI demonstrated the feasibility of this approach, identified safety signals within the trial's framework, and showed measurable brain extracellular exposure with modulation of inflammatory mediator profiles. These findings, in conjunction with preclinical data on neutralization, offer a compelling translational rationale for exploring IL-1β associated innate pathways in mechanistic studies of TBI (Helmy et al., 2014).

4.1.3 Innate effector mechanisms relevant to this chapter's intervention

This chapter targets three innate nodes that are strongly positioned to shape IL-1β linked secondary injury biology.

1. Neutrophils can exacerbate injury through proteolytic and oxidative pathways, as well as through the formation of NETs. These mechanisms have been associated with cerebrovascular dysfunction, hypoperfusion, tissue hypoxia, and exacerbated neurological deficits following experimental TBI, with corroborative human data indicating circulating NET signatures (Vaibhav et al., 2020). Given that these processes can contribute to endothelial stress responses, and cytokine amplification, attenuating neutrophil activity presents a mechanistic approach to investigate whether early innate effector functions influence subsequent immune programming and tissue dysfunction.
2. CCR2-dependent inflammatory monocytes and monocyte-derived macrophages are key innate effectors following TBI (Hsieh et al., 2014; Morganti et al., 2015), positioned to contribute to cytokine production, antigen presentation capacity, and the remodelling of the lesion microenvironment. While the previous chapter focused on dynamics, the mechanistic emphasis here is that CCR2-dependent monocyte programs can act as potent sources and amplifiers of IL-1β linked inflammatory signals. This makes monocyte depletion a direct test of whether this innate axis is necessary for subsequent maladaptive T cells.
3. IL-1β is regulated at both transcriptional and post-translational levels. The conversion of pro- IL-1β to mature IL-1β is classically mediated by inflammasome assembly and caspase-1 activation. In experimental neurotrauma, inflammasome components are engaged following TBI, and therapeutic targeting of inflammasome signalling has been shown to reduce IL-1β processing and tissue damage (de Rivero Vaccari et al., 2009). In parallel, clinical studies have detected inflammasome-related proteins in

the CSF after TBI and proposed them as biomarkers linked to injury severity and outcome, supporting the translational relevance of inflammasome-dependent inflammatory biology (Adamczak et al., 2012). Together, these data provide a strong rationale for targeting inflammasome pathways as a means to modulate IL-1β bioactivity after TBI. A therapeutically attractive inflammasome node in this context is NLRP3. Unlike approaches that target a single innate immune cell population, pharmacological inhibition of NLRP3 inflammasome is designed to limit inflammasome-dependent IL-1β maturation across multiple cellular sources that may contribute to post-traumatic inflammation. MCC950, a selective NLRP3 inhibitor (Coll et al., 2015), has been reported to reduce edema and inflammatory signalling and to improve functional outcomes in experimental TBI (Ismael et al., 2018; Xu et al., 2018). This positions MCC950 as a complementary, pathway-level strategy in this chapter to test whether limiting inflammasome-dependent IL-1β activation can alter downstream immune programming and broader post-traumatic tissue phenotypes (Ismael et al., 2018).

4.1.4 Why IL-1β is a plausible "licensing" signal for adaptive immune pathogenesis?

IL-1β is a strong candidate link between innate activation and adaptive effector programming because it can act directly on T cells and antigen-presenting cells to shape magnitude, trafficking, and effector function. IL-1 signalling enhances antigen-specific $CD8^+$ T cell expansion and effector differentiation and supports tissue localization and memory development (Ben-Sasson et al., 2013). IL-1β is also a key driver of type-17 immune programs. Human Th17 polarization in serum-free conditions requires IL-1β in combination with partner cytokines such as TGF-β and IL-6, IL-21 or IL-23 (Manel et al., 2008). In addition, IL-1β together with IL-23 can rapidly induce IL-17 production from $\gamma\delta^+$ T cells, providing a mechanistic route for early innate cytokines to generate IL-17 biased lymphocyte responses (Sutton et al., 2009). In experimental TBI, the pathological relevance of adaptive immunity is supported by evidence that activated cytotoxic $CD8^+$ T cells accumulate after injury and contribute to long-term neurological impairment, providing a downstream outcome axis that could plausibly be modified by IL-1β linked innate programming (Daglas et al., 2019).

This framework collectively positions IL-1β associated innate signalling as a plausible upstream regulator of subsequent post-traumatic immune trajectories. The experiments detailed in this chapter aim to evaluate whether targeting neutrophils, CCR2-dependent

monocyte/macrophage recruitment, or NLRP3-dependent inflammasome activity can modify downstream secondary injury processes and the subsequent emergence of pathogenic T cell responses. This approach provides a mechanistic link from the descriptive immune atlas developed in the preceding chapter to the causal examination of whether early innate immune pathways contribute to the development of chronic adaptive pathology following TBI (Daglas et al., 2019; Ismael et al., 2018; Xu et al., 2018).

4.2 Results

4.2.1 Validation of neutrophil depletion using an anti-Ly6G piggyback strategy in acute TBI

To assess the efficacy of neutrophil depletion, a previously described "piggy-back" neutrophil depletion strategy was employed (Boivin et al., 2020). In this approach, anti-Ly6G binds Ly6G-expressing neutrophils, while a secondary anti-rat κ antibody binds the rat anti-Ly6G antibody already attached to the neutrophil surface. This amplifies antibody-mediated clearance and improves depletion efficiency compared with anti-Ly6G alone. Adult C57BL/6J mice were administered i.p. injections of anti-Ly6G (100μg) combined with anti-rat κ (100μg) (treatment) or anti-rat κ with IgG2a (isotype control) prior to undergoing either sham surgery or CCI, with tissue collection occurring 12 hpi **(Fig. 4.2.1 A)**. Utilizing flow cytometry with intracellular Ly6G staining enabled the precise identification of Ly6G+ neutrophils **(Fig. 4.2.1 B)**. TBI resulted in a significant elevation of $Ly6G^+$ cells in the ipsilateral hemisphere compared to sham controls, whereas anti-Ly6G treatment reduced the number of $Ly6G^+$ cells in the brain to levels comparable to sham, indicating effective depletion at this acute time point **(Fig. 4.2.1 C)**. In peripheral blood, TBI similarly elevated circulating $Ly6G^+$ cells relative to sham, while anti-Ly6G treatment significantly decreased $Ly6G^+$ counts compared to TBI controls **(Fig. 4.2.1 D)**. Collectively, these findings confirm effective short-term neutrophil depletion in both the injured brain and circulation at 12 hours post-injury.

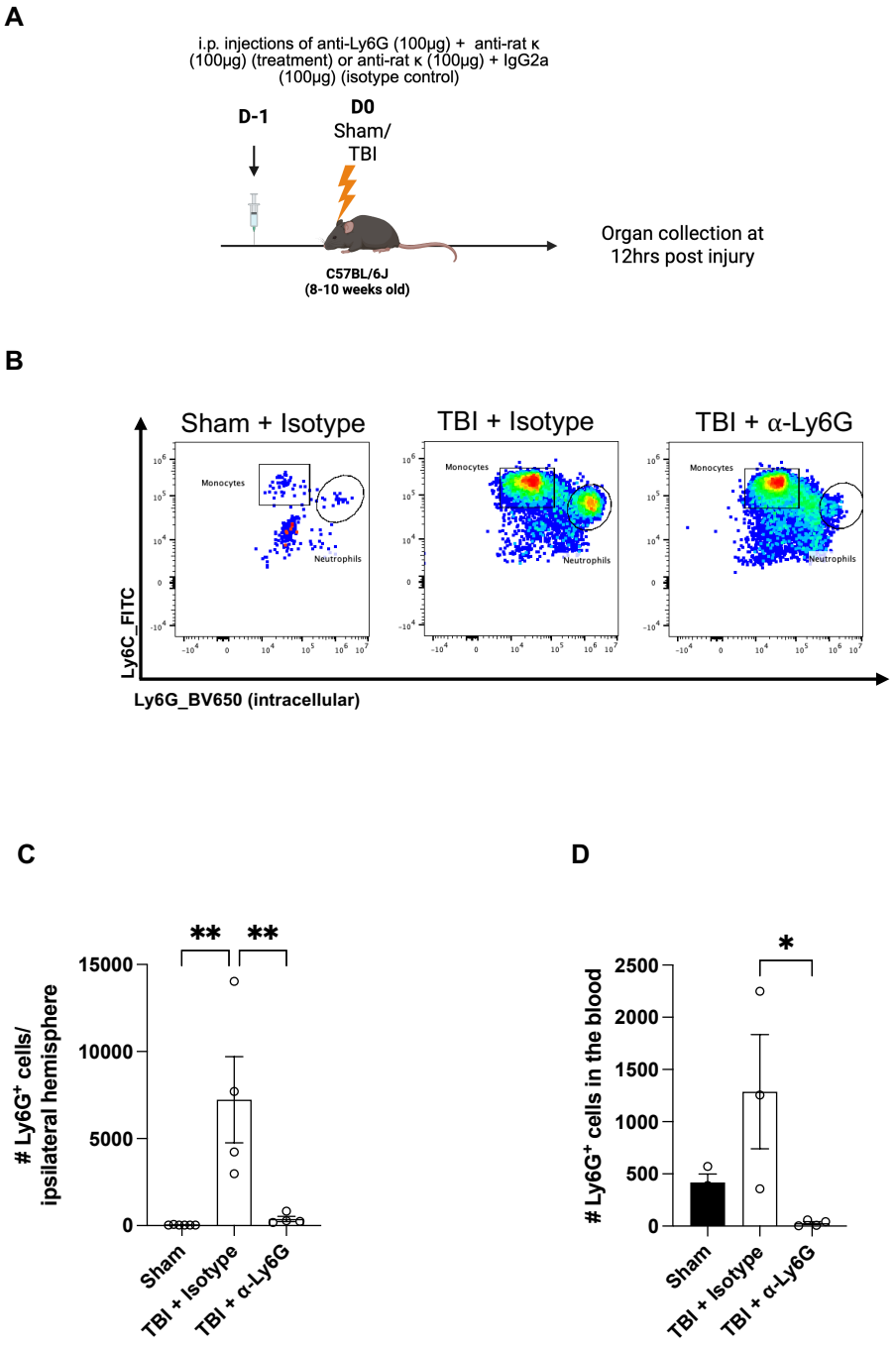

Fig. 4.2.1: Anti-Ly6G plus anti-rat-k efficiently reduces Ly6G+ neutrophils in blood and injured brain 12 h after TBI. (A) Experimental design. (B) Representative flow cytometry plots of the group are presented. (C,D) The bar graph illustrates the absolute number of neutrophils present in the brain (C) and blood (D). Data= Mean ± SEM (n=3-4 per group); *p <0.05, **p <0.01, ***p <0.001 by one-way ANOVA, with Tukey's multiple comparisons.

4.2.2 Validation of CCR2-dependent inflammatory monocyte depletion with MC-21 in acute TBI

To assess the depletion of inflammatory monocytes, a previously described depletion strategy was employed (Mack et al., 2001), C57BL/6J mice were administered a dose of MC-21 (anti-CCR2; 50μg) or an IgG2b isotype control i.p., 24 hours prior to either sham surgery or CCI, with tissue collection occurring 12 hpi **(Fig. 4.2.2 A)**. Representative flow cytometry plots demonstrate that TBI resulted in an increased $Ly6C^+$ population, whereas MC-21 significantly reduced $Ly6C^+$ cells **(Fig. 4.2.2 B**). Quantitative analysis confirmed a substantial increase in $Ly6C^+$ cells in the ipsilateral hemisphere following TBI, which was mitigated by MC-21 treatment, reducing levels to near those observed in sham conditions **(Fig. 4.2.2 C)**. In peripheral blood, TBI similarly elevated circulating $Ly6C^+$ cells, and MC-21 decreased these cells compared to TBI isotype controls **(Fig. 4.2.2 D**), indicating effective systemic depletion. Collectively, these findings demonstrate that pre-injury administration of MC-21 effectively depletes CCR2-dependent $Ly6C^+$ inflammatory monocytes in both the circulation and the injured brain during the acute phase following TBI.

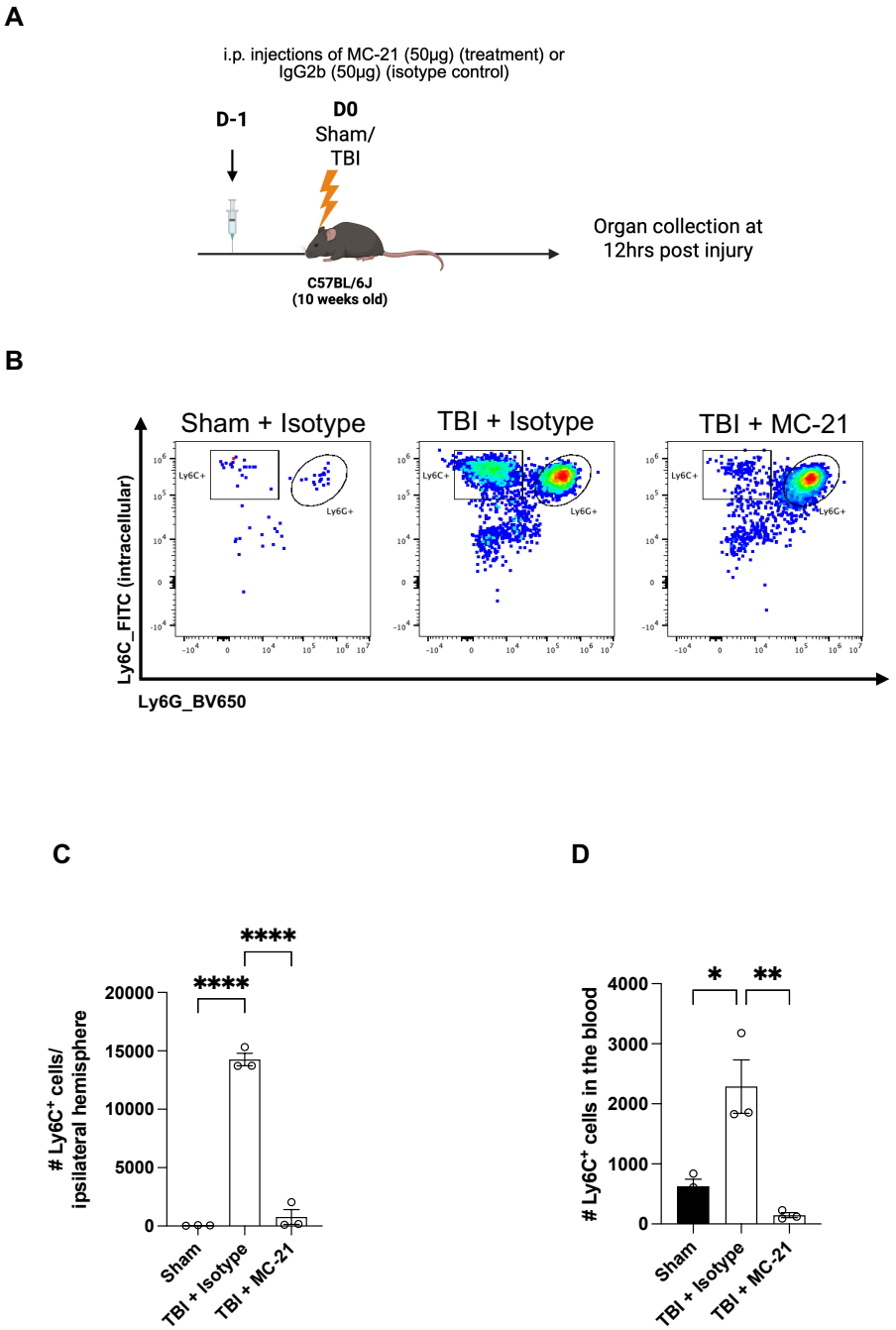

Fig. 4.2.2: MC-21 pre-treatment depletes Ly6C+ inflammatory monocytes in blood and injured brain 12 h after TBI. (A) Experimental design. (B) Representative flow cytometry plots of the group are presented. (C,D) The bar graph illustrates the absolute number of monocytes present in the brain (C) and blood (D). Data= Mean ± SEM (n=3 per group); *p <0.05, **p <0.01, ***p <0.001 by one-way ANOVA, with Tukey's multiple comparisons.

4.2.3 Therapeutic NLRP3 inhibition with MCC950 reduces early microglial accumulation and activation after TBI

To evaluate the impact of pharmacological inhibition of the inflammasome on early microglial responses following TBI, mice were administered either a vehicle or MCC950 (10mg/kg); (Coll et al., 2015) i.p., starting 2 hpi, with subsequent doses at 12 hpi, 1 , and 2 dpi. Tissue samples were collected at 3 dpi **(Fig. 4.2.3 A)**. TBI resulted in a significant increase in the numbers of microglia in the ipsilateral hemisphere compared to sham controls, treatment with MCC950 significantly reduced the total number of microglia relative to vehicle-treated TBI mice, although the counts remained elevated compared to sham levels **(Fig. 4.2.3 B)**. In alignment with these findings, TBI markedly increased the number of IL-1β^+ microglia in the ipsilateral hemisphere, and MCC950 significantly mitigated this IL-1β^+ microglial response compared to the vehicle **(Fig. 4.2.3 C)**. Additionally, microglia expressing major histocompatibility complex class II (MHC-II) increased following TBI, and this activation marker was significantly reduced by MCC950 treatment **(Fig. 4.2.3 D)**. Among the infiltrating myeloid populations, there was a significant increase in IL-1β^+ neutrophils in vehicle treated TBI mice compared to sham controls. In contrast, MCC950-treated mice exhibited a numerically lower response, although this difference was not statistically significant when compared to the vehicle group **(Fig. 4.2.3 E)**. Similarly, IL-1β^+ monocytes were elevated following TBI in both vehicle and MCC950 treated animals relative to sham controls, with no significant reduction observed in the MCC950-treated group **(Fig. 4.2.3 F)**. Collectively, these results indicate that MCC950 mitigates the early accumulation and activation of microglia in the injured hemisphere at 3 dpi, while exerting minimal effects on IL-1β+ infiltrating neutrophil and monocyte populations at this time point.

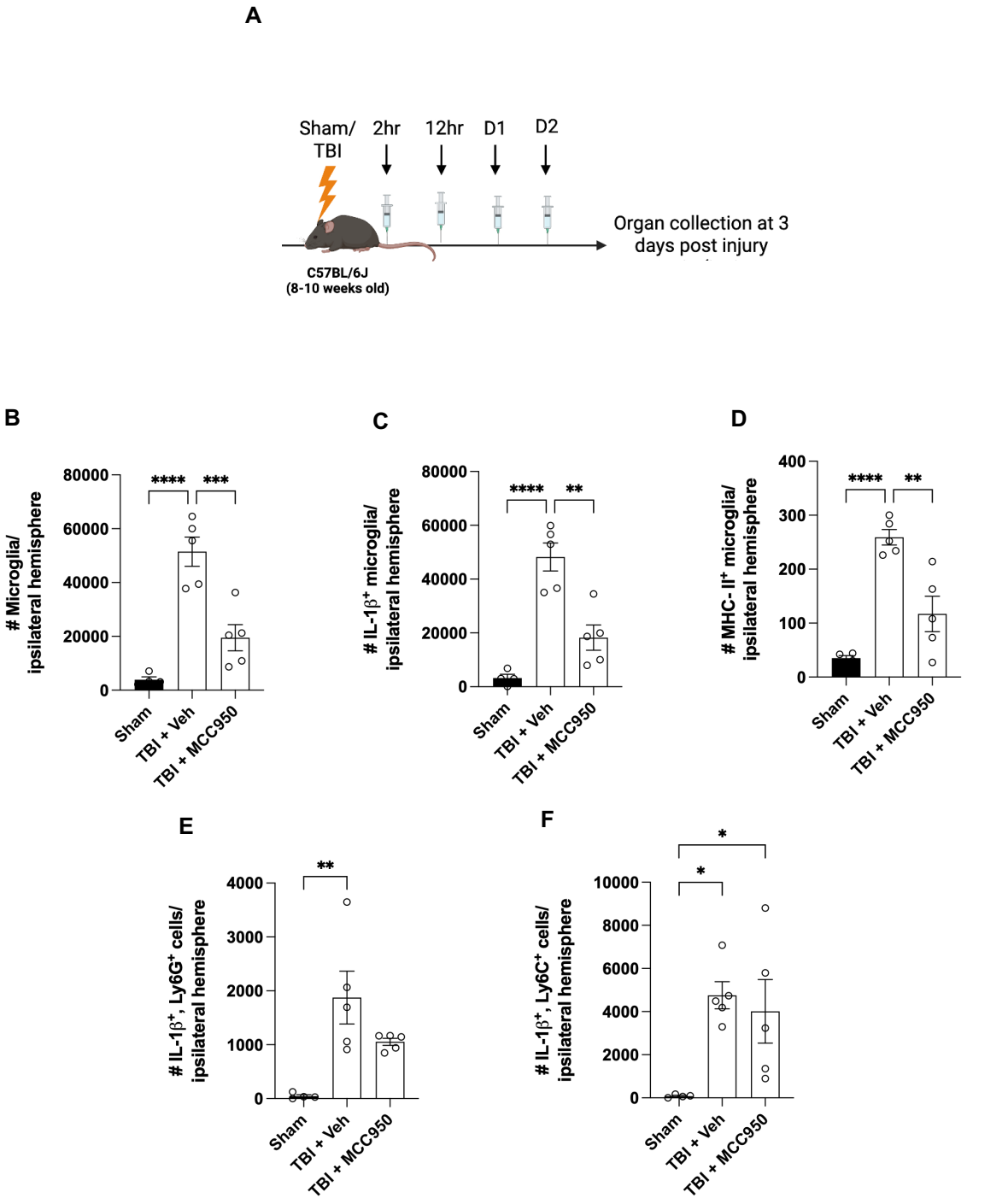

Fig. 4.2.3: MCC950 dosing from 2 h post-injury attenuates total microglia and reduces IL-1β$^+$ and MHC-II$^+$ microglial responses in the ipsilateral hemisphere at 3 days after TBI. (A) Experimental design. (B-F) The bar graph illustrates the absolute number of microglia (B), IL-1β$^+$ microglia (C) , MHC-II$^+$ microglia (D), IL-1β$^+$ Ly6G$^+$ neutrophils (E), IL-1β$^+$ Ly6C$^+$ monocytes (F). Data= Mean ± SEM (n=4-5 per group); *p <0.05, **p <0.01, ***p <0.001 by one-way ANOVA, with Tukey's multiple comparisons.

4.2.4 Neutrophil depletion increases IL-1β$^+$ myeloid cell accumulation and the expansion of IL-17$^+$ γδ$^+$ T cells in the injured brain

Following the rapid recruitment of IL-1β expressing neutrophils to the injured brain, the role of neutrophils in modulating subacute adaptive immune responses following TBI was evaluated through repeated anti-Ly6G treatment. Both sham and CCI mice received anti-Ly6G (or isotype control) i.p., as previously described in section **4.2.1** at 1 and 2 dpi. Brains were collected at 3 dpi for flow cytometric analysis. The isolated brain mononuclear cells were ex vivo stimulated with PMA and ionomycin in the presence of Brefeldin A before staining **(Fig. 4.2.4 A)**. TBI induced a significant increase in Ly6G$^+$ neutrophils compared to sham mice, while anti-Ly6G treatment resulted in only a partial reduction in neutrophil numbers, which did not reach statistical significance ($p = 0.07$**; Fig. 4.2.4 B**), indicating incomplete attenuation of the neutrophil compartment at 3 dpi. Concurrently, Ly6C$^+$ monocytes were elevated following TBI and were further increased in anti-Ly6G-treated TBI mice, suggesting an augmented inflammatory monocyte response in the context of neutrophil targeting **(Fig. 4.2.4 C)**. Consistent with a pro-inflammatory infiltrate, IL-1β$^+$ neutrophils were elevated post-injury **(Fig. 4.2.4 D)**, but was not altered by anti-Ly6G treatment at 3 dpi ($p = 0.4$ vs CCI + isotype), aligning with the incomplete reduction in total neutrophils. In contrast, IL-1β$^+$ monocytes were increased after TBI **(Fig. 4.2.4 E)** and were further augmented by anti-Ly6G treatment, indicating that neutrophil targeting coincided with enhanced IL-1β-associated activation within the monocyte compartment.

Adaptive immune responses were also amplified in anti-Ly6G-treated TBI mice. TBI increased CD4$^+$ T cell numbers in the injured brain **(Fig. 4.2.4 F)** and increased IFN-γ$^+$ CD4$^+$ T cells **(Fig. 4.2.4 G)**, further elevated in the anti-Ly6G group. A similar trend was observed for CD8$^+$ T cells **(Fig. 4.2.4 H)** and IFN-γ$^+$ CD8$^+$ T cells **(Fig. 4.2.4 I)**, again indicating heightened type 1 effector T cell activity following neutrophil targeting. The most pronounced change was observed in the γδ$^+$ T cell compartment, anti-Ly6G-treated TBI mice exhibited an expansion of γδ$^+$ T cells **(Fig. 4.2.4 J)** and a marked increase in IL-17$^+$ γδ$^+$ T cells **(Fig. 4.2.4 K)**, highlighting a shift toward an IL-17-associated inflammatory adaptive profile. Resident innate immune activation was evident at 3 dpi, with TBI increasing microglial numbers **(Fig. 4.2.4 L)** and elevating IL-1β$^+$ microglia **(Fig. 4.2.4 M)**. Notably, IL-1β$^+$ microglia were further increased in anti-Ly6G treated TBI mice compared with isotype-treated TBI mice, consistent with an overall enhancement of IL-1β-linked inflammatory signalling within the injured brain. *Il1b*

mRNA expression in the hippocampus was increased by TBI, and this was partially reduced by anti-Ly6G treatment **(Fig. 4.2.4 N)**.

