Ulm University Hospital

Department of Neurology

Director: Prof. Dr.med. Albert Christian Ludolph

FLT3/FLT3L signaling modulates splenic dendritic cells maturation upon traumatic brain injury and is enhanced by ethanol intoxication

Dissertation

Submitted to the

Medical Faculty of Ulm University

For the Doctoral Degree of Medicine (Dr. med.)

Presented by

Jin Zhang

Born in Zibo city, Shandong Province, P. R. China

2022

Dean of the Faculty of Medicine: Prof. Dr. rer. nat. Thomas Wirth

1st reviewer: Prof. Dr. Francesco Roselli

2nd reviewer: Prof. Dr. Gudrun Strauß

Date of defense: June 15, 2023

Results gained in my thesis have previously been accepted in the following publication:

Zhang J, Li Z, Chandrasekar A, Li S, Ludolph A, Boeckers TM, Huber-Lang M, Roselli F, Olde Heuvel F. **Fast Maturation of Splenic Dendritic Cells Upon TBI Is Associated With FLT3/FLT3L Signaling.** Front Immunol. 2022 Feb 24;13:824459. https://www.frontiersin.org/articles/10.3389/fimmu.2022.824459/full

The article is distributed under the Creative Commons License Attribution 4.0 International (CC BY 4.0), https://creativecommons.org/licenses/by/4.0/

The works herein were covered by an ethical approval by the ethical committee of University of ULM (153/19).

List of Content

List of Content ..... **I**
Abbreviations ..... **III**
1 Introduction **1**
1.1 General introduction of traumatic brain injury 1
1.1.1 Neuroinflammation following traumatic brain injury 3
1.1.2 Apoptosis after TBI 4
1.1.3 The role of oxidative stress, excitatory amino acids and calcium overload in traumatic brain injury 5
1.1.4 Serum biomarkers following TBI 8
1.2 TBI and the systemic inflammatory response 9
1.2.1 The role of the spleen in the systemic inflammatory response 9
1.2.2 Brain-spleen connection 11
1.2.3 The role of dendritic cells in the systemic inflammatory response 13
1.2.4 Effects of ethanol intoxication on traumatic brain injury 14
1.3 Current status of TBI treatment 15
1.4 The aim of this study 17
2 Materials and methods **19**
2.1 Materials 19
2.1.1 Laboratory equipments 19
2.1.2 Plastic materials 21
2.1.3 Equipment for traumatic brain injury model 21
2.1.4 Chemicals and drugs 22
2.1.5 Antibodies for immunofluorescence staining 24
2.2 Methods 25
2.2.1 Animals, traumatic brain injury model and ethanol treatment 25
2.2.2 Tissue isolation 27
2.2.3 RNA isolation and Quantitative RT-PCR 28
2.2.4 Tissue sectioning and immunofluorescence staining 31
2.2.5 Single mRNA in situ hybridization 31
2.2.6 Image acquisition and image analysis 32
2.2.7 Statistical analysis 35

3 Results 36
3.1 TBI rapidly and selectively induces cytokine expression in the spleen and is enhanced by concomitant EI 36
3.2 Rapid modulation of the splenic chemokine pattern by TBI and by EI/TBI 38
3.3 TBI induces the phosphorylation of FLT3 and its downstream signaling partner BTK in DCs, which are both enhanced by EI 40
3.4 TBI and EI-TBI strongly induce protein synthesis in splenic DCs..... 45
3.5 TBI and EI cooperate to enhance the maturation of splenic DCs..... 48
3.6 TBI strongly induces immunogenic function of splenic DCs 52
4 Discussion..... 56
4.1 Induction of maturation markers in splenic DCs occurs rapidly after TBI..... ...56
4.2 DCs are participants in the immunomodulation that occurs upon EI in TBI. ...60
5 Summary..... 63
6 Reference..... 65
7 Acknowledgements..... 93
8 Curriculum vitae 95

Abbreviations

3R	Replacement, reduction,refinement
℃	Degree Celsius

A

AD	Alzheimer‘s disease
ADRB2	Adrenergic beta-2 receptor
AIS	Acute ischemic stroke
ALS	Amyotrophic lateral sclerosis
AMP	Amplify
ANOVA	Analysis of variance
APC	Antigen presenting cell

B

BAC	Blood alcohol concentration
BAL	Blood alcohol level
BBB	Blood-Brain Barrier
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
BTK	Brutons tyrosine kinase

C

Ca^{2+}	Calcium
CCL	C -C motif chemokine ligand
CCR	C-C motif chemokine receptor
cDNA	Complementary DNA
CeA	Central amygdala
CI	Confidence Interval
CNS	Central nervous system
Con	Control
CRH	Corticotropin releasing hormone
CSF	Cerebral spinal fluid
CT	Computed Tomography
CTE	Chronic traumatic encephalopathy
CT value	Cycle threshold value
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor

D

d	Day
DAMPs	Damage-associated molecular patterns
DAPI	4′,6-diamidino-2-phenylindole
DBS	Deep brain stimulation
DEPC	Diethyl pyrocarbonate

DCs	Dendritic cells
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides

E

E	Ethanol
EI	Ethanol intoxication
eIF2α	α subunit of eukaryotic initiation factor 2
eotaxin	Eosinophil chemotactic factor
ERS	Endoplasmic reticulum stress
et al.	And others

F

FGF-basic	Fibroblast growth factor-basic
FLT3	Fms-like tyrosine kinase 3
FLT3L	Fms-like tyrosine kinase 3 ligand

G

g	Gram
GCS	Glasgow coma score
GDP	Gross domestic product
GFAP	Glial fibrillary acidic protein

H

h	Hour
HCl	Hydrochloric acid
HMGB1	High mobility group protein box-1

I

ICAM-1	Intercellular adhesion molecule 1
ICU	Intensive care unit
iDC	Immature dendritic cells
i.e.	For example
IFN-α	Interferon α
IFN-γ	Interferon γ
IFN-g	Interferon g
IL	Interleukin
IRES	Internal ribosome entry site

L

l	Litre
Lamp1	Lysosomal associated membrane protein 1
LP	Lipid peroxidation
LSM	Laser scanning microscope

M

MAP2	Microtubule-associated protein 2

MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MCP1	Monocyte chemotactic protein-1
miRNA	MicroRNA
mg	Milligram
MHC-II	Major histocompatibility complex-II
min	Minutes
MIP-1β	Macrophage inflammatory protein-1β
ml	Millilitre
mm	Millimeter
MN	Motor neuron
MODS	Multiple organ dysfunction syndrome
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
mTBI	Mild traumatic brain injury
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1

N

NaCl	Sodium chloride
NFL	Neurofilament light chain
NF-κB	Nuclear factor-κB

NK	Natural killer
Nm	Nanomolar
nm	Nanometre
NSE	Neuron specific enolase

O

O2	Oxygen
O.C.T	Optimal cutting temperature compound
OE	Overexpression
OFR	Oxygen free radicals
OR	Odds ratio

P

PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinase
pS6-RP	Phosphorylated S6 ribosomal protein
PVN	Paraventricular nucleus of hypothalamus

Q

Qrt-PCR	Real-Time quantitative reverse transcription PCR

R

R	Reverse
RNS	reactive nitrogen species
RNA	Ribonucleotide acid
ROS	Reactive oxygen species
RT	Room temperature
RT buffer	Reverse transcription buffer

S

S	Sham
S100β	Calcium binding protein β
SAP	Severe acute pancreatitis
SBDPs	Spectrin degradation products
SCI	spinal cord injury
SIRS	Systemic inflammatory response syndrome
SPX	splenectomy

T

T	Trauma
TBI	Traumatic brain injury
TCR	T cell receptor
TGF-β	Transforming growth factor-β1
Th1	Helper T cell 1
TLR4	Toll-like receptor 4

Tm	Melting temperature
TMS	Transcranial magnetic stimulation
TNFα	Tumor necrosis factor-α

U

UCH-L1	Ubiquitin C-terminal hydrolase-L
µl	Microlitre
µm	Micrometre
USA	United States of America

V

VN	Vagus nerve

W

WT	Wild type

1 Introduction

1.1 Introduction to traumatic brain injury

With the rapid development of modern economy and society, the use of road transportation is increasing day by day, resulting in frequent traffic accidents and fall injuries, which in turn lead to an increase in traumatic brain injury (TBI), causing a heavy economic burden to society and families. According to the Glasgow coma scale (GCS), TBI can be classified as mild (GCS=13-15), moderate (GCS=9-12) and severe (GCS=3-8)[112]. Although the level of diagnosis and treatment has improved significantly, the mortality rate of severe craniocerebral injury is still as high as 25-50% [170]. TBI has become one of the common causes of death and disability. More than 50 million people worldwide experience TBI each year, and about half of the world's population is likely to suffer from one or more TBIs in their lifetime [116]. TBI costs the global economy approximately $400 billion annually, or 0.5% of the world's gross domestic product (GDP) [197]. Epidemiological studies of TBI in Europe have shown that the annual incidence of TBI is 47.3/100,000 to 849/100,000, with a mortality rate of 3.3/100,000 to 28.1/100,000 [67]. TBI is not only highly prevalent, but also highly disabling. TBI has been found to be a risk factor for late onset neurological disorders, causing cognitive impairment and increasing the risk of depression in patients [164]. It is estimated that 3-17 million TBI patients in the United States have permanent sequelae [117, 153]. The combined advantage ratio (OR) for late-onset dementia combined with TBI and impaired consciousness was reported to be 1.58 (95% CI 1.21-2.06) [62]. Based on relevant statistics, the attributable risk of TBI on the overall incidence of dementia is as high as 5-15% [179]. Patients with TBI aged >55 years old have a 44% increased risk of developing Parkinson's disease over the next 5-7 years [66]. It is predicted that globally, between now and 2030, TBI will remain one of the top three neurological disorders causing neurological impairment and that the social burden of its associated complications will continue to increase [116].

The pathogenesis of TBI is a complex process. TBI usually originates from a primary

injury, directly related to an external blow to the brain, which then progresses to a secondary injury. Secondary pathogenesis includes molecular, chemical and inflammatory cascades involving the release of excitatory neurotransmitters, which can lead to temporary or permanent neurological deficits [65]. The biochemical, cellular and physiological events that occur during primary injury often develop into delayed and prolonged secondary injury lasting from hours to years. Individuals with TBI vary widely, and the recovery trajectory after TBI is highly unpredictable [78]. The dysfunction of the blood-brain barrier (BBB), caused by traumatic brain injury, allows the transfer of inflammatory mediators into the injured brain parenchyma [37]. Activated leukocytes, microglia and astrocytes produce reactive oxygen species (ROS) and inflammatory molecules, causing infiltration of leukocytes and macrophages, leading to demyelination and disruption of the axonal cytoskeleton, resulting in axonal swelling and accumulation of transport proteins at the ends, thereby impairing neuronal activity [56]. Progressive axonal damage may lead to neurodegenerative pathologies such as Alzheimer's disease (AD) [34] and chronic traumatic encephalopathy (CTE) [203]. In addition, the proliferation of astrocytes at the lesion site leads to the formation of glial scars, which create an impermissible environment that hinders axonal regeneration [23, 85].

TBI has acute, systemic effects [168]. There are complex interactions between the brain, peripheral and immune system. Even in the absence of systemic disease or infection, TBI may lead to impaired heart, lung, liver, and kidney functions [10, 158]. The systemic response to TBI is characterized by inflammation, accompanied by dysregulation of the autonomic nervous system and systemic immunosuppression [73, 82, 120]. Although brain injury leads to systemic increases in inflammatory mediators and cytokines in patients [51, 159, 190] and in rodent models of TBI [157, 205, 225], there is substantial evidence of systemic immunosuppression following TBI with a reduction in peripheral immune cells [91, 108, 128, 174, 181, 204]. Immunosuppression may lead to secondary infections after trauma and increase the length of hospital stay.

Despite the ongoing search for effective ways to mitigate secondary brain injury, treatment options for traumatic brain injury (TBI) are very limited [189], and there are no known pharmacological treatments that can improve the prognosis of patients. Therefore, it is of great significance to study the effective treatment methods of TBI to reduce its disability rate, fatality rate and social burden.

1.1.1 Neuroinflammation following traumatic brain injury

After TBI, the central nervous system (CNS) undergoes a series of reactions, characterised by cell membrane rupture, vascular damage and BBB destruction, which in turn triggers ionic imbalance, excitatory amino acid release, calcium overload and mitochondrial dysfunction, ultimately resulting in cell death [41, 99, 195]. During this pathological process, a large number of damage-associated molecular patterns (DAMP), cytokines and chemokines are released, microglia and astrocytes are activated, and circulating immune cells are recruited [95, 131, 156]. Following TBI, intracranial cells release large amounts of endogenous factors such as inflammatory vesicles, mitochondrial DNA (mtDNA), heat kinin and high mobility group box-1 protein (HMGB1) [224]. They bind as DAMP to nuclear factor-κB (NF-κB) and Toll-like receptors (TLR) of the mitogen-activated protein kinase pathway, causing the release of a variety of chemokines (CCL), their chemokine receptors (CCR) and pro-inflammatory cytokines, including CCL2/CCR2, CXCL12, CXCR4 and interleukins (IL) like IL-1β, IL-6, IL-12, IL-13, etc.[55, 141, 224]. The inflammatory response is critical for debris clearance, repair and regeneration after TBI, and dysinflammation can cause acute and chronic brain damage [229].

Cytokines can be divided into pro-inflammatory and anti-inflammatory cytokines based on their different modulating effects on the inflammatory response. Both are involved in secondary brain injury and damage repair after TBI, mainly including brain oedema, cerebral blood flow disturbance, altered blood-brain barrier permeability, neuronal death and CNS repair and regeneration processes [126]. IL-1β, IL-6 and TNF-α, which usually represent pro-inflammatory factors, are expressed first, followed by IL-8, then chemokines and adhesion factors represented by ICAM-1, and finally IL-4,

IL-10, TGF-β and other inhibitory inflammatory factors [114, 221]. Following TBI, there are multiple cytokine signalling pathways that mediate inflammation and upon activation have different interactions within the cytoplasm or nucleus [39, 83]. In addition, each signalling pathway can activate its own positive or negative feedback regulatory mechanisms, tending to amplify or blunt the response [221].

1.1.2 Apoptosis after TBI

While necrosis is a long recognized mode of cell death, apoptosis, a concept first introduced by Kerr et al. [87] in 1972, has gradually become more widely recognized as a mode of cell death in the last decade or so. In recent years, a series of studies have confirmed the involvement of apoptosis in the development of secondary brain injury following traumatic brain injury [40]. Apoptosis is the activation of the membrane signalling system of the cell body in response to certain physiological or pathological factors, which in turn transcribes genes which are programmed to regulate apoptosis, leading to self-destruction and cell death. Apoptosis in neuronal cells after TBI is a pathological process that occurs actively in cells as a result of the activation of apoptotic genes by various factors. Shaw et al. [178] found TUNEL (deoxyribonucleic acid end transferase-mediated nick end labelling) positive neurons in the hippocampus and cingulate gyrus of brain specimens from acute traumatic brain injury patients who survived 5h to 10d after fatal craniocerebral trauma, with a peak at 24h-48h post-injury. In fact, Wiliiams et al. [219] found that significant apoptosis was still seen in the white matter of some traumatized brain patients 12 months after the injury.

The early death of nerve cells after traumatic brain injury is a direct result of necrosis, with subsequent cell death occurring through both necrosis and apoptosis [151]. Pike et al. [151] found that calpain and caspases were activated after traumatic brain injury, accompanied by morphological changes of necrosis and apoptosis, suggesting that both are jointly involved in the process of secondary injury after traumatic brain injury. After TBI, cerebral blood flow regulation is impaired, the brain becomes ischemic and hypoxic, nerve cell biofilm dysfunction occurs, vascular-derived and cytotoxic cerebral

edema forms, further aggravating cerebral ischemia. Furthermore, brain tissue ischemia and hypoxia cause impaired energy metabolism in nerve cells, intracellular calcium overload, excessive release of excitatory amino acids, lipid peroxidation and free radical production. The effects of these factors contribute to the up- or down-regulation of apoptosis regulatory genes. Ultimately, the process of apoptosis is initiated [235].

Apoptosis initiation signals following TBI are derived from both intracellular and extracellular sources. The intracellular initiation signal is mainly triggered by mitochondrial dysfunction [187]. However, the extracellular initiation signal is mediated by the TNF receptor superfamily [200]. TNF-α is an important cytokine with a specific role in TBI-mediated neuroinflammatory responses [180]. It is not significantly expressed in brain tissue from mild head injury, but for severe head injury it can be significantly increased within 1-12 hours after injury [7]. In addition to apoptosis, several mechanisms are also involved in the pathophysiology of brain injury, such as autophagy [234], axonal degeneration [85], mitochondrial dysfunction [230] and endoplasmic reticulum stress (ERS)[119]. Mechanisms such as excitatory amino acids, calcium overload, and oxidative stress do not occur in isolation, but occur simultaneously and are interconnected to form a chain reaction of pathophysiology after TBI.

The mechanisms of nerve damage after TBI are complex. The apoptotic response to TBI varies for different degrees of TBI. In addition to necrosis and apoptosis, demyelination, neurodegeneration and neural stem cell differentiation have been widely observed after TBI [147].

1.1.3 The role of oxidative stress, excitatory amino acids and calcium overload in traumatic brain injury

Oxidative stress is a process in which the body is exposed to a variety of harmful stimuli, resulting in excessive production of highly reactive molecules such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), oxidation that exceeds the rate of

removal of oxidants, and an imbalance between the oxidative and antioxidant systems, leading to tissue damage [102, 154]. As early as 1986, Kontos et al. found that there was a significant production of free radicals after TBI [92]. Subsequently, studies have continued to show that oxidative stress plays an important role in the pathophysiological processes following TBI [42]. It is mainly manifested by increased free radical content in brain tissue, accumulation of peroxidation products and a decrease in antioxidant substances in the brain such as reduced glutathione, superoxide dismutase and ascorbic acid [88]. Oxidative stress can cause direct damage to neuronal cells through lipid peroxidation (LP), protein oxidation, nucleic acid oxidation, and inflammation, and indirectly induce apoptosis through mitochondrial and nuclear transcription factor kappa B (NF-κB) pathways [111]. Excess oxygen free radicals (OFR) can also cause DNA fragment damage and impair the blood-brain barrier, leading to further brain tissue damage [139]. In conclusion, the sustained release of highly reactive oxygen radicals in TBI and the associated increase in ROS-mediated lipid peroxidation has a detrimental effect on brain plasticity, cerebral blood flow and promotes immunosuppression [186].

Excitatory amino acids mainly refer to glutamate and aspartate. Numerous studies have confirmed that excitatory amino acids are not only important excitatory neurotransmitters in the central nervous system, but also play an important physiological role in the nutrition, development and transmission of neuronal information in the nervous system [121]. Glutamate is the most abundant excitatory amino acid in brain tissue, with concentrations three to four times higher than those of aspartate. Traumatic brain injury can cause the uncontrolled release of large amounts of excitatory amino acids such as glutamate and aspartate, and excitatory amino acid levels are significantly elevated after brain injury. The resulting brain damage is an important factor in the poor prognosis after traumatic brain injury [35].

It has been found that excitatory amino acid levels in brain tissue of patients following traumatic brain injury are significantly elevated, tens of times higher in areas of injury than in areas without injury [177]. The degree of elevation of excitatory amino acids is

independent of the size of the contusion focus, the level of intracranial pressure and additional ischaemic factors, so it is hypothesized that the excitatory amino acid leakage is due to the presence of some non-specific destruction mechanism in the contusion area of the brain, resulting in many microscopic pores in the cell membrane [177]. Dysregulation of extracellular glutamate and glutamate uptake during the acute phase of TBI, and failure to resolve glutamate homeostatic mechanisms play a causal role in chronic cognitive symptoms following TBI [53]. As a result of impaired glutamate uptake, glutamate diffusion outside the synapse may lead to increased extrasynaptic glutamate signalling, which causes secondary damage through activation of cell death pathways, as well as loss of fidelity and specificity of synaptic transmission [32, 53]. In conclusion, the massive release of excitatory neurotransmitters overstimulates glutamate receptors leading to prolonged post-traumatic oxidative stress and excitatory cell death.

As an important messenger in neuronal cells, calcium ions are involved in the synthesis and release of neurotransmitters, maintenance of neural excitability, synaptic plasticity and enzymatic activity, as well as in pathological processes such as ischemia, hypoxia and neutrophilic cell death [22]. It is believed that intracellular calcium overload in nerve cells after craniocerebral injury plays an important role in the onset and progression of secondary brain injury and is the final common pathway for cell death in a variety of conditions [136]. The exact cause of intracellular calcium overload in neuronal plasma following traumatic brain injury is still unclear. It is now generally accepted that the large influx of extracellular Ca^{2+} into neurons after traumatic brain injury is the main cause of calcium overload in neuronal cells [21]. By inhibiting Ca^{2+} release from the endoplasmic reticulum and the inward flow of extracellular Ca^{2+}, neuronal damage after TBI could be reduced [161]. Deshpande et al. measured Ca^{2+} in rat hippocampal neurons on days 1, 7 and 30 after TBI and found a significant increase in Ca^{2+} on days 1 and 7 after trauma [188]. When the Ca^{2+} level returned to the original level, the cognitive function of the rats also recovered [49]. In addition, a large influx of calcium can decouple the oxidative phosphorylation electron transport chain, which can also activate the caspase apoptotic signalling pathway, leading to cell edema or

apoptosis [69, 223].

1.1.4 Serum biomarkers following TBI

TBI-related biomarkers can not only help to compensate for the shortcomings of traditional diagnostic methods, but can also further elucidate the biological damage mechanisms of TBI [165]. TBI biomarkers mainly include coagulation-related biomarkers, inflammation-related biomarkers, and nervous system injury-related biomarkers. A number of proteins synthesized by glial cells or neurons have been found to be closely associated with damage to the central nervous system. Among them, neurospecific enolase, calcium-binding protein, C-tau protein, glial fibrillary acidic protein (GFAP), myelin basic protein (MBP), ubiquitin C-terminal hydrolase-L1 (UCH-L1), and spectrin degradation products (SBDPs) are representative of these due to their high expression in the nervous system and significantly elevated expression after TBI and are considered to be the most likely specific biomarkers for the diagnosis and prognosis of TBI [214]. There are other TBI biomarker candidates with biofluidic evidence from human animal models of TBI ranging from severe TBI to mTBI. These include dendritic protein microtubule-associated protein 2 (MAP2)[142], brain-derived neurotrophic factor (BDNF)[94], and postsynaptic protein neurogranule protein [226]. However, further evaluation of the utility and TBI specificity of these biomarkers is required. Collective evidence shows that TBI protein biomarkers are a promising diagnostic and prognostic tool for improving patient treatment and management, while providing opportunities for other interventions for patients suffering from the sequelae of TBI [90, 127, 206].

The presence of the BBB makes it difficult for protein biomarkers to migrate from the cerebrospinal fluid (CSF) into the blood during mild TBI, whereas small noncoding RNAs may be more stable and sensitive in the blood. In recent years miRNAs have gained widespread attention as biomarkers of TBI. For example, miR-21 [68], and mirR-155 [76] showed distinct advantages in the diagnosis of TBI.

1.2 TBI and the systemic inflammatory response

Following acute brain injury, increased intracranial production of pro-inflammatory cytokines induces a systemic inflammatory response. Current research on brain oedema after TBI has mostly focused on localised brain tissue, while the role of the body's peripheral tissues and organs in secondary brain injury is unclear. After traumatic brain injury (TBI), activation of the body's immune system promotes the clearance of damaged cells and cellular debris. Severe trauma causes hyperexcitation of the sympathetic nervous system and massive secretion of inflammatory cytokines, which leads to the development of systemic inflammatory response syndrome (SIRS). Chaban V et al. [28] conducted a large prospective cohort study showing that a large number of cytokines of different functions persist for one year after mild TBI (mTBI) and that a low level of systemic inflammatory response persists, i.e., mTBI activates the body's immune system for a long time. The inflammatory response after mTBI is caused not only by the trauma itself or the apparent pathological damage to the brain tissue, but also by the active involvement of the organism's immune system [52]. The systemic inflammatory response syndrome caused by TBI, especially after severe craniocerebral injury, is the result of an over-activated immune system. One study showed [28] that plasma interferon-g (IFN-g), interleukin-8 (IL-8), eosinophil chemotactic factor (eotaxin), macrophage inflammatory protein-1β (MIP-1β), monocyte chemotactic protein-1 (MCP-1), interleukin 17-A (IL17-A), interleukin-9 (IL-9), tumour necrosis factor (TNF) and basic fibroblast growth factor (FGF-basic) were significantly higher in mTBI patients than in controls at various time points of the assay. These cytokines also have an important role in the pathology of secondary brain injury. For severe craniocerebral injury, patients often have multiple traumas, such as hemorrhagic shock, fracture, lung injury and other comorbidities. Various tissue damages lead to systemic hypermetabolism, immune system disorders, and even multiple organ dysfunction syndrome (MODS) [134].

1.2.1 The role of spleen in systemic inflammatory response

The spleen is the largest immune organ in the body and plays a very important role in the systemic inflammatory response. The spleen is involved in promoting the interaction between antigen presenting cells (APCs) and T and B lymphocytes [103]. In systemic inflammatory response syndrome, the spleen is an important effector organ. The spleen accounts for approximately 25% of the body's lymphoid tissue and contains a variety of immunologically active cells, including macrophages, T cells, B cells, natural killer cells, and dendritic cells. Under stressful conditions, they can release IL-1β, IL-6, IL-8, IL-12, TNF-α, IFN-α and other inflammatory mediators, which play an important role in maintaining the body's normal immune response [133]. The spleen is considered to be a direct reservoir of monocytes, which are mobilized directly into the spleen after an injury to the body [228].

In 1996, Okuaki [137] first reported that splenectomy (SPX) prior to hepatic ischemia-reperfusion injury exerted a hepatoprotective effect by reducing leukocyte infiltration in the ischemic zone of liver tissue. SPX also prevented ischemia-reperfusion injury in the small intestine and prevented the induction of inflammatory injury following cellular degeneration in other organs [2, 213]. In the systemic inflammatory response to severe acute pancreatitis (SAP), splenectomy reduces liver and lung damage and intestinal mucosal barrier dysfunction [238]. Recent studies have shown that in addition to the involvement of inflammatory factors in the site of injury, the peripheral immune system is also involved in the process of secondary brain injury after TBI, promoting the formation of traumatic brain oedema [167].

