Cellular and Molecular Responses to Traumatic Brain Injury

LÖÖV CAMILLA
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Abstract

Traumatic brain injury (TBI) is a relatively unknown disease considering the tens of millions of people affected around the world each year. Many TBI patients die from their injuries and survivors often suffer from life-long disabilities. The primary injury initiates a variety of cellular and molecular processes that are both beneficial and detrimental for the brain, but that are not fully understood. The focus of this thesis has been to study the role of astrocytes in clearance of dead cells after TBI and to identify injury specific proteins that may function as biomarkers, by using cell cultures, animal models and in cerebrospinal fluid (CSF) from TBI patients.

The result demonstrates a new function in that astrocytes, the most numerous cell type in the brain, engulf dead cells after injury both in cell cultures and in adult mice and thereby save neurons from contact-induced apoptosis. Astrocytes are effective phagocytes, but degrade the ingested dead cells very slowly. Moreover, astrocytes express the lysosome-alkalizing proteins Rab27a and Nox2 as well as major histocompatibility complex class II, the receptors on which antigens are being presented. By lowering the pH of the lysosomes with acidic nanoparticles, the degradation increases, but the astrocytes still remained less effective than macrophages. Taken together, the data indicates that the low acidification in astrocytes can preserve antigens and that astrocytes may be able to activate T cells.

The expression and secretion of injury-specific proteins was studied in a cell culture model of TBI by separate mass spectrometry analysis of cells and medium. Interestingly, close to 30% of the injury-specific proteins in medium are linked to actin, for example ezrin of the ezrin/radixin/moesin (ERM) protein family. Ezrin, but none of the other ERM proteins or actin, is actively secreted after injury. Extracellular ezrin also increases in CSF in response to experimental TBI in rats and is present in CSF from TBI patients, indicating that ezrin is a potential biomarker for TBI.

Keywords: Traumatic Brain Injury, Astrocyte, Apoptosis, Biomarkers, Ezrin, Actin, Extracellular Proteins, Degradation, Lysosome, Antigen Presentation

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urn:nbn:se:uu:diva-215154 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-215154)
To my granpa, Torsten Jansson, for teaching me about the value of knowledge.
This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


II Lööv, C., Mitchell, C.H., Simonsson, M., Erlandsson, A., Degradation of Ingested Dead Cells in Phagocytic Astrocytes is Tightly Regulated, but can be Enhanced by Lysosomal Acidification. *Manuscript*


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Abbreviations

AA Acquired aplastic anemia
AD Alzheimer’s disease
ADF Actin depolymerizing factor
APC Antigen-presenting cell
BBB Blood-brain-barrier
BDNF Brain-derived neurotrophic factor
bFGF, FGF2 Basic fibroblast growth factor
BrdU 5-bromo-2-deoxyuridine
CCI Control cortical impact
CCL Chemokine (C-C) ligand
ced Cell death abnormal
CH Cerebral ischemia
CNPase 2’,3’-cyclic-nucleotide 3’-phosphodiesterase
CNS Central nervous system
CNTF Ciliary neurotrophic factor
CSF Cerebrospinal fluid
CTL Cytotoxic T cell
CXCL Chemokine (C-X-C) ligand
DAMP Danger-associated molecular pattern
DAPI 4’,6’-diamidino-2-phenylindole
DC Dendritic cell
ECM Extracellular matrix proteins
EGF Epidermal growth factor
ERM Ezrin/Radixin/Moesin
F-actin Filamentous actin
G-actin Globular actin
G-CSF Granulocyte colony-stimulating factor
GDNF Glial cell-line derived neurotrophic factor
GFAP Glial fibrillary acidic protein
GJ Gap junction
GM-CSF Granulocyte-Macrophage colony-stimulating factor
IF Intermediate filament
IL Interleukin
KO Knock-out
Lamp Lysosomal membrane-associated protein
LysoS LysoSensor™ Green DND-153
LysoT LysoTracker® Red DND-99
Introduction

Traumatic brain injury – an overview

Traumatic brain injury (TBI) is a leading cause of death and disability in the developed world. The annual prevalence of TBI in the US was estimated to 1.7 million annually, of which 52,000 die and 275,000 are hospitalized, making it the third most common cause of injury related deaths. In Europe the incidence rate lies between 495,000 to 990,000 annually and with an annual cost of 33 013 million Euros, TBI is estimated as the ninth most expensive brain disorder.