Despite these substantial alterations in infiltrating and resident immune populations, anti-Ly6G treatment did not improve early motor outcomes. Rotarod performance was comparable between anti-Ly6G and isotype-treated TBI mice, and AUC analysis demonstrated no significant treatment effect ($p = 0.6$; **Fig. 4.2.4 O**). Overall, the data indicate that repeated anti-Ly6G treatment did not confer an early functional advantage following TBI, and was associated with an enhanced inflammatory profile rather than suppression of neuroinflammation. This profile was characterized by increased IL-1β^+ monocytes and microglia alongside amplified T cell responses, most prominently the expansion of IL-17 producing $\gamma\delta^+$ T cells within the injured brain.

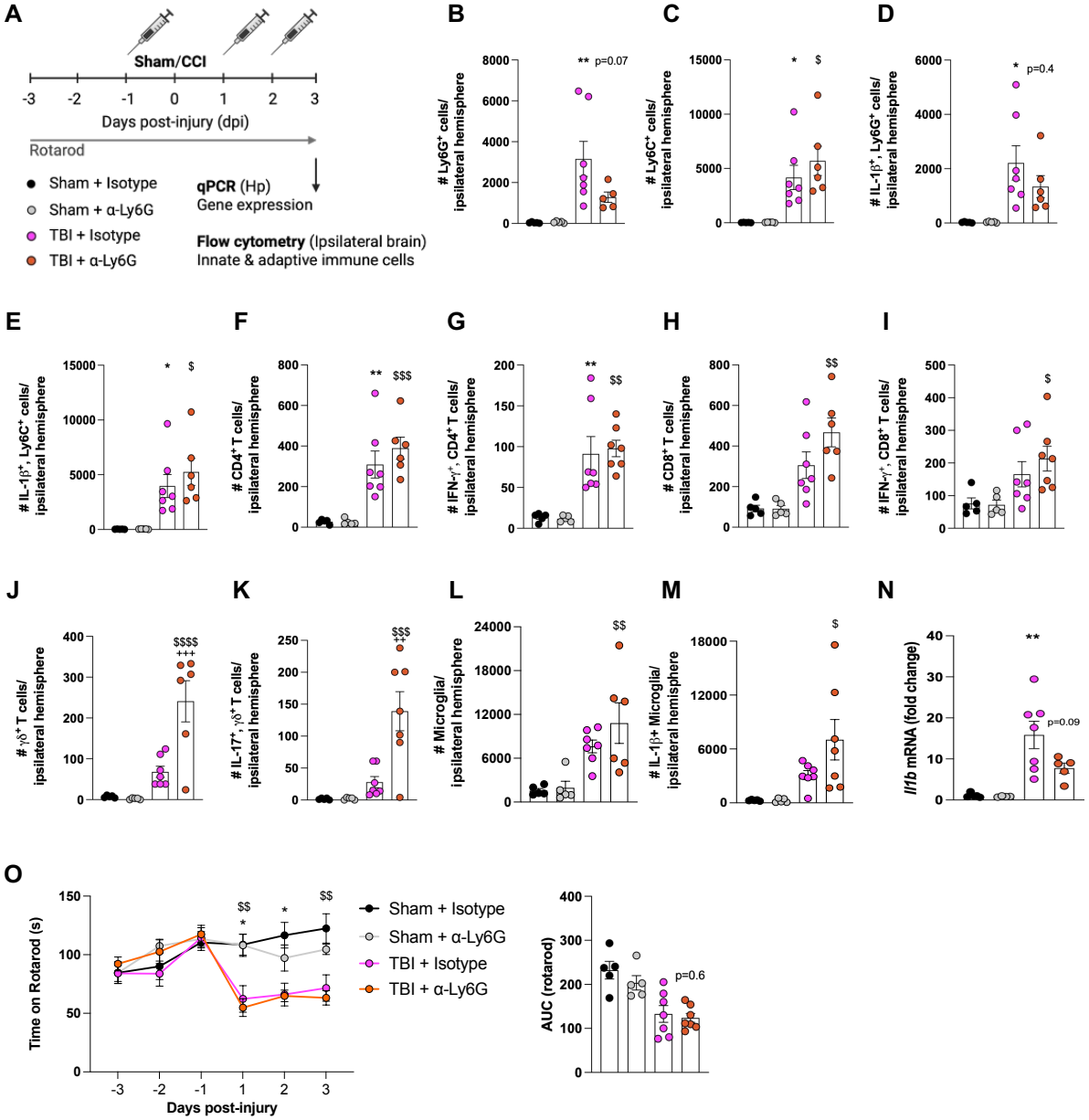

Fig. 4.2.4: Neutrophil depletion does not alter T cell numbers and cytokine production post-TBI. (A) Experimental design. (B-M) Bar graphs illustrate the absolute numbers of Ly6G$^+$ neutrophils (B), Ly6C$^+$ monocytes (C), IL-1β producing neutrophils (D), IL-1β producing monocytes (E), CD4$^+$ T cells (F), IFN-γ producing CD4$^+$ T cells (G), CD8$^+$ T cells (H), IFN-γ producing CD8$^+$ T cells (I), γδ$^+$ T cells (J), IL-17 producing γδ$^+$ T cells (K), microglia (L), and IL-1β producing microglia (M). Relative fold-change expression levels of *Il1b* mRNA in hippocampus (N). Neurobehavioural tests: Rotarod (O). Data presented as Mean ± SEM (n=5-7 per group); Sham + Isotype vs. TBI + Isotype *p<0.05, **p<0.01, ***p<0.001; Sham + anti-Ly6G (α-Ly6G) vs. TBI + α-Ly6G $^{\$}$p<0.05, $^{\$\$}$p<0.01, $^{\$\$\$}$p<0.001; TBI + Isotype vs. TBI + α-Ly6G $^{+}$p<0.05, $^{++}$p<0.01, $^{+++}$p<0.001 by two-way ANOVA with post-hoc Tukey's test to correct for multiple comparisons.

4.2.5 Neutrophil depletion does not alter T cell effector phenotypes or neurobehavioral outcomes at 10 dpi

Building upon the initial findings at 3 dpi, this study subsequently investigated whether early neutrophil intervention influenced the subsequent T cell landscape and functional outcomes up to 10 dpi, where the isolated brain mononuclear cells were ex vivo stimulated with PMA and ionomycin in the presence of Brefeldin A before staining **(Fig. 4.2.5 A)**. At 10 dpi, TBI mice demonstrated a significant accumulation of $CD4^+$ T cells within the injured brain **(Fig. 4.2.5 B)**, however, anti-Ly6G treatment did not reduce the total number of $CD4^+$ T cells compared to isotype-treated TBI controls, suggesting that early neutrophil attenuation did not result in a sustained reduction in $CD4^+$ T cell recruitment or persistence. Concurrently, IFN-γ^+ **(Fig. 4.2.5 C)** and $GzmB^+$ **(Fig. 4.2.5 D)** $CD4^+$ T cells were elevated at 10 dpi, yet these effector-associated subsets were present at similar levels in both anti-Ly6G and isotype-treated TBI mice, further supporting the conclusion that neutrophil attenuation did not alter the magnitude of the sub-acute $CD4^+$ response. A similar pattern was observed in the CD8 T cell compartment. Total $CD8^+$ T cells remained elevated at 10 dpi following injury **(Fig. 4.2.5 E)**, and anti-Ly6G treatment did not diminish this response. Similarly, IFN-γ^+ **(Fig. 4.2.5 F)** and $GzmB^+$ **(Fig. 4.2.5 G)** $CD8^+$ T cells were elevated post-injury regardless of neutrophil attenuation, indicating that the broader $\alpha\beta$ T cell response at 10 dpi was largely independent of early neutrophil depletion. In contrast, the $\gamma\delta^+$ T cell compartment remained distinctly altered at 10 dpi and exhibited selective sensitivity to anti-Ly6G treatment. $\gamma\delta^+$ T cells were increased post-injury **(Fig. 4.2.5 H)** and were further elevated in anti-Ly6G-treated TBI mice, accompanied by a marked increase in IL-17^+ $\gamma\delta^+$ T cells **(Fig. 4.2.5 I)**. In comparison, IFN-γ^+ $\gamma\delta^+$ T cells were detected at very low levels at 10 dpi **(Fig. 4.2.5 J)**, and anti-Ly6G treatment did not measurably alter this subset. Thus, while early neutrophil attenuation did not suppress the dominant $CD4^+$ or $CD8^+$ effector responses at 10 dpi, it was associated with a selective enhancement of the IL-17 producing $\gamma\delta$ T cell axis.

To determine whether these immunological changes were accompanied by functional benefits, cognitive and motor recovery were assessed using established neurobehavioral paradigms. In the NOR task, discrimination indices did not differ between anti-Ly6G and isotype-treated TBI mice **(Fig. 4.2.5 K)**, suggesting no detectable improvement in recognition memory with neutrophil attenuation. Motor coordination was evaluated longitudinally using the beam walk test over the first 10 dpi. As expected, TBI resulted in significant deficits in beam walk performance relative to sham animals **(Fig. 4.2.5 L)**. However, anti-Ly6G

treatment did not improve motor performance compared with isotype-treated TBI mice, the AUC analysis across the testing period similarly showed no statistically significant difference between TBI + anti-Ly6G and TBI + isotype groups (p = 0.6). Overall, these data indicate that acute neutrophil attenuation did not prevent the establishment of a robust adaptive immune response by 10 dpi, it was associated with a sustained and selective amplification of the IL-17^{+} γδ^{+} T cell response, without measurable improvements in cognitive or motor outcomes over this time frame.

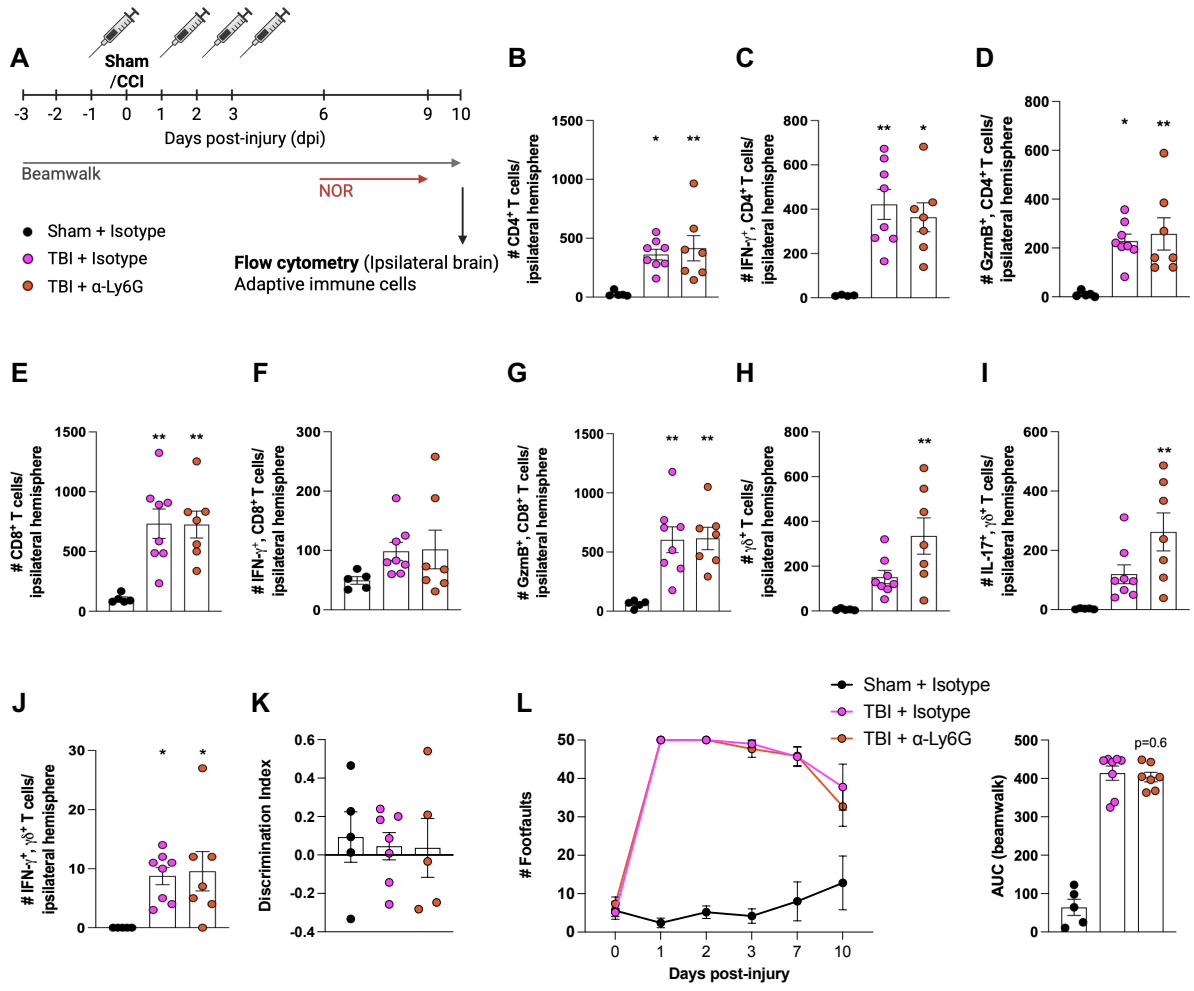

Fig. 4.2.5: Neutrophil depletion does not alter T cell numbers or their effector phenotype and does not improve neurobehavioural outcomes. (A) Experimental design. (B-J) Bar graphs illustrate the absolute numbers of CD4^{+} T cells (B), IFN-γ producing CD4^{+} T cells (C), GzmB producing CD4^{+} T cells (D), CD8^{+} T cells (E), IFN-γ producing CD8^{+} T cells (F), GzmB producing CD8^{+} T cells (G), γδ^{+} T cells (H), IL-17 producing γδ^{+} T cells (I), and IFN-γ producing γδ^{+} T cells (J). Neurobehavioral tests: Novel object recognition test (K), Beam walk (L). Data = Mean ± SEM (n=10 per group); Sham vs. TBI *p<0.05, **p<0.01, ***p<0.001; TBI + Isotype vs. TBI + α-Ly6G $^{+}$p<0.05, $^{++}$p<0.01, $^{+++}$p<0.001 by one-way ANOVA with Tukey's multiple comparisons test.

4.2.6 Monocyte depletion suppresses the recruitment and effector activation of CD8$^+$ and γδ$^+$ T cell subsets in the injured brain

Monocytes constituted a prominent secondary wave of IL-1β$^+$ peripheral leukocyte recruitment to the injured brain following the initial neutrophil response. Therefore, their contribution to acute post-injury inflammation was assessed by depleting inflammatory monocytes using MC-21 antibody (anti-CCR2) as described in **4.2.2**. This depletion strategy was then applied in sham and CCI mice using repeated MC-21 dosing at day −1, and 1 dpi, with rotarod assessment performed before and after injury, and brain tissue collected at 3 dpi for flow cytometric analysis. The isolated brain mononuclear cells were ex vivo stimulated with PMA and ionomycin in the presence of Brefeldin A before staining **(Fig. 4.2.6 A)**.

At 3 dpi, TBI significantly increased Ly6G$^+$ neutrophil numbers in the injured brain, and MC-21 did not attenuate this neutrophil accumulation **(Fig. 4.2.6 B)**; however, the marked TBI-induced increase in Ly6C$^+$ monocytes was completely abrogated by MC-21 treatment **(Fig. 4.2.6 C)**. Consistent with selective targeting of the monocyte compartment, IL-1β$^+$ monocytes were robustly increased after TBI and were reduced to near-baseline levels with MC-21 **(Fig. 4.2.6 E)**, whereas IL-1β$^+$ neutrophils remained elevated despite monocyte depletion **(Fig. 4.2.6 D)**. Monocyte depletion also modified early adaptive immune responses, with total CD4$^+$ T cells increased after TBI but not significantly altered by MC-21, alongside a trend towards reduced IFN-γ$^+$ CD4$^+$ T cells **(Fig. 4.2.6 F,G)**. In contrast, MC-21 reduced total CD8+ T cell accumulation and IFN-γ$^+$ CD8$^+$ T cells relative to TBI + isotype controls **(Fig. 4.2.6 H,I)**, and similarly reduced total γδ$^+$ T cells and IL-17$^+$ γδ$^+$ T cells **(Fig. 4.2.6 J,K)**.

Resident innate immune activation remained evident, as TBI increased total microglia and IL-1β$^+$ microglia, with MC-21 associated with a further increase in IL-1β$^+$ microglia that did not reach statistical significance **(Fig. 4.2.6 L,M)**, in addition TBI-induced MHC-II$^+$ microglia were not altered by MC-21 **(Fig. 4.2.6 N**). Despite these cellular profiles, hippocampal *Il1b* mRNA expression was reduced by MC-21 compared with TBI + isotype controls **(Fig. 4.2.6 O)**, indicating that CCR2-dependent monocytes contribute substantially to early *Il1b* transcriptional output after TBI. Functionally, TBI-induced rotarod impairments were not improved by monocyte depletion, and AUC analysis showed no difference between the TBI + MC-21 and TBI + isotype groups ($p = 0.5$) **(Fig. 4.2.6 P)**. Overall, these data indicate that CCR2$^+$ inflammatory monocytes act as key regulators of acute neuroinflammation after TBI by supporting early *Il1b* transcription and promoting early recruitment and effector activation of

CD8⁺ and γδ⁺ T cell subsets, but that depletion of this monocyte wave is insufficient to improve early gross motor recovery following TBI.

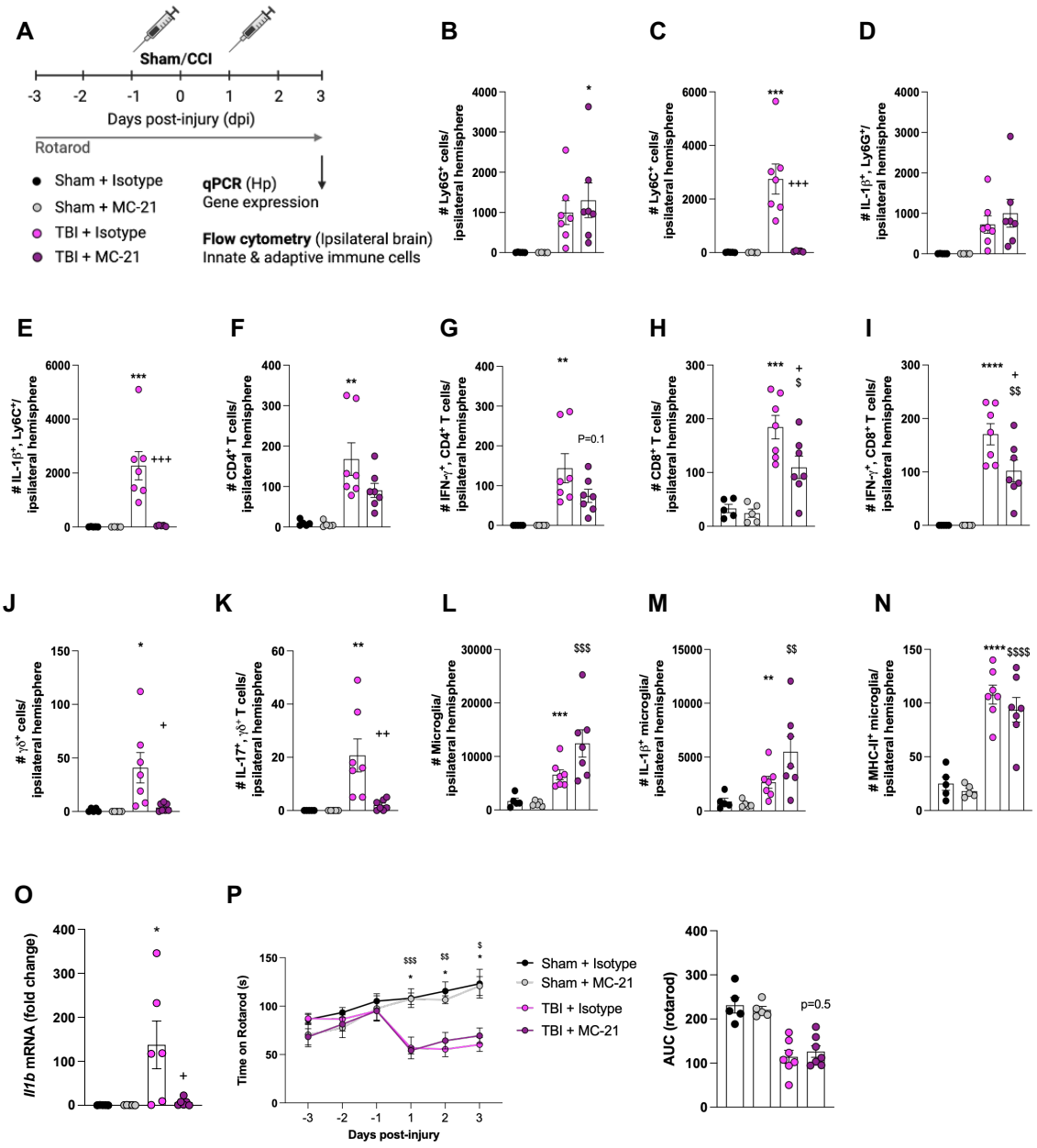

Fig. 4.2.6: Monocyte depletion acutely alters T cell numbers and their effector phenotype post-TBI. (A) Experimental design. (B-N) Bar graphs illustrate the absolute numbers of Ly6G⁺ neutrophils (B), Ly6C⁺ monocytes (C), IL-1β producing neutrophils (D), IL-1β producing monocytes (E), CD4⁺ T cells (F), IFN-γ producing CD4⁺ T cells (G), CD8⁺ T cells (H), IFN-γ producing CD8⁺ T cells (I), γδ⁺ T cells (J), IL-17 producing γδ⁺ T cells (K), microglia (L), IL-1β producing microglia (M), MHC-II⁺ microglia (N). Relative fold-change expression levels of *Il1b* mRNA in hippocampus (O). Neurobehavioural tests: Rotarod (P). Data are presented as Mean ± SEM (n=5-7 per group); Sham + Isotype vs. TBI + Isotype *p<0.05, **p<0.01, ***p<0.001; Sham + MC-21 vs. TBI + MC-21 $^{\$}$p<0.05, $^{\$\$}$p<0.01, $^{\$\$\$}$p<0.001; TBI + Isotype vs. TBI + MC-21 $^{+}$p<0.05, $^{++}$p<0.01, $^{+++}$p<0.001 by two-way ANOVA with post-hoc Tukey's test to correct for multiple comparisons.

4.2.7 Monocyte depletions results in partial motor function recovery but fails to alter T cell effector phenotypes at 10 dpi

This study next examined whether MC-21 mediated monocyte depletion altered adaptive immune responses and neurobehavioural recovery during the sub-acute phase after TBI, focusing on outcomes up to 10 dpi, where the isolated brain mononuclear cells were ex vivo stimulated with PMA and ionomycin in the presence of Brefeldin A before staining **(Fig. 4.2.7 A)**. Consistent with a sustained post-injury lymphocytic response, TBI induced a robust accumulation of $CD4^+$ T cells in the injured brain at 10 dpi **(Fig. 4.2.7 B**), accompanied by increased numbers of IFN-γ^+ **(Fig. 4.2.7 C)** and $GzmB^+$ **(Fig. 4.2.7 D)** $CD4^+$ T cells, indicating persistence of an activated and cytotoxic effector phenotype, these were not attenuated by the MC-21 treatment. A similar pattern was observed for $CD8^+$ T cells, where TBI produced significant increases in total $CD8^+$ T cell numbers **(Fig. 4.2.7 E)** as well as IFN-γ^+ **(Fig. 4.2.7 F)**and $GzmB^+$ **(Fig. 4.2.7 G)** $CD8^+$ T cells at 10 dpi. Again, these $CD8^+$ T cell responses were not reduced by monocyte depletion. $\gamma\delta$ T cell compartment also remained markedly altered at this time point, total $\gamma\delta^+$ T cells were increased following injury **(Fig. 4.2.7 H)**, alongside elevated IL-17^+ **(Fig. 4.2.7 I)** and IFN-γ^+ **(Fig. 4.2.7 J)** $\gamma\delta^+$ T cell populations, yet MC-21 treatment again failed to reduce total or cytokine-producing $\gamma\delta^+$ T cell numbers.