Studies have shown that the spleen can release large amounts of inflammatory factors such as TNF-α after TBI, further promoting the inflammatory response and aggravating the extent of the organism's injury, and that splenectomy can protect the organism by hindering the progression of the pathological process to some extent [6, 213]. Seifert et al.[228] indicated that an important pathway for the action of splenic-derived inflammatory mediators after ischemic brain injury may be the MAPK signalling pathway. The above studies suggest that the spleen plays an important role in the systemic inflammatory response.

1.2.2 Brain-spleen connection

The brain and viscera interact within the autonomic nervous system, with the vagus nerve (VN) containing approximately 80% of the afferent fibres and 20% of the efferent fibres, playing a variety of key roles in the homeostatic regulation of visceral function [15]. New research shows that lymph nodes are innervated by distinct sensory neuron populations with immunomodulatory potential [81]. Recent physiological and anatomical studies demonstrated that inflammation is regulated by neuroimmune polysynaptic interactions between the vagus and visceral nerves to regulate splenic function [8]. Anatomically, there is extensive distribution of nerve fibers in all splenic compartments, including splenic nodules [80]. The activity of the spleen is controlled by the autonomic nervous system (part of the nervous system that regulates the organ), and more specifically, the spleen is controlled primarily through the sympathetic branch of the autonomic nervous system, which is associated with the 'attack and flight' response [27]. However, little is currently known about the upstream brain regions that may be connected to the splenic autonomic nerves to control the spleen, and the resulting acquired immunity.

Recent studies have shown that systemic immune function is rapidly regulated through the development of neurological diseases through a pathway conceptualized as the "brain-spleen axis" [24, 217, 236]. A study in mice showed that stimulation of the ventral tegmental area of the brain (part of the brain's reward circuit) enhanced the immune response and helped to defend against harmful bacteria [237]. Although the exact mechanisms underlying the initiation of splenocyte responses to the traumatized brain have yet to be identified, several events, including chemokine/chemokine receptor interactions, autonomic nervous system activation, and the release of CNS antigen have been documented to be essential for efficient brain–spleen connection.

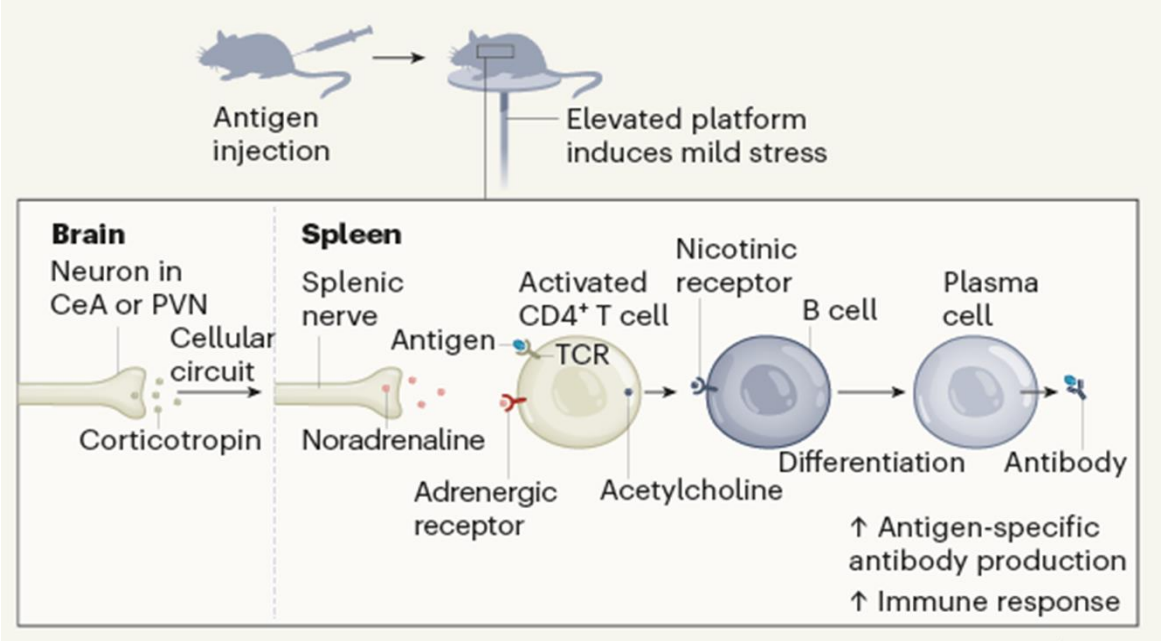

Figure 1. Brain control of adaptive immunity. Mice are made to stand on an elevated platform, leading to activation of CeA and PVN CRH neurons and increased plasma cell formation. In immunized mice, the elevated platform regimen induces an increase in antigen-specific IgG antibodies in a manner that depends on CRH neurons in the CeA and PVN, an intact splenic nerve, and B cell expression of the α9 acetylcholine receptor. By identifying a specific brain-spleen neural connection that autonomically enhances humoral responses and demonstrating immune stimulation by a bodily behaviour. Reprinted from [237] with permission from Nature Publishing Group for Nature.

Recent studies have shown that the immune action of the spleen is regulated by the central nervous system [232]. Previous studies on the brain-spleen axis confirmed that the nervous system initiates the interaction and modulation of spleen responses through circulating catecholamines [1] or autonomic nerve fibers associated with spleen immune cells [80]. In fact, sympathetic and parasympathetic fibers have been reported to innervate dendritic cells (DCs) in the spleen via neurotransmitters, which influence DCs function to modulate immune responses [194]. Furthermore, stimulation of the vagus nerve can rapidly reduce macrophage-induced release of TNFα in the spleen through a so-called cholinergic anti-inflammatory pathway [212]. More and more studies have shown that the innervation of immune organs is not static,

and the density of nerve endings in immune organs changes dynamically with the degree of inflammation [166].

There is growing evidence that dysregulation of the immune system contributes bottom-up to behaviours associated with several neuropsychiatric disorders [26]. It has recently been shown that multi-organ inflammatory responses involving the autonomic nervous system and spleno-intestinal-brain axis dysfunction exacerbate the pathogenesis of TBI in the brain [20]. The pathway of action of neurons in the central nucleus of the amygdala and the paraventricular nucleus of the hypothalamus causes the adrenal glands to secrete glucocorticoids in response to stress, and glucocorticoids may have immunosuppressive effects [43]. These studies suggest a broad link between the brain and the spleen in the systemic inflammatory response.

1.2.3 The role of dendritic cells in the systemic inflammatory response

The systemic inflammatory response is closely related to immune dysfunction [79]. Dendritic cells (DCs) are the most important antigen-presenting cells (APCs) in the body and play a key role in the initiation and regulation of the immune response. DCs play an important role in both the innate and adaptive immune systems. Under normal conditions, the majority of DCs are immature and express low levels of costimulatory molecules (e.g. CD80, CD86, etc.). In response to antigenic or inflammatory stimuli, DCs mature, take up and process antigens, increase the expression of surface costimulatory molecules and present them to effector cells such as T cells to induce an immune response [124]. During the maturation of DCs, a variety of cytokines and receptors play a role in promoting [72, 115, 143, 215, 220]. The receptor tyrosine kinase FLT3 and its ligands are essential for DCs homeostasis by activating downstream effectors [125].

DCs present antigens to T effector cells in the form of antigenic peptides and major histocompatibility complex (MHC) complexes, while secreting cytokines to regulate T cell function [48]. Chemokines also synergistically promote T cell-dendritic cell interaction to enhance immunity [25]. DCs secrete high levels of IL-12 to induce the differentiation of helper T (Th) cells from Th0 to Th1, producing Th1-type cytokines

such as interferon-gamma (IFN-γ) [201], TNF-α and interleukin-2 (IL-2), which mediate the Than1-type immune response. When DCs secrete high levels of IL-4 and less IL-12, they induce the differentiation of helper T cells into Th2, producing Th2-type cytokines such as IL-4, IL-10 and other anti-inflammatory factors [124]. It is evident that DCs can act on different T cells to induce and regulate the immune response.

The number and functional status of DCs in immune organs are dynamically altered during systemic inflammatory responses. One study found a significant increase in DCs in the splenic T-cell area of mice 6h after intravenous injection of lipopolysaccharide, however, the number of DCs decreased significantly after 48h, presumably as a result of the immune system's own negative feedback [48]. Using a systemically infected mouse model, Tinsley et al. [198] also found that the number of splenic follicular DCs increased significantly early in the infection, then peaked at 36h of infection and decreased significantly at 48 h of infection. It was found that splenic DCs in systemically infected mice were mature in the early stages, with significantly higher expression of major histocompatibility complex-II (MHC-II), CD80 and CD86. Tinsley et al. [198] also demonstrated increased expression of co-stimulatory molecules in DCs early in systemically infected mice, with subsequent functional inhibition of DCs.

The maturation of DCs is regulated by a variety of cytokines, among which the FLT3/FLT3L signaling pathway plays an important role. This signaling pathway not only regulates the differentiation and mobilization of progenitor DCs, but also plays a role in the homeostatic division of DCs in peripheral lymphoid organs. FLT3/FLT3L also plays an important role in maintaining the mature state of DCs [173]. The maturation of DCs is accompanied by changes in leukocyte differentiation antigens, such as CD40, CD80, CD83, and CD86 [12], which are often upregulated together with MHC-II [152, 155, 210].

1.2.4 Effects of ethanol intoxication on traumatic brain injury

Ethanol intoxication (EI) is a frequent comorbidity in traumatic brain injury, with almost 40% of patients having a positive blood alcohol level (BAL) on admission to hospital [13]. The reality is that in the context of traumatic brain injury, most patients who exhibit EI are not chronic drinkers but occasional heavy drinkers. Whether coexistence of EI and TBI is harmful or beneficial is debated. Recent studies have shown that acute EI can have beneficial effects on the neuroimmune response after experimental TBI [30, 70, 138], and these findings have been supported by clinical evidence that TBI patients with EI have shorter ICU stays, shorter hospital stays, and lower overall in-hospital mortality [18, 19, 160]. However, some clinical studies have reported the opposite [176, 231]. Animal models have demonstrated that high doses of EI could reduce the volume of spleen [109] and decrease the pro-inflammatory cytokines IL-1β and IL-6, but increase the anti-inflammatory cytokine IL-10 [110]. In addition, EI has been shown to suppress antigenic expression of DCs [57], reflecting the immunosuppressive effects of ethanol intoxication on the spleen. In experimental TBI, for distal organs, EI can attenuate TBI-induced pro-inflammatory responses in the lung and liver via the HMGB1-RAGE axis [225]. It has also been shown that alcohol intoxication depresses respiratory function [98], decreases cerebral perfusion blood pressure [149], excites the sympathetic nervous system [71]and increases oxygen radical production [239], which in turn exacerbates secondary brain damage. So far, it is unclear whether EI also detects any beneficial effects on plasma biomarkers of neuronal or glial damage that occur in TBI. A clinical study found that ethanol intoxication was associated with reduced in-hospital mortality after isolated moderate-to-severe TBI [18].

There is no doubt that regular ethanol exposure increases the risk of multiple traumas, including TBI. Controversies regarding the effect of EI upon TBI may be related to the dose of ethanol, the duration of exposure associated with the injury, and the nature of the injury.

1.3 Current status of TBI treatment

Current clinical treatment for TBI is mainly supportive, with no specific

neuroprotective drugs available [240]. The most important factor determining the prognosis of patients with acute craniocerebral injury is the severity of the primary craniocerebral injury and, to a lesser extent, the availability of timely and effective lifesaving treatment. The treatment of TBI is a systemic project, and the key to reducing the rate of disability and death from TBI is to adopt a simple, safe and effective approach to the assessment and emergency treatment of TBI patients within the "golden hour" of emergency trauma to avoid secondary brain damage [202]. TBI is a major global health challenge and priority. However, it is worth exploring how to rationalize the allocation of medical resources for different levels of TBI [17]. Current promising research directions aim to optimise neurocritical care protocols, develop an evidence base for surgical interventions, translate promising neuroprotective drug therapies into applications for human pathology, and actively promote personalised and combination therapies [89]. Newly developed imaging techniques are also providing significant assistance in the diagnosis and treatment of TBI [183]. For clinical diagnosis, the radiological examination of choice in emergencies is computed tomography (CT): it is fast, accurate, and widely used. During hospitalization, follow-up CT scans can determine whether damage has progressed. MRI is often used to complement CT, but for mild TBI and chronic TBI, MRI offers additional advantages [129].

Physical, cognitive and emotional sequelae usually occur in patients after severe TBI [9]. To date, pharmacological and rehabilitation therapies to treat these sequelae are limited [96]. Non-invasive methods such as transcranial magnetic stimulation (TMS) and transcranial direct current stimulation have been extensively studied [106]. Deep brain stimulation (DBS) has been used to treat a variety of conditions, and it also has the potential to improve cognitive hypofunction after TBI [96]. Nutritional supplements and alternative therapies may have some degree of improvement in cognitive function and psychiatric symptoms in patients during rehabilitation after TBI, but more clinical trial evidence is still needed [113]. Recent studies suggest that targeting neural stem cells may be a potential strategy for repairing and regenerating the injured brain in the future [16, 218]. For severe TBI (sTBI), there is still further

research work need to be done.

1.4 The aim of this study

Systemic inflammatory response remains a major complication after traumatic brain injury (TBI), with nearly all organs affected. This response consists of post-injury inflammation and concurrent immunosuppression. The spleen is one of the major immunoregulatory organs and has numerous interactions with the brain. This communication between the brain and spleen is known as the brain-spleen axis, and both nerve fibers and brain-derived circulating mediators have been shown to interact directly with splenic immune cells. One of the most important comorbidities in TBI is ethanol intoxication (EI), with almost 40% of patients having a positive blood alcohol concentration (BAC) at the time of injury. EI itself has been shown to reduce pro-inflammatory mediators and enhance anti-inflammatory mediators in the spleen. Notably, in the context of traumatic brain injury, the majority of patients who show EI are not chronic drinkers, but younger, often occasional drinkers on weekends. Studies have demonstrated that acute alcoholism can have a beneficial effect on the immune response after experimental TBI [30, 138]. Clinical studies have also reached the same conclusion that TBI patients with alcoholic intoxication have shorter ICU stays and lower overall in-hospital mortality [18, 19, 160]. However, some clinical studies have found the opposite [231], or that alcoholism is not associated with mortality in TBI patients [176]. Furthermore, EI has been shown to inhibit antigen expression in DCs [57], which reflects the immunosuppressive effect of ethanol intoxication on the spleen. Does acute EI interfere with or amplify TBI-induced systemic immune regulation, specifically does EI modulate TBI-induced splenic immune responses? Given that alcoholism is the most common comorbidity in TBI patients, especially those who drive after drinking, this issue has immediate clinical implications. How EI affects spleen immune regulation is unclear, and does EI affect the crosstalk regulation of immune function by the autonomic system? In this doctoral dissertation, I investigate the effects of a single high-dose ethanol exposure in adult mice prior to experimental TBI on the

splenic immune response, focusing on the effect on the rapid maturation of splenic dendritic cells after TBI.

2 Materials and methods

2.1 Materials

2.1.1 Laboratory equipments

Item	Company
-80°C Deep freezer	Kaltis, Taiwan
-20°C Freezer	Kirsch, Germany
Cryostat	Leica, Germany
ACD HybEZ Hybridization System	Advanced Cell Diagnostics, USA
Block thermostat	Thomas scientific, USA
BZ-II analyzer software	Keyence, Japan
BZ-X800 microscope	Keyence, Japan
Desk centrifuge	VWR International GmbH, Germany
Disposable hypodermic needle 0.4 x 20 mm	B.Braun Melsungen AG, Germany
Disposable hypodermic needle 0.9 x 40 mm	B.Braun Melsungen AG, Germany
Disposable hypodermic needle 0.45 x 12 mm	Henke sass wolf, Germany
Electronic thermometer	IKA® Werke GmbH, Germany
Excel 2013	Microsoft, USA
Fume cupboard	Wesemann, Germany

GraphPad Prism 8	GraphPad Software, USA
Heating pad	Stoelting, Ireland
Image J	National Institutes of Health
Light Cycler 480 II	Roche, Germany
LSM-710 Confocal laser scanning microscope	Carl Zeiss AG, Oberkochen, Germany
Magnetic hotplate stirrer	VWR International GmbH, Germany
Microwave oven	Severin, Germany
Microscope slides (Ground edges frosted)	VWR International GmbH, Germany
Microscope coverslips 24x50 mm	VWR International GmbH, Germany
Microscope coverslips 21x 26 mm	VWR International GmbH, Germany
Microscope slides folder	VWR International GmbH, Germany
Microtome blades	Feather, Japan
Mini microcentrifuge	Biozym, Germany
Mini vortex shaker	Greiner Bio-one GmbH, Germany
Perfusion tubing pump	Ismatec, Germany
Scalpel	Aesculap AG, Germany
Scales	Sartorius, Germany
Shaker	IKA® Werke GmbH, Germany
Sterile nitril gloves	B.Braun Melsungen AG, Germany
Tissue-Tek O.C.T compound	Sakura Finetek, Netherlands
Water bath	VWR International GmbH, Germany
Word 2013	Microsoft, USA

2.1.2 Plastic materials

Item	Company
15ml FALCON conical tube	BD Falcon Biosciences, Germany
50ml FALCON conical tube	BD Falcon Biosciences, Germany
96-well plate	Greiner Bio-one GmbH, Germany
Adhesive Clear PCR Seal	Greiner Bio-one GmbH, Germany
Eppendorf tubes 0.5 ml	Eppendorf, Germany
Eppendorf tubes 1.5 ml	Eppendorf, Germany
Eppendorf tubes 2 ml	Eppendorf, Germany
Eppendorf tubes 5 ml	Eppendorf, Germany
Microscope slides	VWR international bvba, Belgium
Ocular gel	Bepanthen, Bayer Leverkusen Germany
Peel-A-Way® disposable plastic tissue embedding molds (22×22×20 mm deep)	Polyscience, Inc. U.S.A.
Pipettes tips	Sarstedt, Germany
Sarstedt serological pipettes 5 ml	Sarstedt, Germany
Sarstedt serological pipettes 10 ml	Sarstedt, Germany
Sarstedt serological pipettes 25 ml	Sarstedt, Germany
Syringe 1ml	BD Plastipak, Spain

2.1.3 Equipment for traumatic brain injury model

Item	Company
Active charcoal absorption filter	UNO BV, Netherlands
Bepanthen® wound and healing ointment	Bayer, Germany
Cotton-tipped applicator	Paul-Hartmann, Germany
Fine scissors	Fine science tools, Germany
Gas anesthesia head holder	David Kopf Instruments, USA
Gas exhaust unit	UNO BV, Netherlands
Heating pad	Stoelting, Ireland
Induction box for anesthesia	UNO BV, Netherlands
Microloader pipette tips	Eppendorf, Germany
Micro needle holder	Fine science tools, Germany
Prolene 6-0 suture thread	Ethicon, Germany
Sigma-delta sevoflurance vaporizer	Penlon, UK
Stereotactic frame	Consultants GmbH D-40211, Germany
Vannas-Tübingen spring scissor	Fine science tools, Germany
Zoom stereo microscope for surgery	Olympus, Japan

2.1.4 Chemicals and drugs

Chemicals and drugs	Company	Reference number
2-Propanol	Applichem Panreac, Germany	A3928
Albumine Bovine Fract V (BSA)	Applichem Panreac, Germany	A1391

Chloroform	Applichem Panreac, Germany	32211-1L-M
Distilled water	Fresenius Kabi, Germany	7151-5
EtOH	Applichem Panreac, Germany	A3678
Fluorogold prolong antifade mounting medium	Invitrogen, Germany	P36934
Hydrochloric acid (HCl)	Honeywell, Germany	35328
Natriumhydrogenphosphat (Na2HPO4)	Carl Roth GmbH, Germany	P030.2
Paraformaldehyde (PFA)	Sigma-Aldrich Chemie GmbH, Germany	441244
Phosphate buffered saline (PBS) 1X w/o Ca2+/Mg2+	Gibco, Thermo Fisher Scientific, Germany	14190-094
Sodium chloride (NaCl)	Sigma-aldrich Chemie GmbH, Germany	31434
Sodium hydroxide(NaOH)	Carl Roth GmbH, Germany	6771.3
Temgesic ampullen Buprenorphine	Reckitt Benckiser Healthcare, UK	-
Tri-Sodium citrate	Sigma-Aldrich Chemie GmbH, Germany	71405
Triton-X-100	Sigma-Aldrich Chemie GmbH, Germany	T8787
QIAzol	Qiagen, Germany	56602181

2.1.5 Antibodies for immunofluorescence staining

Primary antibody	Dilution	Company	Reference number
Mouse anti CD11c	1:100	Abcam	ab11029
Mouse anti CD68	1:200	Abcam	Ab31630
Mouse anti MHC-II	1:200	Invitrogen	12-5321-82
Rabbit anti CD11c	1:500	Synaptic Systems	375 003
Rabbit anti eIF2α (phospho)	1:50	CST	3597S
Rabbit anti FLT3 (phospho)	1:100	Bioss	bs-3149R
Rabbit anti BTK (phospho)	1:200	Novus	NBP1-78295
Rabbit anti S6-RP (phospho)	1:200	CST	2211S
Rat anti CD45	1:50	BD Bioscience	550539
Rat anti LAMP1	1:30	DSHB	1D4B

Secondary antibody	Dilution	Company	Reference number
DAPI	1:1000	Thermo	62247
Donkey anti-mouse IgG (H+L) Alexa Fluor® 488	1:500	Invitrogen	A21202
Donkey anti-mouse IgG (H+L) Alexa Fluor® 568	1:500	Invitrogen	A10037
Donkey anti-mouse IgG (H+L) Alexa Fluor® 647	1:500	Invitrogen	A31571
Donkey anti-rabbit IgG (H+L) Alexa Fluor® 488	1:500	Invitrogen	A21206
Donkey anti-rabbit IgG (H+L) Alexa Fluor® 568	1:500	Invitrogen	A10042
Donkey anti-rabbit IgG (H+L) Alexa Fluor® 647	1:500	Invitrogen	A31573
Donkey anti-rat IgG (H+L) Alexa Fluor® 568	1:500	Invitrogen	A21472

2.2 Methods

2.2.1 Animals, traumatic brain injury model and ethanol treatment

This study represents a post-hoc analysis of spleen samples obtained from previous studies [29, 30, 31, 138]. Investigations with these samples have never been reported before and this study was undertaken in accordance with the 3R-principle, to reduce the number of mice in animal experimentation but increase the scientific output from

animal sacrifice. These experiments have been approved by Ulm University animal experimentation oversight committee and by the Regierungspräsidium Tübingen (license number 1222). Male wild-type mice (B6-SJL) were bred locally under standard housing conditions (24°C, 60-8% humidity, 12/12 light/dark cycle, with ad libitum access to food and water). TBI was performed on wild-type (WT) male mice aged p60-90, in agreement with epidemiological data on human TBI [61, 84, 148]. Currently commonly used TBI models include fluid percussion injury model, control cortical brain injury model and free fall-brain injury model. The free-fall brain injury model was used in this experiment. This method is easy to operate, has a single injury mechanism, can simulate clinical TBI well, and has high reliability. Experimental TBI was performed as previously reported [29, 30, 31, 138]. All TBI operations were performed in the operating room of the centre of biomedical research (ZBF), University of Ulm. Briefly, mice were administered buprenorphin (0.1mg/kg by subcutaneous injection), after which the mice were anesthetized with sevoflurane (2.5% in 97.5% O2). The head was shaved and disinfected with ethanol, followed by a skin incision (8-10mm) on the midline. The skin was gently pushed sideway (5 mm) to expose the parietal bone. Scalp skin incision did not involve the manipulation of major vessels or sensory structures and did not result in macroscopic blood loss. To avoid damage to the cornea, ocular gel (Bepanthen, Bayer Leverkusen Germany) was placed on the corneal surfaces before the procedure. Then the mice were subjected to a closed head weight drop TBI model. Animals were positioned in the weight-drop apparatus, and the TBI was delivered by a weight of 333 g free falling from a height of 2cm, targeting the parietal bone [63]. It can be calculated that the kinetic energy transferred to the skull is 0.065J, and the impact force is about 21N. Directly after the TBI, mice were administered 100% O_2 and apnea time was monitored. After spontaneous breathing was restored, the scalp skin of the anesthetized mice was stitched with Prolene 6-0 surgical thread and the animals were transferred to a recovery cage (single-housed) containing a warmed pad and ad libitum access to food and water. Control mice (sham group) had the same treatment and procedures (analgesia, skin incision and handling), but without the trauma being administered. Ethanol treatment was performed as previously described [64, 209].

Briefly, 100% synthesis grade ethanol was diluted in 0.9% NaCl saline to a final dilution of 32% volume/volume (32 µl of 100% ethanol and 68 µl of saline). Mice (20-25 g) were administered a volume 400-500 µl of diluted ethanol (to obtain a concentration of 5 g/kg) by oral gavage 30 min before TBI. Four experimental groups were investigated; saline-administered, subjected to sham surgery (saline-sham, SS), saline-administered, subjected to TBI (saline-TBI, ST), ethanol administered, subjected to sham surgery (ethanol-sham, ES), ethanol administered, subjected to TBI(ethanol-TBI, ET).