TBI is mostly caused by falls, especially in the very young and the very old, and due to the rise in the old population the incidence of TBI will most likely increase. The highest rate in TBI-related deaths are seen after motor vehicle accidents, which are overrepresented in young adults.

Approximately 80 % of TBI-related hospitalizations are released after treatment, but an estimated 5.3 million Americans and 6.3 million Europeans live with disability as a result of a TBI. These numbers are probably underestimated due to the high rate of untreated mild TBIs, for which the long-term effects have not been fully elucidated. Moreover, TBI patients have a 2.25 times increased likelihood of premature death and a shorten lifespan of between five and nine years. Epidemiological studies of persons surviving an earlier TBI have also shown an increased risk of developing dementia, perhaps making TBI the most reliable environmental predictor of acquired neurodegenerative disease.

Secondary injury processes

Masel and DeWitt designated TBI as “a disease process, not an event” in which the primary injury, “the event”, is caused by external forces. The primary injury initiates the secondary injury, “the disease process”, that can continue for as long as years after the primary insult and includes, but is not limited to, blood-brain-barrier (BBB) disruption, edema, cell death, oxidative stress, mitochondrial disruption, excitotoxicity, autophagy, ischemia, inflammation, and hematomas. The secondary injuries presented after TBI are like dominoes, in which one leads to the other and the result is greater damage than the initial insult. The primary injury cannot be counte-
extracted once it has happened, but the secondary injury mechanisms have the potential to be modified to limit or even restore brain functions. Unfortunately, to this day, all Phase III clinical trials to limit secondary injuries in patients with TBI have failed, although several studies have shown promising results pre-clinically\textsuperscript{14,16-17}.

**Astrocytes in health and disease**

**History and functions**

The neuropathologist Rudolf Virchow was the first to identify glial cells in 1856\textsuperscript{18-19}. However, it was Ramón y Cajal, who named the fibrous and protoplasmic glia “astrocytes” and proposed that these cells functioned as supporting cells in the central nervous system (CNS)\textsuperscript{18}. The astrocyte is the most abundant cell type in the CNS and it is becoming more and more evident that astrocytes have an active role both in health and disease.

In the healthy brain, astrocytes are organized in essentially non-overlapping microdomains where each astrocyte occupies a set anatomical space with little overlap between neighboring cells and where all neuronal cell bodies and synapses only have contact with the processes from a single astrocyte\textsuperscript{20-22}. Since the discovery of astrocytes, the list of functions have grown longer and longer and include, but is not limited to; promotion of neurogenesis\textsuperscript{23}, influence of neuronal differentiation\textsuperscript{24}, and modification of synaptic signaling\textsuperscript{25-26}. Astrocytes both strengthens synapses\textsuperscript{27} and eliminate unwanted ones\textsuperscript{28} and buffers excess neurotransmitters and ions\textsuperscript{29-30}. As a vital part of the BBB and regulators of cerebral blood flow, they control the microenvironment as well as provide metabolic support to neurons\textsuperscript{31-32}.