To evaluate the impact of monocyte depletion on functional recovery, despite ongoing adaptive immune activation, cognitive and motor outcomes were assessed using established behavioural assays. In the 2-arm Y maze, none of the groups exhibited a significant preference for the novel arm, as within-group paired t-tests comparing time spent in the novel versus old arm were non-significant for sham plus isotype ($p=0.1$), TBI plus isotype ($p=0.9$), and TBI plus MC-21 ($p=0.6$) **(Fig. 4.2.7 K)**. This suggests no detectable improvement in spatial recognition memory at 10 dpi. Neurological scoring using the SNAP test identified clear TBI-induced impairments in gross motor function (**Fig. 4.2.7 L;** $p<0.001$ versus sham)**,** but MC-21 treatment did not alleviate this deficit. Conversely, performance on a once-off accelerating rotarod test at 10 dpi revealed a differential effect, isotype-treated TBI mice displayed reduced latency to fall compared with sham animals (**Fig. 4.2.7 M**; $p<0.01$ versus sham). Notably, MC-21 treated TBI mice showed improved motor performance, remaining on the rotarod for significantly longer than isotype-treated TBI mice ($p<0.05$ versus TBI plus isotype). Fine motor coordination was monitored longitudinally using the beam walk test across the first 10 dpi. Isotype-treated TBI mice exhibited robust and sustained impairments relative to sham controls, whereas MC-21 treated TBI mice showed small

reductions in foot-fault scores at 7 and 10 dpi **(Fig. 4.2.7 N)**. These improvements did not reach statistical significance, as reflected by the AUC analysis (p=0.2). Collectively, these findings indicate that MC-21 mediated monocyte depletion does not significantly alter the magnitude or effector composition of the T cell response at 10 dpi, yet it is associated with subtle functional benefits, most notably improved rotarod performance, with more modest non-significant trends toward improved fine motor coordination.

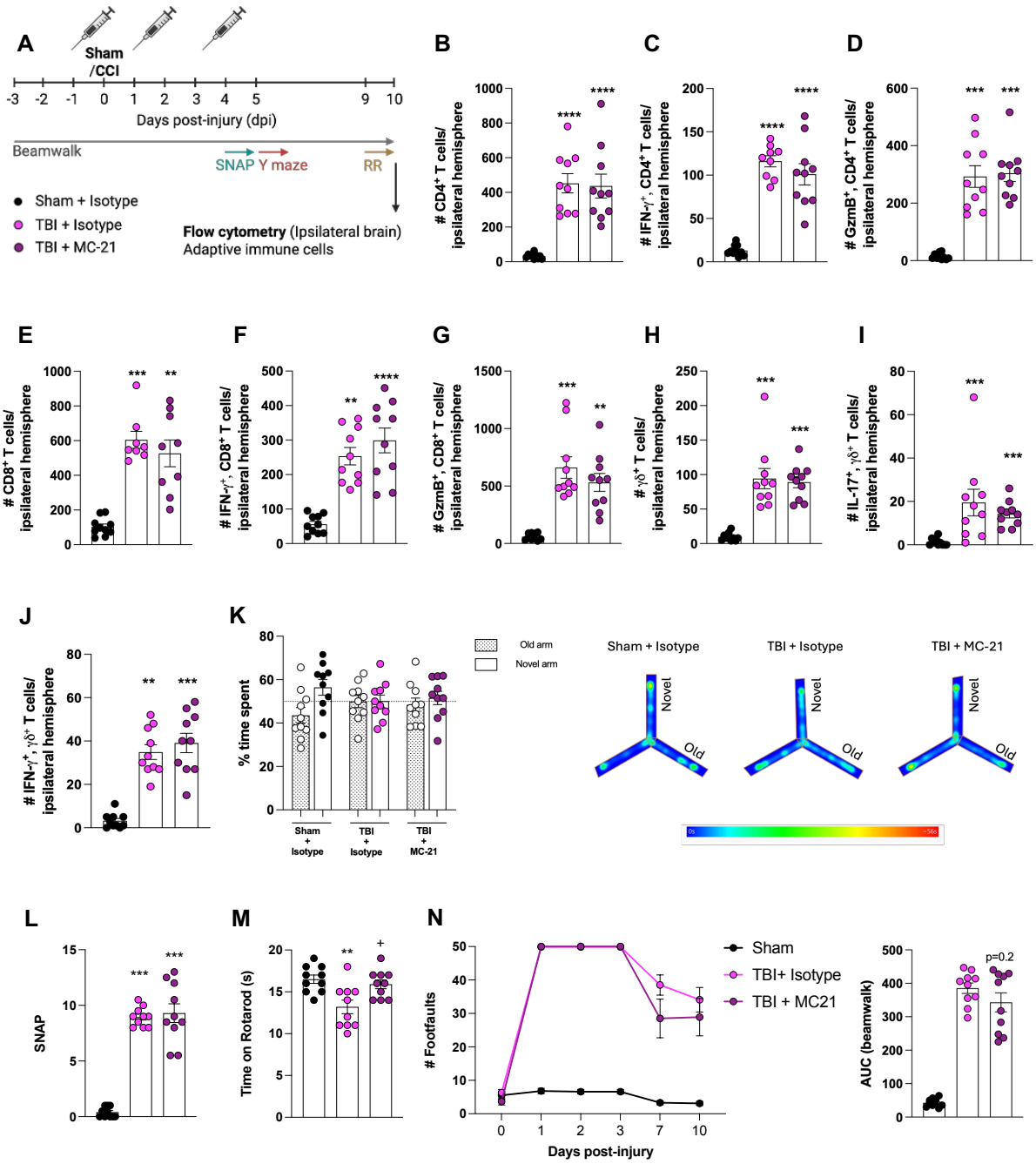

Fig. 4.2.7: Partial motor function recovery is observed at 10 dpi following monocyte depletion despite persistent T cell infiltration. (A) Experimental design. (B-J) Bar graphs illustrate the absolute numbers of $CD4^+$ T cells (B), IFN-γ producing $CD4^+$ T cells (C), GzmB producing $CD4^+$ T cells (D), $CD8^+$ T cells (E), IFN-γ producing $CD8^+$ T cells (F), GzmB producing $CD8^+$ T cells (G), $γδ^+$ T cells (H), IL-17 producing $γδ^+$ T cells (I), and IFN-γ producing $γδ^+$ T cells (J). (K-N) Neurobehavioral tests: Y-maze (K), SNAP score (L), Forced rotarod (M), Beam walk (N). Data = Mean ± SEM (n=10 per group); Sham vs. TBI *p<0.05, **p<0.01, ***p<0.001; TBI + Isotype vs. TBI + MC-21 $^+$p<0.05, $^{++}$p<0.01, $^{+++}$p<0.001 by one-way ANOVA with Tukey's multiple comparisons test.

4.2.8 NLRP3 inflammasome inhibition reduces microglial IL-1β without altering T cell effector phenotype

Considering the limited effect of neutrophil and monocyte depletion on adaptive immune responses following TBI, it was hypothesized that inflammasome-dependent IL-1β signalling through NLRP3 inflammasome might serve as a primary mechanism influencing the T cell landscape after injury. To investigate this, sham and CCI mice were treated with the selective NLRP3 inflammasome inhibitor MCC950, as outlined in section **4.2.3**, for immune profiling via flow cytometry, where the isolated brain mononuclear cells were ex vivo stimulated with PMA and ionomycin in the presence of Brefeldin A before staining, and evaluation of motor function using the rotarod test **(Fig. 4.2.8 A)**. At 3 dpi, TBI led to a marked increase in the total numbers of infiltrating Ly6G$^+$ neutrophils **(Fig. 4.2.8 B)** and Ly6C$^+$ monocytes **(Fig. 4.2.8 C)**, as well as an increase in IL-1β$^+$ neutrophils **(Fig. 4.2.8 D)** and IL-1β$^+$ monocytes **(Fig. 4.2.8 E)** compared to sham controls. Despite this significant infiltration and IL-1β expression in peripheral myeloid subsets, MCC950 treatment did not reduce the accumulation of neutrophils or monocytes, nor did it significantly change the IL-1β$^+$ fractions of these populations **(Fig. 4.2.8 B-E)**.

In the adaptive immune compartment, TBI resulted in an increase in total CD4$^+$ T cells **(Fig. 4.2.8 F)** and CD8$^+$ T cells **(Fig. 4.2.8 H)**, along with increased IFN-γ$^+$ CD4$^+$ T cells **(Fig. 4.2.8 G)** and IFN-γ$^+$ CD8$^+$ T cells **(Fig. 4.2.8 I)** compared to sham mice; however, MCC950 did not decrease overall T cell numbers at this early stage, and there was a non-significant trend toward increased IFN-γ$^+$ cells within the CD8 T cell compartment in MCC950-treated TBI mice **(Fig. 4.2.8 I)**. Additionally, total γδ$^+$ T cells **(Fig. 4.2.8 J)** and IL-17$^+$ γδ$^+$ T cells **(Fig. 4.2.8 K)** were elevated following TBI but were not significantly affected by MCC950 treatment **(Fig. 4.2.8 J,K)**.

In contrast MCC950 treatment did not change the numbers of infiltrating leukocytes, but it reduced the TBI-induced increase in total microglia **(Fig. 4.2.8 L)** and lowered IL-1β$^+$ producing microglia **(Fig. 4.2.8 M)**. It also decreased MHC-II$^+$ microglia, but was not statistical significance **(Fig. 4.2.8 N)**. At the tissue level, hippocampal *Il1b* mRNA expression did not differ between vehicle-treated and MCC950-treated TBI mice despite the cellular suppression of microglial IL-1β$^+$ cells **(Fig. 4.2.8 O)**.

Functionally, TBI induced deficits in rotarod performance during the early post-injury period were not significantly altered by MCC950 treatment. However, AUC analysis revealed a significant improvements due to MCC950 treatment (p=0.02), indicating an overall benefit in

motor performance over the testing period **(Fig. 4.2.8 P)**. Overall, MCC950 exhibited limited effects on early infiltrating myeloid and T cell responses at 3 dpi. However, it significantly reduced microglial activation, including IL-1β^{+} microglia, with improved early motor function.

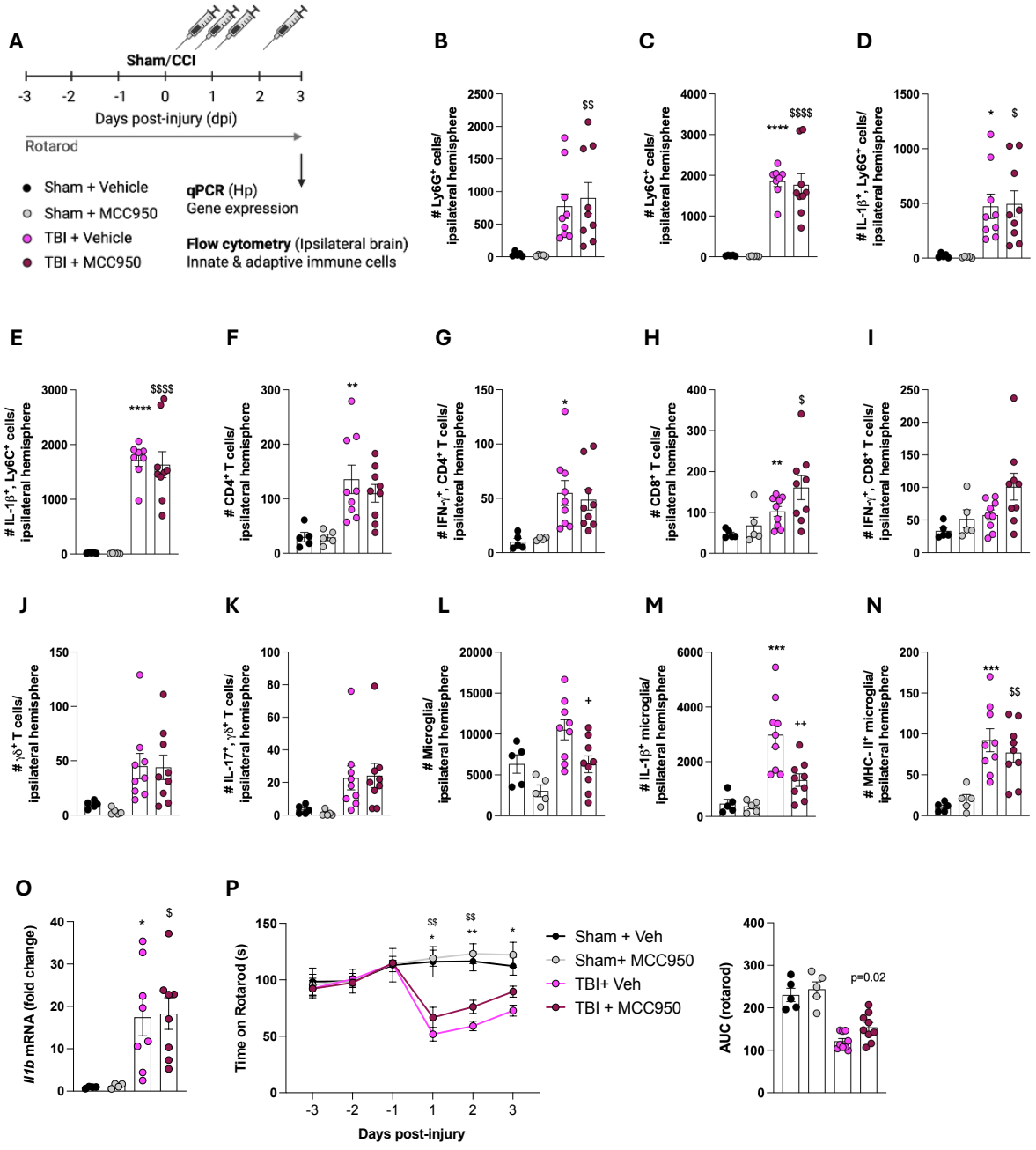

Fig. 4.2.8: MCC950 treatment selectively reduces microglial IL-1β at 3dpi without altering T cell effector phenotype. (A) Experimental design. (B-N) Bar graphs illustrate the absolute numbers of Ly6G^{+} neutrophils (B), Ly6C^{+} monocytes (C), IL-1β producing neutrophils (D), IL-1β producing monocytes (E), CD4^{+} T cells (F), IFN-γ producing CD4^{+} T cells (G), CD8^{+} T cells (H), IFN-γ producing CD8^{+} T cells (I), γδ^{+} T cells (J), IL-17 producing γδ^{+} T cells (K), microglia (L), IL-1β producing microglia (M), and MHC-II^{+} microglia (N). Relative fold-change expression levels of *Il1b* mRNA in hippocampus (O). Neurobehavioural tests: Rotarod (P). Data are presented as Mean ± SEM (n=5-9 per group); Sham + Vehicle vs. TBI + Vehicle *p<0.05, **p<0.01, ***p<0.001; Sham + MCC950 vs. TBI + MCC950 $^{\$}$p<0.05, $^{\$\$}$p<0.01, $^{\$\$\$}$p<0.001; TBI + Vehicle vs. TBI + MCC950 $^{+}$p<0.05, $^{++}$p<0.01, $^{+++}$p<0.001 by two-way ANOVA with post-hoc Tukey's test to correct for multiple comparisons.

4.2.9 NLRP3 inflammasome inhibition improves motor and cognitive function after TBI without altering T cell effector phenotypes

The study evaluated the impact of MCC950 on NLRP3 inflammasome inhibition, focusing on its potential to modulate adaptive immune responses and improve neurobehavioral recovery during the sub-acute phase following TBI, up to 10 dpi, where the isolated brain mononuclear cells were ex vivo stimulated with PMA and ionomycin in the presence of Brefeldin A before staining **(Fig. 4.2.9 A)**. At this time-point, TBI was linked to a pronounced and sustained increase in brain-infiltrating T cells, marked by a significant rise in total $CD4^+$ T cell numbers **(Fig. 4.2.9 B)**, accompanied by increased numbers of IFN-γ^+ **(Fig. 4.2.9 C)** and $GzmB^+$ **(Fig. 4.2.9 D)** $CD4^+$ T cells. Treatment with MCC950 did not result in a reduction in the overall accumulation of $CD4^+$ T cells or the proportion of IFN-γ or GzmB expressing $CD4^+$ T cells at this stage **(Fig. 4.2.9 B-D)**. A similar trend was observed in the $CD8^+$ T cell compartment, where TBI led to elevated total $CD8^+$ T cell numbers **(Fig. 4.2.9 E)** and increased IFN-γ^+ **(Fig. 4.2.9 F)** and $GzmB^+$ **(Fig. 4.2.9 G)** $CD8^+$ T cells at 10 dpi, indicating a persistent cytotoxic T cell response in the injured brain. MCC950 did not significantly alter these $CD8^+$ T cell responses, IFN-γ^+ $CD8^+$ T cells remained unchanged **(Fig. 4.2.9 F)**, and although there was a decrease in $GzmB^+$ $CD8^+$ T cells following MCC950 treatment, this reduction did not reach statistical significance **(Fig. 4.2.9 G)**. Additionally, TBI led to an increase in the total $\gamma\delta^+$ T cell numbers **(Fig. 4.2.9 H)** and elevating both IL-17^+ and IFN-γ^+ **(Fig. 4.2.9 I,J)** $\gamma\delta^+$ T cell subsets at 10 dpi. However, MCC950 treatment did not reduce total $\gamma\delta^+$ T cell accumulation or cytokine-positive $\gamma\delta^+$ T cell subsets **(Fig. 4.2.9 H-J)**.

Although there was no measurable impact on T cell recruitment or effector phenotype, the functional outcomes indicated modest benefits from NLRP3 inflammasome inhibition. Cognitive performance was assessed using the 2-arm Y-maze, where sham mice showed a clear preference for the novel arm (paired t-test novel vs. old: $p = 0.0004$). In contrast, vehicle-treated TBI mice did not demonstrate a preference for novelty ($p = 0.5$), consistent with impaired spatial recognition memory. Notably, MCC950-treated TBI mice exhibited a restored preference for the novel arm ($p = 0.0015$), suggesting improved spatial learning or memory performance **(Fig. 4.2.9 K**). Gross neurological function, evaluated by SNAP scoring, revealed significant TBI-induced deficits compared to sham ($p < 0.001$), while MCC950 treatment was associated with a trend toward improved scores, indicating partial functional recovery **(Fig. 4.2.9 L)**. Motor performance, assessed at 10 dpi using a once-off accelerating rotarod test. Vehicle-treated TBI mice remained impaired relative to sham ($p = 0.1$), while

MCC950-treated TBI mice did not demonstrate improved performance compared to vehicle-treated injured animals ($p = 0.3$) **(Fig. 4.2.9 M)**. Fine motor coordination was further examined using the beam walk test, where TBI induced significant deficits in vehicle-treated mice compared to sham **(Fig. 4.2.9 N)**; MCC950-treated mice exhibited improved foot fault performance at 10 dpi, but these improvements did not achieve statistical significance in AUC analysis ($p = 0.07$) **(Fig. 4.2.9 N)**. Collectively, these findings demonstrate that MCC950-mediated NLRP3 inflammasome inhibition did not alter the magnitude or effector polarization of the T cell response at 10 dpi, including $CD4^+$, $CD8^+$, and $\gamma\delta^+$ T cells. However, MCC950 treatment was associated with subtle functional improvements, most evident in the restoration of novelty preference in the Y-maze and trends toward improved motor coordination following TBI.

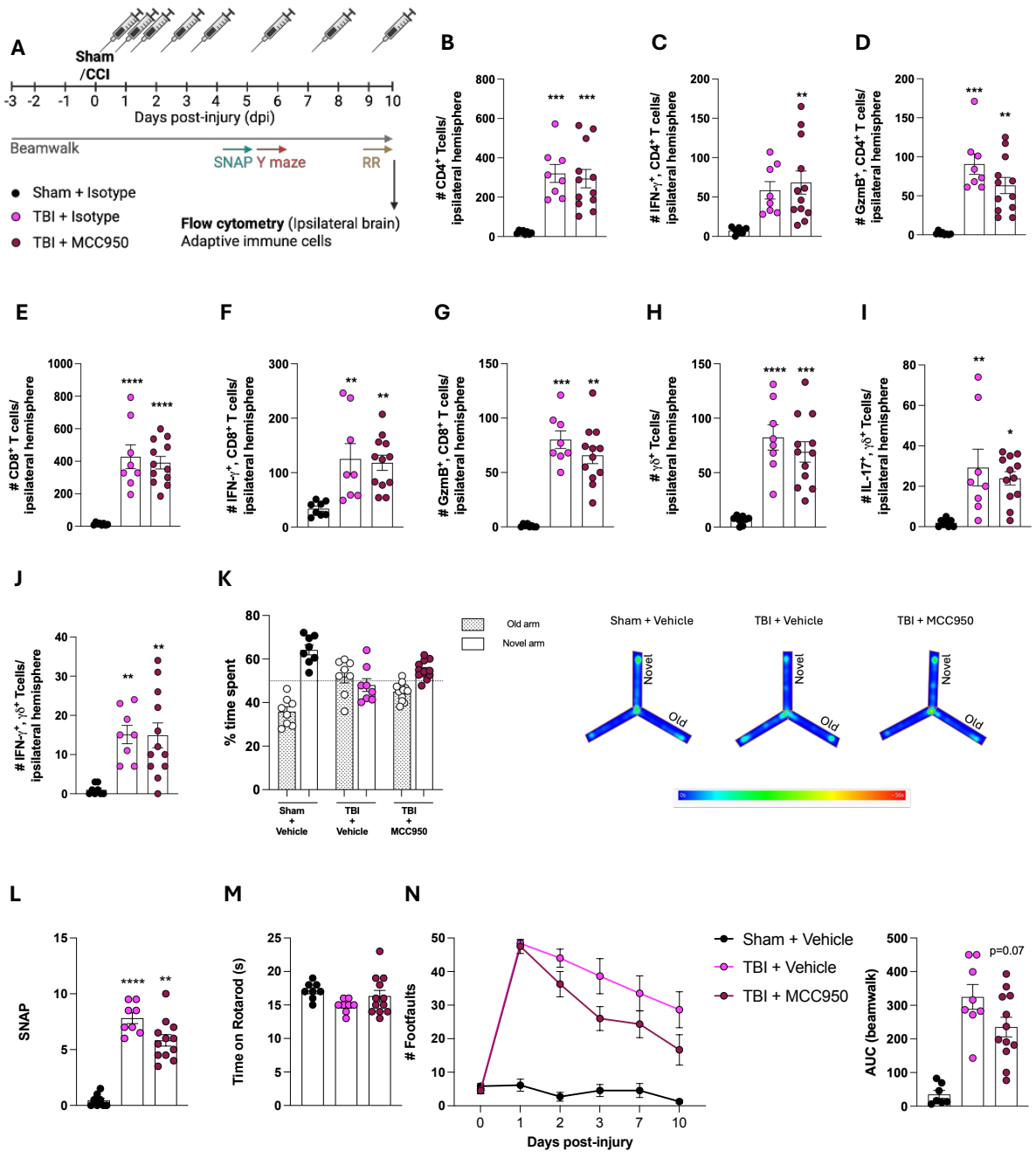

Fig. 4.2.9: MCC950 does not alter T cell effector phenotype but had beneficial effects on motor and cognitive function at 10 dpi. (A) Experimental design. (B-J) Bar graphs illustrate the absolute numbers of $CD4^+$ T cells (B), IFN-γ producing $CD4^+$ T cells (C), GzmB producing $CD4^+$ T cells (D), $CD8^+$ T cells (E), IFN-γ producing $CD8^+$ T cells (F), GzmB producing $CD8^+$ T cells (G), $γδ^+$ T cells (H), IL-17 producing $γδ^+$ T cells (I), IFN-γ producing $γδ^+$ T cells (J). (K-N) Neurobehavioral tests: Y-maze (K), SNAP score (L), Forced rotarod (M), Beam walk (N). Data= Mean ± SEM (n=8-12 per group); Sham + Vehicle vs. TBI + Vehicle *p<0.05, **p<0.01,***p<0.001 by one-way ANOVA with Tukey's multiple comparisons test.

4.3 Discussion

This chapter evaluates a mechanistic hypothesis rather than a descriptive one, specifically, whether early IL-1β competent innate programs function as upstream regulators of subsequent T cell states following TBI. Through three complementary perturbations targeting distinct "entry points" into this biological framework, namely, neutrophil attenuation, CCR2-dependent monocyte depletion, and NLRP3 inflammasome inhibition with MCC950, the overarching observation was that innate immunobiology could be significantly modified. However, the subsequent T cell effector signatures and behavioural outcomes were only modestly and selectively influenced. This finding is significant as it suggests that the post-traumatic IL-1β axis does not operate as a linear relay from a singular innate source to a singular adaptive output. Rather, it functions as a distributed network with multiple cellular sources, diverse processing pathways, and various downstream targets within the injured brain.

Targeting neutrophils altered the early inflammatory composition but did not result in a sustained reprogramming of subacute inflammation or behaviour. Moreover, it enhanced IL-17 production by $\gamma\delta^+$ T cells and IFN-γ by $CD4^+$ and $CD8^+$ T cells that were sustained up to 10 dpi. This is consistent with reports showing that neutrophils restrain IL-17 producing $\gamma\delta^+$ T cells through a contact dependent mechanism, such that neutrophil depletion can release this brake and amplify IL-17 responses (Yu et al., 2025). Neutrophils are recognized as early amplifiers of secondary injury, and their depletion or impaired recruitment can mitigate edema and tissue pathology in experimental TBI (Kenne et al., 2012). However, functional recovery often remains relatively unaffected, as demonstrated in CCI and closed head injury models, including those with CXCR2 disruption (Semple et al., 2010).