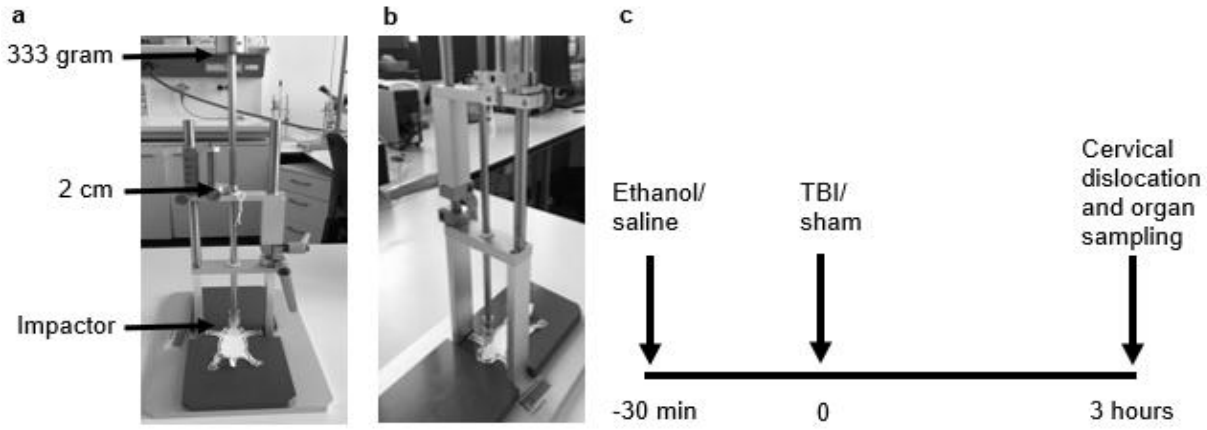

Figure 2. TBI model and ethanol treatment.

Four treatment groups were used: saline-sham (SS), ethanol-sham (ES), saline-TBI (ST) and ethanol-TBI (ET). (a, b) Picture of the blunt weight-drop model used in this study, depicting the impactor, distance and weight. (c) Workflow of ethanol/saline administration, TBI/sham operation and collection of organs post-surgery. SS=Saline Sham, ES=Ethanol Sham, ST=Saline Traumatic brain injury, ET=Ethanol Traumatic brain injury. Modified from (Li Z, Zhang J et al[107], 2021), CC BY 4.0, https://creativecommons.org/licenses/by/4.0/.

2.2.2 Tissue isolation

Three hours post trauma, mice were euthanized by cervical dislocation and organs were harvested for further processing. The spleen was dissected quickly and snap frozen in dry ice for further processing. Tissue was used for either RNA isolation and Quantitative RT-PCR, for tissue sectioning and immunofluorescence staining.

2.2.3 RNA isolation and Quantitative RT-PCR

RNA was isolated from the spleen using QIAzol (Qiagen, Germany) by disrupting and homogenizing the tissue in 1mL QIAzol, after which 200µl of chloroform was added and vortexed for 15 sec. The samples were placed at RT for 10 min and centrifuged for 10 min 12,000g at 4^0C to achieve the phase separation. The top layer (containing RNA) was moved to another tube and precipitated with the same amount of isopropanol. The samples were placed at RT for 10 min and centrifuged for 10 min 12,000g at 4^0C. The isopropanol was removed and 1mL of 75% ethanol in DEPC treated dH_2O was added and mixed. The samples were centrifuged for 10 min 8,000 x g at 4^0C, ethanol was removed and the samples air dried. The RNA pellet was redissolved in 20µl RNAse-free dH_2O. RNA concentration was determined by Nanodrop. Reverse transcription was performed by adding 5µl random hexamers (Biomers, Germany) to 0.75µg RNA (total volume 40µl diluted in dH_2O) and incubated for 10 min at 70^0C. The samples were placed on ice and a mastermix of 0.5µl reverse transcriptase (Promega, germany), 0.5µl RNase Inhibitor (RiboLock, Thermo Scientific, Germany), 2µl dNTPs (genaxxon, Germany) and 12µl reverse transcriptase buffer (Promega, Germany). The samples were incubated for 10 min at RT and placed in a water bath for 45 min at 42^0C. The samples were incubated for 3 min at 99^0C, placed on ice and frozen until further use.

The primers used in the present study were designed using the primer blast tool from NCBI (National Center for Biotechnology Information, USA) and sequences were found through the NCBI nucleotide search tool, GenBank. Sequences were copied into the primer blast tool and parameters were set to achieve the most optimal PCR product for our in house lightcycler (Roche Lightcycler 480 II). Briefly, PCR product size was set from minimum 70 to maximum 140, the primer melting temperature (Tm) was set at 60 ± 3^0C, the exon junction span was set at no preference and finally the correct organism (Mus musculus) was set. The primer pair with the most optimal Tm, self complementarity and self 3' complementarity was chosen. The chosen primer pairs were double checked in the primer blast tool, to ensure specificity for the target gene.

Primers were ordered from Biomers (Biomers, Germany) and were validated, by performing a run on test samples together with corresponding controls (samples without RNA and samples without reverse transcriptase) to verify the Ct values and thereby the selectivity and functioning of the primer before using them for the experiments. The detailed list of the primer sequences for each gene tested is reported in Table 1 .

RT-qPCR was performed on the Light Cycler 480 II (Roche, Germany) with the Power PCR TB green PCR master mix (Takara, Japan). 2µl of sample cDNA was used in a total volume of 10µl (3µl primer mix and 5µl of TB green) in a 96-well plate, all samples were duplicated and the housekeeping gene GAPDH was used as a control (for a complete overview of cytokine sequences see Table 1). The Ct values obtained from the lightcycler were normalized according to the following equation: 2-ΔCt (ΔCt = Cttarget gene - CtGapdh) = relative mRNA.

Table 1: List of primer sequences.

Gene	**Sequence**
CCL1	forward: 5’ -tgc cgt gtg gat aca gga tg- 3’ reverse: 5’ -aca gga gga gcc cat ctt tc- 3’
CCL2	forward: 5’ -caa atg tgc ttg cct gac cc- 3’ reverse: 5’ -tcc tca ttt ggg gcc tga ac- 3’
CCL3	forward: 5’ -tgc cct tgc tgt tct tct ct- 3’ reverse: 5’ -gtg gaa tct tcc ggc tgt ag- 3’
CCL4	forward: 5’ -ccc agc tct gtg caa acc ta- 3’ reverse: 5’ -cca ttg gtg ctg aga acc ct- 3’
CCL6	forward: 5’ - tga gaa act cca aga ctg cca t reverse: 5’ -tgg agg gtt ata gcg acg atc t
CCL12	forward: 5’ -aat cac aag cag cca gtg tcc -3' reverse: 5’ -gtc agc aca gat ctc ctt atc cag t -3'
CCL20	forward: 5’ – gtg ggt ttc aca aga cag atg gc - 3’ reverse: 5’ -cca gtt ctg ctt tgg atc agc g- 3’

CCL22	forward: 5’ -gtg gaa gac agt atc tgc tgc c- 3’ reverse: 5’ -agg ctt gcg gca gga ttt tga g- 3’
CCL24	forward: 5’ – cag cct tct aaa ggg gcc aa - 3’ reverse: 5’ -cta aac ctc ggt gct att gcc- 3’
CX3CL1	forward: 5’ -gca agt ttg aga agc ggg tg- 3’ reverse: 5’ -att cag gct ttg tca ggg ca- 3’
CX3CR1	forward: 5’ -tca ccg tca tca gca tcg ac- 3’ reverse: 5’ -cgc cca gac taa tgg tga ca- 3’
CXCL3	forward: 5‘ -gaa agg agg aag ccc ctc ac- 3‘ reverse: 5‘ -aca cat cca gac acc gtt gg- 3‘
CXCL10	forward: 5` -gct gcc gtc att ttc tgc- 3´ reverse: 5´-tct cac tgg ccc gtc atc- 3´
CXCL16	forward: 5‘ -gca ggg tac ttt gga tca cat cc- 3‘ reverse: 5‘ -agt tca cgg acc cac tgg tct t- 3‘
FLT3	forward: 5’ -tgt cag taa tga ttc ttg aga ccg- 3’ reverse: 5’ -ctt ctg ggg atc ctc gca c- 3’
FLT3L	forward: 5’ -gct ctg aag ccc tgt atc gg- 3’ reverse: 5’ -tgg ctt cta ggg cta tgg ga- 3’
GAPDH	forward: 5‘ - tgg atc tga cgt gcc gc- 3‘ reverse: 5‘ - tgc ctg ctt cac cac ctt c- 3‘
IFN-y	forward: 5’ -ttc aag act tca aag agt ctg agg- 3’ reverse: 5’ -tct gga gga act ggc aaa ag- 3’
IL-4	forward: 5’ -gag aga tca tcg gca ttt tga - 3’ reverse: 5’ -agc cct aca gac gag ctc ac- 3’
IL-10	forward: 5‘ -agg cgc tgt cat cga ttt ctc- 3‘ reverse: 5‘ - gcc ttg tag aca cct tgg tct t- 3‘
IL-12	forward: 5’ -cct gtg cct tgg tag cat ct- 3’ reverse: 5’ -ctg aag tgc tgc gtt gat gg- 3’
IL-13	forward: 5’ -acc cag agg ata ttg cat gg - 3’ reverse: 5’ - tgg gct act tcg att ttg gt - 3’
IL-17	forward: 5’ -gga cag ccc ttc ttt gtc tg- 3’ reverse: 5’ -tgc ttt tta tat ttc att acg tgg tt- 3’

IL-19	forward: 5' -gga gca ata gac agc ccc tc- 3' reverse: 5' -tgc act aca gca cac cac aa- 3'
IL-23a	forward: 5' -gag caa ctt cac acc tcc cta- 3' reverse: 5' -tag aac tca ggc tgg gca tc- 3'

2.2.4 Tissue sectioning and immunofluorescence staining

Frozen spleen tissue was embedded in O.C.T (Tissue Tek, The Netherlands), 10 micron sections were cut with the cryostat and mounted on glass slides. Slides were air dried for 15 min at RT and then stored at -80^{0}C for 24h. On the day of performing the assay the slides were washed with 100 ml 1×PBS in a Tissue-Tek slide rack for 5 min while they rocked up and down to remove O.C.T., followed by a 10 min fixation step of the sections in 4% PFA. Target retrieval was performed in sodium citrate buffer pH 8.5 (Table 9) for 30 min at 85^{0}C, followed by blocking of the sections in blocking buffer (3% BSA, 0.3% triton X-100; PBS) for 2h at RT. Primary antibodies (for a complete overview of antibodies used see Antibodies for immunofluorescence staining) were diluted in blocking buffer and incubated for 48h at 4^{0}C, followed by 3 x 30min washes in PBS at RT. Secondary antibodies were diluted in blocking buffer and incubated for 2h at RT, followed by 3 x 30 min washes in PBS.

Excess PBS was aspired from the sections and left to dry for 10-15 min at RT. Samples were mounted by adding ProLong Gold anti-fade mounting medium and covered with a coverslip. Samples were dried and sealed with nail polish.

2.2.5 Single mRNA in situ hybridization

Fluorescence in situ mRNA hybridisation was used as previously reported [211] in agreement to the manufacturer's instructions (ACDBio, RNAscope, Fluorescence In Situ Hybridisation for fresh frozen tissue, all reagents and buffers were provided by ACDBio), with minor modifications [138]. Briefly, frozen spleen tissue was embedded in O.C.T (Tissue Tek, the Netherlands), 10 micron sections were cut with the cryostat and mounted on super frost plus glass slides. Slides were stored at -80^{0}C for 24h. On the day of performing the assay, wash the slides with 100 ml 1x PBS for 5 min in a Tissue-Tek

slide holder while moving the slides up and down to remove the O.C.T. And then post-fixed the slides by immersing them in 4% PFA for 10 min at 4^0C. After removing the slides from 4% PFA, immersed them in 50% EtOH for 5 min at RT and then took them out, immersed slides in 70% EtOH for 5 min at RT and then took out, and immersed slides in 100% EtOH for 5 min at RT and repeat once. Removed the slides from 100% EtOH and let them air dry for 5 min at RT. Sections were covered with Protease IV and incubated at 40^0C for 30 min followed by 2 x 2 min washing in PBS. Then the probe (TNFα and ADRB2) was added and incubated for 4.5h at 40^0C followed by 2 x 2 min washing step with wash buffer. Then, amplification 1 was added to the sections and incubated for 30 min at 40^0C followed by 2 x 2 min washing step with wash buffer. After which, amplification 2 was added to the sections and incubated for 15 min at 40^0C followed by 2 x 2 min washing step with wash buffer. As a final amplification step, amplification 3 was added and then incubated for 30 min at 40^0C followed by 2 x 2 min washing step with wash buffer. Care was taken to avoid drying the slides throughout the process. Finally, the detection step was performed by adding detection reagent 4A to the sections and then incubating for 45 min at 40^0C followed by 2 x 2 min washing step with wash buffer. After which the sections were blocked in blocking buffer (3% BSA, 0.3% triton X-100; PBS) for 1h at RT followed by overnight incubation with primary antibodies diluted in blocking buffer. The sections were washed 3 x 30 min in PBST and incubated with secondary antibodies diluted in blocking buffer for 2h at RT. A final wash step of 3 x 30 min was performed in PBST, and the sections were counterstained with DAPI and mounted using Fluorogold prolong antifade mounting medium (Invitrogen, Germany).

2.2.6 Image acquisition and image analysis

Immunofluorescence staining was imaged with a keyence BZ-X800 microscope (Keyence, Japan) equipped with a 100x oil objective. After starting the microscope and software system, load each microscope slide containing the sections onto the microscope stage. A single optical section with 3x3 tile scan was made spanning an area covering a splenic follicle and the red pulp. Acquisition parameters were set to avoid

hyper- or hypo-saturation, and imaging parameters were kept constant from specimen to specimen throughout the acquisition process. Images were merged with the BZ-II analyzer software (Keyence, Japan) and analyzed using Image-J. Fluorescence intensity was assessed by manually tracing the CD11c+ cells and measuring the mean gray value. Density was assessed by using the Image-J plugin cell counter. The analyzed marker was assessed as high or low expressing, by thresholding the signal. Each picture was analyzed by making a ratio between total CD11c+/CD45+ cells and the imaged marker or the ratio between CD11c-/CD45+ cells and the imaged markers. A minimum of 3 sections per mouse were analysed.

Single mRNA in situ Hybridization images were acquired using an LSM-710 (Carl Zeiss, Germany) microscope with an 40x oil objective. Mount each microscope slide containing the section on the microscope stage, manually adjust the focus, and adjust the optical thickness to the optimal value. A z-stack of 8 images has been acquired at 1024x1024 pixel resolution and 16-bit depth. Acquisition parameters were set to avoid over and under-saturation, and imaging parameters were kept constant from specimen to specimen throughout the acquisition. TNFα and ADRB2 mRNA density in CD11c+ cells were assessed by using the Image-J plugin cell counter. A minimum of 3 sections per mouse were analysed.

Table 2. Preparation of Phosphate buffered saline (PBS) 10X

PBS 10X (1000 ml)	
14.4 g	Na_2HPO_4
2.4 g	KH_2PO_4
80 g	NaCl
2 g	KCl
fill up to 1000 ml with	Distilled water
Adjusted to pH 7.4	1M HCl or NaOH

Table 3. Preparation of phosphate buffered saline (PBS) 1X

PBS 1X (1000 ml)	
100 ml	PBS 10X
900 ml	Distilled water

Table 4. Preparation of 4% paraformaldehyde (PFA)

4% PFA (100 ml)	
4 g	PFA
fill up to 100 ml with	PBS 1X
Adjust pH to 7.4	1M HCl or NaOH

Table 5. Preparation of 10% bovine serum albumin (BSA)

10% BSA (100 ml)	
10 mg	Albumine Bovine Fract V
fill up to 100 ml with	PBS 1X

Table 6. Preparation of 10% Triton solution

10% Triton solution (50 ml)	
5 ml	Triton-X-100
45 ml	PBS 1X

Table 7. Preparation of Phosphate Buffered Saline with Tween (PBST)

PBST (1000 ml)	
1 ml	Triton-X-100
999 ml	PBS 1X

Table 8. Preparation of Blocking buffer

Blocking buffer (6.67 ml)	
2000 µl	10% BSA
200 µl	10% Triton solution
4.47 ml	PBS 1X

Table 9. Preparation of Tri-sodium citrate buffer (pH 8.5)

Sodium citrate buffer (pH 8.5)	
2.94 g	Tri-sodium citrate
1000 ml	Distilled water

2.2.7 Statistical analysis

Statistical analysis for the gene expression data sets (Fig. 3 and Fig 4.) was performed using the IBM software suite, using a Two-way Multivariate ANOVA (with Wilk's λ parameter), because the experiment included multiple dependent variables (cytokines or chemokines) and two independent variables (TBI and ethanol treatment). The post-hoc comparisons were performed with Two-way ANOVA with Tukey post-hoc correction. All groups were tested for normality using the Shapiro-Wilk test. Correlation matrices of gene expression data were made with the prism analysis suite (GraphPad Prism version 8), Pearson r coefficient and p-values for every correlation were assessed. For the histological datasets, Two-way ANOVA was performed with Tukey post-hoc comparison, since two independent treatments were done (saline/ethanol and sham/TBI). Statistical significance was set at $p < 0.05$ after multiple-comparison correction.

3 Results

3.1 TBI rapidly and selectively induces cytokine expression in the spleen and is enhanced by concomitant EI.

The spleen has a large number of immune cells that play an important role in innate and adaptive immunity. We set out to verify if any rapid modification in the splenic immune responses would take place upon mild/moderate TBI and, most importantly, if the comorbidity of EI would significantly interact with such responses.

In this study, mice were pre-treated with ethanol (5 g/kg) or a similar volume of saline 30 min before TBI or sham surgery. Considering that the cytokines in the spleen change with the immune response, as an entry point, we screened whole spleen extracts for expression of several cytokines 3 hours after TBI or sham surgery.

We considered three sets of cytokines: prototypical Th2-B-cells-directed, anti-inflammatory mediators (IL-13, IL-19, IL-4 and IL-10), prototypical Th1-cellular-immunity-directed, pro- inflammatory mediators (IFN-γ, IL-12, IL-17 and IL-23a) and prototypical DCs-monocytes mediators (FLT3, FLT3L, CX3CR1 and CX3CL1). Interestingly, we found a significant effect of both the TBI itself and EI (Two-way MANOVA; TBI: $F= 7.379$; Wilks $\lambda= 0.253$; $p = 0.0001$; EI: $F = 4.599$; Wilks $\lambda = 0.352$; $p= 0.0001$). However, the interaction of TBI and EI showed no significance in the Two-way MANOVA ($F= 1.801$; Wilks $\lambda = 0.581$; $p= 0.09$). This could be characterised by (Two-way ANOVA, Tukey corrected) the significantly increased expression upon TBI alone of FLT3L ($F(3, 34)= 9.771$; SS vs ST; 100±27 vs 163±44; $p = 0.045$; Fig. 3J), FLT3 ($F(3, 36) = 4.266$; SS vs ST; 100±46 vs 239±119; $p = 0.037$; Fig. 3I) and CX3CR1 ($F(3, 41) = 6.825$; SS vs ST; 100±16 vs 134±18; $p= 0.01$; Fig. 3K), whereas IL-13 was significantly downregulated ($F(3, 37)= 7.499$; SS vs ST; 100±35 vs 42±16; $p = 0.0005$; Fig. 3A). Ethanol intoxication prior to TBI resulted in a significant further increase of the expression of FLT3L (ST vs ET; 163±44 vs 233±56; $p = 0.026$; Fig. 3J) and CX3CL1 (ST vs ET; 98±25 vs 168±53; $p = 0.006$; Fig. 3L), whereas FLT3 and CX3CR1 did not change in ET group compared to ST group (FLT3: ST vs ET; 239±119 vs 266±124; $p=0.93$; Fig. 3I; CX3CR1: ST vs ET; 134±18 vs 146±34; $p = 0.52$; Fig. 3K). Surprisingly, no effect was

observed on any of the other cytokines considered.

This initial screening did not only point towards a fast activation of splenic immune cells shortly after TBI, but reveal a pattern compatible with a selective effect on innate immunity.

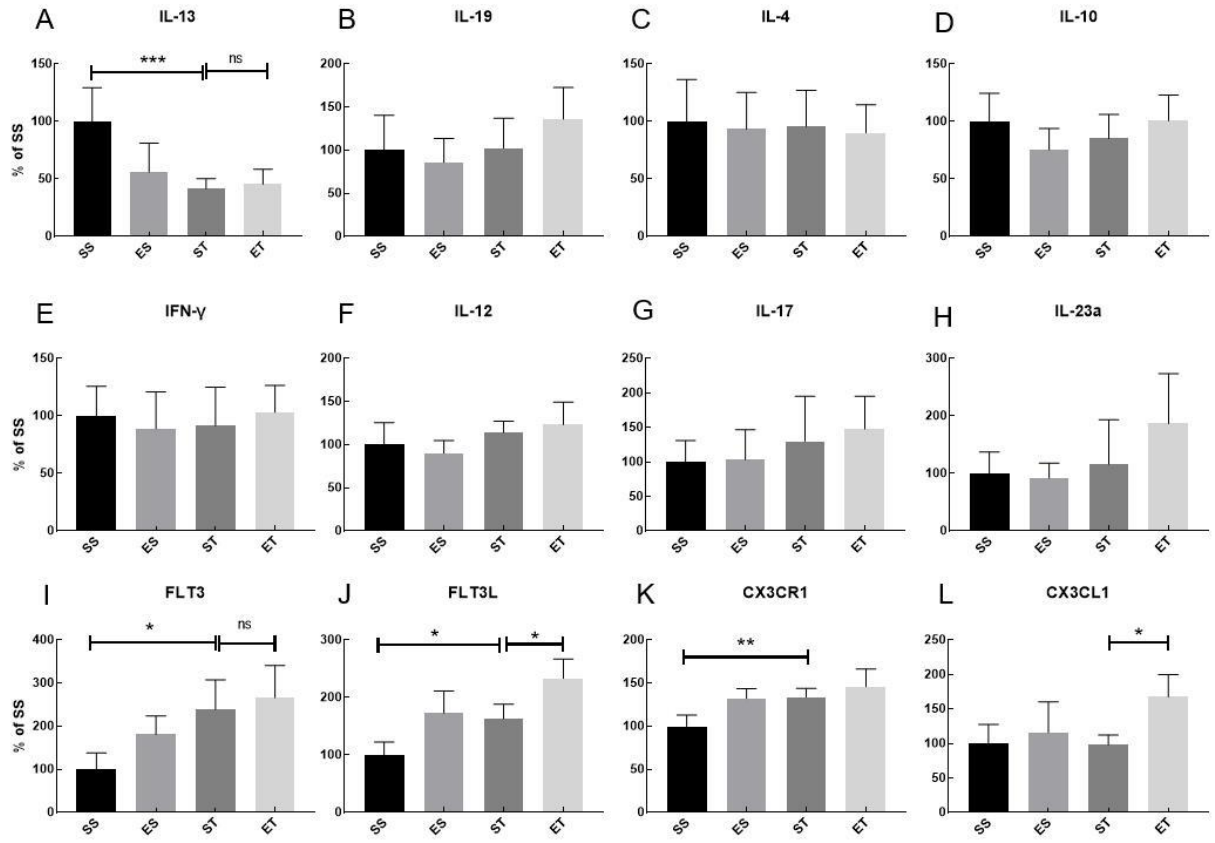

Figure 3: EI enhances the selective cytokine expression post TBI.

Cytokine expression screening in the spleen of saline sham (SS), ethanol sham (ES), saline TBI (ST) and ethanol TBI (ET) treated mice 3h after trauma. A-D) Barplots show relative expression of Th2-cell, anti- inflammatory markers; IL-13, IL-19, IL-4 and IL-10. IL-13 expression showed a significant downregulation after TBI (SS vs ST; $P < 0.001$), ethanol pretreatment did not alter the TBI induced effect on IL-13 (ST vs ET; $p > 0.05$). IL-19, IL-4 and IL-10 were not affected by any treatment. E-H) Barplots show relative expression of Th1-cell, pro-inflammatory markers; IFN-γ, IL-12, IL-17 and IL-23a. TBI with or without ethanol pretreatment showed no significant differences. I-L) Barplots show relative expression of DCs-monocyte specific mediators; FLT3, FLT3L, CX3CR1, CX3CL1. TBI resulted in a significant increase of FLT3 (SS vs ST; $p < 0.037$), FLT3L (SS vs ST; $p < 0.045$) and CX3CR1 (SS vs ST; $p < 0.01$). Ethanol pretreatment resulted in a further

significant enhancement of FLT3 (ST vs ET; $p < 0.026$) and CX3CL1 (ST vs ET; $p < 0.006$). Data shown as barplots. Group size: SS N = 8, ES N = 10, ST N = 14, ET N = 13. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. TBI= Traumatic brain injury, IL= Interleukin, IFN-γ= Interferon γ, FLT3= Fms-like tyrosine kinase 3, FLT3L= Fms-like tyrosine kinase 3 ligand, CX3CR1= C-X3-C motif chemokine receptor 1, CX3CL1= C-X3-C motif chemokine ligand 1. SS=Saline Sham, ES=Ethanol Sham, ST=Saline Traumatic brain injury, ET=Ethanol Traumatic brain injury. Modified from (Zhang J, Li Z et al[233], 2022), CC BY 4.0, https://creativecommons.org/licenses/by/4.0/.