The astrocytes are usually characterized by their intermediate filament (IF) mostly comprised by glial fibrillary acidic protein (GFAP), first identified in 1971\textsuperscript{33}. GFAP exists in eight different splice variants, where GFAP\textalpha{} is the most abundantly expressed isoform\textsuperscript{34}. GFAP is also expressed by other cell types in the brain besides astrocytes, for example by ependymal and the astrocyte-like radial cells\textsuperscript{18,35}. Moreover, it has been shown that not all astrocytes express GFAP or express it at undetectable levels\textsuperscript{30} and several authors have voiced their frustration over the lack of markers that can detect all types of astrocytes\textsuperscript{18,29-30}. Perhaps it is the multitude of functions, their pleomorphism and the identification of astrocytic subclasses that make it improbable to find one marker for all types of astrocytes\textsuperscript{18,29-30,36}. Markers that have been proposed as good complements to GFAP include S100\textbeta{}, glutamate transporter GLT1 (EEA1 in humans), aquaporin 4 and Aldh1L1\textsuperscript{18,29-30,37}. Some protein that have been suggested as markers of astrocyte subclasses include S100\textbeta{}, Sox2\textsuperscript{38} and GFAP\textdelta{}\textsuperscript{39-42}. GFAP\textdelta{}\textsuperscript{41}, 14-3-3\textepsilon{}\textsuperscript{43-44} and 14-3-3\textsigma{}\textsuperscript{44} is ex-
pressed by multinuclear astrocytes, which could represent a separate subgroup.

Reactive astrocytes and the glial scar

All CNS pathologies lead to an activation of astrocytes, a process referred to as reactive gliosis\textsuperscript{30,45}. A hallmark for reactive astrogliosis is the increased cells size and up-regulation of IF components such as GFAP, vimentin (Vim), S100\textbeta and nestin\textsuperscript{45-49}. TBI also increase proliferation, primarily of astrocytes\textsuperscript{50-52}, which have been shown to dedifferentiate to more immature cells with stem cell potential\textsuperscript{52}. In contrast, the reactive astrocytes seen in Alzheimer’s disease (AD) do not show the same dedifferentiation\textsuperscript{53} indicating that, although the reactive astrocytes appear identical, their response is different dependent on insult.

Contusive trauma to the CNS not only results in reactive gliosis, but also formation of the long-lasting glial scar. The scar is made up of newly formed or adjacent astrocytes, pericytes and meningeal cells, which create a border around severely damaged tissue by rearranging the otherwise domain-organized astrocytes\textsuperscript{30,45}. However, it is still not elucidated whether the scar is beneficial or detrimental for TBI outcome\textsuperscript{30,45,47-48}. Studies have used several different approaches to study the effect of the glial scar, for example ablation of dividing, reactive astrocytes or knock-out (KO) of GFAP and/or Vim. Specific ablation under the GFAP promoter has shown a negative effect on outcome in both spinal cord injury and TBI. The worsened outcome was attributed to an excessive infiltration of immune cells, increased BBB leakage and damage of neurons\textsuperscript{49,54-55}, which exacerbated the secondary injury (see below). In contrast, double KO of GFAP and Vim was shown to result in some positive effects, including increased differentiation and survival of astrocytes and neurons\textsuperscript{56-57} and the rescue of synapses\textsuperscript{58}, compared to wild-type (WT) mice. However, double-KO of GFAP and Vim, showed an approximate 300\% increase in tissue loss after ischemia compared to WT mice\textsuperscript{59}, indicating that the scar can have beneficial as well as detrimental effects depending on the type of injury.

One of the most studied negative effects of the glial scar is the inhibition of neurite regeneration. Activated astrocytes secrete proteoglycans and other extracellular matrix proteins (ECM), which have shown a more prominent negative effect on neurite regeneration than the physical barrier comprised of the compacted cells in the scar\textsuperscript{60-62}. The reactive astrocytes also secrete matrix metalloproteinases (MMPs), that degrade the ECM and can rescue the inhibitory effect on axon growth, but some MMPs are toxic to neurons\textsuperscript{61}. Astrocytes that have been activated by macrophages can contribute additional to neurite damage by physically tearing and stretching the neurites as they migrate away from the cavity formed in contusive TBI\textsuperscript{60}. Taken together, the glial scar may have positive effects initially, especially in for example
ischemia that leads to great cell loss, but detrimental effects later on. A time-dependent disassembly of the scar may therefore be a great treatment target to increase regeneration and patient outcome.