Monocyte targeting influenced early programming but did not durably suppress subacute inflammation. CCR2-dependent monocytes and monocyte-derived macrophages contribute to post-TBI inflammatory polarization and cognitive impairment, while CCR2 antagonism (CCX872) reduces inflammatory macrophage accumulation and improves cognition, supporting a causal CCR2 axis (Morganti et al., 2015). In our study, early monocyte depletion did not consistently attenuate inflammation by 10 dpi, consistent with compensatory recruitment and resident glial adaptation as CCR2-linked monocyte availability shapes microglial subset trajectories such that reduced influx can shift inflammatory execution toward resident microglia rather than lower total inflammatory burden (Somebang et al., 2021). In comparison to depletion strategies that focus on a single cellular compartment, the

behavioural sensitivity to the NLRP3 inflammasome, MCC950, observed in our research work aligns with the decoupling of leukocyte recruitment from inflammasome-associated IL-1β output. Our findings indicate that MCC950 specifically attenuated the IL-1β signal within the microglial population, while leukocyte recruitment remained largely unaffected. This pattern supports the hypothesis that inhibiting an upstream inflammasome node can significantly reduce the parenchymal IL-1β signal by limiting the microglial contribution, even when peripheral myeloid cells continue to be recruited. TBI studies have similarly demonstrated that NLRP3 inhibition with MCC950 enhances neurological outcomes and decreases edema, lesion volume, and neuroinflammation (Ismael et al., 2018; Xu et al., 2018). A parsimonious interpretation is that recruitment alone is insufficient to sustain IL-1β signalling if microglial inflammasome activity, a major local amplifier, is suppressed.

In this chapter, compensatory remodelling is identified as a mechanistic constraint rather than a secondary observation. This is because it suggests that suppressing one myeloid compartment can lead to the redistribution of effector functions to another, rather than diminishing the overall inflammatory capacity (Makinde et al., 2017; Soehnlein & Lindbom, 2010). In various contexts of traumatic CNS injury, reciprocal interactions between microglia and macrophages have been demonstrated to adjust microglial inflammatory programs and phagocytic behaviour when the availability of infiltrating monocyte-derived macrophages is altered (Greenhalgh et al., 2018). A similar principle is observed in CNS demyelination and repair biology, where the depletion of microglia does not necessarily impede myelin debris clearance, as monocyte-derived macrophages can compensate, maintaining clearance while altering the executing compartment (Baaklini et al., 2023). From this perspective, the increase in monocytes following neutrophil depletion and the increase in microglia following monocyte depletion are best understood as network-level reallocations of IL-1β competent myeloid capacity. This is directly linked to our hypothesis, as such source switching can sustain the early IL-1β environment that predisposes subsequent IL-17 and IFN-γ linked T cell programs, thereby elucidating why shifts in immune composition may not correlate with behavioural recovery.

Significant limitations are associated with this network issue, our immune phenotyping was optimized for T cell readouts and likely sub-optimal for the diversity of microglial states. The network effects are further influenced by the practical limitations of antibody-based depletion, which is frequently incomplete and challenging to sustain over time. This complicates the differentiation between true pathway suppression and rebound or replacement phenomena. The validation of anti-Ly6G through flow cytometry is particularly

problematic, as antibody binding can obscure epitopes and alter perceived marker intensity, with efficacy varying according to context (Pollenus et al., 2019; Stackowicz et al., 2019). Similarly, MC-21 is most effective within short dosing intervals, since extended administration may elicit host antibody response against the depleting antibody, thereby diminishing its efficacy over time (Mack et al., 2001). Consequently, null effects observed a later time points may be indicative of compensatory mechanisms and declining antibody performance, rather than a true disengagement of the targeted pathway. Additionally, the use of male-only cohorts, the restricted time window could influence adaptive priming collectively limit generalizability and mechanistic specificity (Doran et al., 2019).

T cell responses following TBI are increasingly recognized as a persistent, context-dependent contributor to chronic neuroinflammation, rather than a transient bystander. Longitudinal studies utilizing CCI models demonstrate the sustained presence of lymphocytes months post-injury, accompanied by persistent gliosis. These studies further suggest that restricting immune cell trafficking can mitigate chronic accumulation of macrophages and lymphocytes, with sex-dependent functional protection (Ertürk et al., 2016). However, the literature emphasizes a critical point for interpretation: the reduction of lymphocytes does not inherently confer neuroprotection in the acute phase, as evidenced by Rag1-/- mice showing no early neuroprotection following closed head injury (Weckbach et al., 2012). Additionally, modulation of the sphingosine-1-phosphate receptor with FTY720 reduces circulating lymphocytes and brain invasion without improving lesion size or behavioural outcomes (Mencl et al., 2014). However, another study showed that FTY720 partially improved neurobehavioural recovery and reduced brain edema and apoptotic cell death after TBI (Zhang et al., 2016). Our study extends this research by investigating a more mechanistic, hypothesis-driven question, whether early innate perturbations that modify the IL-1β axis can reprogram the subsequent T cell phenotype (rather than merely reducing immune presence) and thereby influence chronic microglial states and behaviour. Evidence supporting the concept that the quality of T cells can direct microglial activity is provided by intranasal anti-CD3, which induces IL-10 dependent FoxP3+ regulatory T cells that engage microglia, enhance phagocytosis, and improve recovery (Izzy et al., 2025). Therefore, it suggests that the timing and cellular source of early IL-1β could influence later adaptive immunity, biasing it toward pathogenic IL-17/IFN-γ states, thereby shaping chronic microglial trajectories and functional outcomes. A crucial next step involves the temporal mapping and source-specific manipulation of IL-1β producing cells, utilizing advanced genetic access and lineage-tracing techniques. This strategy allows to identify which initial

IL-1β sources are essential and adequate for initiating adaptive polarization and recovery following TBI (Nemeth et al., 2026).

Chapter 5

Brain tissue-resident memory T cells evolve after TBI and can be reactivated by systemic inflammation

Hypothesis: TBI establishes a persistent brain T_{RM} compartment, which predisposes the injured brain to subsequent systemic inflammatory challenges. The degree to which LPS-induced amplification is mitigated by CD49d/VLA-4 blockade will differentiate between effects dependent on peripheral trafficking and those resulting from resident immune reactivation.

5.1 Introduction

5.1.1 Neuroimmune communication: why “peripheral” inflammation becomes a brain problem

The CNS is safeguarded by layered barriers and has traditionally been characterized as "immune privileged"; however, it is not devoid of immunological activity. Under physiological conditions, the brain maintains tightly regulated immune surveillance and rapid innate sensing while minimizing collateral tissue damage (Engelhardt & Ransohoff, 2012; Ousman & Kubes, 2012). A fundamental organizing principle is compartmentalization, when inflammatory circuits become sustained or self-perpetuating behind the BBB, CNS pathology can become independent of peripheral immune activity (Tan et al., 2024). Building on this transition from "immune privilege" to compartmentalized CNS immunity, contemporary neuroimmunology underscores that immune populations can persist locally, adapt to the brain niche, and influence outcomes long after a peripheral trigger or local insult has resolved. Within this framework, tissue-resident memory T (T_{RM}) cells have emerged as a particularly significant concept because they combine three mechanistically decisive properties in the CNS, (i) retention behind barriers, (ii) rapid reactivation potential, and (iii) the capacity to amplify neuroinflammation through cytokines, cytotoxicity, and interactions with glia and vascular interfaces (Mix & Harty, 2022; Smolders et al., 2018). More broadly, the T_{RM} cell framework highlights that multiple immune lineages can acquire residency programs and diverge functionally from circulating counterparts, establishing durable, tissue-specific "immune setpoints" that can be protective or pathogenic depending on context (Li et al., 2025).

Microglia, the quintessential tissue-resident macrophages of the brain, originate from embryonic progenitors and are predominantly sustained through self-renewal, guided by specialized transcriptional programs (Ajami et al., 2007). These cells are strategically positioned to interpret systemic inflammatory signals and translate them into synaptic and neuronal outcomes, partly through interactions with barrier structures and neurovascular units (Bisht et al., 2021). However, microglia represent only a component of the resident immune milieu. Emerging research indicates that the CNS also contains resident adaptive immune cells, notably T_{RM} cells with tissue-resident phenotypes, which persist across various states of health, aging, infection history, and neurological disease (Smolders et al., 2018).

5.1.2 Defining CNS T_{RM}: identity, markers, and the logic of residency

In peripheral tissues, T_{RM} cells are characterized by their long-term persistence at sites of previous challenges and limited recirculation, facilitated by transcriptional programs that decrease egress and enhance retention (Skon et al., 2013; Szabo et al., 2019). In the CNS, defining residency necessitates additional caution, as acute inflammation can temporarily induce "resident" phenotypes in recently infiltrated cells. Consequently, the field relies on convergent criteria, including anatomical localization, exclusion of intravascular cells, durable persistence, and transcriptional features indicative of reduced egress (Skon et al., 2013; Szabo et al., 2019). The CNS-focused robust identification typically integrates intravascular labelling strategies with marker and transcriptional profiling, rather than depending on any single surface marker. Across tissues, prototypical T_{RM} cells often express CD69 and variably CD103, along with additional molecules such as CXCR6, CD49a, and P2RX7, depending on the tissue and subset (Mix & Harty, 2022). In CNS autoimmunity models and human CNS inflammatory tissue, significant fractions of infiltrating T cells can exhibit CD69 and CD103 expression, consistent with a resident program (Frieser et al., 2022). A systematic analysis of brain T_{RM} responses in mice demonstrated that $CD69^{+}$ $CD103^{-}$ T_{RM} populations are detectable in naïve brains and expand rapidly following various neurological insults, while $CD69^{+}$ $CD103^{+}$ populations emerge later and can persist long after the initial insult (Ayasoufi et al., 2023).

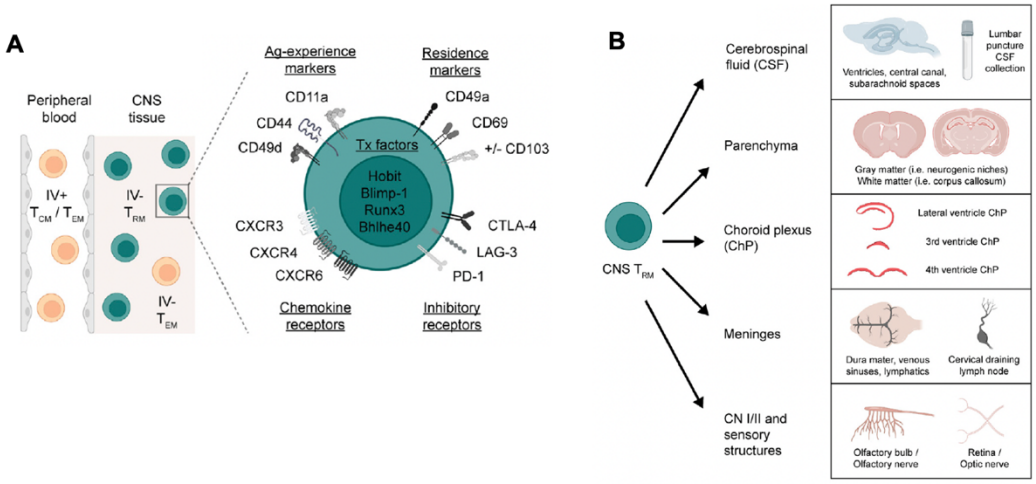

Fig. 5.1.2 (A) Identification. Tissue-localized T_{RM} can be distinguished from circulating T central memory (T_{CM}) and T effector memory (T_{EM}) in part by their resistance to labelling via intravenously injected antibodies (IV–) in mice. IV– T_{RM} in the CNS can be further distinguished from transiently surveilling IV– T_{EM} through a combination of surface markers and transcription (Tx) factors, (B) Localization T_{RM} can seed multiple niches of the murine and/or human CNS, including the parenchyma, cerebrospinal fluid (CSF), choroid plexus (ChP), meninges, and cranial nerves (CN) I/II with associated sensory structures (*Mix and Harty, Trends in Immunology, December 2022, Vol. 43, No. 12)*

5.1.3 Evolution of brain T_{RM} cells after an initial CNS insult

Systematic profiling reveals that the brains of naïve mice inherently contain T cells exhibiting resident-memory phenotypes, indicating that these populations may be resident rather than merely reflecting vascular contamination (Ayasoufi et al., 2023). The naïve brains possess populations of $CD4^+$ and $CD8^+$ T cells with a resident memory phenotype, which respond swiftly to neurological insults (Ayasoufi et al., 2023). Their conclusions are strengthened by methodologies frequently employed to ascertain CNS residency, such as intravascular labelling to exclude intravascular T cells and parabiosis to differentiate resident from circulating cell populations (Ayasoufi et al., 2023; Mix & Harty, 2022). A characteristic aspect of T_{RM} biology is the capacity for local expansion, with early post-insult expansion being substantiated by direct evidence of in situ proliferation within the brain. In the same study, the initial increase in $CD69^+CD103^-$ cells occurs prior to the infiltration of antigen-specific $CD8^+$ T cells and is ascribed to local proliferation, as demonstrated by experiments employing trafficking blockade (FTY720) in conjunction with proliferation labelling (Ayasoufi et al., 2023). The early expansion phase following neurological insults is supported by evidence of proliferation within the brain and is crucial for this chapter's narrative as it provides a mechanistic bridge from descriptive characterization to functional autonomy, if brain T_{RM} cells can expand in situ, then the CNS can rapidly develop enlarged resident compartments, serving as an important factor for the secondary challenge responses.

Beyond early responders, CNS T_{RM} biology encompasses the formation of durable antigen-experienced resident compartments following neurotropic infections and other inflammatory events. A review on T_{RM} emphasizes that antigen-specific CD8 T_{RM} can populate parenchymal and meningeal niches post-infection, providing long-term immune surveillance (Mix & Harty, 2022). It also supports the principle that brain specific T_{RM} cells can function as a locally retained protective barrier against viral infection, underscoring their stability as immune populations (Urban et al., 2020). This temporal evolution provides the conceptual framework for the first part of this chapter's results, mapping the temporal changes in the brain T_{RM} compartment, identifying markers and subsets that distinguish early responsive pools from later, more memory-oriented pools, and establishing the baseline upon which subsequent "second hit" experiments can be interpreted.

5.1.4 Brain T_{RM} cells as a double-edged compartment, local protection versus collateral tissue injury

T_{RM} cells should not be inherently considered pathogenic, as their primary biological role is to facilitate rapid, localized immune recall in tissues where reliance on recirculating

lymphocytes could allow for unchecked pathogen proliferation (Mix & Harty, 2022; Paik & Farber, 2021). In the CNS, this protective mechanism is particularly crucial following neurotropic infections, where $CD8^+$ T_{RM} populations can persist within the brain parenchyma, rapidly detect antigen re-encounter, and initiate local effector responses prior to the activation of a broader peripheral response (Urban et al., 2020; Wakim et al., 2012). These advantageous effects are not merely theoretical; brain T_{RM} cells have been demonstrated to confer protection during subsequent brain infections and to enhance tissue-wide antiviral programs through rapid cytokine production, notably interferon-γ associated responses (Paik & Farber, 2021; Urban et al., 2020). Consequently, in the context of infection, brain T_{RM} can be perceived as a strategically positioned layer of immune memory that enhances the speed and anatomical precision of local host defence (Mix & Harty, 2022; Prasad & Lokensgard, 2019). However, the same attributes that render T_{RM} beneficial during infection can become detrimental when recall activity occurs in neural tissue with limited regenerative capacity, as persistent or excessive T cell signalling can alter glial behaviour and cause collateral damage rather than effective pathogen control (Mix & Harty, 2022; Reagin & Funk, 2022).

This is well exemplified in post-viral models, where retained brain T cells promote microglia-dependent synaptic elimination and cognitive dysfunction during recovery from flavivirus encephalitis, illustrating that successful pathogen control does not necessarily equate to benign immune resolution (Garber et al., 2019; Reagin & Funk, 2022). Mechanistic reactivation studies reinforce this concern by demonstrating that antigen-specific brain T_{RM} are sufficient to initiate a rapid local alarm program, including the recruitment of additional inflammatory cells and activation of neighbouring brain-resident immune compartments, even when circulating T cell participation is minimized (Ayasoufi et al., 2023; Musial et al., 2024). This protective-versus-destructive duality is highly relevant to TBI, as sterile tissue damage results in barrier dysfunction, myelin abnormalities, persistent glial priming, and a chronically altered inflammatory milieu that may lower the threshold at which tissue-adapted T cell responses become maladaptive (Cáceres et al., 2024; Simon et al., 2017). Consistent with this notion, chronic experimental TBI is associated with sustained accumulation of GzmB expressing effector/memory $CD8^+$ T cells in the injured brain, and reducing this CD8 response correlates with less myelin pathology and improved long-term neurological outcomes (Daglas et al., 2019). Collectively, the critical issue is not whether brain T_{RM} are beneficial or harmful in absolute terms, but rather which post-infectious or post-traumatic cues determine whether they remain a focused protective memory population or

transition into a persistent driver of cytokine-mediated, cytotoxic, and glia-amplified tissue injury (Ayasoufi et al., 2023; Garber et al., 2019; Mix & Harty, 2022).

5.1.5 Aging as a modifier of brain T_{RM} abundance, phenotype, and inflammatory potential

Aging is a well-established risk factor for adverse neurological outcomes following systemic inflammatory insults, significantly altering immune composition at barrier interfaces and within tissues (Baruch et al., 2013; Godbout et al., 2005). Within a second-hit framework focused on resident immune memory, aging is anticipated to affect both (i) the size of resident compartments and (ii) their activation thresholds. Empirical evidence supports the presence of age-associated resident memory $CD8^+$ T cells in the CNS, indicating their potential to enhance inflammation following subsequent insults (Ritzel et al., 2016). The study documents an increase in CNS-resident $CD8^+$ T cells in aged mice, suggesting these cells are primed to exacerbate inflammation post-ischemic brain injury (Ritzel et al., 2016). Beyond mere changes in abundance, aging modifies the CNS environment in ways that can intensify T cell-driven immune responses. Recent research on aging white matter reveals that $CD8^+$ T cells can induce interferon-responsive oligodendrocyte and microglial responses, and that checkpoint modulation can exacerbate these responses (Kaya et al., 2022). Although not confined to classical T_{RM} definitions, these findings underscore the broader premise that aging brains may become increasingly influenced by adaptive immune cells residing in or entering CNS compartments, thereby supporting the rationale for explicitly investigating the effects of aging on T_{RM} evolution in the context of TBI.

5.1.6 Why brain T_{RM} cells are a plausible mechanistic bridge between post-TBI infections and worsened CNS outcomes

Hospital-acquired infections (HAIs) frequently occur as complications following significant neurological events and are consistently associated with adverse outcomes (Caceres et al., 2023; Gandasasmita et al., 2024). Within stroke cohorts, infections complicate a considerable proportion of admissions, with pneumonia, in particular, being linked to significantly increased mortality and diminished functional recovery (Teh et al., 2018). In cases of TBI, nosocomial infections, such as ventilator-associated pneumonia, are prevalent and contribute to extended intensive care, secondary complications, and an elevated risk of mortality (Kesinger et al., 2015; Kumar et al., 2020). Experimental evidence further supports

a "second hit" model, wherein superimposed bacterial lung infection following TBI results in increased mortality compared to infection alone (Doran et al., 2020).

Systemic inflammatory triggers, such as LPS, offer a viable experimental entry point into this issue, as they induce a reproducible "sickness program" (characterized by hypo locomotion, anorexia, altered thermoregulation, and motivational changes) alongside the induction of brain cytokines and glial activation (Norden et al., 2016; Skelly et al., 2013). These responses are not merely peripheral; they encompass brain cytokine transcription and translation, microglial activation states, and functional outcomes that become exaggerated in vulnerable brains (Jeong et al., 2010; Skelly et al., 2013). The most compelling mechanistic evidence supporting the role of resident-memory T cells in driving "second hit" pathology is derived from models demonstrating that resident T cells can sustain CNS inflammation from within the BBB. Two complementary studies published in Science Translational Medicine have shown that tissue-resident $CD8^+$ T cells are responsible for compartmentalized and chronic autoimmune damage within the CNS, independent of recirculating CD8 T cells, with $CD4^+$ T cells facilitating persistence and effector programming (Vincenti et al., 2022). Extending this beyond $CD8^+$ T cells, a 2025 study in Science Translational Medicine identifies CNS $CD4^+$ T_{RM} in progressive MS tissue and demonstrates that targeting resident $CD4^+$ compartments alleviates chronic neuroinflammation in a mouse model (Pignata et al., 2025). Although these autoimmune and reactivation models do not directly pertain to TBI, they establish a crucial biological principle that underpins the hypothesis of this chapter, once resident T cell programs are established in the CNS, they can exacerbate pathology in a manner partially independent of peripheral influences. This principle positions brain T_{RM} as a compelling mechanistic link to the clinical observation that infections acquired post-injury exacerbate outcomes. A hospital-acquired infection may provide inflammatory cues that re-engage the injury-shaped resident compartment, thereby amplifying neuroinflammation and contributing to clinical deterioration.

As a "second hit," LPS is particularly valuable in TBI research because it operationalizes a clinically relevant scenario; a peripheral inflammatory surge imposed on a brain that has already been immunologically altered by injury. Reviews of TBI neuroinflammation explicitly discuss secondary insults (including systemic immune challenges) as drivers of amplified CNS responses after injury, consistent with a second-hit framework (Gandasasmita et al., 2024; Sharma et al., 2021; Sun et al., 2018). In the context of this chapter, LPS is deliberately positioned as a proof-of-concept secondary challenge, rather than a comprehensive model of pneumonia or sepsis. This rationale is supported by a study which demonstrated that

peripheral LPS induces exaggerated and prolonged CNS cytokine responses and more persistent sickness behaviour in aged rodents compared to young adults (Godbout et al., 2005). Subsequent studies further revealed that aged animals exhibit microglia-centred hyper-responsiveness following peripheral LPS exposure, characterized by enhanced induction of inflammatory mediators, prolonged depressive-like and motivational disturbances, and greater disruption of hippocampal-dependent working memory (Chen et al., 2008; Henry et al., 2009). The experimental objective is to evaluate whether a systemic inflammatory event can engage and amplify post-injury neuroimmune compartments that have evolved over time, including resident adaptive immunity.

5.2 Results

5.2.1 TBI induces a progressive and persistent increase in brain T_{RM} cells cells

To investigate whether TBI facilitates the development and persistence of tissue-resident memory T cells (T_{RM}) within the brain, mice were analyzed at acute, subacute, and chronic intervals post-injury **(Fig. 5.2.1 A)**. The experimental framework included sham and CCI groups, with samples collected at 3, 10, 21, and 60 dpi. Intravascular leukocytes were excluded through intravenous anti-CD45 labelling 10 minutes prior to euthanasia, followed by flow cytometric analysis of brain **(Fig. 5.2.1 A),** using the gating strategy described in the **Fig 2.5** of the methods section. T_{RM} populations were evaluated within antigen-experienced compartments defined by $CD44^+$ $CD62L^-$ gating, and residency-associated phenotypes were identified by CD69 and CD103 expression.

Representative flow plots of CD69 versus CD103 demonstrate the injury-associated progression towards residency-marker expression over time **(Fig. 5.2.1 B)**. Quantitative analysis of these populations revealed that the qualitative patterns observed in the plots corresponded to a significant, time-dependent increase in T_{RM} cells **(Fig. 5.2.1 D-I)**. The numbers of $CD4^+$ $CD69^+$ $CD103^{+/-}$ T_{RM} were low in sham animals and remained relatively modest at 3 dpi, but increased substantially by 10 dpi and reached their highest levels at 21 dpi **(Fig. 5.2.1 D)**. $CD8^+$ $CD69^+$ $CD103^{+/-}$ T_{RM} exhibited a similar trajectory, with minimal counts in sham animals, a modest early rise at 3 dpi, a pronounced increase by 10 dpi, and sustained elevation at 21 dpi **(Fig. 5.2.1 E)**. The overall $\gamma\delta^+$ $CD69^+CD103^{+/-}$ population also increased throughout the post-injury period **(Fig. 5.2.1 F)**. The NK-T cell-associated $CD69^+$ $CD103^{+/-}$ counts increased after injury **(Fig. 5.2.1 G)**, but remained comparatively lower than those in

the conventional $CD4^+$ and $CD8^+$ compartments, indicating that the dominant injury-associated shift occurred within conventional T cells.