3.2 Rapid modulation of the splenic chemokine pattern by TBI and by EI/TBI

Dendritic cells (DCs) are the most robust antigen-presenting cells and play a pivotal role in the immune response. Mature DCs deliver antigens, initiate and induce T cell differentiation to generate immune responses or directly activate B cells and generate immune memory. We wanted to see if the rapid effects of TBI on splenic immune cells, particularly DCs, could be confirmed and further expanded. We evaluated the expression of a set of chemokines known to be strong modulators of the innate immune response. The focus was set, in particular on DC function (and other APC: CCl3, CCL2, CCl12 and CCL4); Th cells (CCL22, CXCL10, CCL1 and CCL20); or leukocytes and NK cells (CXCL3, CCL6, CCL24, and CXCL16). We could identify a significant effect of TBI (Two-way MANOVA: F= 4.494; Wilks λ = 0.357; p= 0.0001) and EI (F= 4.956; Wilks λ= 0.335; p= 0.0001), the interaction of TBI and EI showed no significant effect in the Two-way MANOVA (F= 1.776; Wilks λ= 0.585; p= 0.099). However, post-hoc analysis (Tukey corrected) showed significantly increased expression upon TBI alone of CCL3 ($F(3, 37) = 8.910$; SS vs ST; 100±13 vs 161±42; p= 0.028; Fig. 4A), CCL1 ($F(3, 39) = 4.156$; SS vs ST; 100±63 vs 244±116; p= 0.019; Fig. 4G), and CCL22 ($F(3, 37) = 7.483$; SS vs ST; 100±30 vs 159±47; p= 0.039; Fig. 4E). On the other side, concomitant EI significantly downregulated CCL3 (ST vs ET; 161±42 vs 84±42; p= 0.0006; Fig. 4A) and CCL1 (ST vs ET; 244±116 vs 134±101; p= 0.037; Fig. 4G), whereas CCL22 was unaffected by EI and remained upregulated (ST vs ET; 159±47 vs 128±50; p= 0.35; Fig. 4E). Ethanol intoxication prior to TBI presented a distinct chemokine profile: a significant

upregulation of CCL4 was observed only in the ET group, but not in the ST and ES groups alone (F(3, 39) = 6.093; SS vs ET; 100±35 vs 155±54; p= 0.042; Fig. 4D). On the contrary, the ET group displayed the downregulation of CXCL10 expression (F(3, 41)= 3.458; SS vs ET; 100±78 vs 37±18; p= 0.014; Fig. 4F). Finally, ethanol exposure alone caused a downregulation of CCL2 in both sham and TBI groups (F(3, 36)= 9.679; SS vs ES; 100±61 vs 29±25; p = 0.0021; ST vs ET; 66±37 vs 17±8; p= 0.014; Fig. 4B).

To sum up the above, this data confirm the rapid involvement of the brain-spleen axis in TBI. It was also shown that ethanol intoxication prior to TBI has a significant regulatory effect on innate and adaptive immunity, especially on APCs.

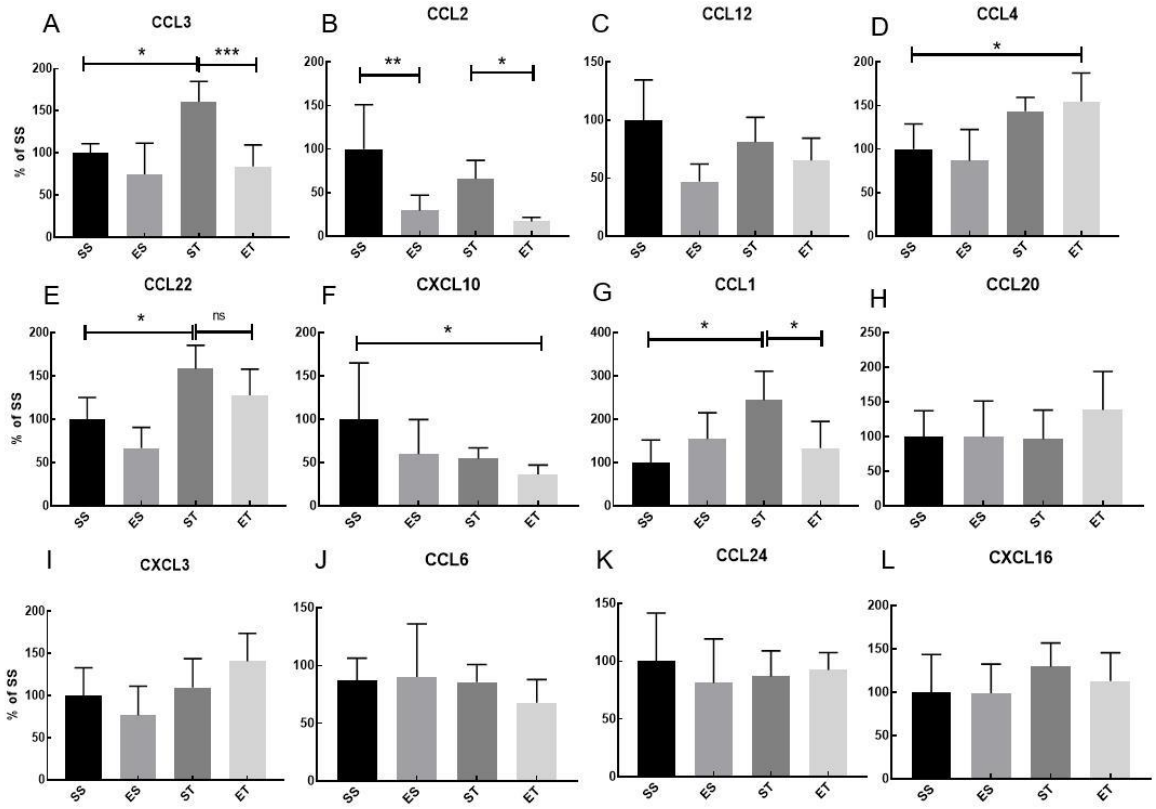

Figure 4: Splenic chemokine expression shows a fast response to TBI and EI.

Chemokine expression data in the spleen of saline sham (SS), ethanol sham (ES), saline TBI (ST) and ethanol TBI (ET) treated mice 3h after trauma. A-D) Barplots show relative expression of modulators of DC biology; CCL3, CCL2, CCL12 and CCL4. TBI resulted in a significant upregulation of CCL3 (SS vs ST; p = 0.028), ethanol pretreatment significantly reduced the TBI induced CCL3 expression (ST vs ET; p= 0.0006). Ethanol by itself resulted in a significant

downregulation of CCL2, independent of TBI (SS vs ES; p= 0.0021; ST vs ET; p= 0.014). ET resulted in a significant upregulation of CCL4 compared to SS (SS vs ET; p= 0.042). CCL12 expression was not affected by any treatment. E-H) Barplots show relative expression of modulators of Th-cells; CCL22, CXCL10, CCL1 and CCL20. TBI resulted in a significant upregulation of CCL1 (SS vs ST; p = 0.019) and CCL22 (SS vs ST; p = 0.039), ethanol pretreatment significantly reduced the TBI induced CCL1 expression (ST vs ET; p = 0.037) but not for CCL22 (ST vs ET; p = 0.35). ET resulted in a significant downregulation of CXCL10 compared to SS (SS vs ET; p = 0.014). CCL10 expression was not affected by any treatment. I-L) Barplots show relative expression of modulators of leukocytes and NK-cells; CXCL3, CCL6, CCL24 and CXCL16. TBI with or without ethanol pretreatment showed no significant differences. Data shown as barplots. Group size: SS N = 8, ES N = 10, ST N = 14, ET N = 13. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. TBI= Traumatic brain injury, CCL= C-C motif chemokine ligand, CXCL= C-X-C motif chemokine ligand. NK= natural killer. SS=Saline Sham, ES=Ethanol Sham, ST=Saline Traumatic brain injury, ET=Ethanol Traumatic brain injury. Modified from (Zhang J, Li Z et al[233], 2022), CC BY 4.0, https://creativecommons.org/licenses/by/4.0/.

3.3 TBI induces the phosphorylation of FLT3 and its downstream signaling partner BTK in DCs, which are both enhanced by EI.

Our expression screenings suggested that FLT3 signaling may be upregulated in association with a cytokine pattern compatible with DC engagement. Considering that FLT3/FLT3L plays an important role in regulating DCs maturation and immune function, we sought to directly confirm that FLT3 enhances splenic DCs maturation and immune function after TBI and during EI. For this aim, we immunostained thin sections of the spleen from the four treatment groups for the pan-DCs marker CD11c, for the pan-leukocyte marker CD45 and for phosphorylated FLT3 (pFLT3, Y589/591; Fig. 6A). Immunostaining results showed that more than 95% of CD11c+ cells were also CD45+(Fig. 5D-E) consistent with the expression of CD45 in the DC subpopulations. Phospho-FLT3 immunoreactivity was highly heterogeneous in the spleen sections, with a comparatively small number of highly pFLT3-positive cells located around the follicles and a minor number of cells with moderate pFLT3 immunoreactivity scattered in the parenchyma. Initial immunostainings revealed that CD11c density was not

altered by TBI or concomitant EI (Two-way ANOVA, F(3, 12)= 0.9360; p= 0.45; Fig. 5B-C). Co-immunostaining with CD11c and CD45 revealed that nearly all the pFLT3-high cells (upon binning) were CD45+/CD11c+ (Fig . 5F-G), in agreement with their DCs identity. However, we investigated the density of pFLT3-high cells (labeling leukocytes other than DCs) in CD45+/CD11c- subpopulations and we found no significant difference among the 4 groups (Two-way ANOVA, F(3, 12) = 0.8446; p = 0.50; Fig. 5F-G). Although the fluorescence intensity of pFLT3 in CD11c+ DCs was not altered across the four treatment groups (Two-way ANOVA, F(3, 588) = 2.541; p= 0.06; Fig. 6C), the number of pFLT3-high/CD11c+ cells was significantly affected by TBI and treatment (Two-way ANOVA, F(3, 12) = 45.84; p< 0.0001). Post-hoc comparison (Tukey corrected) revealed a significant increase in pFLT3-high /CD11c+ cells upon TBI alone (SS vs ST; 20±1% vs 48±4%; p< 0.0001; Fig. 6B) and interestingly, ethanol intoxication prior to TBI induced a substantial further enhancement (ST vs ET; 48±4% vs 65±3%; p = 0.0061; Fig. 6B), confirming that TBI alone could strongly induce FLT3 signaling in DCs. Ethanol intoxication prior to TBI, in particular, further enhanced FLT3 signaling in DCs. In order to verify that the phosphorylation of the FLT3 receptor corresponded to the functional engagement of signal transduction pathways, we monitored the phosphorylation of the Bruton's tyrosine kinase (BTK), an established downstream target of FLT3. Immunostaining of thin spleen sections for phosphorylated BTK (pBTK, Y223; Fig. 6D) revealed a pattern very similar to that of pFLT3. Fluorescence intensity of pBTK in CD11c+ cells showed a significant difference between treatment groups (Two-way ANOVA, F(3, 444) = 123.5; p< 0.0001) and a post-hoc comparison (Tukey corrected) showed a significant increase in pBTK fluorescence intensity after TBI (SS vs ST; 44±9 vs 48±9; p= 0.0008; Fig. 6F). Ethanol intoxication prior to TBI resulted in a further increase of pBTK fluorescence intensity (ST vs ET; 48±9 vs 62±11; p < 0.0001; Fig. 6E). Similarly, quantification of the number of pBTK-high/CD11c+ cells revealed a significant effect in the treatment groups (Two-way ANOVA, F(3, 12)= 68.07; p< 0.0001), due to the massive increase of pBTK-high/CD11c+ cells in the ST compared to the SS group (Tukey's post-hoc; SS vs ST; 31±6% vs 58±5%; p < 0.0001; Fig. 6E), which was further increased in the ET group (ST vs ET; 58±5% vs 67±2%; p= 0.022; Fig. 6E).

To sum up the above, these data demonstrate that TBI leads to a rapid (3h) increase in phosphorylation of the FLT3 receptor (and its downstream target BTK) in splenic DCs, and that this effect is substantially amplified by concomitant EI. Since CD11c+ cells were nearly always CD45+, we performed further investigations using the CD11c marker alone to identify DCs.

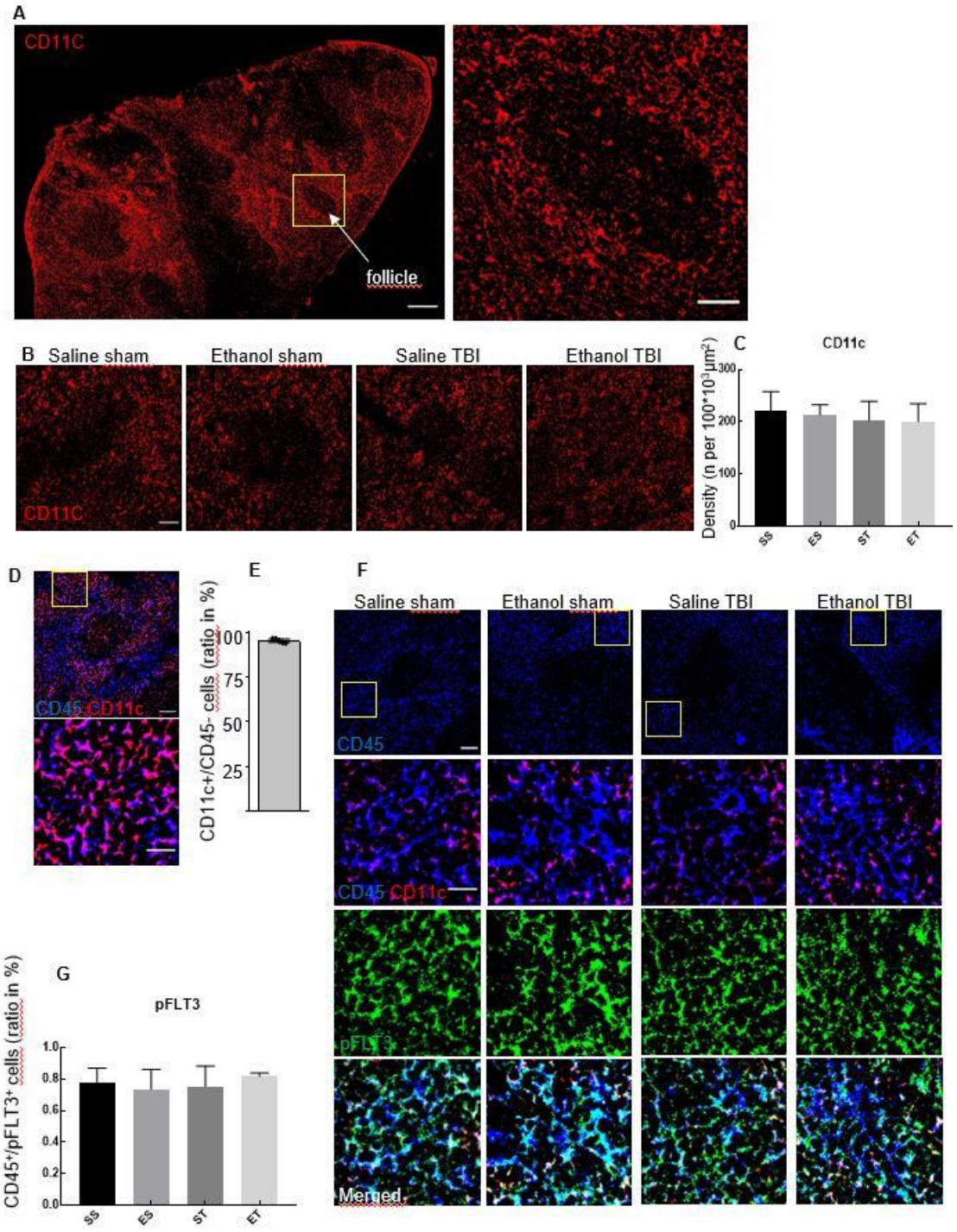

Fig.5: Phospho-FLT3 immunoreactivity was highly inhomogeneous in the spleen sections.

CD11c density was not altered by TBI or concomitant ethanol intoxication (Two-way ANOVA, F(3,

12) = 0.9360; p = 0.45;). Co-immunostaining with CD11c and CD45 revealed that nearly all the pFLT3-highcells (upon binning) were CD45+. Nevertheless, when investigating the density of pFLT3-high cells in CD45+/CD11c- cells, we found no significant difference among treatment groups (Two-way ANOVA, F(3, 12) = 0.8446; p = 0.50. Data shown as barplots. Group size: SS N= 5, ES N= 5, ST N= 5, ET N= 5. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$. Scale bar overview: 50 µm; scale bar insert: 20 µm. CD=Cluster of differentiation, pFLT3= Phospho Fms-like tyrosine kinase 3, ANOVA= Analysis of variance. SS=Saline Sham, ES=Ethanol Sham, ST=Saline Traumatic brain injury, ET=Ethanol Traumatic brain injury. Modified from (Zhang J, Li Z et al[233], 2022), CC BY 4.0, https://creativecommons.org/licenses/by/4.0/.

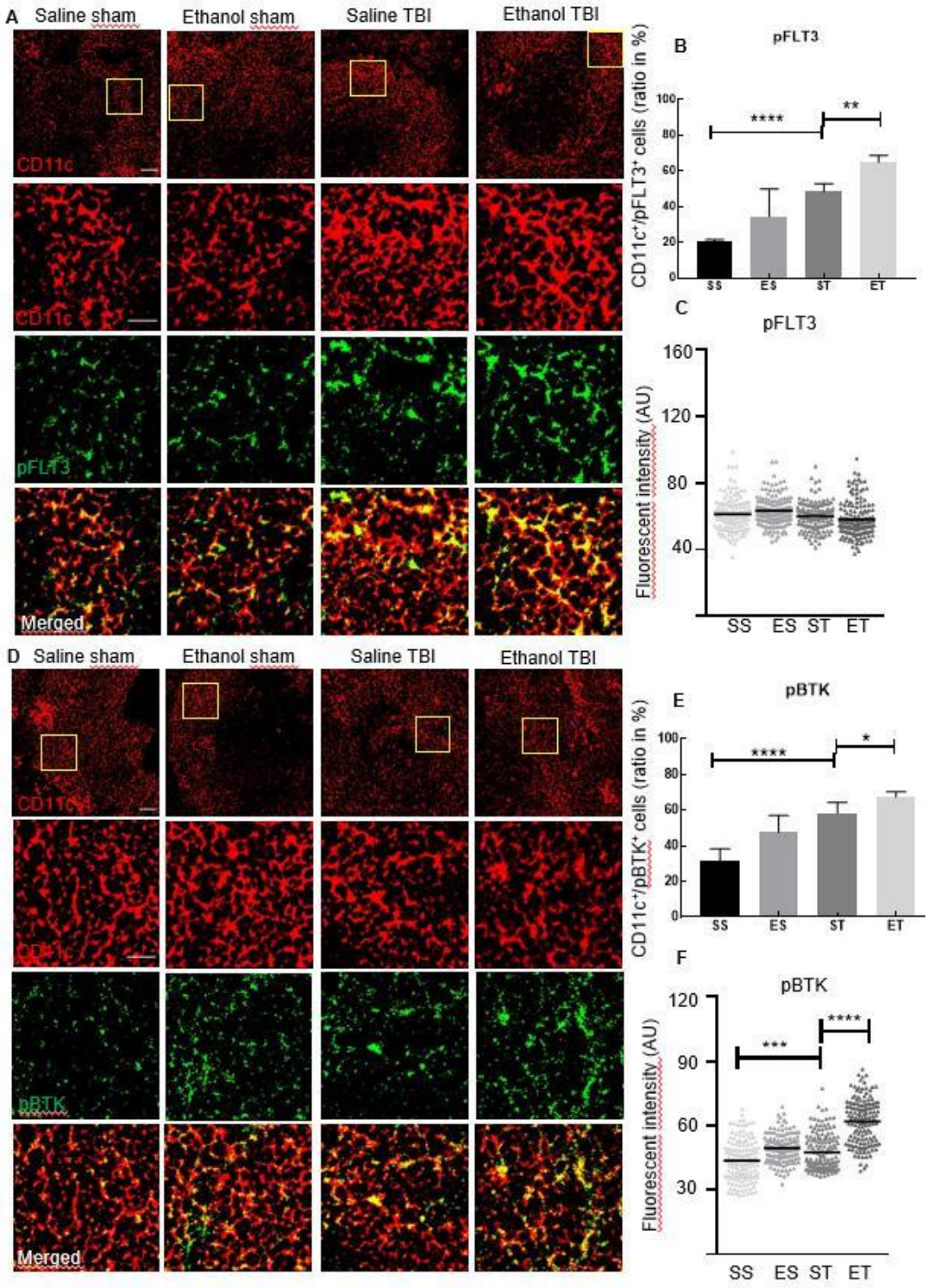

Figure 6: TBI induced phosphorylation of FLT3 and downstream signaling partner BTK is further enhanced by EI.

Immunofluorescence staining of pFLT3 and pBTK with DC marker CD11c on thin spleen sections of saline sham (SS), ethanol sham (ES), saline TBI (ST) and ethanol TBI (ET) treated mice 3h after trauma. A-C) immunofluorescence staining of pFLT3 colocalized with CD11c resulted in no significant difference in fluorescence intensity between treatment groups (p= 0.06). However, the

amount of FLT3-high/CD11c+ cells revealed a significant increase after TBI (SS vs ST; p< 0.0001) and a further significant enhancement in the ET group (ST vs ET; p=0.006). D-F) immunofluorescence staining of pBTK colocalized with CD11c resulted in a significant increase in fluorescence intensity upon TBI (SS vs ST; p= 0.0008), with a further significant increase in the ET group (ST vs ET; p= 0.006). Likewise, the number of BTK-high/CD11c+ cells revealed a significant increase after TBI (SS vs ST; p< 0.0001) with a further significant increase in the ET group (ST vs ET; p < 0.0001). Data shown as barplots or scatterplots. Group size: SS N= 5, ES N= 5, ST N= 5, ET N= 5. *: p< 0.05; **: p< 0.01; ***: p< 0.001; ****: p< 0.0001. Scale bar overview: 50 µm; scale bar insert: 20 µm. TBI= Traumatic brain injury, pFLT3= Phospho Fms-like tyrosine kinase 3, pBTK= Phospho brutons tyrosine kinase. SS=Saline Sham, ES=Ethanol Sham, ST=Saline Traumatic brain injury, ET=Ethanol Traumatic brain injury. Modified from (Zhang J, Li Z et al[233], 2022), CC BY 4.0, https://creativecommons.org/licenses/by/4.0/.

3.4 TBI and EI-TBI strongly induce protein synthesis in splenic DCs

The levels of MHC-II molecules, co-stimulatory molecules and adhesion molecules expressed by DCs are increased significantly upon maturation, showing strong antigen uptake and processing capabilities. We therefore set out to determine whether the observed upregulation of FLT3 signaling on TBI is accompanied by an activation-related increase in protein synthesis, and whether ET further enhances the increased protein synthesis in DCs. First, we considered the levels of phosphorylated S6 ribosomal protein (pS6), a proxy of mTOR activation directly involved in increasing ribosomal translation of mRNA. Immunostaining of thin spleen sections for phosphorylated-S6 (pS6, Ser235/236; Fig. 7A) revealed a diffuse immunopositivity in the cytoplasm of close to each cell; however, upon TBI, CD11c+ cells stood out due to a massive increase in pS6 immunofluorescence intensity (Two- way ANOVA, $F(3, 465) = 418.3$; $p < 0.0001$). The post-hoc comparison (Tukey corrected) showed a significant increase after TBI (SS vs ST; 44±6 vs 70±13; p< 0.0001; Fig. 7C), with further enhancement of pS6 immunofluorescence intensity in the ET group (ST vs ET; 70±13 vs 98±23; p< 0.0001; Fig. 7C). This effect corresponded to the significant increase in

the amount of CD11c+ showing high levels of pS6 (Two-way ANOVA, $F(3, 12)= 26.09$; $p< 0.0001$) as pS6 was significantly elevated after TBI (Tukey's post-hoc; SS vs ST; 32±4% vs 58±8%; $p= 0.0002$; Fig. 7B) but not further increased in ET group compared to ST alone (ST vs ET; 58±8% vs 65±7%; $p = 0.34$; Fig. 7B). Thus, Ethanol intoxication prior to TBI amplified the increased pS6 levels in all sensitive CD11c+ cells following TBI, but did not increase the number of cells responding to TBI itself.

We considered the levels of the cellular stress-related phospho-eIF2α. Cellular stress often leads to phosphorylation of eIF2α, which in turn regulates the transcription and translation of cell-related genes to enhance cellular protection. Immunostaining of thin spleen sections show a significant difference in fluorescence intensity in CD11c+ cells within treatment groups (Two-way ANOVA, $F(3, 444) = 8.553$; $p< 0.0001$) due to a significant increase upon TBI (Tukey's post-hoc; SS vs ST; 48±10 vs 54±10; $p< 0.0001$; Fig. 7F), again with no difference between ET and ST (ST vs ET; 54±10 vs 52±11; $p= 0.78$; Fig. 7F). Colocalization of peIF2α-high cells with CD11c+ cells revealed a significant difference in fluorescence intensity within treatment groups (Two-way ANOVA, $F(3, 12)= 40.50$; $p< 0.0001$). The post-hoc comparison (Tukey corrected) shows a significant increase after TBI (SS vs ST; 39±8% vs 60±4%; $p= 0.0001$; Fig. 7E) with a further increase in ET group compared to ST alone (ST vs ET; 60±4% vs 67±6%; $p= 0.01$; Fig. 7E). Therefore, TBI induces a substantial increase in protein synthesis, with some alteration of cap-dependent translation in DCs, and this effect is amplified by EI.