To ascertain whether these injury-associated T_{RM} populations persisted beyond the subacute phase, animals were also examined at 60 dpi. Representative plots at this chronic time point demonstrate that $CD69^+$ $CD103^{+/-}$ events remained readily detectable within both $CD4^+$ and $CD8^+$ $CD44^+$ $CD62L^-$ gates in CCI animals compared with sham, indicating that residency-marker co-expression is maintained long after the initial injury-associated inflammatory period **(Fig. 5.2.1 C)**. Both $CD4^+$ $CD69^-$ $CD103^{+/-}$ and $CD8^+$ $CD69^-$ $CD103^{+/-}$ populations were significantly higher in injured animals, confirming an overall injury-associated increase in brain T_{RM} cells at 60 dpi **(Fig. 5.2.1 H,I)**. Collectively, this data demonstrates that CCI induces a progressive accumulation of brain T_{RM} cells, evident by 10 dpi, peaking in the subacute phase, and persisting to at least 60 dpi, thereby supporting the durable establishment of the T_{RM} compartment in the injured brain.

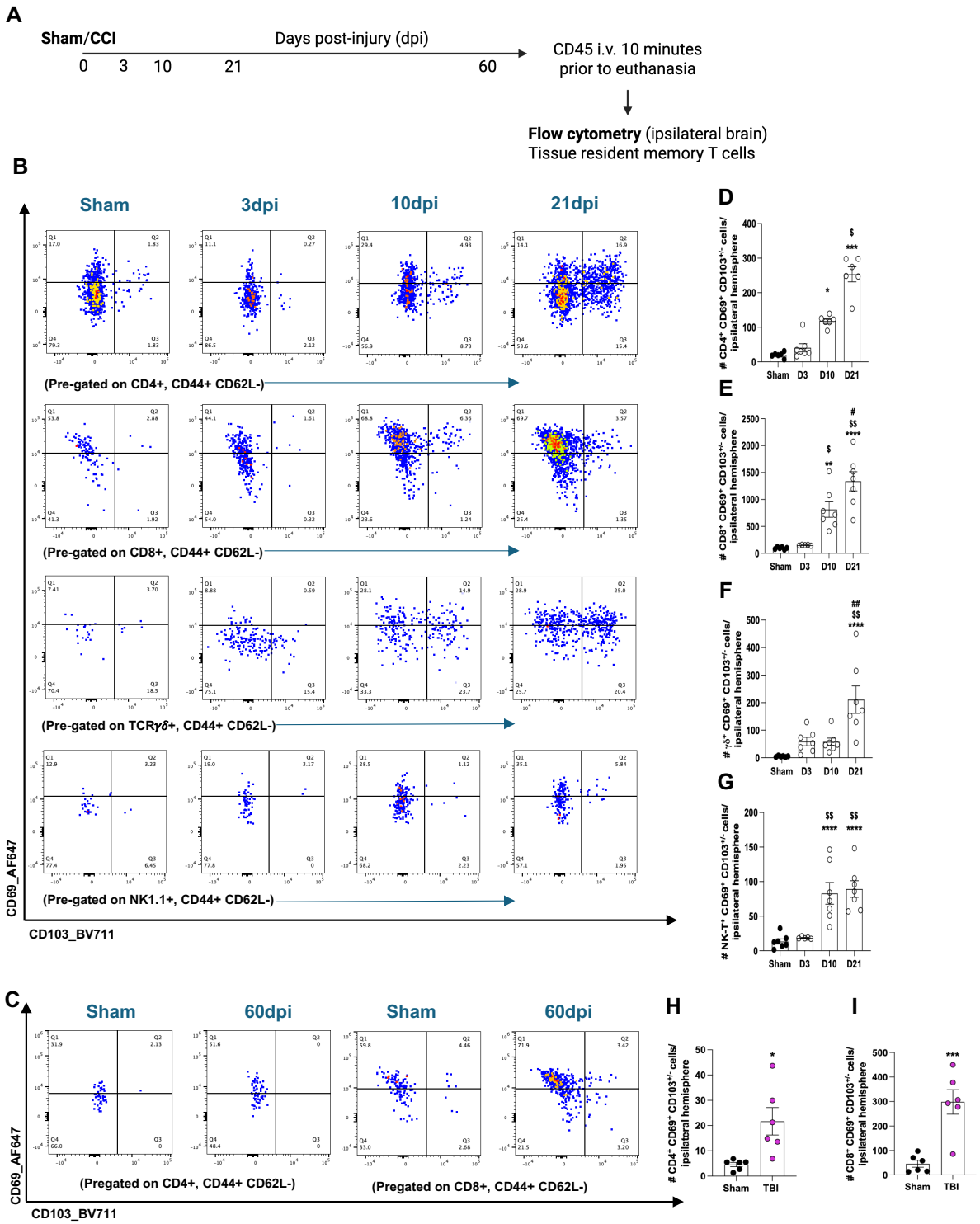

Fig. 5.2.1: TBI drives progressive accumulation and long-term persistence of brain T_{RM} cells. (A) Experimental design. (B,C) Representative flow cytometry plots for different T_{RM} cell subsets in the brain. (D-G) Bar graphs illustrate the absolute numbers at acute and sub-acute timepoints of $CD4^+$ T_{RM} cells (D), $CD8^+$ T_{RM} cells (E), $\gamma\delta^+$ T_{RM} cells (F), NK-T^+ T_{RM} cells (G). (H-I) Bar graphs illustrate the absolute numbers at chronic timepoint of $CD4^+$ T_{RM} cells (H), $CD8^+$ T_{RM} cells (I). Data = Mean ± SEM (n=6-7 per time point); differences vs. Sham *p<0.05, **p<0.01, ***p<0.001; differences vs. 3dpi $^{\$}$p<0.05, $^{\$\$}$p<0.01, $^{\$\$\$}$p<0.001; differences vs. 10dpi $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001; by one-way ANOVA with Tukey's multiple comparisons.

5.2.2 TBI differentially alters meningeal resident T_{RM} at 7 dpi

At 7dpi, intravascular CD45 labelling was used immediately prior to tissue collection to differentiate vascularly accessible leukocytes from tissue-resident meningeal cells, followed by flow-cytometric analysis of $CD69^+CD103^{+/-}$ T_{RM} populations **(Fig. 5.2.2 A)**. Within the $CD45^+$ circulating compartment, $CD69^+CD103^{+/-}$ cells were infrequent across the $CD4^+$ and $CD8^+$ memory gates in both sham and CCI groups, suggesting that meningeal T_{RM} cells were not represented within the vascularly labelled pool **(Fig. 5.2.2 B, C)**. A small circulating $CD69^+CD103^{+/-}$ population was detectable within the gd^+ compartment, but this remained limited relative to the resident fraction **(Fig. 5.2.2 D)**. In contrast, the $CD45^-$ resident compartment exhibited distinct $CD69^+CD103^{+/-}$populations across $CD4^+$, $CD8^+$, and gd^+ T cell subsets, consistent with the presence of a substantial meningeal T_{RM} pool at this time point **(Fig. 5.2.2 B-D)**. Following TBI, resident $CD4^+$ and $CD8^+$ $CD69^+CD103^{+/-}$ populations appeared increased relative to sham, supporting an injury-associated enrichment of T_{RM} cells within the meninges **(Fig. 5.2.2 B-D)**. Resident gd^+ cells also displayed a prominent $CD69^+CD103^{+/-}$ phenotype in both groups. However, unlike the $CD4^+$ and $CD8^+$ compartments, the absolute abundance of resident gd^+ T cells with this phenotype appeared reduced after TBI **(Fig. 5.2.2 D)**. Collectively, this data indicates that meningeal $CD69^+CD103^{+/-}$ T cells are predominantly localized within the tissue-resident rather than circulating compartment, and TBI differentially remodels resident meningeal T cell subsets.

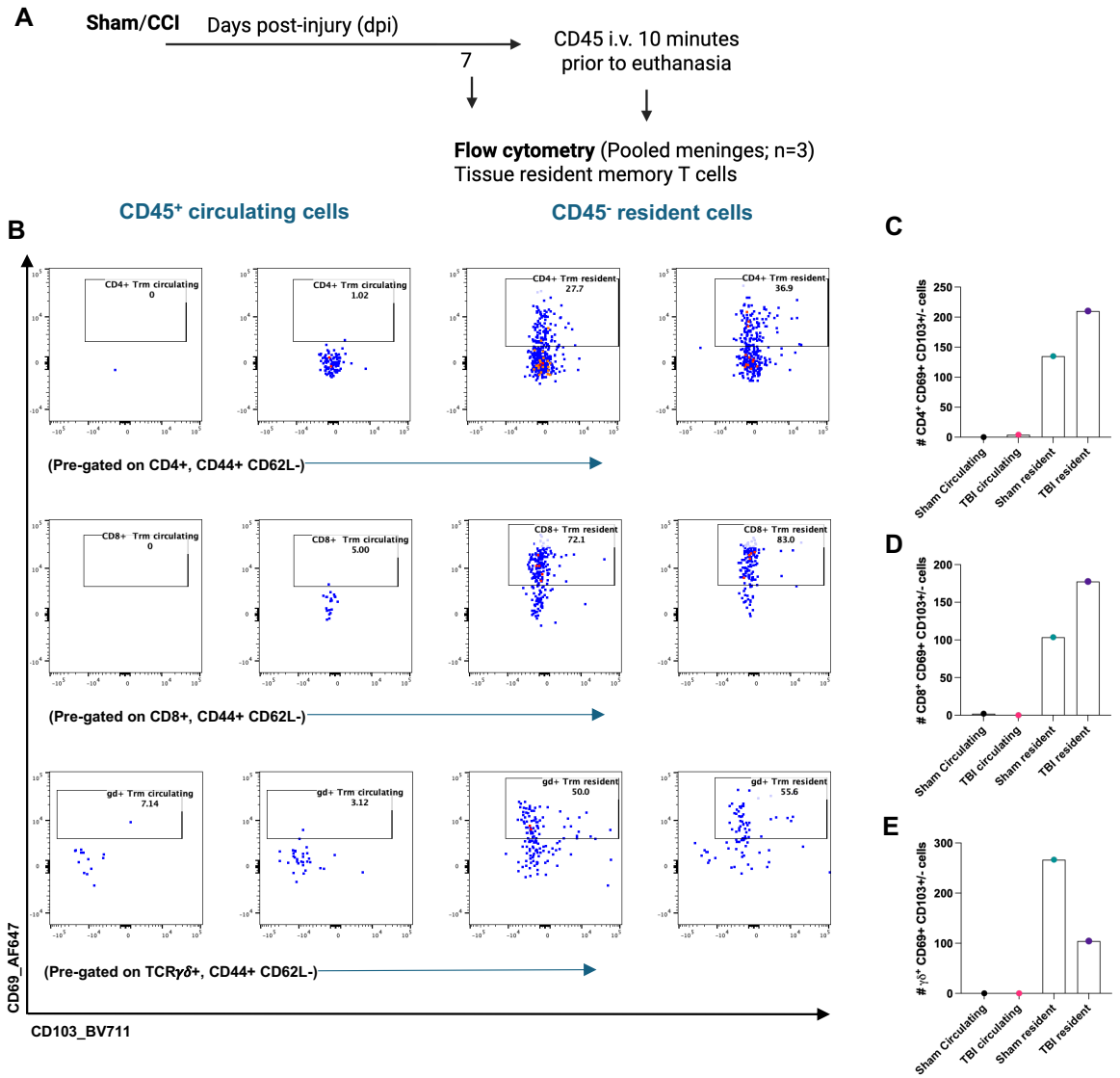

Fig. 5.2.2: Intravascular CD45 labelling identifies predominantly resident meningeal CD69$^+$CD103$^+$ T cell populations post CCI. (A) Experimental design. (B) Representative flow cytometry plots for different T_{RM} cell subsets in the brain. (D-G) Bar graphs illustrate the absolute numbers of CD4$^+$ T_{RM} cells (C), CD8$^+$ T_{RM} cells (D), γδ$^+$ T_{RM} cells (E). Data is represented for pooled samples (n=3 per group)

5.2.3 Systemic LPS amplifies ipsilateral T_{RM} accumulation and effector activation after TBI, paralleling prolonged sickness behaviour

To test whether an acute systemic inflammatory hit exacerbates post-injury sickness and reshapes brain T_{RM} responses, mice underwent sham or CCI surgery at day 0 and were then challenged at 10 dpi with an i.p. administration of LPS (0.5 mg/kg) or saline **(Fig. 5.2.3.1 A)**. The isolated brain mononuclear cells were ex vivo stimulated with PMA and ionomycin in the presence of Brefeldin A before staining. The flow cytometry plots illustrate the identification of Ki67 expression within $CD4^+$, $CD8^+$, and $\gamma\delta^+$ T_{RM} cell populations **(Fig. 5.2.3.1 B-D)**. Systemic LPS induced a rapid decrease in body weight in both sham and injured animals, whereas saline-treated groups continued to gain weight during the same period **(Fig. 5.2.3.2 A)**. The SNAP sickness scoring system was employed as a composite, observer-based measure of acute illness behaviour following the systemic challenge. Each mouse was assessed across multiple sickness-related features, such as reduced spontaneous activity, abnormal posture/piloerection, and reduced exploratory engagement. These component scores were aggregated to produce a single total SNAP score, which served as the primary parameter for plotting and analysis at 3 hours and 3 days post-injection. SNAP scores elevated due to injury and further exacerbated by LPS, with the highest scores observed in the TBI + LPS group at both 3 hours post-LPS (Mean ± SEM of each group are; Sham + Saline = 0 ± 0, Sham + LPS = 1.25 ± 0.75, TBI + Saline = 3.43 ± 0.84, TBI + LPS = 5.94 ± 0.85) and returned to baseline at 3 days post-LPS Mean ± SEM of each group are; Sham + Saline = 0.87 ± 0.59, Sham + LPS = 1 ± 0, TBI + Saline = 6.21 ± 1.84, TBI + LPS = 6.94 ± 0.66) **(Fig. 5.2.3.2 B,C)**.

At the immune-cell level, T_{RM} cells were significantly increased in the injured hemisphere following TBI. The numbers of $CD4^+$ $CD69^+$ $CD103^{+/-}$ T_{RM} cells were markedly increased ipsilaterally in injured mice compared to sham controls and the contralateral side, with evidence that LPS further amplified this response **(Fig. 5.2.3.2 D)**. Injury also increased the proliferative ($Ki67^+$) fraction of these cells in the ipsilateral hemisphere, which was further enhanced following LPS administration **(Fig. 5.2.3.2 E)**. Regarding effector readouts, ipsilateral $CD4^+$ T_{RM} IFN-γ^+, $GzmB^+$ and TNF-α^+ cells were elevated post-injury, demonstrating clear lateralization to the injured side, but LPS-dependent modulation was not evident for these effector subsets in injured mice **(Fig. 5.2.3.2 F-H)**. A parallel and often more pronounced pattern was observed for $CD8^+$ T_{RM} cells. $CD8^+$ $CD69^+$ $CD103^{+/-}$ T_{RM} cells

accumulated significantly in the ipsilateral hemisphere following TBI, with minimal changes observed contralaterally or in sham mice **(Fig. 5.2.3.2 I)**. This population exhibited increased proliferation ($Ki67^+$) post-injury, which was further enhanced LPS administration **(Fig. 5.2.3.2 J)**. Effector $CD8^+$ T_{RM} subsets also expanded ipsilaterally post-injury, including IFN-γ^+, $GzmB^+$ and TNF-α^+ populations, with additional LPS-induced increases within the injured hemisphere in IFN-γ^+ producing population and trends towards increase in the $GzmB^+$ and TNF-α^+ **(Fig. 5.2.3.2 K-M)**. $\gamma\delta^+$ $CD69^+$ $CD103^{+/-}$ cells increased predominantly in the ipsilateral hemisphere following TBI, exhibiting injury effects, and ipsilateral-contralateral asymmetry of both total and proliferating ($Ki67^+$) $\gamma\delta^+$ resident populations **(Fig. 5.2.3.2 N,O)**. In contrast, $\gamma\delta^+$ T_{RM} cells showed no difference in the effector cytokine production of IFN-γ^+, $GzmB^+$ and TNF-α^+ **(Fig. 5.2.3.2 P-R)**.

Finally, microglial numbers increased following injury and LPS-associated changes were evident in injured animals **(Fig. 5.2.3.2 S)**. Overall, this data indicates that TBI induces a highly lateralized neuroimmune environment, characterized by the accumulation and activation of ipsilateral brain T_{RM} populations. A subsequent systemic LPS challenge shows a tendency towards sickness behaviour and further enhances proliferative and effector T_{RM} responses within the injured hemisphere. This supports the hypothesis that peripheral inflammatory episodes can amplify and extend post-traumatic neuroimmune activation.

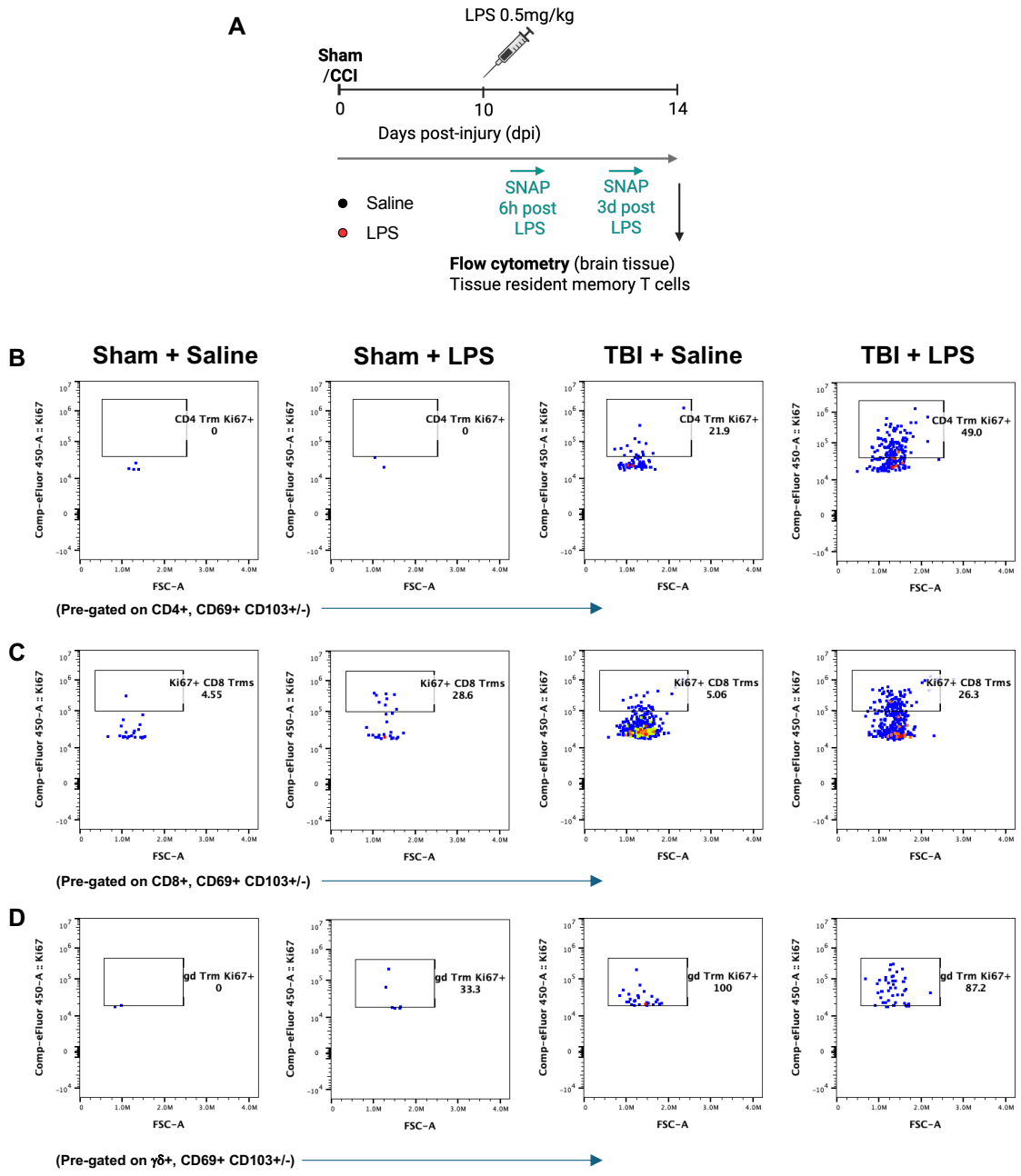

Fig. 5.2.3.1: Experimental design and representative flow cytometry plots showing proliferating brain T_{RM} cells after systemic LPS challenge following TBI. (A) Experimental design. Ki67 expression was assessed to identify proliferating T_{RM} populations within $CD4^+$ $CD69^+CD103^{+/-}$ T_{RM} cells (B), $CD8^+$ $CD69^+CD103^{+/-}$ T_{RM} cells (C), and $\gamma\delta^+$ $CD69^+CD103^{+/-}$ T_{RM} cells (D).

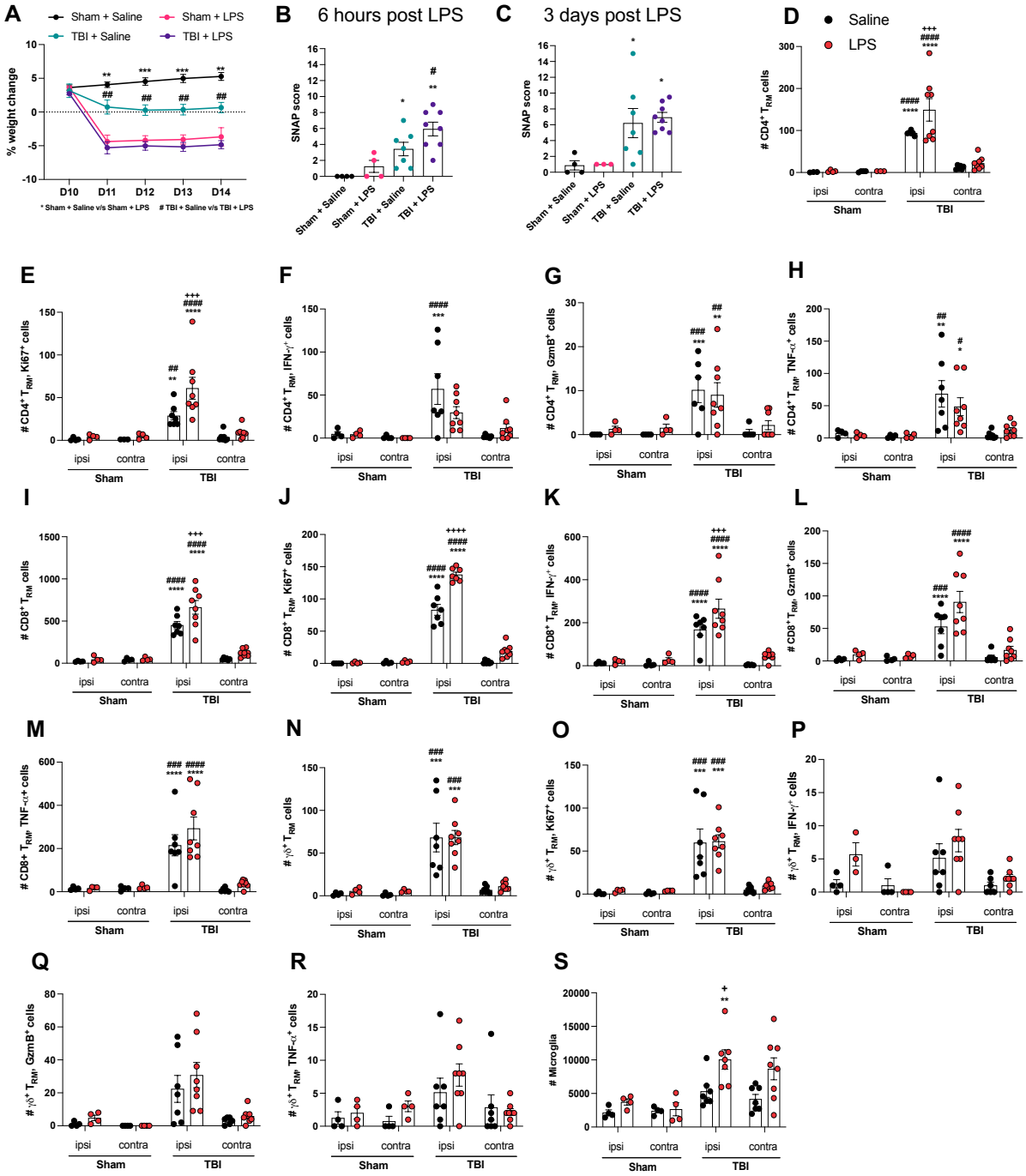

Fig. 5.2.3.2: Peripheral LPS challenge worsens sickness behaviour and boosts ipsilateral brain T_{RM} cell responses after TBI. (A) Percent body-weight change across days 10–14. (B,C) Neurobehavioral tests: SNAP scoring at 6 hours and 3 days post LPS injection. (D-S) Bar graphs illustrate the absolute numbers of $CD4^+$ T_{RM} cells (D), Proliferating $CD4^+$ T_{RM} cells (E), IFN-γ producing $CD4^+$ T_{RM} cells (F), GzmB producing $CD4^+$ T_{RM} cells (G), TNF-α producing $CD4^+$ T_{RM} cells (H), $CD8^+$ T_{RM} cells (I), Proliferating $CD8^+$ T_{RM} cells (J), IFN-γ producing $CD8^+$ T_{RM} cells (K), GzmB producing $CD8^+$ T_{RM} cells (L), TNF-α producing $CD8^+$ T_{RM} cells (M), $γδ^+$ T_{RM} cells (N), Proliferating $γδ^+$ T_{RM} cells (O), IFN-γ producing $γδ^+$ T_{RM} cells (P), GzmB producing $γδ^+$ T_{RM} cells (Q), TNF-α producing $γδ^+$ T_{RM} cells (R), microglia (S). Data= Mean ± SEM (n=4-8 per group); * represents injury effects *p <0.05, **p <0.01, ***p <0.001, # represents side effects #p < 0.05, ##p < 0.01, ###p < 0.001, + represents LPS effects +p < 0.05, ++p < 0.01, +++p < 0.001 by three-way ANOVA with correction for multiple comparisons by Benjamini, Kreiger and Yekutieli test.

5.2.4 Persistent ipsilateral immune signatures after TBI are not amplified by LPS in the chronic phase post-injury

To evaluate whether prior systemic administration of LPS influences neuroimmune composition at a chronic post-injury stage, mice were subjected to either sham or CCI surgery. Subsequently, CCI mice received either LPS (0.5 mg/kg) or saline at 10 dpi i.p., and immune populations were quantified via flow cytometry at 60 dpi, where the isolated brain mononuclear cells were ex vivo stimulated with PMA and ionomycin in the presence of Brefeldin A before staining **(Fig. 5.2.4 A)**. Within $CD4^+$ T_{RM} populations, no statistically significant effects were observed at the chronic endpoint, although several measures exhibited directional trends towards elevated values in injured and/or ipsilateral tissue. Total $CD4^+$ T_{RM} counts demonstrated variability, with an overall inclination towards higher numbers in the ipsilateral hemisphere post-injury **(Fig. 5.2.4 B)**. Similarly, proliferating ($Ki67^+$) $CD4^+$ T_{RM} cells and PD-1^+ $CD4^+$ T_{RM} cells did not display clear injury, side, or LPS induced differences, but some comparisons indicated trends towards increased ipsilateral values in the injured groups **(Fig. 5.2.4 C,D)**. IFN-γ+ and $GzmB^+$ $CD4^+$ T_{RM} cells also did not exhibit consistent chronic augmentation by LPS, with any apparent elevations remaining modest and variable **(Fig. 5.2.4 E)**. Collectively, these findings suggest that, at 60 dpi, $CD4^+$ T_{RM} readouts do not provide evidence for sustained immune activation driven by prior LPS exposure, despite trends towards higher values in injured tissue.

In contrast, the $CD8^+$ T_{RM} compartment demonstrated more pronounced injury and hemisphere associated effects at the chronic time point. Total $CD8^+$ T_{RM} counts were elevated in the ipsilateral hemisphere in injured mice **(Fig. 5.2.4 F)**. While proliferating ($Ki67^+$) $CD8^+$ T_{RM} cells did not indicate sustained proliferative activation attributable to prior LPS administration **(Fig. 5.2.4 G)**, activation-associated $CD8^+$ T_{RM} subsets were enriched ipsilaterally, including PD-1^+ cells **(Fig. 5.2.4 H)** and cytotoxic effector cells IFN-γ+ and $GzmB^+$ **(Fig. 5.2.4 I).** Notably, these effects were primarily driven by injury and hemisphere, rather than by chronic elevation in the LPS-treated group.

Myeloid profiling further corroborated a persistent ipsilateral injury signature. Microglial numbers were increased in the ipsilateral hemisphere following TBI **(Fig. 5.2.4 J)**. The number of MHC-II^+ microglia was observed to be higher in the TBI + saline group compared to the TBI + LPS group at the chronic endpoint **(Fig. 5.2.4 K)**; however, this difference did not achieve statistical significance. These findings indicate that the chronic neuroimmune composition

following TBI is primarily influenced by injury and hemisphere specific factors, with no evidence of prolonged LPS associated activation at 60 dpi.

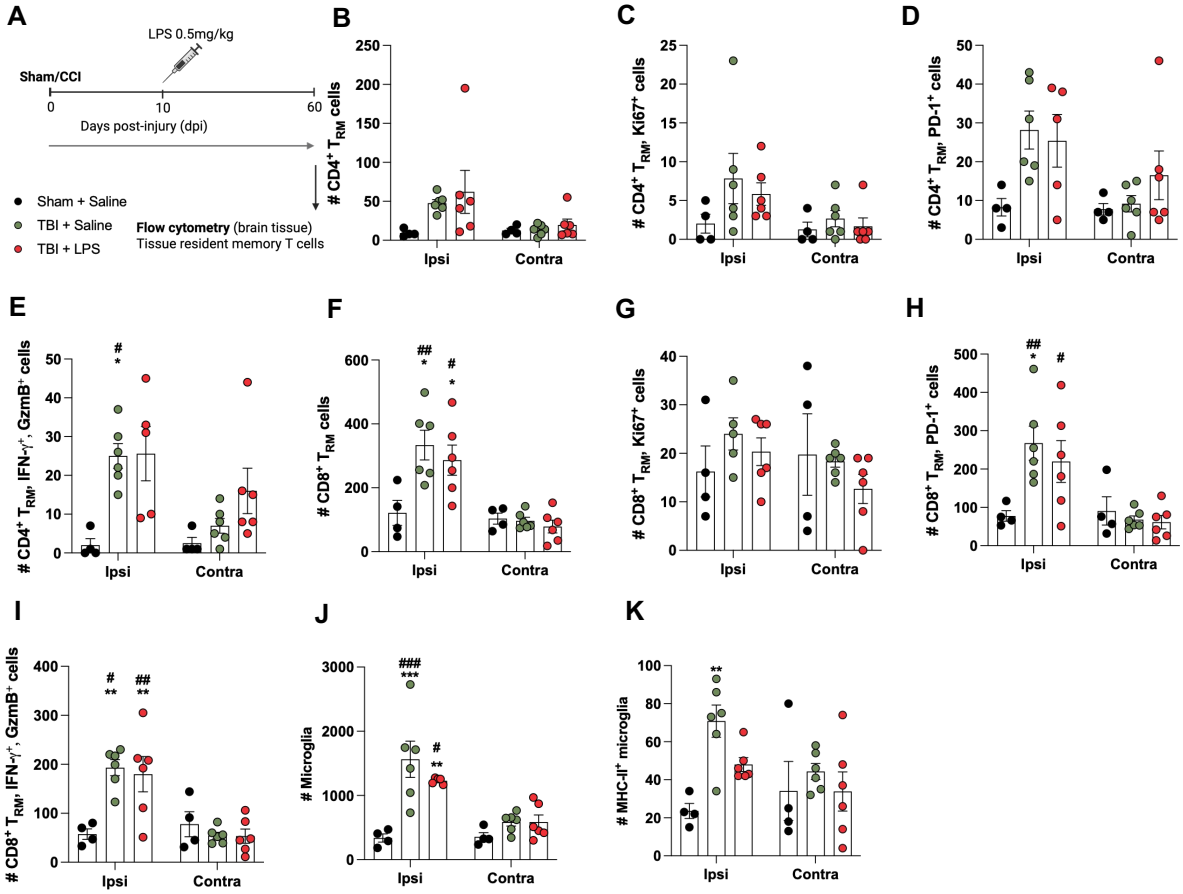

Fig. 5.2.4: Chronic neuroimmune composition after TBI is not chronically activated by LPS. (A) Experimental design. (B-K) Bar graphs illustrate the absolute numbers of $CD4^+$ T_{RM} cells (B), Proliferating $CD4^+$ T_{RM} cells (C), PD-1^+CD4^+ T_{RM} cells (D), IFN-γ and GzmB producing $CD4^+$ T_{RM} cells (E), $CD8^+$ T_{RM} cells (F), Proliferating $CD8^+$ T_{RM} cells (G), PD-1^+CD8^+ T_{RM} cells (H), IFN-γ and GzmB producing $CD8^+$ T_{RM} cells (I), mircoglia (J), MHC-II^+ microglia (K). Data= Mean ± SEM (n=6 per group); * represents injury effects *p <0.05, **p <0.01, ***p <0.001, # represents side effects #p < 0.05, ##p < 0.01, ###p < 0.001, by two-way ANOVA with post-hoc Tukey's test to correct for multiple comparisons.

5.2.5 Aging amplifies post-injury sickness behaviour and brain T_{RM} accumulation

In this experiment, young (10–12 weeks) and aged (22–24 months) C57BL/6J mice were subjected to either sham surgery or CCI surgery and evaluated at 3 and 7 dpi using the SNAP sickness score **(Fig. 5.2.5.1 A)**. This was followed by flow cytometric analysis to quantify brain T_{RM} cells in ipsilateral tissue, where the isolated brain mononuclear cells were ex vivo stimulated with PMA and ionomycin in the presence of Brefeldin A before staining. The flow cytometry plots are presented to demonstrate the identification of $CD4^+$ and $CD8^+$ T_{RM} cell populations. These populations are characterized by the expression of CD69 and CD103 within pre-gated $CD44^+CD62L^-$ T cells. Subsequently, the expression of Ki67 is assessed within these T_{RM} gates **(Fig. 5.2.5.1 B-E)**.

TBI resulted in a significant increase in sickness behaviour at 3 dpi (Mean ± SEM of each group are; Young Sham = 0.16 ± 0.16, Young TBI = 4.75 ± 1.06, Aged Sham = 0.25 ± 0.16, Aged TBI = 9.83 ± 0.67), with a more pronounced response observed in aged mice **(Fig. 5.2.5.2 A)**. By 7 dpi (Mean ± SEM of each group are; Young Sham = 0.16 ± 0.10, Young TBI = 3.50 ± 1.13, Aged Sham = 0.56 ± 0.23, Aged TBI = 9.16 ± 0.71), SNAP scores remained elevated post-injury, with aged mice continuing to exhibit a greater sickness burden compared to young animals **(Fig. 5.2.5.2 B)**.

At the cellular level, TBI led to an increase in the number of ipsilateral $CD4^+$ T_{RM} cells, with a marked amplification in aged mice **(Fig. 5.2.5.2 C)**. Within the $CD4^+$ T_{RM} compartment, the expansion driven by injury was most prominent in the $CD69^+$ $CD103^-$ subset, with a larger increase in aged animals **(Fig. 5.2.5.2 D)**, whereas the $CD69^+$ $CD103^+$ subset exhibited a more modest injury-related increase with less pronounced age dependence **(Fig. 5.2.5.2 E)**. Markers of activation and effector differentiation in $CD4^+$ T_{RM} cells were also elevated following TBI, including proliferation ($Ki67^+$) **(Fig. 5.2.5.2 F)** and effector readouts such as IFN-γ^+, $GzmB^+$ and TNF-α^+ cells, with several measures showing stronger responses in aged mice **(Fig. 5.2.5.2 G-I)**.

$CD8^+$ T_{RM} responses were even more significantly influenced by age. Aged mice demonstrated higher baseline and post-injury numbers of $CD8^+$ T_{RM} cells, with TBI further augmenting this population **(Fig. 5.2.5.2 J)**. Injury-associated increases were also observed in $CD8^+$ $CD69^+$ $CD103^-$ and $CD69^+$ $CD103^+$ subsets **(Fig. 5.2.5.2 K,L)**. Functionally, TBI enhanced the proliferation ($Ki67^+$) of $CD8^+$ T_{RM}, with a notable elevation in aged mice **(Fig. 5.2.5.2 M)**. Similarly, $CD8^+$ T_{RM} effector programs were augmented post-injury, including IFN-γ^+, $GzmB^+$

and TNF-α^+, with these effector responses consistently larger in aged animals **(Fig. 5.2.5.2 N-P)**.

Finally, microglial numbers on the ipsilateral side varied with age, where aged mice displayed a reduction in microglial numbers compared to young animals across conditions **(Fig. 5.2.5.2 Q)**. Overall, these findings suggest that aging exacerbates both acute sickness behaviour following TBI and the accumulation and effector polarization of ipsilateral brain T_{RM} cells, particularly within the CD8 T cell compartment.

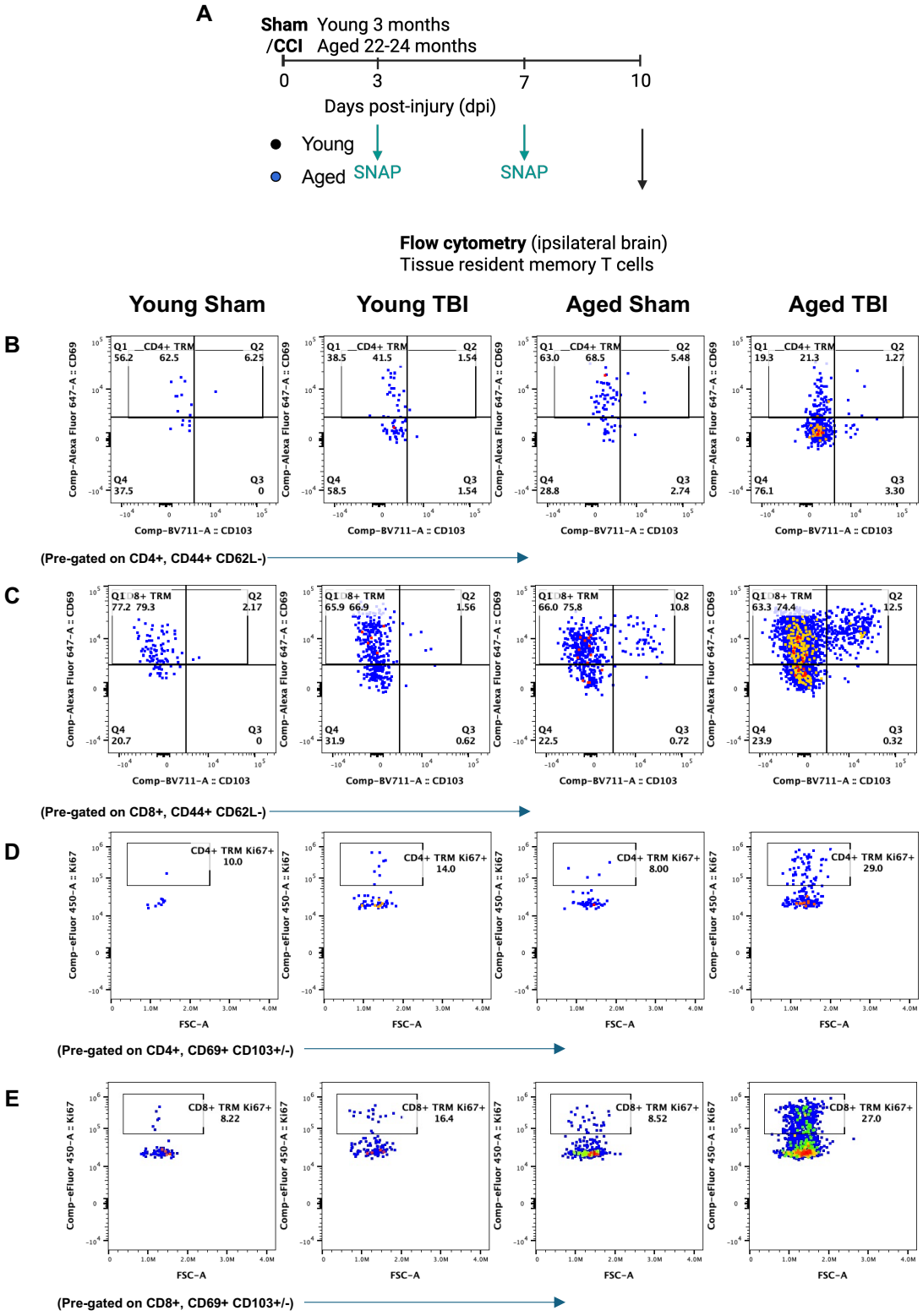

Fig. 5.2.5.1: Experimental design and representative flow cytometry plots showing age and injury associated changes in brain T_{RM} cells. (A) Experimental design. Representative plots show CD69 and CD103 expression within pre-gated $CD4^+CD44^+CD62L^-$ T cells (B) and $CD8^+CD44^+CD62L^-$ T cells (C). Ki67 expression was assessed to identify proliferating T_{RM} populations within $CD4^+$ $CD69^+CD103^{+/-}$ T_{RM} cells (D) and $CD8^+$ $CD69^+CD103^{+/-}$ T_{RM} cells (E).

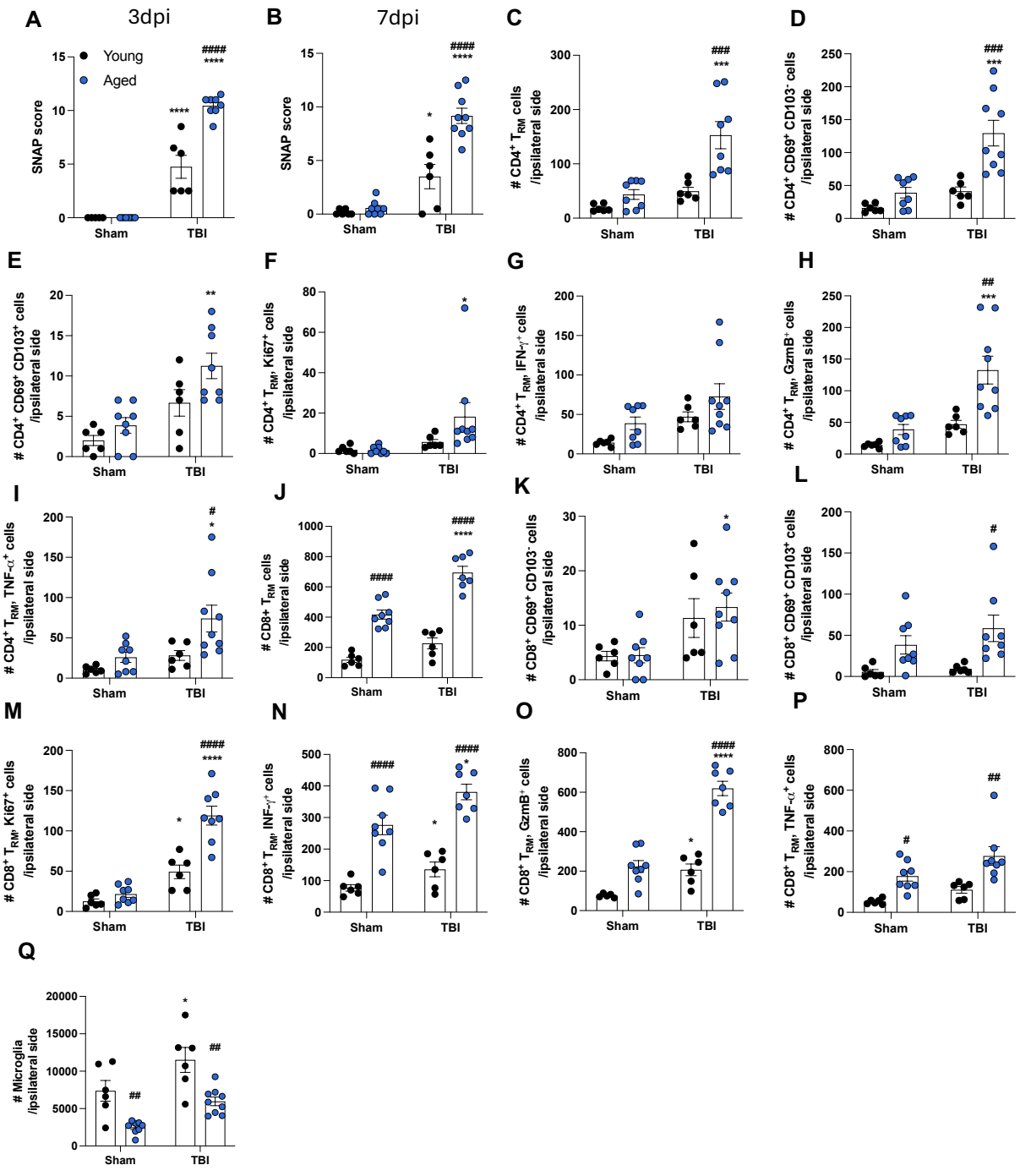

Fig. 5.2.5.2: Aging enhances injury-induced accumulation, proliferation, and effector phenotype of ipsilateral brain T_{RM}. (A,B) Neurobehavioral tests: SNAP scoring at 3 and 7 dpi. (C-Q) Bar graphs illustrate the absolute numbers of $CD4^+$ T_{RM} cells (C), $CD4^+$ $CD69^+$ $CD103^-$ cells (D), $CD4^+$ $CD69^+$ $CD103^+$ cells (E), Proliferating $CD4^+$ T_{RM} cells (F), IFN-γ producing $CD4^+$ T_{RM} cells (G), GzmB producing $CD4^+$ T_{RM} cells (H), TNF-α producing $CD4^+$ T_{RM} cells (I), $CD8^+$ T_{RM} cells (J), $CD8^+$ $CD69^+$ $CD103^-$ cells (K), $CD8^+$ $CD69^+$ $CD103^+$ cells (L), Proliferating $CD8^+$ T_{RM} cells (M), IFN-γ producing $CD8^+$ T_{RM} cells (N), GzmB producing $CD8^+$ T_{RM} cells (O), TNF-α producing $CD8^+$ T_{RM} cells (P), microglia (Q). Data= Mean ± SEM (n=6-9 per group); * represents injury effects *p <0.05, **p <0.01, ***p <0.001, # represents aging effects #p < 0.05, ##p < 0.01, ###p < 0.001, by two-way ANOVA with post-hoc Tukey's test to correct for multiple comparisons.

5.2.6 T_{RM} cells respond robustly after TBI, while continued trafficking contributes to the magnitude of the response

This experiment aimed to determine whether the LPS-induced alterations observed in brain T_{RM} cells following CCI are contingent upon the recruitment of additional peripheral leukocytes or if the resident compartment can independently respond within the injured hemisphere. To investigate this, mice subjected to CCI were administered i.p. with LPS at 10 dpi, and CD49d (VLA-4) blocking antibody (αCD49d; 200μg/animal) or an isotype control at 9 dpi and 13 dpi, and brain immune populations were quantified via flow cytometry in both ipsilateral and contralateral hemispheres, where the isolated brain mononuclear cells were ex vivo stimulated with PMA and ionomycin in the presence of Brefeldin A before staining **(Fig. 5.2.6.1 A)**. Representative plots show $CD4^+$ and $CD8^+$ T cell identification, Ki67 detection within $CD4^+$ and $CD8^+$ T_{RM} gates, and MHC-II expression within the microglial population **(Fig. 5.2.6.1 B-E)**.

The beam walk foot-faults followed the anticipated post-CCI deficit and recovery trajectory, with no significant evidence that αCD49d fundamentally altered the overall recovery pattern during the LPS challenge window **(Fig. 5.2.6.2 A)**. Sickness behaviour was evaluated at 6 hours (Mean ± SEM of each group are; Isotype + Saline = 6.0 ± 1.55, Isotype + LPS = 5.83 ± 0.82, αCD49d + Saline = 4.08 ± 1.04, αCD49d + LPS = 6.41 ± 1.75), and 3 days post-LPS (Mean ± SEM of each group are; Isotype + Saline = 5.58 ± 0.91, Isotype + LPS = 5.91 ± 1.24, αCD49d + Saline = 6.17 ± 1.18, αCD49d + LPS = 3.25 ± 0.80) **(Fig. 5.2.6.2 B,C)**.There were no significant differences in SNAP scores between saline and LPS-treated mice at either 6 hours or 3 days post-treatment, additionally, αCD49d did not alter behavioural sickness at either time point **(Fig. 5.2.6 B,C)**.

To confirm that αCD49d engaged the intended biological processes in the brain, we initially examined total T cell accumulation across hemispheres. At the cellular level, significant side-dependent differences were observed, with immune alterations predominantly localized to the ipsilateral hemisphere. Total $CD4^+$ T cells were more abundant ipsilaterally than contralaterally, and LPS further increased ipsilateral $CD4^+$ T cell accumulation **(Fig. 5.2.6.2 D)**. This LPS-induced increase was mitigated by αCD49d, indicating a role for VLA-4 (CD49d) in T cell recruitment into the inflamed or injured brain. A similar pattern was noted for $CD4^+$ T_{RM}, with greater ipsilateral abundance, enhancement following LPS, and reduction with αCD49d **(Fig. 5.2.6.2 E)**. Functional profiling supported an LPS-driven activation state within $CD4^+$ T_{RM} proliferation ($Ki67^+$) was enriched ipsilaterally, increased following LPS, and

was attenuated by αCD49d **(Fig. 5.2.6.2 F)**. Concurrently, inhibitory/checkpoint and effector phenotypes within $CD4^+$ T_{RM} increased following LPS, including PD-1^+ cells **(Fig. 5.2.6.2 G)** and IFN-γ^+, $GzmB^+$ subsets, with predominant ipsilateral increase and partial suppression by αCD49d **(Fig. 5.2.6.2 H,I)**.