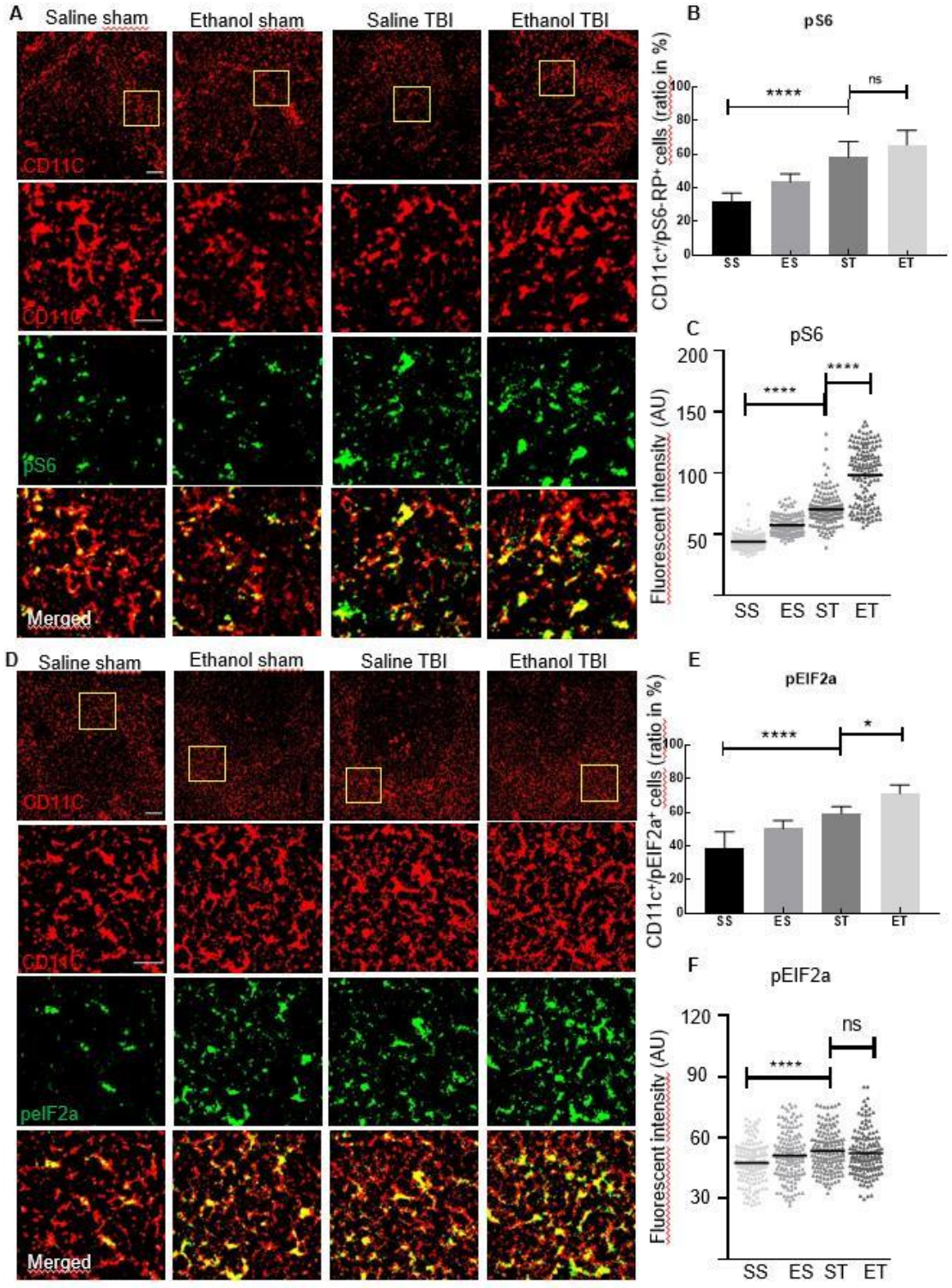

Figure 7: TBI induced metabolic rate and protein synthesis is further enhanced by EI.
Immunofluorescence staining of pS6 and peIF2α with DC marker CD11c on thin spleen sections of saline sham (SS), ethanol sham (ES), saline TBI (ST) and ethanol TBI (ET) treated mice 3h after trauma. A-C) immunofluorescence staining of pS6 colocalized with CD11c resulted in a significant increase in fluorescence intensity upon TBI (SS vs ST; $p < 0.0001$), with a significant enhancement in the ET group (ST vs ET; $p < 0.0001$). The amount of pS6 -high/CD11c+ cells revealed a significant

increase after TBI (SS vs ST; p< 0.0001) however, no significant difference in the ET group (ST vs ET; p= 0.34). D-F) immunofluorescence staining of peIF2α colocalized with CD11c resulted in a significant increase in fluorescence intensity upon TBI (SS vs ST; p< 0.0001), with no difference in the ET group (ST vs ET; p= 0.78). The number of peIF2α-high/CD11c+ cells revealed a significance increase after TBI (SS vs ST; p= 0.0001) with a further significant increase in the ET group (ST vs ET; p= 0.0107). Data shown as barplots or scatterplots. Group size: SS N = 5, ES N = 5, ST N = 5, ET N = 5. *: p< 0.05; **: p < 0.01; ***: p< 0.001; ****: p < 0.0001. Scale bar overview: 50 µm; scale bar insert: 20 µm. TBI= Traumatic brain injury, pS6= Phosphorylated S6, peIF2α= Phospho α subunit of eukaryotic initiation factor 2. SS=Saline Sham, ES=Ethanol Sham, ST=Saline Traumatic brain injury, ET=Ethanol Traumatic brain injury. Modified from (Zhang J, Li Z et al[233], 2022), CC BY 4.0, https://creativecommons.org/licenses/by/4.0/.

3.5 TBI and EI cooperate to enhance the maturation of splenic DCs

We wondered if the signaling events and metabolic reprogramming observed in splenic DCs after TBI also corresponded to the appearance of a “mature” APC phenotype. Maturation of DCs is accompanied by an increase of antigen presentation and by lysosomal activity. Antigen presentation of DCs (and macrophages) can be visualized by investigating MHC-complexes in CD11c+ cells. Furthermore, enhanced antigen presentation in DCs was associated with upregulation of phagocytic and lysosomal markers including CD68 and LAMP1. Spleen sections were stained for MHC-II and CD11c (Fig. 8A), and the presence of large amounts of MHC-II in DCs following TBI was found to result in a significant increase in fluorescence intensity within treatment groups (Two-way ANOVA, F(3, 444)= 118.0; p< 0.0001). Post-hoc analysis (Tukey corrected) indicated a significant increase after TBI (SS vs ST; 26±9 vs 41±11; p < 0.0001; Fig. 8C) with a further increase in the ET group (ST vs ET; 41±11 vs 45±13; p < 0.0001; Fig. 8C). Furthermore, when assessing the density of colocalizing MHC-II-high with CD11c+ cells, we found a significant increase within treatment groups (Two-way ANOVA, F(3, 12)= 60.83; p< 0.0001) due to a large increase upon TBI (Tukey corrected; SS vs ST; 44±6% vs 74±5%; p < 0.0001; Fig. 8B) and a further increase in the ET group (ST vs ET; 74±5% vs 84±4%; p = 0.045; Fig. 8B). Therefore, both the number of CD11c+ cells expressing MHC-II and the levels of such expression increase upon TBI and are

further increased by EI.

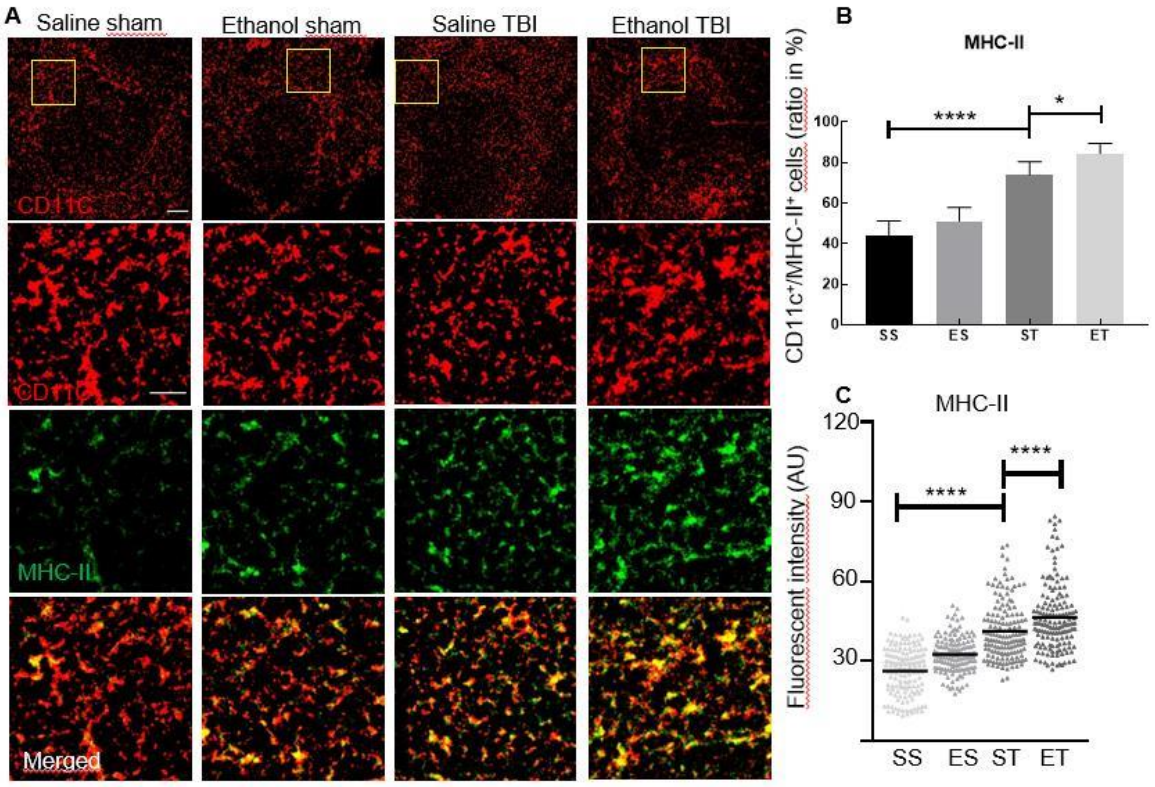

Figure 8: TBI induces antigen presentation on DCs, which is further enhanced by EI.

Immunofluorescence staining of MHC-II with DC marker CD11c on thin spleen sections of saline sham (SS), ethanol sham (ES), saline TBI (ST) and ethanol TBI (ET) treated mice 3h after trauma. A-C) immunofluorescence staining of MHC-II colocalized with CD11c resulted in a significant increase in fluorescence intensity upon TBI (SS vs ST; $p < 0.0001$), with a significant enhancement in the ET group (ST vs ET; $p < 0.0001$). Likewise, the amount of MHC-II-high/CD11c+ cells revealed a significant increase after TBI (SS vs ST; $p< 0.0001$) with a significant enhancement in the ET group (ST vs ET; $p = 0.045$). Data shown as barplots or scatterplots. Group size: SS N = 5, ES N = 5, ST N = 5, ET N = 5. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$. Scale bar overview: 50 µm; scale bar insert: 20 µm. TBI= Traumatic brain injury, DCs= Dendritic cells, MHC-II= Major histocompatibility complex-II, CD=Cluster of differentiation. SS=Saline Sham, ES=Ethanol Sham, ST=Saline Traumatic brain injury, ET=Ethanol Traumatic brain injury. Modified from (Zhang J, Li Z et al[233], 2022), CC BY 4.0, https://creativecommons.org/licenses/by/4.0/.

We attempted to assess the lysosomal activity of splenic DCs by studying LAMP1 and CD68 in the samples. LAMPs are a family of glycosylated proteins mainly present on

the lysosomal membrane and are involved in a variety of cellular activities, such as phagocytosis, autophagy, and lipid transport. LAMP1 is a highly glycosylated glycoprotein mainly present on the lysosomal membrane. CD68 is also a member of the LAMP family. Immunostaining of CD68 and CD11c (Fig. 9A) on spleen sections showed a significant difference in intensity within treatment groups (Two-way ANOVA, $F(3, 444)= 79.68$; $p < 0.0001$) and increased significantly after TBI (Tukey corrected, SS vs ST; 36±13 vs 51±13; $p< 0.0001$; Fig. 9C) and an even further enhancement in the ET group (ST vs ET; 51±13 vs 63±22; $p < 0.0001$; Fig. 9C). When assessing the density of CD68-high cells colocalized with CD11c+ DCs, the treatment resulted in a significant difference within groups (Two-way ANOVA, $F(3, 12) = 41.25$; $p< 0.0001$) due to TBI which resulted in an increase in the amount of CD68-high/CD11c+ cells in the spleen (SS vs ST; 25±9% vs 36±6%; $p= 0.0007$; Fig. 9B) and a further significant increase in ET group (ST vs ET; 36±6% vs 46±9%; $p= 0.002$; Fig. 9B). Interestingly, immunostaining of LAMP1 and CD11c (Fig. 9D) on spleen sections also showed a significant effect in intensity within treatment groups (Two-way ANOVA, $F(3, 444) = 214.9$; $p < 0.0001$) due to a significant increase upon TBI (Tukey corrected; SS v ST; 24±6 vs 38±12; $p < 0.0001$; Fig. 9F) and a further increase was detected in the ET group (ST vs ET; 38±12.3 vs 49.2±7.6; $p< 0.0001$; Fig. 9F). The density of LAMP1-high cells colocalized with CD11c+ cells exhibited a significant effect within the treatment groups (Two-way ANOVA, $F(3, 12)= 43.02$; $p< 0.0001$). The post-hoc comparison within the treatment groups (Tukey corrected) revealed a strong significant upregulation after TBI (SS vs ST; 30±4% vs 54±6%; $p = 0.0007$; Fig. 9E) and a further enhancement in ET group (ST vs ET; 54±6% vs 67±8%; $p= 0.01$; Fig. 9E). The convergence of MHC-II, LAMP-1 and CD68 upregulation indicate that upon TBI, APCs functions are upregulated in splenic DCs and they are further enhanced by concomitant EI.

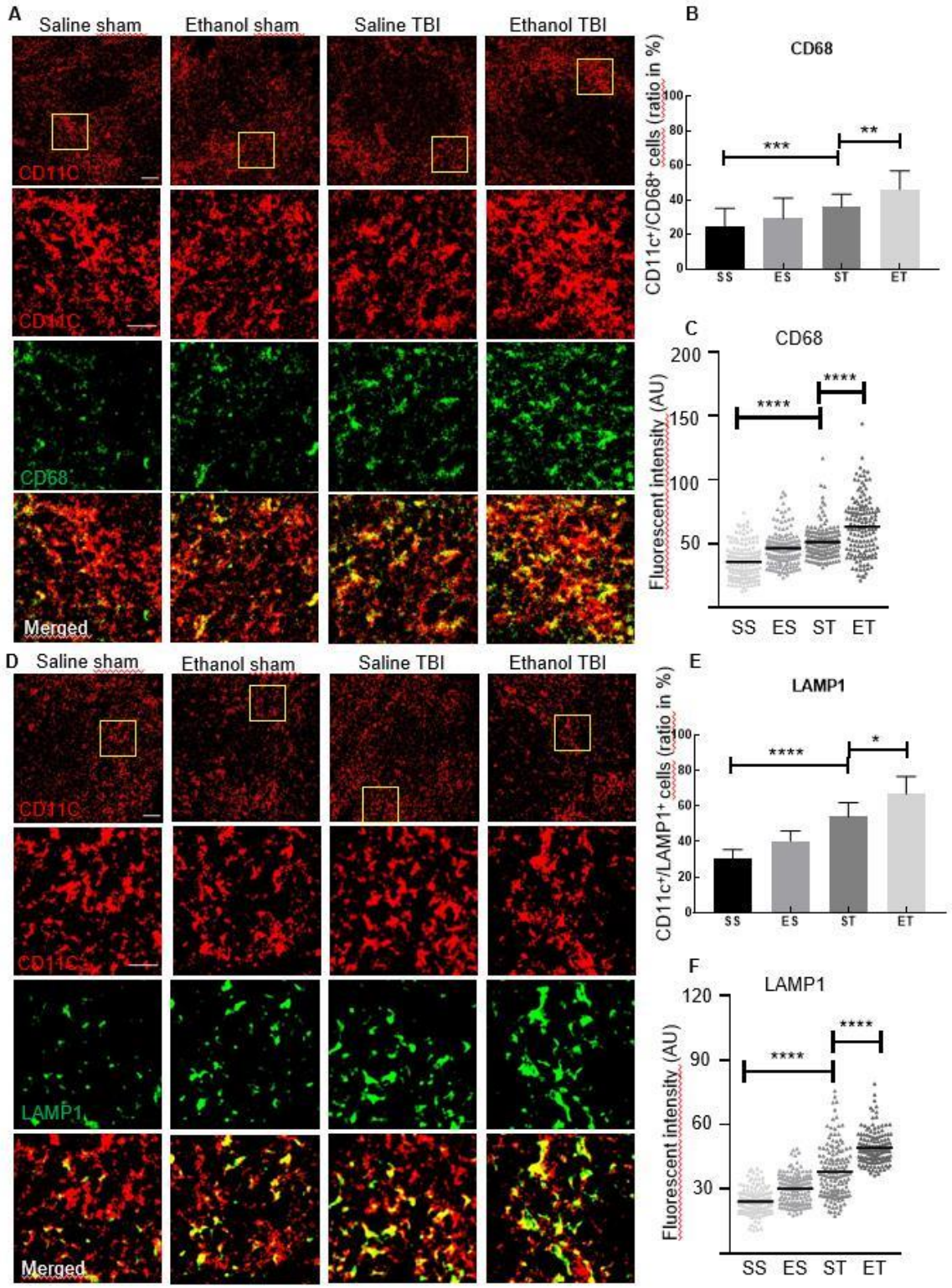

Figure 9: Lysosomal activity in DCs increases by TBI and is further enhanced by EI.

Immunofluorescence staining of CD68 and LAMP1 with DC marker CD11c on thin spleen sections of saline sham (SS), ethanol sham (ES), saline TBI (ST) and ethanol TBI (ET) treated mice 3h after trauma. A-C) immunofluorescence staining of CD68 colocalized with CD11c resulted in a significant increase in fluorescence intensity upon TBI (SS vs ST; $p < 0.0001$), with a significant enhancement

in the ET group (ST vs ET; p< 0.0001). The amount of CD68-high/CD11c+ cells revealed a significant increase after TBI (SS vs ST; p= 0.0007), with a significant enhancement in the ET group (ST vs ET; p= 0.002). D-F) immunofluorescence staining of LAMP1 colocalized with CD11c resulted in a significant increase in fluorescence intensity upon TBI (SS vs ST; p< 0.0001), with a significant increase in the ET group (ST vs ET; p< 0.0001). The number of LAMP1-high/CD11c+ cells revealed a significant increase after TBI (SS vs ST; p= 0.0007) with a further significant increase in the ET group (ST vs ET; p= 0.01). Data shown as barplots or scatterplots. Group size: SS N= 5, ES N= 5, ST N= 5, ET N= 5. *: p < 0.05; **: p< 0.01; ***: p< 0.001; ****: p< 0.0001. Scale bar overview: 50 µm; scale bar insert: 20 µm. DCs= Dendritic cells, LAMP1= Lysosomal associated membrane protein 1, CD=Cluster of differentiation. SS=Saline Sham, ES=Ethanol Sham, ST=Saline Traumatic brain injury, ET=Ethanol Traumatic brain injury. Modified from (Zhang J, Li Z et al[233], 2022), CC BY 4.0, https://creativecommons.org/licenses/by/4.0/.

3.6 TBI strongly induces immunogenic function of splenic DCs

The increased antigen presentation and lysosomal activity on CD11c+ DCs, shown by the increase in MHC-II, CD68 and LAMP1, strongly suggests that TBI and EI induces a rapid maturation of splenic DCs. However, functional aspects of these mature DCs after TBI remain unclear, including the effect of EI on the maturation phenotype of these DCs post TBI. To further explore the immunostimulatory phenotype of splenic DCs in trauma, we assessed the expression levels of TNFα in CD11c+ DCs. In fact, TNFα is upregulated in DCs by the interaction with antigens and by the stimulation of TLRs and it is a major inducer of T-cell responses.

We also considered the expression of the beta-2 adrenergic receptor, a marker associated with tolerogenic DCs, which shows immunosuppressive phenotype, under the hypothesis that expression of beta-2 adrenergic receptor and TNFα should be inversely correlated. Fluorescence single mRNA in situ hybridization was performed on spleen sections for beta-2 adrenergic receptor (ADRB2) and TNFα (Fig. 10A). Density analysis of single mRNA TNFα in CD11c+ DCs showed a significant difference within treatment groups (Two-way ANOVA, $F(3,267)= 79.5$; p< 0.0001), due to a strong increase after TBI (Tukey corrected, SS vs ST; 13±6 vs 22±8; p < 0.0001; Fig. 10B), and ethanol intoxication prior to TBI further increased it (ST vs ET; 22±8 vs 29±10; p<

0.0001; Fig. 10B). Furthermore, when assessing ADRB2 in CD11c+ DCs, density analysis revealed a significant difference within treatment groups (Two-way ANOVA, $F(3,267) = 18.99$; $p < 0.0001$), due to a significant decrease after TBI (Tukey corrected, SS vs ST; 14±5 vs 10±4; $p< 0.0001$; Fig. 10C). Interestingly, the ET group showed a strong increase in ADRB2 compared to the ST group (ST vs ET; 10±4 vs 16±7; $p < 0.0001$; Fig. 10C). This data suggests, that TBI induces an increase in immunogenic function of splenic DCs, shown by the increased TNFα expression and decreased ADRB2 expression. Upon EI, the immunogenic function is further enhanced shown by the increased TNFα expression, however, the increase of ADRB2 suggests a simultaneous high sensitivity to adrenergic dampening of inflammation after EI.

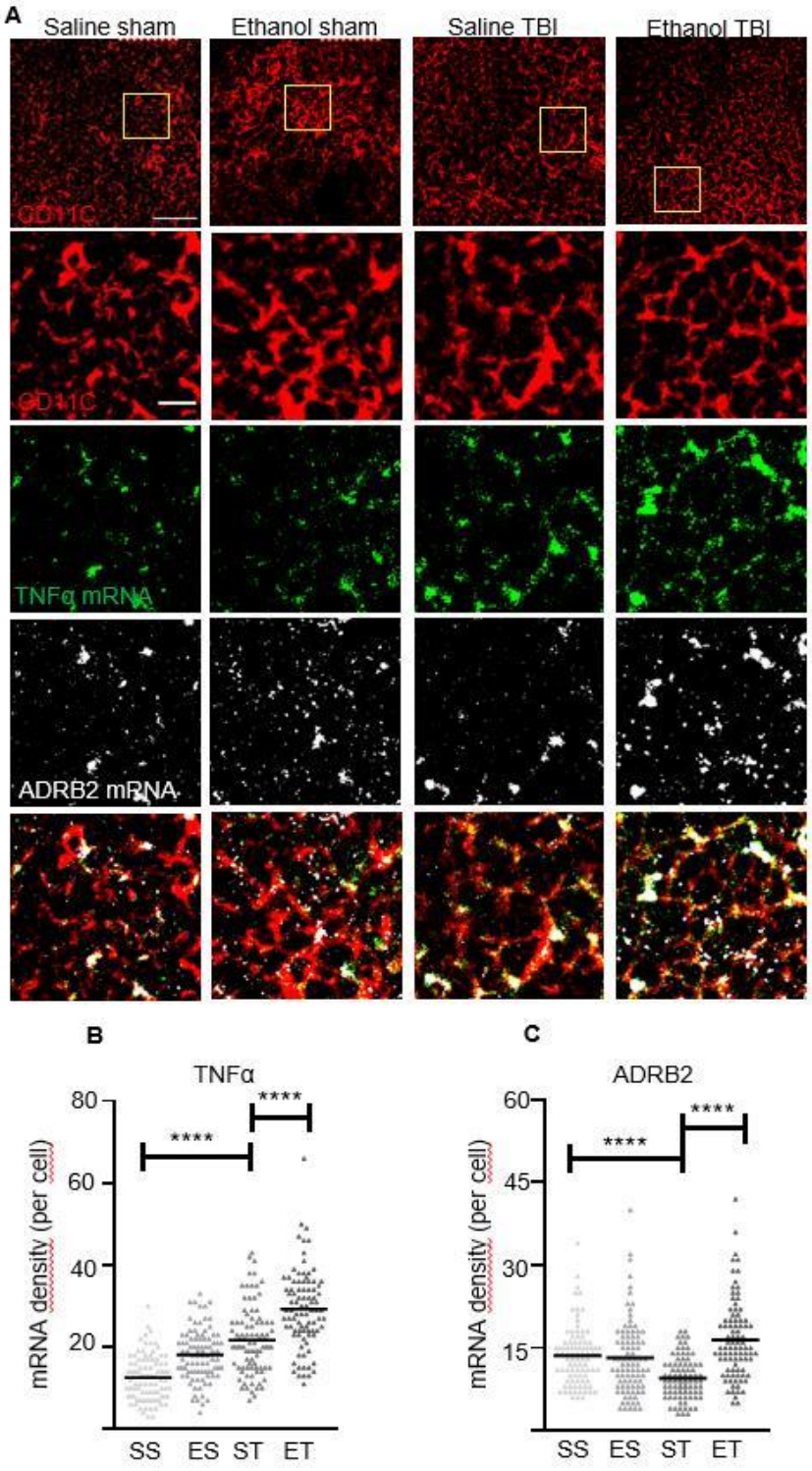

Figure 10: TBI enhances TNFα expression in splenic DCs and EI shows high sensitivity to adrenergic dampening.

Fluorescence in situ mRNA hybridization of TNFα and beta-2 adrenergic receptor (ADRB2) with co- staining of DC marker CD11c on thin spleen sections of saline sham (SS), ethanol sham (ES), saline TBI (ST) and ethanol TBI (ET) treated mice 3h after trauma. A-B) Fluorescence in situ hybridization of TNFα resulted in a significant increase in mRNA density upon TBI (SS vs ST; $p <$

0.0001), with a significant enhancement in the ET group (ST vs ET; p< 0.0001). A-C) Fluorescence in situ hybridization of ADRB2 resulted in a significant decrease in mRNA density upon TBI (SS vs ST; p < 0.0001), however the ET group shows a significant increase (ST vs ET; p < 0.0001). Data shown as scatterplots. Group size: SS N = 5, ES N = 5, ST N = 5, ET N = 5. *: p < 0.05; **: p < 0.01; ***: p < 0.001; ****: p < 0.0001. Scale bar overview: 50 µm; scale bar insert: 10 µm. DCs= Dendritic cells, TNFα= tumor necrosis factor-α, TBI= Traumatic brain injury ADRB2= SS=Saline Sham, ES=Ethanol Sham, ST=Saline Traumatic brain injury, ET=Ethanol Traumatic brain injury. Modified from (Zhang J, Li Z et al[233], 2022), CC BY 4.0, https://creativecommons.org/licenses/by/4.0/.

4 Discussion

Traumatic brain injury (TBI) is the leading cause of mortality and morbidity in young people worldwide. The annual incidence rate is reported to be around 300/100,000 or even higher. Traumatic brain injury causes a systemic inflammatory response and the spleen plays an important role in the systemic inflammatory response. The spleen can release a variety of inflammatory mediators that increase the level of inflammatory response in the periphery of the body and at the site of brain tissue injury. Splenectomy after injury can reduce the level of local inflammatory factors in the blood and brain tissue, reduce brain tissue edema and reduce mortality [44]. Alcohol can reduce spleen volume, and previous studies have demonstrated that alcohol preconditioning can reduce neuroinflammation after trauma [30, 70, 138]. DCs are potent APCs and play an important role in linking innate and adaptive immune responses. The production of a distinct set of cytokines by DCs upon different types of stimulation is a central feature in determining the generation of diverse effector adaptive immune responses.