$CD8^+$ T cell responses exhibited a similar core logic but were often more pronounced. Total $CD8^+$ T cells and $CD8^+$ T_{RM} were elevated in the ipsilateral hemisphere, increased following LPS, and reduced by αCD49d **(Fig. 5.2.6.2 J,K)**. Within $CD8^+$ T_{RM}, LPS increased proliferating ($Ki67^+$) cells in a strongly side-biased manner, and this proliferative expansion was diminished by αCD49d **(Fig. 5.2.6.2 L)**. LPS also increased PD-1^+ $CD8^+$ T_{RM} **(Fig. 5.2.6.2 M)** and enhanced effector programming, including IFN-γ^+ and $GzmB^+$ $CD8^+$ T_{RM} subsets, with dominant effects on the ipsilateral side and consistent attenuation by αCD49d **(Fig. 5.2.6.2 N,O)**.

Finally, microglial readouts demonstrated clear hemisphere bias and inflammatory modulation. Total microglia were elevated ipsilaterally relative to contralaterally, and LPS increased ipsilateral microglial numbers and activation state, which was attenuated by αCD49d **(Fig. 5.2.6.2 P)**. Specifically, antigen presentation and activation markers (MHC-II^+ and $CD68^+$ microglia) were elevated in response to LPS with a strong ipsilateral predominance, and these activation signatures were reduced by αCD49d **(Fig. 5.2.6.2 Q,R)**. Collectively, these data substantiate the interpretation that brain-resident immune compartments, including T_{RM} and microglia, are capable of initiating an LPS-responsive program within the injured hemisphere without necessitating a substantial additional influx of infiltrating T cells. Meanwhile, CD49d-dependent trafficking modulates the magnitude of the overall inflammatory cellular burden.

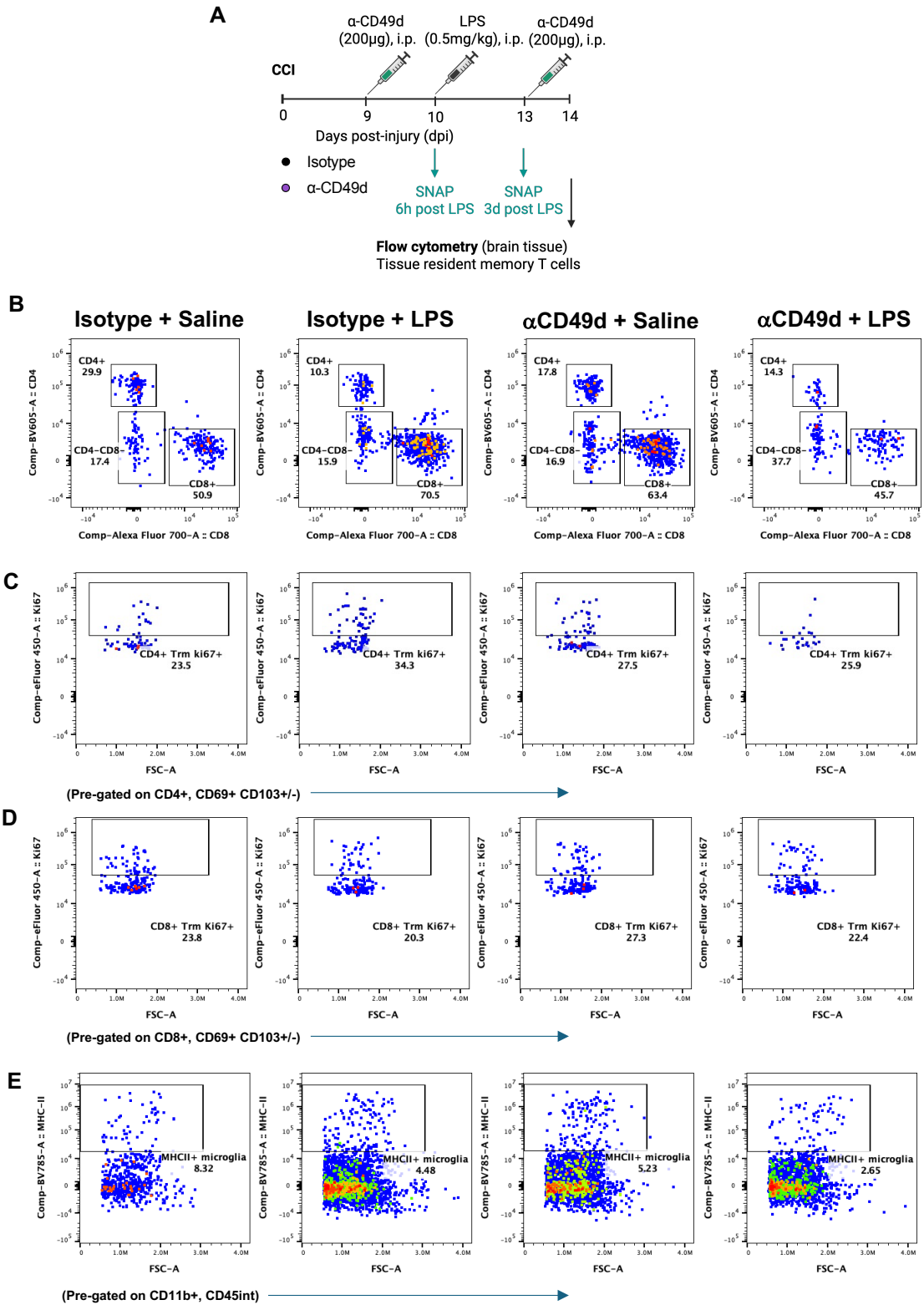

Fig. 5.2.6.1: Experimental design and representative flow cytometry plots showing the effect of CD49d blockade on brain T cell and microglial responses after systemic LPS challenge following TBI. (A) Experimental design. (B) Representative flow cytometry plots showing $CD4^+$ and $CD8^+$ T cell populations. Ki67 expression was assessed to identify proliferating T_{RM} populations within $CD4^+$ $CD69^+CD103^{+/-}$ T_{RM} cells (C) and $CD8^+$ $CD69^+CD103^{+/-}$ T_{RM} cells (D). (E) Representative plots showing MHC-II^+ microglial population.

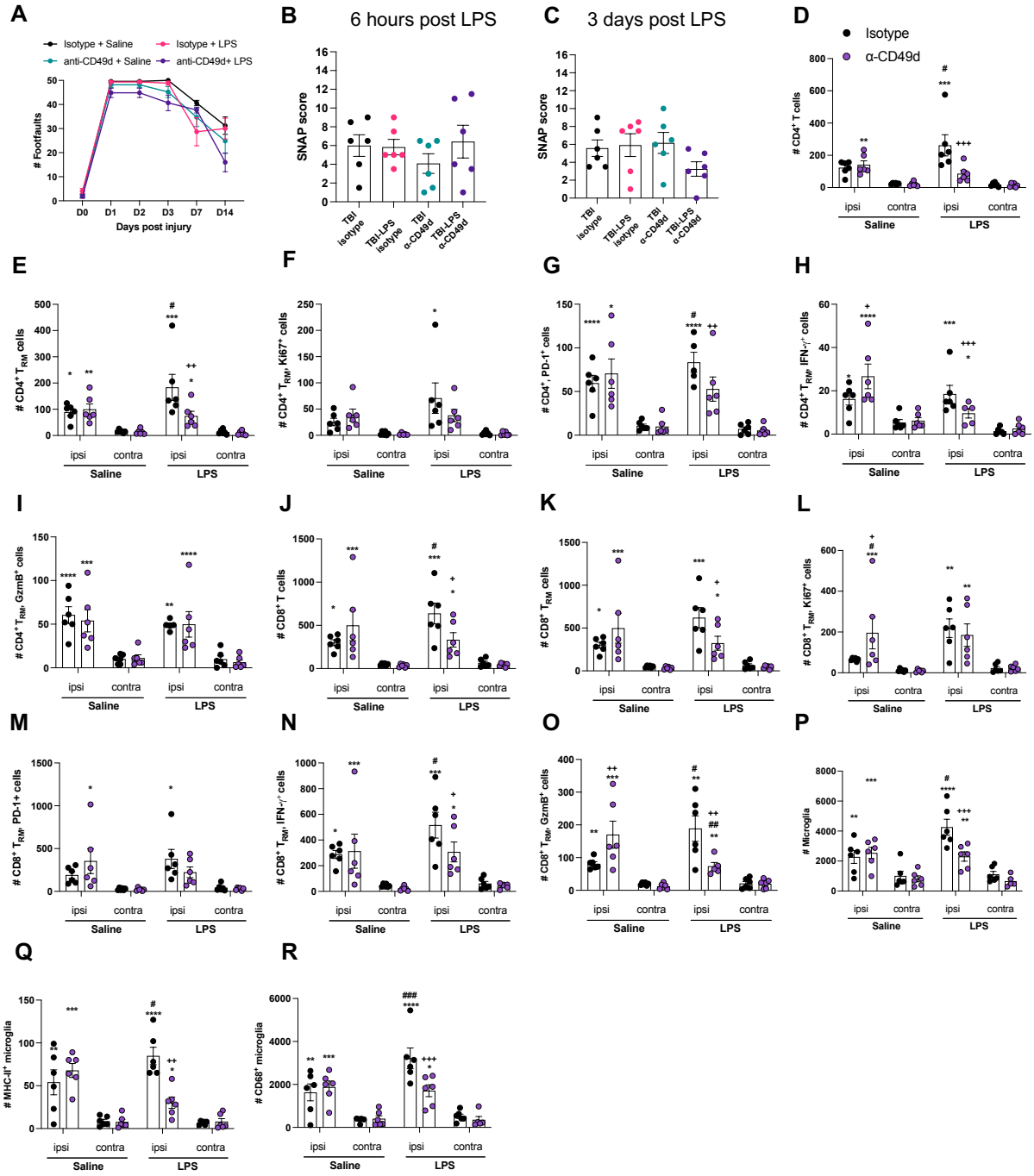

Fig. 5.2.6.2: CD49d blockade reduces delayed LPS-associated sickness and dampens ipsilateral T_{RM} and microglial activation after TBI. (A-C) Neurobehavioral tests: Beam walk (A), SNAP scoring at 6 hours and 3 days post LPS injection (B,C). (D-R) Bar graphs illustrate the absolute numbers of $CD4^+$T cells (D), $CD4^+$ T_{RM} cells (E), Proliferating $CD4^+$ T_{RM} cells (F), PD-1^+CD4^+ T_{RM} cells (G), IFN-γ producing $CD4^+$ T_{RM} cells (H), GzmB producing $CD4^+$ T_{RM} cells (I), $CD8^+$T cells (J), $CD8^+$ T_{RM} cells (K), Proliferating $CD8^+$ T_{RM} cells (L), PD-1^+CD8^+ T_{RM} cells (M), IFN-γ producing $CD8^+$ T_{RM} cells (N), GzmB producing $CD8^+$ T_{RM} cells (O), microglia (P), MHC-II^+ microglia (Q), $CD68^+$ microglia (R). Data= Mean ± SEM (n=6 per group); * represents side effects *p <0.05, **p <0.01, ***p <0.001, # represents LPS effects #p < 0.05, ##p < 0.01, ###p < 0.001, + represents antibody effects +p < 0.05, ++p < 0.01, +++p < 0.001 by three-way ANOVA with correction for multiple comparisons by Benjamini, Kreiger and Yekutieli test.

5.3 Discussion

This chapter demonstrates that TBI can impart a lasting "footprint" on the brain's immune landscape. The primary observation is that immune alterations can persist long after the initial phase of injury which reside within tissue rather than circulating in the bloodstream on the injured side (Ayasoufi et al., 2023). These cells can potentially be reactivated by a significant systemic inflammatory event (Ayasoufi et al., 2023). Over time post-injury, the results show a consistent rise in the T_{RM} cells, becoming evident approximately at 10 dpi , peaking during the subacute phase, and persisting at a later time point (60 dpi). The interpretation derived from this pattern is that injury does not merely induce a transient influx of immune cells. Instead, the injured tissue appears to become a niche where memory T cells can accumulate and persist (Ayasoufi et al., 2023; Wakim et al., 2012). They are often underrepresented in blood samples, which can lead to their significance being overlooked if only blood is analyzed (Schenkel et al., 2014). Conceptually, this reinterprets post-traumatic immunity from being merely a transient infiltrative event to a model where the injured hemisphere undergoes durable remodelling into an immune-permissive compartment capable of sustaining local memory populations.

This interpretation is notably reinforced by the application of intravascular anti-CD45 labelling prior to tissue harvest, which facilitated the exclusion of blood-exposed leukocytes and allowed for the classification of CD45 i.v. negative cells as non-intravascular at the time of collection (Ayasoufi et al., 2023; Mix & Harty, 2022). Consequently, the T_{RM} populations identified herein are unlikely to represent mere vascular contamination and are more accurately interpreted as tissue-associated cells within the injured hemisphere. The phenotypic markers such as CD69, CD44, and CD103 enhances rather than diminishes the current findings, as the persistence of CD45 negative T_{RM} cells in the injured hemisphere over weeks to months remains biologically significant and supports the conclusion that TBI establishes a durable tissue-associated adaptive immune compartment (Masopust & Soerens, 2019; Schenkel et al., 2014).

This interpretation is further corroborated by human data, as donor human white matter contains $CD8^+$ T cells exhibiting $CD69^+CD103^-$ and $CD69^+CD103^+$ phenotypes, along with transcription-factor profiles indicative of T_{RM} differentiation (Smolders et al., 2018). Notably, this suggests that resident programs are not exclusive to experimental injury models, supporting the interpretation of post-injury enrichment as an amplification and reshaping of a biologically plausible compartment rather than a purely artefactual phenotype (Smolders et al., 2018). Further evidence is provided by human MS tissue, where compartmentalized

inflammatory responses are predominantly characterized by $CD8^+$ T cells (Machado-Santos et al., 2018). These cells acquire characteristics of tissue-resident memory cells and may be locally reactivated within lesions, indicating that persistent resident CD8 programs can exist in diseased human brain tissue (Machado-Santos et al., 2018). Moreover, $CD69^+CD103^+$ $CD8^+$ T_{RM} cells are found to be enriched in the CSF of patients with chronic neuroinflammatory diseases (Kimura et al., 2024). Although these studies do not focus on TBI, they establish a principle directly related to this chapter's findings, once the brain provides an appropriate niche, durable tissue-adapted memory compartments can be maintained and reactivated in situ. In summary, the most straightforward interpretation is that TBI does not merely result in residual inflammation. Instead, it reprograms the injured hemisphere into a site capable of sustaining long-term, compartmentalized adaptive immune surveillance, with a strong bias towards CD8-associated tissue adaptation (Ayasoufi et al., 2023; Daglas et al., 2019).

The experiment involving meningeal T_{RM} cells presented in this chapter further indicates that post-traumatic immune remodelling is not confined to the injured parenchyma but also encompasses CNS border tissues. This finding aligns with the well-established perspective that the meninges serve as an active neuroimmune interface rather than merely a passive covering of the brain (Buenaventura et al., 2023). This is mechanistically significant because TBI can impair meningeal lymphatic drainage for extended periods, and pre-existing dysfunction in meningeal lymphatics exacerbates neuroinflammation and worsens outcomes post-injury (Bolte et al., 2020). Consequently, these meningeal findings supports a broader model wherein TBI establishes a distributed tissue-associated adaptive immune niche across both the injured hemisphere and its border compartments, thereby creating additional sites capable of retaining and shaping T_{RM} responses following injury (Bolte et al., 2020; Buenaventura et al., 2023).

In addition to the meninges, the skull bone marrow should be regarded as a component of the post-traumatic neuroimmune environment, given its anatomical connection to the dura via specialized channels (Herisson et al., 2018). This connection allows it to receive signals originating from the CNS and to supply immune cells to border tissues during inflammatory responses (Cugurra et al., 2021; Mazzitelli et al., 2022). Although direct evidence of its specific role in harbouring post-traumatic T_{RM} cells is currently limited, its close association with the meninges and its recognized function as a niche for long-lived memory T cells suggest it may contribute to the maintenance or modulation of T_{RM} responses following TBI (Di Rosa, 2016; Okhrimenko et al., 2014). Collectively, these observations support a comprehensive model in which post-traumatic adaptive immune remodelling occurs across

an integrated skull-meninges-brain axis, rather than being restricted solely to the injured parenchyma (Cugurra et al., 2021; Herisson et al., 2018; Kolabas et al., 2023; Mazzitelli et al., 2022; Okhrimenko et al., 2014).

The aging experiment demonstrates that older animals exhibit more pronounced sickness behaviour following TBI and a greater accumulation of T_{RM} cells in the injured hemisphere. This is accompanied by heightened indicators of cell proliferation and inflammatory or cytotoxic characteristics. This chapter further elucidates that the age effect is particularly pronounced in $CD8^+$ T_{RM} cells. This age-dependent amplification aligns closely with an expanding body of research indicating that $CD8^+$ T cells may contribute to age-related deterioration in the brain's white matter and can induce inflammatory states in adjacent brain cells (Kaya et al., 2022). Their study also revealed that $CD8^+$ T cells can instigate interferon-responsive states in oligodendrocytes and microglia, with these alterations serving as significant modifiers of white matter aging (Kaya et al., 2022). Another study described a mechanism whereby microglial activation facilitates the accumulation of deleterious $CD8^+$ T cells via a chemokine pathway (CXCL10), linking this to the degeneration of myelinated axons and declining function in aging (Groh et al., 2025).

The association becomes particularly significant in the context of TBI, as brain injury has been demonstrated to expedite the development of an age-related microglial phenotype linked to inflammatory neurodegeneration (Ritzel et al., 2023). This suggests that trauma and aging may converge into a shared state of chronic vulnerability, rather than functioning as distinct biological processes (Ritzel et al., 2023). Additionally, there is direct evidence associating aging with resident memory $CD8^+$ T cells in the CNS that are "primed" to amplify inflammation following brain injury, as demonstrated in a study on ischemic injury (Ritzel et al., 2016). The convergence of these findings renders the age-related effects discussed in the current chapter particularly compelling. They indicate that aging not only amplifies the extent of post-traumatic inflammation but also modifies its cellular structure, resulting in a more persistent, compartmentalized, and CD8-dominated state (Chen et al., 2024; Moro et al., 2022; Ritzel et al., 2019). This suggests that the aged brain may approach injury with a partially preconfigured immunological landscape predisposed to resident memory accumulation and heightened secondary responses (Buenaventura et al., 2023; Macheda et al., 2024). This interpretation further elucidates why the behavioural consequences of injury may exacerbate with age, even when the initial traumatic event is similar, as the host tissue is no longer immunologically equivalent prior to the occurrence of injury (Macheda et al., 2024; Moro et al., 2022; Susman et al., 2002). In summary, these studies support this

chapter's primary age-related implication, older brains appear predisposed to generate more robust and prolonged immune responses post-injury, with T_{RM} cells likely contributing to this shift.

In this chapter, the systemic inflammatory episode induced post-injury elicits a more pronounced response in the injured hemisphere of the brain, particularly among T_{RM} cells, which exhibit increased proliferation and activation of inflammatory and cytotoxic functions. The LPS challenge serves as a controlled method to examine how an infection-like inflammatory surge interacts with a brain already biologically altered by injury, an approach explicitly employed to model hospital-acquired infection following TBI in this experimental study (Gandasasmita et al., 2024; Sharma et al., 2021). The LPS second-hit experiment is enhanced by clearly differentiating between antigen-specific reactivation and non-specific or bystander activation, as these processes are mechanistically related yet distinct (Kim & Shin, 2019; Maurice et al., 2021). In conceptual terms, antigen-specific reactivation involves the recognition of cognate peptide-MHC by a pre-existing T cell receptor. In contrast, bystander activation pertains to the cytokine-driven effector activation of previously primed or memory T cells, occurring in the absence of confirmed cognate antigen engagement at the time of the subsequent insult (Kim & Shin, 2019; Maurice et al., 2021).

The novelty in the current chapter is the association of the second hit not only with T_{RM} activation but also with T_{RM} proliferation. This finding implies a local expansion of an injury-conditioned resident pool, rather than merely a transient alteration in activation phenotype (Ayasoufi et al., 2023). This observation is particularly significant because, in viral models of CNS disease, brain T_{RM} cells have been shown to undergo rapid expansion following a secondary challenge (Ayasoufi et al., 2023). Recent research has also demonstrated that resident brain T cells exhibit early expansion and proliferation within 24 hours of neurotropic viral infection, even prior to the accumulation of detectable antigen-specific virus-reactive $CD8^+$ T cells in the brain (Ayasoufi et al., 2023). Similarly, brain T_{RM} cells undergo spontaneous homeostatic proliferation and subsequently rapidly acquire cytotoxic effector functions following viral reinfection (Steinbach et al., 2016). This finding supports the hypothesis that resident cells can respond through local numeric expansion and rapid recall, independent of circulating memory cells (Steinbach et al., 2016).

Additional support comes from congenital cytomegalovirus (CMV) infection, brain $CD8^+$ T_{RM} cells have been characterized as long-lived and slowly proliferating entities that respond to

local reinfection (Brizić et al., 2018). This observation further supports the notion that proliferative re-engagement is a biologically plausible characteristic of brain-resident memory cell populations (Brizić et al., 2018). Collectively, these studies on viral infections underscore the significance of the proliferative burst observed following LPS exposure in the injured brain. This phenomenon is particularly noteworthy as it situates the event within a broader context, wherein a quiescent brain T_{RM} compartment can be transformed into an expanding effector pool due to a secondary inflammatory or infectious stimulus (Ayasoufi et al., 2023; Steinbach et al., 2016). This distinction is particularly crucial in this context, as bona-fide antigen-specific reactivation of brain T_{RM} has been demonstrated in peptide-restimulation paradigms to induce rapid neuroinflammation, microglial activation, disruption of the BBB, and extensive remodelling of the local immune environment, and serves as a crucial comparator for understanding the capabilities of resident cells when they encounter a cognate stimulus (Ayasoufi et al., 2023; Musial et al., 2024). Given that systemic LPS does not inherently offer a specific T cell receptor ligand, the most straightforward interpretation of the LPS+TBI paradigm presented in this chapter is that it primarily uncovers non-cognate, cytokine-mediated activation of an injury-conditioned T_{RM} compartment. Nonetheless, previous antigen exposure may have played a role in the establishment or shaping of this resident pool (Kim & Shin, 2019; Maurice et al., 2021).

This interpretation is further substantiated by research conducted outside the CNS, which demonstrates that established T_{RM} populations are capable of proliferating and producing effector cytokines following exposure to LPS or unrelated bacterial stimuli, without the necessity for cognate antigen recognition (Curham et al., 2023). This "second hit" model has a clear clinical parallel, as infections acquired during hospital care are prevalent following moderate to severe TBI, especially pulmonary infections such as ventilator-associated pneumonia, which are associated with prolonged intensive care unit stays and poorer recovery outcomes in numerous cohorts (Caceres et al., 2023; Doran et al., 2020; Gandasasmita et al., 2024; Kesinger et al., 2015; Kumar et al., 2020). The current findings do not definitively demonstrate antigen-specific recall in the LPS condition. However, they provide substantial support for the hypothesis that post-traumatic resident immune compartments reduce the threshold for secondary clinically significant inflammatory deterioration and, notably, may undergo proliferative expansion following a systemic secondary insult (Ayasoufi et al., 2023; Kim & Shin, 2019; Maurice et al., 2021).

At the chronic endpoint of 60 days, the results indicate that the injured side continues to exhibit elevated numbers of T_{RM} cells and increased levels of "activated/cell-killing" markers.

However, prior exposure to LPS does not further elevate these T cell measures. In contrast, there is a persistent LPS-associated alteration in microglia, characterized by a reduction in MHC-II$^+$ microglia, which are microglia with enhanced antigen-presentation capabilities, compared to saline. This demonstrates that a single inflammatory episode can impart a long-lasting "memory" in microglia, even if it does not maintain T cells in a permanently heightened state (Schaafsma et al., 2015; Wendeln et al., 2018).

The divergence between the T cell compartment and the microglial compartment is particularly informative, as it indicates that chronic post-traumatic immune memory is not uniformly distributed across different cell types. One interpretation of this finding is that the T cell response to a transient systemic insult is temporally limited, whereas microglia, as long-lived resident myeloid cells, maintain a more enduring record of inflammatory history through epigenetic and transcriptional reprogramming (Schaafsma et al., 2015). This interpretation is consistent with substantial evidence suggesting that microglia can be "reprogrammed" by previous inflammatory events in a manner that persists for months and can subsequently influence disease outcomes (Muccigrosso et al., 2016; Schaafsma et al., 2015).

Accordingly, the observed reduction in MHC-II$^+$ microglia following prior LPS exposure should not be automatically interpreted as a state of quiescence. Instead, it may indicate a qualitatively altered resident myeloid condition, wherein antigen-presentation behaviour and secondary responsiveness have been durably reconfigured due to the history of inflammation (Henry et al., 2009; Neher & Cunningham, 2019). This issue is particularly relevant in the context of aging, as peripheral innate immune activation leads to heightened and prolonged neuroinflammation and sickness behaviour in aged mice compared to adult mice (Godbout et al., 2005). Furthermore, it was shown that aged microglia display increased MHC-II expression along with enhanced IL-1β and IL-10 responses following systemic LPS challenge (Godbout et al., 2005; Henry et al., 2009).