4.1. Induction of maturation markers in splenic DCs occurs rapidly after TBI.

This data shows that DCs in the spleen show an increased maturation after experimental TBI, shown by increased FLT3/FLT3L signaling, increased protein synthesis, increased MHC-II and lysosomal activity and finally, an increased inflammation, shown by increased TNFa expression [233]. Most notably, the DC maturation process was enhanced with concomitant high-dose EI, expression of pFLT3 and its downstream targets was increased, protein synthesis was further enhanced, phagocytosis and lysosomal activity were further increased, and more CD11c+ cell fractions showed signs of immune function activation. However, ADRB2 expression was increased at the same time, suggesting a dampening of the immune response. Thus, TBI initiates events that affect an important part of the systemic immune response, and the simultaneous occurrence of EI can substantially amplify these cascades. However, ethanol pretreatment elicited rapid autonomic innervation.

DCs are the most robust professional antigen-presenting cells (APCs) discovered so far. The maturation state of DCs determines the outcome of the immune response. Immature dendritic cells (iDCs) have efficient antigen uptake and processing capacity, but show a low expression of MHC-II, co-stimulatory factors and a decreased activation of T cell responses [118]. After encountering stimulatory signals, such as infection or inflammation, iDC captures the antigen, then processes the antigen, and at the same time migrates to the lymphoid organs, gradually matures, proliferates and secretes a variety of cytokines, increases expression of MHC molecules and co-stimulatory molecules such as CD40, CD80, CD86 and CD58. DCs gradually increases the ability to present antigens, while the ability to absorb and process antigens gradually weakens, and finally develop into mature dendritic cells [196]. Initial evidence suggested that immature DCs are tolerogenic to T cells and that mature DCs increase T cell responses and immunity [46, 50], however other evidences point toward that mature DCs have a tolerogenic function [123, 184]. Furthermore, the maturation of DCs also involves substantial remodeling of their metabolism, which occurs primarily at the translational level by proteomic remodeling, which in turn leads to long-term adaptive changes in cellular metabolism to immune function [86]. DC maturation leads to an increase in mTOR-dependent protein synthesis, which is largely dependent on the PI3K-mTORC1 pathway [101, 171], and an increased use of glycolytic pathways [58]. Activation of the mTOR pathway promotes the maintenance of the mature state of DCs. DC maturation is also associated with major alterations in protein degradation flux, accompanied by an upregulation of lysosomal markers such as LAMP3 [47], but decreased autophagy, which is associated with increased mTORC1 activity [185]. Maturation of DCs is also accompanied by phosphorylation of important translation initiation factors. mTORC1 can phosphorylate the protein translation inhibitors 4E-BP-1 and 4E-BP-2, releasing the initiation factor eIF4E and thus inhibiting cap-dependent translation, which is associated with major changes in the ability of mature DCs to prevent cell death [101, 122]. In addition, the maturation process is intertwined with the upregulation of cytokine secretion, such as TNF-α [100] and IL-12 [130], and IL-10, and simultaneously promotes the activation of helper T cells. Furthermore, enhanced antigen presentation

in mature DCs is associated with increased phagocytic and lysosomal activity, and upregulation of lysosomal markers, including CD68 and LAMP1, has been associated with increased antigen presentation and induction of T cell responses [5, 11, 47, 199]. Therefore, my research shows that with the demonstration of increased FLT3 phosphorylation, upregulation of protein synthesis markers, increased expression of MHC-II and of lysosomal proteins LAMP1 and CD68, we believe to provide substantial evidence to state that TBI induces a rapid upregulation of the splenic DCs maturation process, from steady-state cells to effective APC [233].

TBI causes rapid maturation of splenic DCs. The maturation process of DCs is initiated by PAMPs or DAMPs [60, 140], PAMPs are proteins released by bacterial or viral pathogens, and DAMPs are proteins released by damaged tissues of the body. After TBI, intracranial cells release numerous endogenous factors such as inflammatory vesicles, mitochondrial DNA (mtDNA), heat shock peptides, and HMGB1 [224]. In fact, the levels of brain tissue proteins and injury markers such as GFAP, NSE, S100β and NFL [31, 107] in the serum of TBI-affected mice were already elevated at 3 h. In particular, HMGB1, a damage-associated molecular pattern also a highly conserved nuclear protein that is released when brain tissue is damaged [144, 146], is rapidly elevated in serum after TBI [6, 216]. Studies have shown that HMGB1 not only promotes local inflammatory responses after neurotrauma [97, 227], but also contributes to systemic inflammation and secondary brain injury after TBI [225]. Furthermore, HMGB1 is also a major inducer of DCs maturation, an effect that appears to be associated with lung injury (via mTOR signaling [105]) and liver injury [33]. Furthermore, HMGB1 also regulates the function of DCs through the toll-like receptor 4 (TLR4) signaling pathway.

The nervous system is involved in the regulation of spleen immune responses in the early stages of TBI. It is well established that a crosstalk exists between the nervous and the immune systems, which is with important implications for immune homeostasis maintenance [4]. DCs play an important role in the induction and maintenance of peripheral tolerance and immune regulation. Recent evidence suggests that the autonomic nervous system is strongly involved in the control of splenic responses through adrenergic and cholinergic inputs, and that the nervous system

maintains functional interactions with immune cells through nerve endings that are widespread [1, 93, 166, 194, 236]. Furthermore, adrenergic activated DCs were instead associated with limited expression of MHC-II, CD86, and promoted immune tolerance, but strongly increased IL-10 secretion [222]. Likewise, adrenergic stimulation of DCs significantly reduced IL-12 release, which in turn inhibited Th1 lymphocyte secretion of IFN-γ, while promoting Th17 lymphocyte secretion of IL-17A [193], suggesting that autonomic activity affects the cytokine profile of DCs. Therefore, the effect of autonomic innervation on TBI-induced maturation of splenic DCs may occur together with the effects of systemic, blood-borne cytokines, co-regulating the immune response of splenic dendritic cells in the early stages of TBI [233].

TBI-induced maturation of splenic DCs is involved in systemic immune activation. The direct consequence of activation of the brain-spleen axis in TBI appears to be detrimental, either through circulating cytokines or through the autonomic disturbances associated with TBI. As demonstrated in experimental rat TBI, immediate removal of the spleen resulted in improved survival, reduced cerebral and systemic cytokine responses, improved brain edema and preserved cognitive performance [82, 104]. These effects were correlated with the decrease in NF-κB activation at the injury site. It was shown that TBI significantly upregulated MAPK signaling in damaged brain regions and that immediate splenectomy downregulated the MAPK-NF-κB signaling pathway in the brains of rats after severe TBI [38]. Furthermore, similar beneficial effects of splenectomy have been reported in the setting of spinal cord injury (SCI) [14] and acute ischemic stroke (AIS) [1], which may be related to the activation of alpha and beta adrenergic receptors by circulating catecholamines. Some clinical studies have shown that splenectomy does not benefit trauma patients [175]. However, the actual contribution of DC maturation is not necessarily detrimental and may largely depend on its activation state (immature, semi-mature or mature [196]). It was shown that DCs pulsed with myelin-basic protein could actually drive a protective T-cell response in SCI [74]; on the other hand, complex autoimmune responses following TBI are associated with detrimental outcomes, and the impairment of such autoimmune responses can persist for years and is associated with neurodegeneration [132].

Interestingly, maturation and function of DCs is impaired in SCI patients, whereas autoimmune responses in the acute phase may have neuroprotective or repairing effects [150]. Thus, our findings suggest that TBI leads to a rapid recruitment of DCs in the spleen and enhances antigen-presenting capacity and T-cell induction [233]. Given the central role of these cells as APCs, they may be substantial contributors to the systemic imbalance of immune functions after TBI.

4.2. DCs are participants in the immunomodulation that occurs upon EI in TBI.

EI-driven enhanced DCs maturation upon TBI may contribute to attenuating inflammatory responses. In a mouse model of TBI, EI has been reported to be dose-dependently associated with reduced inflammation at the injury site [30, 70, 138]. Importantly, EI also suppressed systemic inflammatory responses early in TBI (within 3 hours of trauma). Studies have shown that the levels of HMGB1 and IL-6 decreased in the liver of mice receiving EI/TBI, and in the lungs, EI decreased the levels of HMGB1, IL-6, IL-1β, and TNF-α, while modestly increasing IL-10 [225]. Consistent with this evidence, EI is associated with decreased systemic IL-6 levels and leukopenia and increased IL-10 [208] levels in human TBI patients [207] and patients following major trauma (including TBI) [162]. However, some studies have found that the effects of chronic ethanol exposure on DCs are time- and organ-specific [3]. In the skin, chronic ethanol feeding can lead to dysfunction of cutaneous DCs and reduced migratory responses, leading to immunosuppression, but does not affect the maturation of DCs [145]. Our results show that EI leads to an accelerated maturation of DCs [233]. This was driven by increased phosphorylation of FLT3 [45] and evidenced by a larger fraction of CD11c+ cells showing upregulation of protein synthesis (pS6) and lysosomal markers [36, 169] (LAMP-1, CD68). Also TNFα, which is essential for the survival of DCs during maturation, showed high levels of TNFα mRNA induction [100].

Chronic alcohol abuse inhibits the antigen-presenting capacity of DCs, reduces the number of bone marrow and circulating DCs, and reduces the expression of costimulatory molecules in DCs [59, 182]. Our model is based on acute ethanol

intoxication, and studies have shown that acute ethanol intoxication reduces the expression of co-stimulatory molecules CD80 and CD86 in DCs [191, 192]. However, a combination of in vitro and in vivo data about the effects of EI on trauma-related inflammation suggests that EI has an overall immunosuppressive effect that reduces infection-related complications after TBI, consistent with the effects of chronic ethanol on DC function. Based on this inference, the enhanced maturation of splenic DCs observed in the EI/TBI group may contribute to a DC phenotype with reduced inflammation properties [233]. As a marker associated with tolerogenic DCs, the β-2 adrenergic receptor expression changes also support this inference [54, 75]. Compared with ST group, ADRB2 expression was increased in ET group. This hypothesis is supported by the observed upregulation of β-2 adrenergic receptors in DCs. Nevertheless, the combination of increased TNFα and ADRB2 mRNA (not previously described), may correspond to a peculiar state of activation characterized by high immune-stimulatory properties and, at the same time, quick dampening from autonomic innervation [233]. Thus, the impact of EI-driven expansion of DCs maturation may ultimately contribute to the reduced systemic immune reactivity seen in TBI upon ethanol intoxication [233].

Although all experiments have been done according to the research design, there are still some limitations in this thesis. First, the ultimate evaluation of the DCs function would require an active immunization protocol in vivo or an ex-vivo naive T-cell stimulation assay, which was beyond the technical scope of the present project. In follow-up studies, this may serve as a separate research direction. Second, the use of CD11c+ marker does not distinguish the subpopulation of myeloid DCs or plasmacytoid DCs. We have nevertheless maintained a consistent selection of the region of interest in correspondence with the marginal zone [77]. Third, we performed the analysis 3 hours after TBI, therefore, we could only study the early immune response of the spleen after EI combined with TBI. In the future, additional experiments, such as studies of in vitro co-culture of DCs with immature T cells, and the long-term effects of EI on the phenotype of spleen DCs after TBI should be performed.

In summary, these findings suggest that induction of maturation markers in splenic DCs takes place rapidly after TBI and is highly correlated with the phosphorylation of FLT3; we further demonstrate that concomitant EI amplifies the maturation process of splenic DCs post TBI. Thus, the above findings identify the DCs as a new player in the immunomodulation occurring upon EI in TBI.

5 Summary

Background and purpose: Systemic inflammatory consequences remain an important burden after traumatic brain injury (TBI), with almost all organs affected. The spleen is connected to the brain through autonomic innervation and soluble mediators, and cross-talk between the brain and spleen may be important in establishing a systemic inflammatory response to TBI. Ethanol intoxication is the most common comorbidity of TBI and is thought to affect the peripheral inflammatory response either directly or through brain-spleen cross-talk. Animal models have demonstrated that high doses of ethanol intoxication (EI), which reduce spleen size, decrease the pro-inflammatory cytokines IL-1β and IL-6 but increase the anti-inflammatory cytokine IL-10. In addition, EI has been shown to inhibit antigen expression in DCs, reflecting the immunosuppressive effects of ethanol intoxication on the spleen. Is acute EI hypothesized to interfere with or amplify TBI-induced systemic immune regulation, and in particular, does EI modulate TBI-induced immune responses in the spleen? Given the high prevalence of EI in patients with TBI, this question has direct clinical relevance.

Methods: We obtained spleens using a mouse TBI animal model previously established in the laboratory. Four experimental groups were investigated; administered saline and received sham surgery (saline-sham, SS); administered saline and received TBI (saline-TBI, ST); administered ethanol and received sham surgery (ethanol-sham, ES); and administered ethanol and received TBI (ethanol-TBI, ET). Three hours after trauma, mice were euthanized by cervical dislocation and organs were harvested for further processing. We used RNA isolation and quantitative RT-PCR to detect typical Th1 cell immune-directed pro-inflammatory mediators (IL-12, IL-17, IL-23a and IFN-y), typical Th2-B cell-directed anti-inflammatory mediators (IL-4, IL-10, IL-13 and IL-19) and typical DCs-monocytes in spleen tissue mediators (FLT3, FLT3L, CX3CL1 and CX3CR1). as well as DC biology (and other APCs: CCL2, CCL3, CCL4 and CCL12;) Th cells (CCL1, CCL20, CCL22 and CXCL10;) or leukocytes and NK cells (CCL6, CCL24, CXCL3

and CXCL16;). We subsequently performed immunostaining for the pan-DCs marker CD11c, the pan-leukocyte marker CD45 and phosphorylated FLT3 on thin sections of spleens from the four treatment groups. To verify that phosphorylation of FLT3 receptors corresponds to functional involvement of signal transduction pathways, we monitored phosphorylation of Bruton's tyrosine kinase (BTK). Using the same immunostaining, we examined phosphorylated S6 ribosomal protein (pS6), phospho-eIF2α, major histocompatibility complex-II (MHC-II), CD68 and lysosomal associated membrane protein 1 (LAMP1). To further explore the immunostimulatory phenotype of splenic DCs in trauma, splenic sections were stained for β-2 adrenergic receptors (ADRB2) and TNFα in fluorescent single mRNA in situ hybridization.

Results: Here we show that TBI leads to substantial changes in the transcription of genes associated with dendritic cell activation in the spleen, in particular the induction of FLT3/FLT3L 3h after TBI, which is enhanced in EI. TBI induces phosphorylation of the FLT3 downstream signalling partner BTK in DCs and is enhanced in EI. The induction of FLT3L is associated with CD11c+ dendritic cells in which phosphorylation of FLT3 receptors was associated with enhanced protein synthesis, as indicated by increased levels of pS6, peIF2α in dendritic cells. This corresponds to the upregulation of proteins associated with the maturation process and immunostimulatory properties such as MHC-II, LAMP1 and CD68, as well as pro-inflammatory cytokines such as TNFα. Notably, EI enhanced the maturation of dendritic cells. However, while TBI reduced the expression of the ADRB2 on dendritic cells, EI increased it, thereby increasing the opportunity for cross-regulation of immune function by the autonomic nervous system.

Conclusion: Induction of maturation markers of splenic DC occurs rapidly after TBI and is highly correlated with phosphorylation of FLT3; concomitant EI amplifies the maturation process of splenic DC after TBI. Thus, our findings identify DC as a novel player in the immune regulation of EI occurrence in TBI.

6 Reference

[1]. Ajmo CT Jr, Collier LA, Leonardo CC, Hall AA, Green SM, Womble TA, Cuevas J, Willing AE, Pennypacker KR. Blockade of adrenoreceptors inhibits the splenic response to stroke. Exp Neurol. 218: 47-55(2009).

[2]. Ajmo CT Jr, Vernon DO, Collier L, Hall AA, Garbuzova-Davis S, Willing A, Pennypacker KR. The spleen contributes to stroke-induced neurodegeneration. J Neurosci Res. 86:2227-2234(2008).

[3]. Alharshawi K, Fey H, Vogle A, Klenk T, Kim M, Aloman C. Sex specific effect of alcohol on hepatic plasmacytoid dendritic cells. Int Immunopharmacol. 90:107166(2007).

[4]. Andersson U, Tracey KJ. Reflex principles of immunological homeostasis. Annu Rev Immunol. 30:313-335(2012).

[5]. Arruda LB, Sim D, Chikhlikar PR, Maciel M Jr, Akasaki K, August JT, Marques ET. Dendritic cell-lysosomal-associated membrane protein (LAMP) and LAMP-1-HIV-1 gag chimeras have distinct cellular trafficking pathways and prime T and B cell responses to a diverse repertoire of epitopes. J Immunol. 177: 2265-2275(2006).

[6]. Au AK, Aneja RK, Bell MJ, Bayir H, Feldman K, Adelson PD, Fink EL, Kochanek PM, Clark RS. Cerebrospinal fluid levels of high-mobility group box 1 and cytochrome C predict outcome after pediatric traumatic brain injury. J Neurotrauma. 29:2013-2021(2012).

[7]. Baratz R, Tweedie D, Wang JY, Rubovitch V. Transiently lowering tumor necrosis factor-α synthesis ameliorates neuronal cell loss and cognitive impairments induced by minimal traumatic brain injury in mice. J Neuroinflammation. 12:45(2015).

[8]. Bassi GS, Kanashiro A, Coimbra NC, Terrando N, Maixner W, Ulloa L. Anatomical and clinical implications of vagal modulation of the spleen. Neurosci Biobehav Rev. 112:363-373(2020).

[9]. Bell C, Hackett J, Hall B, Pülhorn H, McMahon C, Bavikatte G. Symptomatology following traumatic brain injury in a multidisciplinary clinic: experiences from a tertiary centre. Br J Neurosurg. 32:495-500(2018).

[10]. Berthiaume L, Zygun D. Non-neurologic organ dysfunction in acute brain injury. Crit Care Clin. 22:753-66(2006).

[11]. Bettiol E, Van de Hoef DL, Carapau D, Rodriguez A. Efficient phagosomal maturation and degradation of Plasmodium-infected erythrocytes by dendritic cells and macrophages. Parasite Immunol. 32: 389-398(2010).

[12]. Bhatia S, Edidin M, Almo SC, Nathenson SG. Nathenson, B7-1 and B7-2: similar costimulatory ligands with different biochemical, oligomeric and signaling properties. Immunol Lett. 104: 70-75(2006).

[13]. Bjarkø VV, Skandsen T, Moen KG, Gulati S, Helseth E, Nilsen TIL, Vik A. Time of Injury and Relation to Alcohol Intoxication in Moderate-to-Severe Traumatic Brain Injury: A Decade- Long Prospective Study. World Neurosurg. 122: e684-e689(2019).

[14]. Blomster LV, Brennan FH, Lao HW, Harle DW, Harvey AR, Ruitenberg MJ. Mobilisation of the splenic monocyte reservoir and peripheral CX3CR1 deficiency adversely affects recovery from spinal cord injury. Exp Neurol. 247: 226-240(2013).

[15]. Bonaz B, Sinniger V, Pellissier S. Anti-inflammatory properties of the vagus nerve: potential therapeutic implications of vagus nerve stimulation. J Physiol. 594:5781-5790(2016).

[16]. Bonilla C, Zurita M. Cell-Based Therapies for Traumatic Brain Injury: Therapeutic Treatments and Clinical Trials. Biomedicines. 9:669(2021).

[17]. Bonow RH, Quistberg A, Rivara FP, Vavilala MS. Intensive Care Unit Admission Patterns for Mild Traumatic Brain Injury in the USA. Neurocrit Care.30:157-170(2019).

[18]. Brennan JH, Bernard S, Cameron PA, Rosenfeld JV, Mitra B. Ethanol and isolated traumatic brain injury. J Clin Neurosci. 22: 1375-1381(2015).

[19]. Brigode W, Cohan C, Beattie G, Victorino G. Alcohol in Traumatic Brain Injury: Toxic or Therapeutic? J Surg Res. 244: 196-204(2019).

[20]. Buchmann Godinho D, da Silva Fiorin F, Schneider Oliveira M, Furian AF, Rechia Fighera M, Freire Royes LF. The immunological influence of physical exercise on TBI-induced pathophysiology: Crosstalk between the spleen, gut, and brain. Neurosci Biobehav Rev. 130:15-30(2021).

[21]. Büki A, Povlishock JT. All roads lead to disconnection?--Traumatic axonal injury revisited. Acta Neurochir (Wien). 148:181-93; discussion 193-4 (2006).

[22]. Burgoyne RD, Haynes LP. Sense and specificity in neuronal calcium signalling. Biochim Biophys Acta. 1853:1921-1932 (2015).

[23]. Byrnes KR, Loane DJ, Stoica BA, Zhang J, Faden AI. Delayed mGluR5 activation limits neuroinflammation and neurodegeneration after traumatic brain injury. J Neuroinflammation. 9:43 (2012).

[24]. Carnevale D. Neural Control of Immunity in Hypertension: Council on Hypertension Mid Career Award for Research Excellence, 2019. Hypertension. 76: 622-628(2020).

[25]. Castellino F, Huang AY, Altan-Bonnet G, Stoll S, Scheinecker C, Germain RN. Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. Nature. 440: 890-895(2006).

[26]. Cathomas F, Murrough JW, Nestler EJ, Han MH, Russo SJ. Neurobiology of Resilience: Interface Between Mind and Body. Biol Psychiatry. 86: 410-420 (2019).

[27]. Cathomas F, Russo SJ. Brain-spleen connection aids antibody production. Nature. 581:142-143 (2020).

[28]. Chaban V, Clarke GJB, Skandsen T, Islam R, Einarsen CE, Vik A, Damås JK, Mollnes TE, Håberg AK, Pischke SE. Systemic Inflammation Persists the First Year after Mild Traumatic Brain Injury: Results from the Prospective Trondheim Mild Traumatic Brain Injury Study. J Neurotrauma. 37:2120-2130(2020).

[29]. Chandrasekar A, Aksan B, Heuvel FO, Förstner P, Sinske D, Rehman R, Palmer A, Ludolph A, Huber-Lang M, Böckers T, Mauceri D, Knöll B, Roselli F. Neuroprotective effect of acute ethanol intoxication in TBI is associated to the hierarchical modulation of early transcriptional responses. Exp Neurol. 302: 34-45(2018).

[30]. Chandrasekar A, Heuvel FO, Palmer A, Linkus B, Ludolph AC, Boeckers TM, Relja B, Huber-Lang M, Roselli F. Acute ethanol administration results in a protective cytokine and neuroinflammatory profile in traumatic brain injury. Int Immunopharmacol. 51: 66-75 (2017).

[31]. Chandrasekar A, Olde Heuvel F, Wepler M, Rehman R, Palmer A, Catanese A, Linkus B, Ludolph A, Boeckers T, Huber-Lang M, Radermacher P, Roselli F. The Neuroprotective Effect of Ethanol Intoxication in Traumatic Brain Injury Is Associated with the Suppression of ERBB Signaling in Parvalbumin-Positive Interneurons. J Neurotrauma. 35: 2718-2735(2018).

[32]. Chen T, Fei F, Jiang XF, Zhang L, Qu Y, Huo K, Fei Z. Down-regulation of Homer1b/c attenuates glutamate-mediated excitotoxicity through endoplasmic reticulum and mitochondria pathways in rat cortical neurons. Free Radic Biol Med. 52:208-217(2012).

[33]. Chen Y, Zhang W, Bao H, He W, Chen L. High Mobility Group Box 1 Contributes to the Acute Rejection of Liver Allografts by Activating Dendritic Cells. Front Immunol. 12 :679398 (2021).

[34]. Cheng H, Deaton LM, Qiu M, Ha S. Tau overexpression exacerbates neuropathology after repeated mild head impacts in male mice. Neurobiol Dis. 134:1046-1083(2020).

[35]. Chi OZ, Hunter C, Liu X, Weiss HR. Effects of exogenous excitatory amino acid neurotransmitters on blood-brain barrier disruption in focal cerebral ischemia. Neurochem Res. 34:1249-54(2009).

[36]. Chistiakov DA, Killingsworth MC, Myasoedova VA, Orekhov AN, Bobryshev YV.

CD68/macrosialin: Not just a histochemical marker. Lab Investig. 97: 4-13(2017).

[37]. Chodobski A, Zink BJ, Szmydynger-Chodobska J. Blood-brain barrier pathophysiology in traumatic brain injury. J Transl Stroke Res. 2:492-516(2011).

[38]. Chu W, Li M, Li F, Hu R, Chen Z, Lin J, Feng H. Immediate splenectomy down-regulates the MAPKYNF-JB signaling pathway in rat brain after severe traumatic brain injury. J Trauma Acute Care Surg. 74: 1446-1453(2013).

[39]. Coelho AL, Schaller MA, Benjamim CF, Orlofsky AZ, Hogaboam CM, Kunkel SL. The Chemokine CCL6 Promotes Innate Immunity via Immune Cell Activation and Recruitment. J Immunol. 179: 5474-5482(2007).

[40]. Colicos MA, Dash PK. Apoptotic of dentate gyros granule cells following experimental cortical impact injury in rats: possible role in spatialmemory deficits. Brain Res. 739: 120-131(1996).

[41]. Corbett JM, Ho KM, Honeybul S. Prognostic significance of abnormal hematological parameters in severe traumatic brain injury requiring decompressive craniectomy. J Neurosurg. 132:545-551(2019).

[42]. Cornelius C, Crupi R, Calabrese V, Graziano A, Milone P, Pennisi G, Radak Z, Calabrese EJ, Cuzzocrea S. Traumatic brain injury: oxidative stress and neuroprotection. Antioxid Redox Signal. 19:836-53(2013).

[43]. Coutinho AE, Chapman KE. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. Mol Cell Endocrinol. 335:2-13(2011).

[44]. Crandall M, Shapiro MB, West MA. Does splenectomy protect against immune-mediated complications in blunt trauma patients? Mol Med. 15:263-7(2009).