More broadly, microglial priming is increasingly recognized as a common characteristic of aging, TBI, and neurodegenerative diseases. In these contexts, heightened secondary inflammatory responses may detrimentally affect cognitive function and synaptic plasticity, thereby accelerating neurodegenerative processes. This is particularly evident when secondary systemic inflammation exacerbates an already compromised CNS environment (Norden et al., 2015; Perry & Holmes, 2014). This interpretation is further corroborated by research on diffuse TBI, which demonstrates that delayed cognitive deficits emerge one month post-injury, coinciding with sustained microglial reactivity, characterized by elevated

MHC-II and IL-1β levels (Muccigrosso et al., 2016). Additionally, a subsequent peripheral immune challenge exacerbates memory consolidation disruptions specifically in injured mice, aligning with the hypothesis that chronically primed microglia can transform previous inflammatory exposure into subsequent functional vulnerability (Muccigrosso et al., 2016) This state could be further exacerbated in aged brains and may serve to link post-traumatic immune history to subsequent neurodegenerative susceptibility (Ritzel et al., 2023). This is crucial given recent evidence suggesting that TBI accelerates the development of an age-related microglial phenotype associated with inflammatory neurodegeneration (Ritzel et al., 2019; Ritzel et al., 2023). Collectively, these sources facilitate the contextualization of this chapter's chronic pattern, TBI establishes the long-term baseline in the injured side, while a systemic inflammatory event may leave its most enduring impact on the brain's resident myeloid cells.

A pivotal question within the field is whether the changes associated with LPS are primarily attributable to the infiltration of new immune cells from the bloodstream into the brain, or to the local response of cells already present within the brain. This chapter addresses this issue by employing an antibody that inhibits CD49d (VLA-4), a molecule crucial for the migration of T cells into inflamed tissues (Jurberg et al., 2021). The findings indicate that this inhibition diminishes the LPS-associated increases in both total T cells and T_{RM} cells within the injured hemisphere, as well as attenuates activation markers. This mechanistic finding is significant as it suggests that the post-LPS amplification observed in the injured hemisphere is not solely attributable to the local reactivation of pre-existing tissue cells (Chen et al., 2024). Instead, it remains at least partially reliant on the continued entry of leukocytes from the periphery (Ayasoufi et al., 2023). Consequently, the data support a hybrid model in which enduring tissue adaptation and renewed recruitment coexist rather than exclude one another (Ayasoufi et al., 2023).

This interpretation is reinforced by research conducted on aged TBI mice, which demonstrated that repeated administration of anti-CD49d significantly diminished T cell accumulation in the injured brain (Chen et al., 2024). This reduction was particularly pronounced in $CD8^+$ T cells and activated cytotoxic $CD8^+$ subsets (Chen et al., 2024). Furthermore, this intervention was associated with enhanced survival rates, as well as improvements in contextual and recognition memory, anxiety-related behaviour, and gait outcomes at two months post-injury (Chen et al., 2024). Importantly, these benefits were predominantly age-dependent, with minimal effects observed in young mice, indicating that recruitment-dependent adaptive pathology may hold particular significance in the aged

injured brain (Chen et al., 2024). It is also noteworthy that the TBI literature provides direct evidence that activated $CD8^+$ T cells contribute to long-term complications following brain injury (Daglas et al., 2019). The study documented the accumulation of $GzmB^+$ $CD8^+$ T cells following TBI and linked these cells to chronic neurological impairment (Daglas et al., 2019). Within this framework, the CD49d experiment transcends its role as a mere trafficking control, establishing a functional link between cell entry pathways and the persistence of a deleterious CD8-skewed immune response (Chen et al., 2024). This finding holds therapeutic promise, as it implies that the late-stage adaptive pathology following TBI can potentially be altered even after the initial acute innate immune response has diminished (Chen et al., 2024). This is particularly relevant for older patients or in scenarios involving systemic inflammatory complications (Chen et al., 2024).

Chapter 6

Overall discussion

What this thesis ultimately argues is that the most enduring consequence of TBI may be the creation of an altered immune state rather than a short-lived inflammatory episode. In this context, TBI should not be perceived merely as a lesion followed by a temporary clean-up response. Instead, it should be understood as an event that reorganizes the communication between the injured CNS and circulating leukocytes, border tissues, and long-lived immune cells over time. This perspective aligns with extensive research indicating that post-traumatic inflammation can persist for months to years, that white matter degeneration often continues beyond the initial mechanical insult, and that meningeal and vascular interfaces actively shape the immune consequences of TBI rather than merely reflecting them (Armstrong et al., 2024; Cáceres et al., 2024; Johnson et al., 2013; Ramlackhansingh et al., 2011; Theus, 2024). Significantly, this reframing elucidates why patients with similar primary injuries can exhibit markedly different long-term outcomes. This is because the biological issue extends beyond the initial brain lesion to include the trajectory of immune remodelling that ensues (Brett et al., 2022; Sharma et al., 2025).

An extension of this thesis, with greater mechanistic precision, supports the notion that TBI not only induces inflammation within the parenchyma but also remodels the brain-border immune system itself. This remodelling particularly affects the meningeal lymphatic, antigen-presenting, and regulatory niches, which play a crucial role in determining the persistence of adaptive programs over time (Bolte et al., 2020; Buenaventura et al., 2023; Goddery et al., 2021). An additional implication is that the immune restraint within the meninges is inherently biologically active and may become compromised following TBI. This is evidenced by the rapid unleashing of IFN-γ production, increased access to the parenchyma, induction of reactive glial states, and impairment of hippocampal-dependent memory upon depletion of meningeal regulatory T cells (Krämer et al., 2019). These findings demonstrate that border immune regulation can significantly influence deep brain physiology, even when there is no apparent infection present (Marin-Rodero et al., 2025).

Within the context of the neurotrauma field, this thesis argues that the post-traumatic immune response is more accurately conceptualized as a dynamic sequence of cellular programs rather than a singular entity termed "neuroinflammation" **(Fig. 6.1)**. An important extension of this framework is the interpretation of T cell involvement following TBI, which should consider the subset, activation state, anatomical compartment, and timing post-injury. Early research demonstrated that activated or effector $CD4^+$ T cells can exacerbate acute traumatic damage (Fee et al., 2003). In contrast, the absence of mature B and T cells in a different closed-head model resulted in minimal pathological changes during the first

week (Weckbach et al., 2012). This suggests that adaptive immunity is not uniformly pathogenic but is highly dependent on context and phase (Fee et al., 2003; Weckbach et al., 2012).

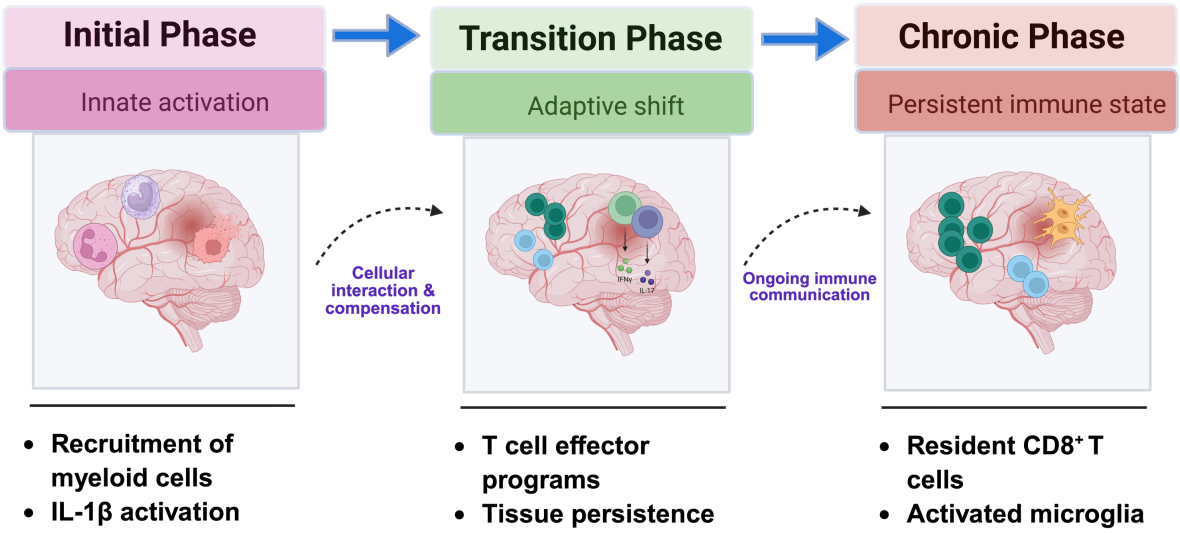

Fig. 6.1: Schematic representation of immune cell dynamics following TBI

Among the detrimental mechanisms, cytotoxic $CD8^+$ T cells are most robustly supported by direct experimental evidence (Daglas et al., 2019). This is evidenced by the accumulation of GzmB expressing effector or memory $CD8^+$ cells in the injured brain for several months post-trauma (Daglas et al., 2019). Notably, mitigating this response correlates with reduced myelin pathology and enhanced long-term neurological recovery (Daglas et al., 2019). Recent studies have more directly elucidated the pathogenic role of CD4 biology, demonstrating that a population of $CD4^+$ T cells can migrate to the injured brain via an anatomical signalling axis between the brain and cervical lymph nodes (Jiang et al., 2024). This migration exacerbates secondary injury, thereby establishing a connection between lymphatic communication and detrimental adaptive amplification following trauma (Jiang et al., 2024). The role of $\gamma\delta^+$ T cells in the context of T cell responses following TBI is complex and not uniform. Specifically, Vγ4 $\gamma\delta^+$ T cells exacerbate neuroinflammation through the production of IL-17 and IFN-γ, whereas Vγ1 $\gamma\delta^+$ T cells mitigate microglial activation via TGF-β, thereby offering a protective effect (Abou-El-Hassan et al., 2023).

The protective effects of T cells are most convincingly demonstrated in the context of Treg programs. Elevated levels of circulating Tregs in patients have been correlated with improved neurological recovery (Li et al., 2015). Experimental depletion of Tregs results in increased T

cell infiltration into the brain, heightened reactive astrogliosis, and elevated IFN-γ expression following CCI (Krämer et al., 2019). Furthermore, adoptive Treg therapy has been shown to mitigate both central and peripheral inflammation following TBI (Caplan et al., 2020). Recent interventional studies have further substantiated this reparative interpretation by demonstrating that IL-33/ST2 signalling enhances the function of Tregs and improves both lesion and sensorimotor outcomes following TBI (Xie et al., 2022). Additionally, brain-targeted delivery of IL-2 has been shown to expand brain-resident Treg cells, thereby offering protection against pathological neuroinflammation (Yshii et al., 2022).

Furthermore, delayed administration of low-dose IL-2 has been found to reverse chronic cognitive deficits and post-traumatic pain, while preferentially expanding meningeal Tregs (Czerpaniak et al., 2024). Recent findings have substantiated the existence of a reparative T cell axis through demonstrating that intranasal administration of anti-CD3 therapy following experimental TBI induced the production of IL-10 producing regulatory T cells (Izzy et al., 2025). This intervention suppresses chronic neuroinflammation, enhances the phagocytic function of microglia, and improves neurological outcomes, together providing direct evidence that not all post-traumatic T cell responses are detrimental and suggesting that selective immune reprogramming can be advantageous following TBI (Izzy et al., 2025). The primary implication of this study is that post-traumatic T cell immunity should be therapeutically redirected rather than indiscriminately suppressed. This is because the adaptive immune compartment encompasses both pathogenic effector programs and reparative regulatory circuits, which can influence chronic outcomes in opposing directions (Abou-El-Hassan et al., 2023; Daglas et al., 2019; Izzy et al., 2025; Yshii et al., 2022).

A notable conceptual contribution of this thesis lies in its examination of early innate responses, which are not disregarded as biologically insignificant following the emergence of adaptive cells. Concurrently, it refrains from oversimplifying the subsequent immune state as merely a downstream reflection of the initial myeloid phase. The mechanistic experiments presented herein suggest a conditional influence, modifications in neutrophil, monocyte, or inflammasome-associated pathways alter the subsequent inflammatory landscape without eradicating it. This conclusion is pivotal as it indicates that post-traumatic immunity is structured through interaction and compensation rather than being governed by a singular dominant upstream controller. The neutrophil component illustrates this phenomenon most clearly, as targeting early neutrophils does not eliminate subsequent inflammation. Instead, it supports the notion that inflammatory activity can be redirected toward alternative myeloid and lymphoid compartments. This is consistent with the broader literature, which indicates

that neutrophils contribute to early tissue injury while also engaging in phagocyte crosstalk during the progression of inflammation (Kenne et al., 2012; Soehnlein & Lindbom, 2010). The CCR2 axis further refines this model, as CCR2-dependent monocyte recruitment is evidently crucial in TBI (Morganti et al., 2015). However, its primary significance appears to be in shaping the quality of the downstream response. CCR2 deficiency reduces macrophage infiltration and enhances cognitive outcomes, CCR2 antagonism alters macrophage polarization and ameliorates hippocampal dysfunction, and single-cell analysis reveals that reduced CCR2-dependent influx reshapes resident microglial activation states rather than merely silencing neuroinflammation (Hsieh et al., 2014; Morganti et al., 2015; Somebang et al., 2021). In contrast, the inflammasome component highlights a partially distinct control point, as NLRP3 inflammasome inhibition with MCC950 improves neurological outcomes and reduces edema, lesion burden, and neuroinflammation following TBI (Ismael et al., 2018; Xu et al., 2018). This supports the view that local inflammasome-dependent amplification can be attenuated even when leukocyte recruitment is not entirely abolished. Collectively, these mechanisms suggest that post-traumatic immunity is sustained by interacting nodes of recruitment and amplification, such that blocking one compartment does not terminate the response but instead redirects inflammatory execution toward other available cellular pathways (Makinde et al., 2017; Soehnlein & Lindbom, 2010).

When considered from this perspective, the failure of early innate interventions to eliminate the inflammatory response indicates that post-traumatic immunity is gradually redistributed across different compartments, and this redistribution elucidates why adaptive T cell mechanisms become increasingly significant during the chronic phases of injury (Buenaventura et al., 2023; Daglas et al., 2019; Jassam et al., 2017). This concept aligns with stroke studies indicating the delayed infiltration of $CD8^+$ T cells during a subsequent inflammatory phase that contributes to long-term pathology, with intracerebral haemorrhage studies demonstrating that T cell receptor activation exacerbates injury, and with aging studies showing that resident CD8 populations within the CNS can intensify inflammatory injury following ischemia (Ritzel et al., 2016; Selvaraj et al., 2021; Xiu et al., 2025). These parallels are significant as they suggest that the biological rationale developed here is not confined to a single TBI model but is part of a broader spectrum of CNS injury responses in which adaptive immunity assumes increasing importance as the lesion progresses, where chronic injury and aging may function as convergent amplifiers of T cell pathology, persistent post-injury tissue damage which may promote not only prolonged but increasingly deleterious T cell responses over time (Ritzel et al., 2016; Selvaraj et al., 2021; Xiu et al.,

2025). In this broader adaptive context, the T_{RM} findings detailed in this thesis offer a more precise mechanistic explanation for how long-term T cell involvement might be maintained within the damaged CNS.

The novelty of the T_{RM} findings presented in this thesis lies not only in the persistence of T cells following TBI but also in the potential of post-traumatic brain and meningeal niches to facilitate local antigen re-encounter, clonal selection, and tissue retention (Goddery et al., 2021; Hobson et al., 2026). The data from this thesis also suggests that persistent post-traumatic T_{RM} compartments may constitute a mechanism by which an acute injury is translated into a long-lasting inflammatory memory state, thereby increasing susceptibility to chronic degeneration or to exaggerated CNS responses when a secondary systemic insult occurs (Ayasoufi et al., 2023; Hobson et al., 2026; Marin-Rodero et al., 2025). This interpretation is strengthened by research demonstrating that CNS resident myeloid cells can function as antigen-presenting cells for CD8 T cells (Goddery et al., 2021). Furthermore, human leptomeningeal CD8 T_{RM} populations exhibit significant clonal expansion. Paired brain-leptomeningeal analyses reveal overlapping yet distinct T cell receptor (TCR) repertoires, which aligns with the concept of coordinated border-parenchymal immunity rather than mere blood contamination (Hobson et al., 2026).

The observations from the T_{RM} studies in this thesis also hold significant relevance to neurodegenerative vulnerability as they identify a form of compartmentalized adaptive immunity increasingly implicated beyond TBI. Resident memory or tissue-retained $CD8^+$ T cell programs have been documented in chronic neuroinflammatory and neurodegenerative contexts, including MS, AD, Parkinson's disease, and amyotrophic lateral sclerosis (Altendorfer et al., 2022; Frieser et al., 2022; Hobson et al., 2026; Ma et al., 2025; Pignata et al., 2025). Experimental studies indicate that resident CNS $CD8^+$ T cell populations can directly sustain chronic neuronal injury and neurotoxic inflammation (Frieser et al., 2022; Kimura et al., 2024; Terrabuio et al., 2025). Recent neurodegeneration studies further suggest that resident CD8 states are functionally heterogeneous rather than unitary, which is highly relevant for interpreting chronic post-traumatic T cell persistence (Altendorfer et al., 2022; Ohyagi et al., 2025; Su et al., 2023). In AD model systems, brain $CD8^+$ T cells with residency programs can localize near plaque-associated microglia (Su et al., 2023). Depending on the disease stage and phenotype, these cells may either mitigate pathology through CXCR6-dependent tissue positioning or exacerbate amyloid accumulation via CCL5-CCR5 mediated suppression of microglial clearance programs (Ohyagi et al., 2025; Su et al., 2023). Likewise, in chronic MS lesions, CD8 T cells exhibiting a tissue-resident phenotype express markers

such as CD69, CD103, CD44, CD49a, PD-1, and CXCR6 (Fransen et al., 2020; Smolders et al., 2018). These cells show signs of reactivation and are capable of infiltrating the parenchyma of active white matter lesions (Fransen et al., 2020). This indicates that long-lived resident T cell programs are compatible with ongoing chronic tissue injury, rather than being limited to quiescent surveillance. A related point, particularly in the context of trauma, is the susceptibility of white matter, which may inherently promote the chronic retention of adaptive immune responses (Groh et al., 2025). Recent research on aging indicates that maladaptive activation of microglia can recruit and retain deleterious $CD8^+$ T cells within white matter via CXCL10-CXCR3 signalling (Groh et al., 2025). This process connects local myeloid dysfunction to axonal degeneration and prolonged behavioural decline, resembling a chronic post-injury feed-forward loop.

The concept of T_{RM} cells within the brain serves to connect this thesis to models of recurrent brain injuries, particularly those impacting white matter. Recent research demonstrates that the immune response discussed herein extends beyond isolated injuries in adults, it is also important to repeated mild injuries, specific white matter damage, and brain development (Main et al., 2026; Shumilov et al., 2026). This broader framing is supported by recent review literature showing that white matter damage after TBI is not a secondary footnote but a central component of long-term disease progression, with axonal injury, myelin disruption, and glial responses contributing to persistent degeneration and impaired recovery (Armstrong et al., 2024). In such instances, injuries recruit immune cells such as macrophages and T cells, induce inflammation, and result in persistent white matter complications (Main et al., 2026). This is significant as it corroborates the central argument of this thesis; post-injury inflammation is not merely a transient issue but can evolve into a prolonged immune state within the tissue (Needham et al., 2019). Research has also shown that inflammation can persist even after the BBB ceases to leak, indicating that a brief period of immune access can initiate sustained local inflammation (Main et al., 2026). Also, in children with repeated injuries, T cells actively influence inflammation in white matter, rather than merely reacting to damage (Shumilov et al., 2026).

This perspective also relates to the risk of neurodegenerative diseases without asserting a direct causation. This thesis does not claim that brain injury invariably results in a specific disease. Instead, it highlights a chronic immune state that may heighten susceptibility over time; ongoing immune imbalance, persistent T cell states, continuous microglial activation, and renewed inflammatory responses. These findings are consistent with other studies linking brain injury to long-term vulnerability through white matter damage, vascular and

membrane issues, and chronic inflammation (Armstrong et al., 2024; Mokbel et al., 2024; Needham et al., 2019; Sun et al., 2025). In parallel, TBI may expedite the onset of a microglial state resembling that of aging, characterized by sustained inflammatory reactivity and heightened neurodegenerative susceptibility (Ritzel et al., 2023). This supports the hypothesis that trauma can induce a chronically reactive immune phenotype in the brain. Complementary evidence from AD research indicates that activated $CD103^{-}$ $CD8^{+}$ T cells, exhibiting a resident-memory phenotype, can accumulate in brain tissue and instigate neurotoxic inflammation via granzyme K-PAR-1 signalling (Terrabuio et al., 2025). This suggests the biological plausibility that persistent post-traumatic T_{RM} cells could become detrimental in a subsequent degenerative environment.

Within this framework, the systemic LPS challenge employed in this thesis serves as a controlled surrogate for the cytokine-rich peripheral surges associated with respiratory infections. This is because T_{RM} cells are not exclusively re-engaged through cognate antigen recognition; they can also undergo non-cognate, cytokine-driven bystander activation (Curham et al., 2023; Iijima, 2024)(Curham et al., 2023; Iijima, 2024). In respiratory tissues, an LPS or heterologous bacterial challenge is sufficient to reactivate established T_{RM} populations and induce rapid effector cytokine production (Curham et al., 2023). This supports the biological plausibility that a post-traumatic brain T_{RM} compartment could similarly be reawakened by a systemic infectious episode, even in the absence of re-encountering the original CNS antigen (Ayasoufi et al., 2023; Curham et al., 2023; Iijima, 2024). This is clinically significant because pneumonia and other respiratory infections are common secondary insults following TBI (Gandasasmita et al., 2024). Experimental bacterial lung infection following TBI exacerbates mortality, impairs recovery, and enhances cortical inflammatory readouts, thereby supporting respiratory infection as a plausible real-world trigger for renewed neuroimmune escalation post TBI (Doran et al., 2020; Gandasasmita et al., 2024). Collectively, these findings support a model wherein repeated injury, white matter vulnerability, meningeal immune signalling, and the persistence of T_{RM} cells may converge to establish a durable neuroimmune niche. This niche does not predetermine a specific disease outcome but may increase susceptibility to chronic dysfunction and subsequent neurodegenerative changes (Armstrong et al., 2024; Bolte et al., 2020; Daglas et al., 2019; Hobson et al., 2026; Main et al., 2026; Mokbel et al., 2024; Shumilov et al., 2026).

Several critical considerations emerge from this discussion. The similarity in results across different models does not imply their equivalence. The current research, alongside studies on optic-tract injuries, mild brain injuries in children, and stroke, indicate ongoing

communication between adaptive and innate systems, although in varying anatomical locations, age groups, and contexts (Main et al., 2026; Ritzel et al., 2016; Selvaraj et al., 2021; Shumilov et al., 2026). The identification of memory-like cells within tissues remains constrained by existing methodologies. While markers such as CD69 and CD103 facilitate the demonstration of tissue association, they do not conclusively establish the cells historical lineage or permanent residency (Ayasoufi et al., 2023; Kimura et al., 2024; Smolders et al., 2018). The use of antibodies for cell depletion or complete gene knockout is not without limitations. Partial cell removal may underestimate their significance, whereas the elimination of entire cell types can result in compensatory mechanisms by other cells, a factor of importance in T cell-deficient injury models (Shumilov et al., 2026). Another important limitation of this thesis is that the experimental work was conducted exclusively on male mice. Consequently, the temporal architecture and cellular weighting of the post-traumatic immune response may not correspond precisely to those in females. This is due to evidence indicating sex-dependent differences in acute myeloid infiltration and neuroinflammatory programming following TBI (Doran et al., 2020). Models that induce inflammation are beneficial yet overly simplistic as they illustrate how brain responds to a peripheral insult but fail to fully capture real-world conditions such as pneumonia, sepsis, or post-brain injury complications (Cáceres et al., 2024; Kumar et al., 2020; Sharma et al., 2021).

The subsequent phase of research should concentrate on spatial resolution. In instances where injury induces localized immune states, it is imperative to ascertain the precise locations of these states, whether they are proximal to the injury site, within the brain's cortical layers, surrounding blood vessels, in white matter, adjacent to cerebrospinal fluid spaces, or a combination thereof. Another objective is to elucidate the origins of cells. Employing methodologies such as parabiosis, fate mapping, and T cell receptor sequencing is essential to distinguish newly arrived cells that resemble local cells from those that are genuinely local. A further area of focus is the development of precise therapeutic interventions. Current research indicates that, rather than broad-spectrum immune suppression, targeted modulation of immune responses may be more efficacious, such as inhibiting deleterious T cell migration in elderly patients with brain injuries or enhancing beneficial cellular interactions through specific treatments (Chen et al., 2024; Izzy et al., 2025). Finally, it is crucial to examine the association with neurodegeneration in longitudinal studies. These investigations should assess immune alterations, neuroimaging findings, neural connectivity, proteinopathies, and cognitive functions, particularly given evidence

suggesting that brain injuries may predispose individuals to subsequent neurodegenerative diseases (Bigler, 2013; Brett et al., 2022; Poudel et al., 2020).

Chapter 7

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