[45]. Cueto FJ, Sancho D. The Flt3L/Flt3 Axis in Dendritic Cell Biology and Cancer Immunotherapy. Cancers (Basel). 13:1525. (2021).

[46]. De Heusch M, Oldenhove G, Urbain J, Thielemans K, Maliszewski C, Leo O, Moser

M. Depending on their maturation state, splenic dendritic cells induce the differentiation of CD4(+) T lymphocytes into memory and/or effector cells in vivo. Eur J Immunol. 34: 1861-1869(2004).

[47]. De Saint-Vis B, Vincent J, Vandenabeele S, Vanbervliet B, Pin JJ, Aït-Yahia S, Patel S, Mattei MG, Banchereau J, Zurawski S, Davoust J, Caux C, Lebecque S. A novel lysosome-associated membrane glycoprotein, DC-LAMP, induced upon DC maturation, is transiently expressed in MHC class II compartment. Immunity. 9: 325-336(1998).

[48]. De Smedt T, Pajak B, Muraille E, Lespagnard L, Heinen E, De Baetselier P, Urbain J, Leo O, Moser M. Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. J Exp Med. 1184: 1413-1424(1996).

[49]. Deshpande LS, Sun DA, Sombati S, Baranova A, Wilson MS, Attkisson E, Hamm RJ, DeLorenzo RJ. Alterations in neuronal calcium levels are associated with cognitive deficits after traumatic brain injury. Neurosci Lett. 441:115-119(2008).

[50]. Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. J Exp Med. 193: 233-238(2001).

[51]. Di Battista AP, Rhind SG, Hutchison MG, Hassan S, Shiu MY, Inaba K, Topolovec-Vranic J, Neto AC, Rizoli SB, Baker AJ. Inflammatory cytokine and chemokine profiles are associated with patient outcome and the hyperadrenergic state following acute brain injury. J Neuroinflammation. 13: 40 (2016).

[52]. Dong T, Zhi L, Bhayana B, Wu MX. Cortisol-induced immune suppression by a blockade of lymphocyte egress in traumatic brain injury. J Neuroinflammation. 13:197(2016).

[53]. Dorsett CR, McGuire JL, DePasquale EA, Gardner AE, Floyd CL, McCullumsmith RE. Glutamate Neurotransmission in Rodent Models of Traumatic Brain Injury. J Neurotrauma. 34:263-272(2017).

[54]. Doxsee CL, Riter TR, Reiter MJ, Gibson SJ, Vasilakos JP, Kedl RM. The immune

response modifier and Toll-like receptor 7 agonist S-27609 selectively induces IL-12 and TNF- alpha production in CD11c+CD11b+CD8- dendritic cells. J Immunol. 171: 1156-1163(2003).

[55]. Durães FV, Carvalho NB, Melo TT, Oliveira SC, Fonseca CT. IL-12 and TNF-alpha production by dendritic cells stimulated with Schistosoma mansoni schistosomula tegument is TLR4- and MyD88-dependent. Immunol Lett. 125: 72-77(2009).

[56]. Eastman CL, D'Ambrosio R, Ganesh T. Modulating neuroinflammation and oxidative stress to prevent epilepsy and improve outcomes after traumatic brain injury. Neuropharmacology. 172: 107907(2020).

[57]. Eken A, Ortiz V, Wands JR. Ethanol inhibits antigen presentation by dendritic cells, Clin. Vaccine Immunol. 18:1157-1166(2011).

[58]. Everts B, Amiel E, Huang SC, Smith AM, Chang CH, Lam WY, Redmann V, Freitas TC, Blagih J, van der Windt GJ, Artyomov MN, Jones RG, Pearce EL, Pearce EJ. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKε supports the anabolic demands of dendritic cell activation. Nat Immunol. 15: 323-332(2014).

[59]. Fan J, Edsen-Moore MR, Turner LE, Cook RT, Legge KL, Waldschmidt TJ, Schlueter AJ. Mechanisms by which chronic ethanol feeding limits the ability of dendritic cells to stimulate T-cell proliferation. Alcohol Clin Exp Res. 35: 47-59(2011).

[60]. Fang H, Ang B, Xu X, Huang X, Wu Y, Sun Y, Wang W, Li N, Cao X, Wan T. TLR4 is essential for dendritic cell activation and anti-tumor T-cell response enhancement by DAMPs released from chemically stressed cancer cells. Cell Mol Immunol. 11: 150-159(2014).

[61]. Faul M, Coronado V. Epidemiology of traumatic brain injury. Handb Clin Neurol. 127:3-13(2015).

[62]. Fleminger S, Oliver DL, Lovestone S, Rabe-Hesketh S, Giora A. Head injury as a risk factor for Alzheimer's disease: the evidence 10 years on; a partial replication. J Neurol Neurosurg Psychiatry. 74:857-62(2003).

[63]. Flierl MA, Stahel PF, Beauchamp KM, Morgan SJ, Smith WR, Shohami E. Mouse closed head injury model induced by a weight-drop device. Nat Protoc. 4: 1328–1337(2009).

[64]. Franz N, Dieteren S, Köhler K, Mörs K, Sturm R, Marzi I, Perl M, Relja B, Wagner N. Alcohol Binge Reduces Systemic Leukocyte Activation and Pulmonary PMN Infiltration After Blunt Chest Trauma and Hemorrhagic Shock. Inflammation. 42: 690-701(2019).

[65]. Galgano M, Toshkezi G, Qiu X, Russell T, Chin L, Zhao LR. Traumatic Brain Injury: Current Treatment Strategies and Future Endeavors. Cell Transplant. 26:1118-1130(2017).

[66]. Gardner RC, Burke JF, Nettiksimmons J, Goldman S, Tanner CM, Yaffe K. Traumatic brain injury in later life increases risk for Parkinson disease. Ann Neurol. 77:987-995(2015).

[67]. GBD 2016 Traumatic Brain Injury and Spinal Cord Injury Collaborators. Global, regional, and national burden of traumatic brain injury and spinal cord injury, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet Neurol. 18: 459-480 (2019).

[68]. Ge XT, Lei P, Wang HC, Zhang AL, Han ZL, Chen X, Li SH, Jiang RC, Kang CS, Zhang JN. miR-21 improves the neurological outcome after traumatic brain injury in rats. Sci Rep. 4:6718(2014).

[69]. Giorgi C, Baldassari F, Bononi A, Bonora M, De Marchi E, Marchi S, Missiroli S, Patergnani S, Rimessi A, Suski JM, Wieckowski MR, Pinton P. Mitochondrial Ca(2+) and apoptosis. Cell Calcium. 52:36-43(2012).

[70]. Goodman MD, Makley AT, Campion EM, Friend LA, Lentsch AB, Pritts TA. Preinjury alcohol exposure attenuates the neuroinflammatory response to traumatic brain injury. J Surg Res. 184: 1053–1058(2013).

[71]. Greiffenstein P, Mathis KW, Stouwe CV, Molina PE. Alcohol binge before

trauma/hemorrhage impairs integrity of host defense mechanisms during recovery. Alcohol Clin Exp Res. 31:704-15(2007).

[72]. Guermonprez P, Helft J, Claser C, Deroubaix S, Karanje H, Gazumyan A, Darasse-Jèze G, Telerman SB, Breton G, Schreiber HA, Frias-Staheli N, Billerbeck E, Dorner M, Rice CM, Ploss A, Klein F, Swiecki M, Colonna M, Kamphorst AO, Meredith M, Niec R, Takacs C, Mikhail F, Hari A, Bosque D, Eisenreich T, Merad M, Shi Y, Ginhoux F, Rénia L, Urban BC, Nussenzweig MC. Inflammatory Flt3l is essential to mobilize dendritic cells and for T cell responses during Plasmodium infection. Nat Med. 19: 730-738(2013).

[73]. Hanscom M, Loane DJ, Shea-Donohue T. Brain-gut axis dysfunction in the pathogenesis of traumatic brain injury. J Clin Invest. 131: e143777(2021).

[74]. Hauben E, Gothilf A, Cohen A, Butovsky O, Nevo U, Smirnov I, Yoles E, Akselrod S, Schwartz M. Vaccination with dendritic cells pulsed with peptides of myelin basic protein promotes functional recovery from spinal cord injury. J Neurosci. 23: 8808-8819(2003).

[75]. Helbig C, Weber F, Andreas N, Herdegen T, Gaestel M, Kamradt T, Drube S. The IL-33-induced p38-/JNK1/2-TNFα axis is antagonized by activation of β-adrenergic-receptors in dendritic cells, Sci Rep. 10: 8152 (2020).

[76]. Henry RJ, Doran SJ, Barrett JP. Inhibition of miR-155 Limits Neuroinflammation and Improves Functional Recovery After Experimental Traumatic Brain Injury in Mice. Neurotherapeutics. 16:216-230(2019).

[77]. Hey YY, O'Neill HC. Murine spleen contains a diversity of myeloid and dendritic cells distinct in antigen presenting function. J Cell Mol Med. 16:2611-2619(2012).

[78]. Hinson HE, Rowell S, Schreiber M. Clinical evidence of inflammation driving secondary brain injury: a systematic review. J Trauma Acute Care Surg. 78:184-91(2015).

[79]. Hotchkiss RS, Karl IE. The pathophysiology and treatment of sepsis. N Engl J Med. 348:138-150(2003).

[80]. Hu D, Al-Shalan HAM, Shi Z, Wang P, Wu Y, Nicholls PK, Greene WK, Ma B. Distribution of nerve fibers and nerve-immune cell association in mouse spleen revealed by immunofluorescent staining. Sci Rep. 10:9850 (2020).

[81]. Huang S, Ziegler CGK, Austin J, Mannoun N, Vukovic M, Ordovas-Montanes J, Shalek AK, von Andrian UH. Lymph nodes are innervated by a unique population of sensory neurons with immunomodulatory potential. Cell. 184:441-459 (2021).

[82]. Huber-Lang M, Lambris JD, Ward PA. Innate immune responses to trauma review-article. Nat Immunol. 19: 327-341 (2018).

[83]. Hughes CE, Nibbs RJB. A guide to chemokines and their receptors, FEBS J. 285: 2944-2971(2018).

[84]. Iaccarino C, Carretta A, Nicolosi F, Morselli C. Epidemiology of severe traumatic brain injury. J Neurosurg Sci. 62: 535-541(2018).

[85]. Johnson VE, Stewart W, Smith DH. Axonal pathology in traumatic brain injury. Exp Neurol. 246: 35-43(2013).

[86]. Jovanovic M, Rooney MS, Mertins P, Przybylski D, Chevrier N, Satija R, Rodriguez EH, Fields AP, Schwartz S, Raychowdhury R, Mumbach MR, Eisenhaure T, Rabani M, Gennert D, Lu D, Delorey T, Weissman JS, Carr SA, Hacohen N, Regev A. Immunogenetics. Dynamic profiling of the protein life cycle in response to pathogens. Science. 347:1259038 (2015).

[87]. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 26: 239-257(1972).

[88]. Khatri N, Thakur M, Pareek V, Kumar S, Sharma S, Datusalia AK. Oxidative Stress: Major Threat in Traumatic Brain Injury. CNS Neurol Disord Drug Targets. 17:689-695(2018).

[89]. Khellaf A, Khan DZ, Helmy A. Recent advances in traumatic brain injury. J Neurol. 266:2878-2889(2019).

[90]. Kobeissy FH, Guingab-Cagmat JD, Razafsha M, O'Steen L, Zhang Z, Hayes RL, Chiu WT, Wang KK. Leveraging biomarker platforms and systems biology for rehabilomics and biologics effectiveness research. PM R. 3(6 Suppl 1):S139-47(2011).

[91]. Kong XD, Bai S, Chen X, Wei HJ, Jin WN, Li MS, Yan Y, Shi FD. Alterations of natural killer cells in traumatic brain injury. Neurosci Bull. 30: 903-912(2014).

[92]. Kontos HA, Wei EP. Superoxide production in experimental brain injury. J Neurosurg. 64:803-807(1986).

[93]. Kooijman S, Meurs I, van Beek L, Khedoe PP, Giezekamp A, Pike-Overzet K, Cailotto C, van der Vliet J, van Harmelen V, Boeckxstaens G, Berbée JF, Rensen PC. Splenic autonomic denervation increases inflammatory status but does not aggravate atherosclerotic lesion development. Am J Physiol Heart Circ Physiol. 309:H646-H654(2015).

[94]. Korley FK, Diaz-Arrastia R, Wu AH, Yue JK, Manley GT, Sair HI, Van Eyk J, Everett AD; TRACK-TBI investigators, Okonkwo DO, Valadka AB, Gordon WA, Maas AI, Mukherjee P, Yuh EL, Lingsma HF, Puccio AM, Schnyer DM. Circulating Brain-Derived Neurotrophic Factor Has Diagnostic and Prognostic Value in Traumatic Brain Injury. J Neurotrauma. 33:215-25(2016).

[95]. Kumar A, Stoica BA, Loane DJ, Yang M, Abulwerdi G, Khan N, Kumar A, Thom SR, Faden AI. Microglial-derived microparticles mediate neuroinflammation after traumatic brain injury. J Neuroinflammation. 14:47(2017).

[96]. Kundu B, Brock AA, Englot DJ, Butson CR, Rolston JD. Deep brain stimulation for the treatment of disorders of consciousness and cognition in traumatic brain injury patients: a review. Neurosurg Focus. 45:E14(2018).

[97]. Laird MD, Shields JS, Sukumari-Ramesh S, Kimbler DE, Fessler RD, Shakir B, Youssef P, Yanasak N, Vender JR, Dhandapani KM. High mobility group box protein-1 promotes cerebral edema after traumatic brain injury via activation of toll-like receptor 4. Glia. 62: 26–38(2014).

[98]. Langhan ML. Acute alcohol intoxication in adolescents: frequency of respiratory depression. J Emerg Med. 44:1063-9(2013).

[99]. Lee SW, de Rivero Vaccari JP, Truettner JS, Dietrich WD, Keane RW. The role of microglial inflammasome activation in pyroptotic cell death following penetrating traumatic brain injury. J Neuroinflammation. 16:27(2019).

[100]. Lehner M, Kellert B, Proff J, Schmid MA, Diessenbacher P, Ensser A, Dörrie J, Schaft N, Leverkus M, Kämpgen E, Holter W. Autocrine TNF Is Critical for the Survival of Human Dendritic Cells by Regulating BAK, BCL-2, and FLIP L. J Immunol. 188: 4810-4818(2012).

[101]. Lelouard H, Schmidt EK, Camosseto V, Clavarino G, Ceppi M, Hsu HT, Pierre P. Regulation of translation is required for dendritic cell function and survival during activation. J Cell Biol. 179: 1427-1439(2007).

[102]. Lewén A, Matz P, Chan PH. Free radical pathways in CNS injury. J Neurotrauma. 17: 871-90(2000).

[103]. Lewis SM, Williams A, Eisenbarth SC. Structure and function of the immune system in the spleen. Sci Immunol. 4 :eaau6085 (2019).

[104]. Li M, Li F, Luo C, Shan Y, Zhang L, Qian Z, Zhu G, Lin J, Feng H. Immediate splenectomy decreases mortality and improves cognitive function of rats after severe traumatic brain injury. J Trauma. 71: 141-147(2011).

[105]. Li R, Zou X, Huang H, Yu Y, Zhang H, Liu P, Pan S, Ouyang Y, Shang Y. HMGB1/PI3K/Akt/mTOR Signaling Participates in the Pathological Process of Acute Lung Injury by Regulating the Maturation and Function of Dendritic Cells. Front Immunol. 11:1104. (2020).

[106]. Li S, Zaninotto AL, Neville IS, Paiva WS, Nunn D, Fregni F. Clinical utility of brain stimulation modalities following traumatic brain injury: current evidence. Neuropsychiatr Dis Treat. 11:1573-86(2015).

[107]. Li Z, Zhang J, Halbgebauer S, Chandrasekar A, Rehman R, Ludolph A, Boeckers

T, Huber-Lang M, Otto M, Roselli F, Heuvel FO. Differential effect of ethanol intoxication on peripheral markers of cerebral injury in murine blunt traumatic brain injury. Burns Trauma. 9:tkab027(2021).

[108]. Liao Y, Liu P, Guo F, Zhang ZY, Zhang Z. Oxidative Burst of Circulating Neutrophils Following Traumatic Brain Injury in Human. PLoS One. 8:e68963 (2013).

[109]. Liu X, Connaghan KP, Wei Y, Yang Z, Li MD, Chang SL. Involvement of the Hippocampus in Binge Ethanol-Induced Spleen Atrophy in Adolescent Rats. Alcohol Clin Exp Res. 40:1489-1500(2016).

[110]. Liu X, Mao X, Chang SL. Altered gene expression in the spleen of adolescent rats following high ethanol concentration binge drinking. Int J Clin Exp Med. 4:252-257(2011).

[111]. Loh KP, Huang SH, De Silva R, Tan BK, Zhu YZ. Oxidative stress: apoptosis in neuronal injury. Curr Alzheimer Res. 3:327-37(2006).

[112]. Losiniecki A, Shutter L. Management of traumatic brain injury. Curr Treat Options Neurol. 12: 142-54(2010).

[113]. Lucke-Wold BP, Logsdon AF, Nguyen L, Eltanahay A, Turner RC, Bonasso P, Knotts C, Moeck A, Maroon JC, Bailes JE, Rosen CL. Supplements, nutrition, and alternative therapies for the treatment of traumatic brain injury. Nutr Neurosci. 21:79-91(2018).

[114]. Lutton EM, Razmpour R, Andrews AM, Cannella LA, Son YJ, Shuvaev VV, Muzykantov VR, Ramirez SH. Acute administration of catalase targeted to ICAM-1 attenuates neuropathology in experimental traumatic brain injury. Sci Rep. 7: 3846(2017).

[115]. Łyszkiewicz M, Witzlau K, Pommerencke J, Krueger A. Chemokine receptor CX3CR1 promotes dendritic cell development under steady-state conditions. Eur J Immunol. 41: 1256-1265(2011).

[116]. Maas AIR, Menon DK, Adelson PD, Andelic N, Bell MJ, Belli A, Bragge P, Brazinova

A, Büki A, Chesnut RM, Citerio G, Coburn M, Cooper DJ, Crowder AT, Czeiter E, Czosnyka M, Diaz-Arrastia R, Dreier JP, Duhaime AC, Ercole A, van Essen TA, Feigin VL, Gao G, Giacino J, Gonzalez-Lara LE, Gruen RL, Gupta D, Hartings JA, Hill S, Jiang JY, Ketharanathan N, Kompanje EJO, Lanyon L, Laureys S, Lecky F, Levin H, Lingsma HF, Maegele M, Majdan M, Manley G, Marsteller J, Mascia L, McFadyen C, Mondello S, Newcombe V, Palotie A, Parizel PM, Peul W, Piercy J, Polinder S, Puybasset L, Rasmussen TE, Rossaint R, Smielewski P, Söderberg J, Stanworth SJ, Stein MB, von Steinbüchel N, Stewart W, Steyerberg EW, Stocchetti N, Synnot A, Te Ao B, Tenovuo O, Theadom A, Tibboel D, Videtta W, Wang KKW, Williams WH, Wilson L, Yaffe K; InTBIR Participants and Investigators. Traumatic brain injury: integrated approaches to improve prevention, clinical care, and research. Lancet Neurol. 16:987-1048(2017).

[117]. Madsen T, Erlangsen A, Orlovska S, Mofaddy R, Nordentoft M, Benros ME. Association Between Traumatic Brain Injury and Risk of Suicide. JAMA. 320:580-588(2018).

[118]. Mahnke K, Ring S, Enk AH. Antibody Targeting of "Steady-State" Dendritic Cells Induces Tolerance Mediated by Regulatory T Cells. Front Immunol. 7:63(2016).

[119]. Martin-Jiménez C, Gaitán-Vaca DM, Areiza N. Astrocytes Mediate Protective Actions of Estrogenic Compounds after Traumatic Brain Injury. Neuroendocrinology. 108:142-160(2019).

[120]. McDonald SJ, Sharkey JM, Sun M, Kaukas LM, Shultz SR, Turner RJ, Leonard AV, Brady RD, Corrigan F. Beyond the Brain: Peripheral Interactions after Traumatic Brain Injury. J Neurotrauma. 37: 770-781(2020).

[121]. Melani A, Pantoni L, Corsi C, Bianchi L, Monopoli A, Bertorelli R, Pepeu G, Pedata F. Striatal outflow of adenosine, excitatory amino acids, gamma-aminobutyric acid, and taurine in awake freely moving rats after middle cerebral artery occlusion: correlations with neurological deficit and histopathological damage. Stroke. 30:2448-2454; discussion 2455(1999).

[122]. Mendes A, Gigan JP, Rodriguez Rodrigues C, Choteau SA, Sanseau D, Barros D,

Almeida C, Camosseto V, Chasson L, Paton AW, Paton JC, Argüello RJ, Lennon-Duménil AM, Gatti E, Pierre P. Proteostasis in dendritic cells is controlled by the PERK signaling axis independently of ATF4, Life Sci Alliance. 4:e202000865(2021).

[123]. Menges M, Rössner S, Voigtländer C, Schindler H, Kukutsch NA, Bogdan C, Erb K, Schuler G, Lutz MB. Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity. J Exp Med. 195:15-21(2002).

[124]. Merad M, Manz MG. Dendritic cell homeostasis. Blood. 113:3418-27(2009).

[125]. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. Annu Rev Immunol. 31: 563-604(2013).

[126]. Merrill JE, Benveniste EN. Cytokines in inflammatory brain lesions: helpful and harmful. Trends Neurosci. 19:331-8(1996).

[127]. Mondello S, Robicsek SA, Gabrielli A, Brophy GM, Papa L, Tepas J, Robertson C, Buki A, Scharf D, Jixiang M, Akinyi L, Muller U, Wang KK, Hayes RL. αII-spectrin breakdown products (SBDPs): diagnosis and outcome in severe traumatic brain injury patients. J Neurotrauma. 27:1203-13(2010).

[128]. Mrakovcic-Sutic I, Tokmadzic VS, Laskarin G, Mahmutefendic H, Lucin P, Zupan Z, Sustic A. Early changes in frequency of peripheral blood lymphocyte subpopulations in severe traumatic brain-injured patients. Scand J Immunol. 72: 57-65(2010).

[129]. Mutch CA, Talbott JF, Gean A. Imaging Evaluation of Acute Traumatic Brain Injury. Neurosurg Clin N Am. 27:409-39(2016).

[130]. Nagayama H, Sato K, Kawasaki H, Enomoto M, Morimoto C, Tadokoro K, Juji T, Asano S, Takahashi TA. IL-12 Responsiveness and Expression of IL-12 Receptor in Human Peripheral Blood Monocyte-Derived Dendritic Cells. J Immunol. 165: 59-66(2000).

[131]. Needham EJ, Helmy A, Zanier ER, Jones JL, Coles AJ, Menon DK. The

immunological response to traumatic brain injury. J Neuroimmunol. 332:112-125(2019).

[132]. Needham EJ, Stoevesandt O, Thelin EP, Zetterberg H, Zanier ER, Al Nimer F, Ashton NJ, Outtrim JG, Newcombe VFJ, Mousa HS, Simrén J, Blennow K, Yang Z, Hutchinson PJ, Piehl F, Helmy AE, Taussig MJ, Wang KKW, Jones JL, Menon DK, Coles AJ. Complex Autoantibody Responses Occur following Moderate to Severe Traumatic Brain Injury. J Immunol. 207: 90-100(2021).

[133]. Nicholson LB, Raveney BJ, Munder M. Monocyte dependent regulation of autoimmune inflammation. Curr Mol Med. 9:23-29(2009).

[134]. Niemeyer M, Jochems D, Houwert RM, van Es MA, Leenen L, van Wessem K. Mortality in polytrauma patients with moderate to severe TBI on par with isolated TBI patients: TBI as last frontier in polytrauma patients. Injury. 53:1443-1448(2022).

[135]. Nijhuis LE, Olivier BJ, Dhawan S, Hilbers FW, Boon L, Wolkers MC, Samsom JN, de Jonge WJ. Adrenergic β2 receptor activation stimulates anti-inflammatory properties of dendritic cells in vitro. PLoS One. 9:e85086 (2014).

[136]. Oellerich T, Mohr S, Corso J, Beck J, Döbele C, Braun H, Cremer A, Münch S, Wicht J, Oellerich MF, Bug G, Bohnenberger H, Perske C, Schütz E, Urlaub H, Serve H. FLT3-ITD and TLR9 use Bruton tyrosine kinase to activate distinct transcriptional programs mediating AML cell survival and proliferation. Blood. 125: 1936–1947(2015).

[137]. Okuaki Y, Miyazaki H, Zeniya M, Ishikawa T, Ohkawa Y, Tsuno S, Sakaguchi M, Hara M, Takahashi H, Toda G. Splenectomy-reduced hepatic injury induced by ischemia/reperfusion in the rat. Liver. 16: 188-194(1996).

[138]. Olde Heuvel F, Holl S, Chandrasekar A, Li Z, Wang Y, Rehman R, Förstner P, Sinske D, Palmer A, Wiesner D, Ludolph A, Huber-Lang M, Relja B, Wirth T, Röszer T, Baumann B, Boeckers T, Knöll B, Roselli F. STAT6 mediates the effect of ethanol on neuroinflammatory response in TBI. Brain Behav Immun. 81: 228-246(2019).

[139]. Oliver DMA, Reddy PH. Small molecules as therapeutic drugs for Alzheimer's

disease. Mol Cell Neurosci. 96: 47-62(2019).

[140]. Oth T, Vanderlocht J, Van Elssen CH, Bos GM, Germeraad WT. Pathogen-Associated Molecular Patterns Induced Crosstalk between Dendritic Cells, T Helper Cells, and Natural Killer Helper Cells Can Improve Dendritic Cell Vaccination. Mediators Inflamm. 2016:5740373 (2016).

[141]. Palomino DC, Marti LC. Chemokines and immunity, Einstein (Sao Paulo).13: 469–473(2015).

[142]. Papa L, Robicsek SA, Brophy GM, Wang KKW, Hannay HJ, Heaton S, Schmalfuss I, Gabrielli A, Hayes RL, Robertson CS. Temporal Profile of Microtubule-Associated Protein 2: A Novel Indicator of Diffuse Brain Injury Severity and Early Mortality after Brain Trauma. J Neurotrauma. 35:32-40(2018).

[143]. Papadopoulos EJ, Sassetti C, Saeki H, Yamada N, Kawamura T, Fitzhugh DJ, Saraf MA, Schall T, Blauvelt A, Rosen SD, Hwang ST. Fractalkine, a CX3C chemokine, is expressed by dendritic cells and is up-regulated upon dendritic cell maturation. Eur J Immunol. 29: 2551-2559(1999).

[144]. Parker TM, Nguyen AH, Rabang JR, Patil AA, Agrawal DK. The danger zone: Systematic review of the role of HMGB1 danger signalling in traumatic brain injury. Brain Inj. 31: 2-8(2017).

[145]. Parlet CP, Schlueter AJ. Mechanisms by which chronic ethanol feeding impairs the migratory capacity of cutaneous dendritic cells. Alcohol Clin Exp Res. 37:2098-107(2013).

[146]. Paudel YN, Shaikh MF, Chakraborti A, Kumari Y, Aledo-Serrano Á, Aleksovska K, Alvim MKM, Othman I. HMGB1: A common biomarker and potential target for TBI, neuroinflammation, epilepsy, and cognitive dysfunction. Front Neurosci. 12:628. (2018).

[147]. Pavlovic D, Pekic S, Stojanovic M, Popovic V. Traumatic brain injury: neuropathological, neurocognitive and neurobehavioral sequelae. Pituitary. 22:270-

282(2019).

[148]. Peeters W, van den Brande R, Polinder S, Brazinova A, Steyerberg EW, Lingsma HF, Maas AI. Epidemiology of traumatic brain injury in Europe. Acta Neurochir (Wien). 157:1683-1696(2015).

[149]. Phelan H, Stahls P, Hunt J, Bagby GJ, Molina PE. Impact of alcohol intoxication on hemodynamic, metabolic, and cytokine responses to hemorrhagic shock. J Trauma. 52:675-82(2002).

[150]. Picotto G, Morse LR, Nguyen N, Saltzman J, Battaglino R. TMEM176A and TMEM176B Are Candidate Regulators of Inhibition of Dendritic Cell Maturation and Function after Chronic Spinal Cord Injury. J Neurotrauma. 37: 528–533(2020).

[151]. Pike BR, Zhao X, Newcomb JK, Glenn CC, Anderson DK, Hayes RL. Stretch injury causes calpain and caspase-3 and necrotic and apoptotic cell death in septo-hippocampal cell cultures. Neurotrauma. 17: 283-298(2000).

[152]. Pollack KE, Meneveau MO, Melssen MM, Lynch KT, Koeppel AF, Young SJ, Turner S, Kumar P, Sol-Church K, Mauldin IS, Slingluff CL Jr. Incomplete Freund's adjuvant reduces arginase and enhances Th1 dominance, TLR signaling and CD40 ligand expression in the vaccine site microenvironment. J Immunother Cancer. 8:e000544 (2020).

[153]. Ponsford J. Factors contributing to outcome following traumatic brain injury. NeuroRehabilitation. 32:803-15(2013).

[154]. Povlishock JT, Kontos HA. The role of oxygen radicals in the pathobiology of traumatic brain injury. Hum Cell. 5:345-53(1992).

[155]. Praveen C, Bhatia SS, Alaniz RC, Droleskey RE, Cohen ND, Jesudhasan PR, Pillai SD. Assessment of microbiological correlates and immunostimulatory potential of electron beam inactivated metabolically active yet non culturable (MAyNC) Salmonella Typhimurium. PLoS One. 16: e0243417 (2021).

[156]. Puntambekar SS, Saber M, Lamb BT, Kokiko-Cochran ON. Cellular players that

shape evolving pathology and neurodegeneration following traumatic brain injury. Brain Behav Immun. 71:9-17(2018).

[157]. Qian Y, Gao C, Zhao X, Song Y, Luo H, An S, Huang J, Zhang J, Jiang R. Fingolimod Attenuates Lung Injury and Cardiac Dysfunction after Traumatic Brain Injury. J Neurotrauma. 37: 2131-2140(2020).

[158]. Rachfalska N, Putowski Z, Krzych ŁJ. Distant Organ Damage in Acute Brain Injury. Brain Sci. 10:1019(2020).

[159]. Rael LT, Bar-Or R, Mains CW, Slone DS, Levy AS, Bar-Or D. Plasma oxidation-reduction potential and protein oxidation in traumatic brain injury. J Neurotrauma. 26: 1203-1211(2009).

[160]. Raj R, Mikkonen ED, Siironen J, Hernesniemi J, Lappalainen J, Skrifvars MB. Alcohol and mortality after moderate to severe traumatic brain injury: A meta-analysis of observational studies. J Neurosurg. 124: 1684-1692(2016).

[161]. Rao W, Zhang L, Peng C, Hui H. Downregulation of STIM2 improves neuronal survival after traumatic brain injury by alleviating calcium overload and mitochondrial dysfunction. Biochim Biophys Acta. 1852:2402-13(2015).

[162]. Relja B, Menke J, Wagner N, Auner B, Voth M, Nau C, Marzi I. Effects of positive blood alcohol concentration on outcome and systemic interleukin-6 in major trauma patients. Injury. 47: 640-645(2016).

[163]. Rheinländer A, Schraven B, Bommhardt U. CD45 in human physiology and clinical medicine, Immunol Lett. 196: 22-32(2018).

[164]. Rice VJ, Schroeder PJ, Cassenti DN, Boykin GL. The Effect of Traumatic Brain Injury (TBI) on Cognitive Performance in a Sample of Active Duty U.S. Military Service Members. Mil Med. 185:184-189(2020).

[165]. Rohlwink UK, Figaji AA. Biomarkers of brain injury in cerebral infections. Clin Chem. 60:823-34(2014).

[166]. Rosas-Ballina M, Tracey KJ. The Neurology of the Immune System: Neural Reflexes Regulate Immunity. Neuron. 64: 28-32(2009).

[167]. Rowe RK, Ellis GI, Harrison JL, Bachstetter AD. Diffuse traumatic brain injury induces prolonged immune dysregulation and potentiates hyperalgesia following a peripheral immune challenge. Mol Pain. 12: 1744806916647055(2016).

[168]. Sabet N, Soltani Z, Khaksari M. Multipotential and systemic effects of traumatic brain injury. J Neuroimmunol. 357:577619(2021).

[169]. Saftig P, Klumperman J. Lysosome biogenesis and lysosomal membrane proteins: Trafficking meets function. Nat Rev Mol Cell Biol. 10: 623-635(2009).

[170]. Sande A, West C. Traumatic brain injury: a review of pathophysiology and management. J Vet Emerg Crit Care (San Antonio). 20:177-90(2010).

[171]. Sathaliyawala T, O'Gorman WE, Greter M, Bogunovic M, Konjufca V, Hou ZE, Nolan GP, Miller MJ, Merad M, Reizis B. Mammalian target of rapamycin controls dendritic cell development downstream of Flt3 ligand signaling. Immunity. 33: 597–606(2010).

[172]. Schanne FA, Kane AB, Young EE, Farber JL. Calcium dependence of toxic cell death: a final common pathway. Science. 206:700-702(1979).

[173]. Scheffler JM, Sparber F, Tripp CH, Herrmann C, Humenberger A, Blitz J, Romani N, Stoitzner P, Huber LA. LAMTOR2 regulates dendritic cell homeostasis through FLT3-dependent mTOR signalling. Nat Commun. 5:5138(2014).

[174]. Schwulst SJ, Trahanas DM, Saber R, Perlman H. Traumatic brain injury-induced alterations in peripheral immunity. J Trauma Acute Care Surg. 75:780-788(2013).

[175]. Shamim AA, Zafar SN, Nizam W, Zeineddin A, Ortega G, Fullum TM, Tran DD. Laparoscopic Splenectomy for Trauma. JSLS. 22:e2018.00050(2018).

[176]. Shandro JR, Rivara FP, Wang J, Jurkovich GJ, Nathens AB, MacKenzie EJ. Alcohol and risk of mortality in patients with traumatic brain injury. J Trauma. 66:1584-90.

(2009).

[177]. Sharp F, Liu DZ, Zhan X, Ander BP. Intracerebral hemorrhage injury mechanisms: glutamate neurotoxicity, thrombin, and Src. Acta Neurochir Suppl. 105:43-6(2008).

[178]. Shaw K, MacKinnon MA, Raghupathi R, Saatman KE, Mclntosh TK, Graham DI. TUNEL-positive staining in white and grey matter after fatal head injury in man. Clip Neuropathol. 20: 106-112(2001).

[179]. Shively S, Scher AI, Perl DP, Diaz-Arrastia R. Dementia resulting from traumatic brain injury: what is the pathology? Arch Neurol. 69:1245-51(2012).

[180]. Shohami E, Ginis I, Hallenbeck JM. Dual role of tumor necrosis factor alpha in brain injury. Cytokine Growth Factor Rev. 10:119-30(1999).

[181]. Sholkina MN, Lebedev MY, Babaev AA, Baryshnikov AY, Novikov VV. Effect of brain injury on immunophenotype of peripheral blood lymphocytes in rats. Russ J Immunol. 7: 365-370(2002).

[182]. Siggins RW, Bagby GJ, Molina P, Dufour J, Nelson S, Zhang P. Alcohol exposure impairs myeloid dendritic cell function in rhesus macaques. Alcohol Clin Exp Res. 33: 1524– 1531(2009).

[183]. Smith LGF, Milliron E, Ho ML, Hu HH, Rusin J, Leonard J, Sribnick EA. Advanced neuroimaging in traumatic brain injury: an overview. Neurosurg Focus. 47:E17(2019).

[184]. Stoitzner P, Holzmann S, McLellan AD, Ivarsson L, Stössel H, Kapp M, Kämmerer U, Douillard P, Kämpgen E, Koch F, Saeland S, Romani N. Visualization and characterization of migratory Langerhans cells in murine skin and lymph nodes by antibodies against Langerin/CD207. J Invest Dermatol. 120: 266-274(2003).

[185]. Sukhbaatar N, Hengstschläger M, Weichhart T. mTOR-Mediated Regulation of Dendritic Cell Differentiation and Function. Trends Immunol. 37:778-789(2016).

[186]. Sullivan PG, Rabchevsky AG, Waldmeier PC, Springer JE. Mitochondrial

permeability transition in CNS trauma: cause or effect of neuronal cell death? J Neurosci Res. 79:231-239(2005).

[187]. Sullivan PG, Thompson MB, Scheff SW. Cyclosporin A attenuates acute mitochondrial dysfunction following traumatic brain injury. Exp Neurol. 160:226-34(1999).

[188]. Sun DA, Deshpande LS, Sombati S, Baranova A, Wilson MS, Hamm RJ, DeLorenzo RJ. Traumatic brain injury causes a long-lasting calcium (Ca2+)-plateau of elevated intracellular Ca levels and altered Ca2+ homeostatic mechanisms in hippocampal neurons surviving brain injury. Eur J Neurosci. 27:1659-72(2008).

[189]. Sun M, Zhao Y, Gu Y, Zhang Y. Protective effects of taurine against closed head injury in rats. J Neurotrauma. 32:66-74(2015).

[190]. Sun Y, Bai L, Niu X, Wang Z, Yin B, Bai G, Zhang D, Gan S, Sun C, Wang S, Zhu F, Zhang M. Elevated serum levels of inflammation-related cytokines in mild traumatic brain injury are associated with cognitive performance. Front Neurol. 10 :1120 (2019).

[191]. Szabo G, Catalano D, White B, Mandrekar P. Acute alcohol consumption inhibits accessory cell function of monocytes and dendritic cells. Alcohol Clin Exp Res. 28:824-828(2004).

[192]. Szabo G, Dolganiuc A, Mandrekar P, White B. Inhibition of antigen-presenting cell functions by alcohol: implications for hepatitis C virus infection. Alcohol. 33:241-249(2004).

[193]. Takenaka MC, Araujo LP, Maricato JT, Nascimento VM, Guereschi MG, Rezende RM, Quintana FJ, Basso AS. Norepinephrine Controls Effector T Cell Differentiation through β 2 - Adrenergic Receptor–Mediated Inhibition of NF-κB and AP-1 in Dendritic Cells. J Immunol. 196: 637-644(2016).

[194]. Takenaka MC, Guereschi MG, Basso AS. Neuroimmune interactions: dendritic cell modulation by the sympathetic nervous system. Semin Immunopathol. 39:165-176. (2017).

[195]. Tan HP, Guo Q, Hua G, Chen JX, Liang JC. Inhibition of endoplasmic reticulum stress alleviates secondary injury after traumatic brain injury. Neural Regen Res. 13:827-836(2018).

[196]. Tan JK, O'Neill HC. Maturation requirements for dendritic cells in T cell stimulation leading to tolerance versus immunity. J Leukoc Biol. 78: 319-324(2005).

[197]. Taylor CA, Bell JM, Breiding MJ, Xu L. Traumatic Brain Injury-Related Emergency Department Visits, Hospitalizations, and Deaths - United States, 2007 and 2013. MMWR Surveill Summ. 66:1-16. (2017).

[198]. Tinsley KW, Grayson MH, Swanson PE, Drewry AM. Sepsis induces apoptosis and profound depletion of splenic interdigitating and follicular dendritic cells. J Immunol. 171:909-914(2003).

[199]. Trombetta ES, Ebersold M, Garrett W, Pypaert M, Mellman I. Activation of lysosomal function during dendritic cell maturation. Science. 299: 1400-1403(2003).

[200]. Tuttolomondo A, Pecoraro R, Pinto A. Studies of selective TNF inhibitors in the treatment of brain injury from stroke and trauma: a review of the evidence to date. Drug Des Devel Ther. 8:2221-38(2014).

[201]. Veinotte L, Gebremeskel S, Johnston B. CXCL16-positive dendritic cells enhance invariant natural killer T cell-dependent IFNγ production and tumor control, Oncoimmunology. 5:e1160979 (2016).

[202]. Vella MA, Crandall ML, Patel MB. Acute Management of Traumatic Brain Injury. Surg Clin North Am. 97:1015-1030 (2017).

[203]. Verboon LN, Patel HC, Greenhalgh AD. The Immune System's Role in the Consequences of Mild Traumatic Brain Injury (Concussion). Front Immunol. 12:620698 (2021).

[204]. Vermeij JD, Aslami H, Fluiter K, Roelofs JJ, van den Bergh WM, Juffermans NP, Schultz MJ, Van der Sluijs K, van de Beek D, van Westerloo DJ. Traumatic brain injury in rats induces lung injury and systemic immune suppression. J Neurotrauma. 30:

2073-2079(2013).

[205]. Villapol S, Kryndushkin D, Balarezo MG, Campbell AM, Saavedra JM, Shewmaker FP, Symes AJ. Hepatic Expression of Serum Amyloid A1 Is Induced by Traumatic Brain Injury and Modulated by Telmisartan. Am J Pathol. 185: 2641-2652(2015).

[206]. Wagner AK, Zitelli KT. A Rehabilomics focused perspective on molecular mechanisms underlying neurological injury, complications, and recovery after severe TBI. Pathophysiology. 20:39-48(2013).

[207]. Wagner N, Akbarpour A, Mörs K, Voth M, Störmann P, Auner B, Lehnert M, Marzi I, Relja B. Alcohol Intoxication Reduces Systemic Interleukin-6 Levels and Leukocyte Counts after Severe TBI Compared with Not Intoxicated TBI Patients. Shock. 46: 261-269(2016).

[208]. Wagner N, Dieteren S, Franz N, Köhler K, Perl M, Marzi I, Relja B. Alcohol-induced attenuation of post-traumatic inflammation is not necessarily liver-protective following trauma/hemorrhage. Int J Mol Med. 44: 1127-1138(2019).

[209]. Wagner N, Franz N, Dieteren S, Perl M, Mörs K, Marzi I, Relja B. Acute Alcohol Binge Deteriorates Metabolic and Respiratory Compensation Capability After Blunt Chest Trauma Followed by Hemorrhagic Shock-A New Research Model. Alcohol Clin Exp Res. 41:1559-1567(2017).

[210]. Wang C, Liu S, Xu J, Gao M, Qu Y, Liu Y, Yang Y, Cui X. Dissolvable microneedles based on Panax notoginseng polysaccharide for transdermal drug delivery and skin dendritic cell activation. Carbohydr Polym. 268:118211 (2021).

[211].Wang F, Flanagan J, Su N, Wang LC, Bui S, Nielson A, Wu X, Vo HT, Ma XJ, Luo Y. RNAscope: A novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J Mol Diagnostics. 14: 22-29(2012).

[212]. Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, Li JH, Wang H, Yang H, Ulloa L, Al-Abed Y, Czura CJ, Tracey KJ. Nicotinic acetylcholine receptor α7 subunit is an essential regulator of inflammation. Nature. 421: 384-388(2003).

[213]. Wang HX, Yi SQ, Li J, Terayama H. Effects of splenectomy on spontaneously chronic pancreatitis in aly/aly mice. Clin Dev Immunol. 2010:614890(2010).

[214]. Wang KK, Yang Z, Zhu T, Shi Y, Rubenstein R, Tyndall JA, Manley GT. An update on diagnostic and prognostic biomarkers for traumatic brain injury. Expert Rev Mol Diagn. 18:165-180(2018).

[215]. Waskow C, Liu K, Darrasse-Jèze G, Guermonprez P, Ginhoux F, Merad M, Shengelia T, Yao K, Nussenzweig M. The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues. Nat Immunol. 9:676-683(2008).

[216]. Webster KM, Sun M, Crack PJ, O'Brien TJ, Shultz SR, Semple BD. Age-dependent release of high-mobility group box protein-1 and cellular neuroinflammation after traumatic brain injury in mice. J Comp Neurol. 527: 1102-1117(2019).

[217]. Wei Y, Chang L, Hashimoto K. Molecular mechanisms underlying the antidepressant actions of arketamine: beyond the NMDA receptor. Mol Psychiatry. 27:559-573. (2021).

[218]. Weston NM, Sun D. The Potential of Stem Cells in Treatment of Traumatic Brain Injury. Curr Neurol Neurosci Rep. 18:1(2018).

[219]. Williams S, Raghupathi R, MacKinnon MA, McIntosh TK, Saatman KE, Graham DI. In situ DNA fragmentation occurs in whitematter up to 12 months after head injury in man. Acta Neuropathol. 102: 581-590(2001).

[220]. Witte E, Kokolakis G, Witte K, Philipp S, Doecke WD, Babel N, Wittig BM, Warszawska K, Kurek A, Erdmann-Keding M, Kunz S, Asadullah K, Kadin ME, Volk HD, Sterry W, Wolk K, Sabat R. IL-19 is a component of the pathogenetic IL-23/IL-17 cascade in psoriasis. J Invest Dermatol. 134: 2757-2767(2014).

[221]. Woodcock T, Morganti-Kossmann MC. The role of markers of inflammation in traumatic brain injury. Front Neurol. 4: 18(2013).

[222]. Wu H, Chen J, Song S, Yuan P, Liu L, Zhang Y, Zhou A, Chang Y, Zhang L, Wei W.

β2-adrenoceptor signaling reduction in dendritic cells is involved in the inflammatory response in adjuvant-induced arthritic rats. Sci Rep. 6:24548 (2016).

[223]. Xiong Y, Gu Q, Peterson PL, Muizelaar JP, Lee CP. Mitochondrial dysfunction and calcium perturbation induced by traumatic brain injury. J Neurotrauma. 14:23-34(1997).

[224]. Xiong Y, Mahmood A, Chopp M. Current understanding of neuroinflammation after traumatic brain injury and cell-based therapeutic opportunities. Chin J Traumatol. 21:137-151(2018).

[225]. Xu B, Chandrasekar A, Olde Heuvel F, Powerski M, Nowak A, Noack L, Omari J, Huber-Lang M, Roselli F, Relja B. Ethanol intoxication alleviates the inflammatory response of remote organs to experimental traumatic brain injury. Int J Mol Sci. 21: 1-15(2020).

[226]. Yang J, Korley FK, Dai M, Everett AD. Serum neurogranin measurement as a biomarker of acute traumatic brain injury. Clin Biochem. 48:843-848(2015).

[227]. Yang L, Wang F, Yang L, Yuan Y, Chen Y, Zhang G, Fan Z. HMGB1 a-Box Reverses Brain Edema and Deterioration of Neurological Function in a Traumatic Brain Injury Mouse Model. Cell Physiol Biochem. 46: 2532-2542(2018).

[228]. Yang W, Liu Y, Liu B, Tan H, Lu H, Wang H, Yan H. Treatment of surgical brain injury by immune tolerance induced by intrathymic and hepatic portal vein injection of brain antigens. Sci Rep. 6:32030(2016).

[229]. Yang Y, Boza-Serrano A, Dunning CJR, Clausen BH, Lambertsen KL, Deierborg T. Inflammation leads to distinct populations of extracellular vesicles from microglia. J Neuroinflammation. 15: 168(2018).

[230]. Yi L, Juan W, Gang C, Leiming Z, Jianning Z. Seawater Immersion Aggravates Early Mitochondrial Dysfunction and Increases Neuronal Apoptosis After Traumatic Brain Injury. Cell Mol Neurobiol. 40:447-457(2020).

[231]. Yue JK, Ngwenya LB, Upadhyayula PS, Deng H, Winkler EA, Burke JF, Lee YM,

Robinson CK, Ferguson AR, Lingsma HF, Cnossen MC, Pirracchio R, Korley FK, Vassar MJ, Yuh EL, Mukherjee P, Gordon WA, Valadka AB, Okonkwo DO, Manley GT; TRACK-TBI Investigators. Emergency department blood alcohol level associates with injury factors and six-month outcome after uncomplicated mild traumatic brain injury. J Clin Neurosci. 45: 293-298(2017).

[232]. Zhang B, Zhong J, Gao Z. A Brain-Spleen Axis Regulates Humoral Immunity. Neurosci Bull. 37:427-429 (2021).

[233]. Zhang J, Li Z, Chandrasekar A, Li S, Ludolph A, Boeckers TM, Huber-Lang M, Roselli F, Olde Heuvel F. Fast Maturation of Splenic Dendritic Cells Upon TBI Is Associated With FLT3/FLT3L Signaling. Front Immunol. 13:824459(2022).

[234]. Zhang L, Wang H. Autophagy in Traumatic Brain Injury: A New Target for Therapeutic Intervention. Front Mol Neurosci. 11:190 (2018).

[235]. Zhang X, Chen Y, Jenkins LW, Kochanek PM, Clark RS. Bench-to-bedside review: Apoptosis/programmed cell death triggered by traumatic brain injury. Crit Care. 9:66-75 (2005).

[236]. Zhang X, Lei B, Yuan Y, Zhang L, Hu L, Jin S, Kang B, Liao X, Sun W, Xu F, Zhong Y, Hu J, Qi H. Brain control of humoral immune responses amenable to behavioural modulation. Nature. 581: 204–208(2020).

[237]. Zhang X, Lei B, Yuan Y, Zhang L, Hu L. Brain control of humoral immune responses amenable to behavioural modulation. Nature. 581:204-208 (2020).

[238]. Zhou R, Zhang J, Bu W, Zhang W, Duan B, Wang X, Yao L, Li Z, Li J. A New Role for the Spleen: Aggravation of the Systemic Inflammatory Response in Rats with Severe Acute Pancreatitis. Am J Pathol. 189:2233-2245 (2019).

[239]. Zink BJ, Schultz CH, Wang X, Mertz M, Stern SA, Betz AL. Effects of ethanol on brain lactate in experimental traumatic brain injury with hemorrhagic shock. Brain Res. 837:1-7(1999).

[240]. Zoerle T, Carbonara M, Zanier ER, Ortolano F. Rethinking Neuroprotection in

Severe Traumatic Brain Injury: Toward Bedside Neuroprotection. Front Neurol. 8:354 (2017).

7 Acknowledgements

Many people have selflessly provided me with assistance in the completion of this thesis. I send my sincerest regards and best wishes to all those who have supported me in any way during the completion of my studies. I would like take this chance to thank especially:

First of all, I would like to thank my parents for their support during my studies at Ulm University. I would also like to thank my wife, who is studying for a PhD in neuroscience at the University of Tübingen, and we have supported each other academically and in life. She has taken on most of the tasks of raising my son since he arrived in Germany.

I would like to thank the China Scholarship Council for providing me with a scholarship to study in Germany. With this funding, I can devote myself to scientific research without having to worry about financial resources.

I would like to thank the Head of the Department of Neurology, Professor Med Albert Christian Ludolph, for giving me the opportunity to work in the Neural Network Laboratory at Ulm University. I would like to thank him for providing me with a modern laboratory, excellent working conditions and a wonderful learning environment.

I am deeply grateful to Prof. Dr. Francesco Roselli, my doctoral thesis mentor, for having always an open door, for his endless enthusiasm, wisdom and immense knowledge. He is diligent and dedicated, and he has been a lifelong role model for me. His professional guidance and support has helped me throughout all the time I have spent researching and writing this thesis.

I am particularly grateful to my colleague Florian olde Heuvel for his enthusiastic introduction to the project and for his kind guidance and cooperation throughout the experiments. He also taught me some of the experimental techniques to operate. He was very attentive and patient. His rigorous approach to research has given me great help and courage.

I would like to thank all my colleagues in Prof. Francesco Roselli's lab. They have always given me support and care in my work and in my life. I would like to thank all the colleagues in Prof. Markus Huber-Lang's lab who work closely with our lab. I would like to thank them for the selfless help they have given me in my work.

I would like to thank my family for all the help they have given me. My wife, WeipingWang, and my son, Jingduo Zhang, accompany me to study hard and grow together.

I am particularly grateful to Linyun Tang, Zhenghui Li, Jialei Song, Shun Li and all my Chinese friends in Ulm, whose names I have not listed, for their friendship and lots of encouragement during my studies in Germany. We have helped each other in life and built a strong friendship. I would also like to thank my relatives and friends in China who, despite the distance, still gave me a lot of support and always encouraged me.

8 Curriculum vitae

Personal Data

Name: Jin Zhang

Date of birth: 1989 in Zibo, China