Cell death

Neural cell death is paramount to the developing brain and removal of dead or damaged cells is important for normal brain homeostasis. Two terms are commonly used to characterize cell death, apoptosis and necrosis. The former being defined as a controlled, energy dependent cellular event characterized by condensation of the nucleus and DNA fragmentation, whereas the latter commonly associates with uncontrolled disruption of the cell membrane, cytosolic leakage and organelar breakdown\(^63-67\). The leakage of cytosolic material has been considered a “danger signal” which augments the inflammatory response\(^68\). The truthfulness of this is still contestable since it has been shown that necrotic cells alone are insufficient to activate macrophages\(^69\), although lysed neutrophils have shown a pro-inflammatory effect\(^68\), which indicate specific responses dependent on the cell type. It has also been shown that necrosis is far from the uncontrolled cell demise previously believed and that it shares several features with apoptosis as for example DNA fragmentation and the flip of phosphatidylserine (PS) from the intracellular to the extracellular side of the cell membrane\(^65,67,69\).

Trauma leads to a considerable amount of dead cells\(^70-71\). Moreover, the dead cells that die from the actual impact, or primary injury, can induce apoptosis in nearby cells in a process called bystander cell death and thereby multiply the number of dead cells and propagate the injury well beyond the initial site. Two of the proposed mechanisms of bystander cell death involves diffusion of chemical death signals from the dead cell through the extracellular space or by a direct binding of the necrotic/apoptotic cell to death receptors on the healthy cell\(^72\). Lack of functional Fas, a death receptor, has been demonstrated to reduce cell death after closed head trauma and leads to faster neurological recovery\(^71\). Bystander cell death can also be propagated through astrocytic gap junctions (GJ) by which noxious signals reach near and making them “fall like dominoes”\(^73-74\). However, the ablation of connexin 43, the prevalent connexin in astrocytic GJs, also has a detrimental effect on outcome after ischemia by increasing apoptosis and inflammation\(^75\). Likely there are multiple mechanisms in the induction of bystander cell death as inhibition of GJs have been shown to reduce bystander cell death, but not completely abolish it\(^76\).
Engulfment of dead cells

Clearing of dead cells is vital during the development and for proper tissue homeostasis and, if the cells are not cleared quickly, can cause autoimmune disease\textsuperscript{77} and or neurodegeneration\textsuperscript{78}.

Programmed cell death induces the expression of dead cell-associated molecular patterns on the surface of the dead cells, which are recognized by receptors on phagocytic cells\textsuperscript{63,66,79-82}. The most well-known dead cell marker is PS, which can be found on both apoptotic and necrotic cells, and is recognized by a multitude of receptors involved in phagocytosis\textsuperscript{79,82}. In traditional phagocytosis, receptors on the phagocytic cell interact directly or indirectly by bridging proteins, with markers exposed on the dead cell and guide the membrane around the apoptotic cell in a “zipper-like” fashion (Figure 1). This creates a very tight fitting phagosome\textsuperscript{83-84}, in contrast to engulfment via macropinocytosis, which creates a spacious phagosome in which surrounding liquid is taken in with the cell material\textsuperscript{85} (Figure 1). The zipper-like phagocytosis is commonly used by professional phagocytes when ingesting apoptotic cells\textsuperscript{86}, but macrophages can also use macropinocytosis when engulfing apoptotic\textsuperscript{82} or necrotic cells\textsuperscript{86}. Macropinocytic “phagocytosis” has been hypothesized to be independent of receptors\textsuperscript{85}, but it has been shown that the digestion is PS dependent, which indicates that the initial hypothesis is incorrect\textsuperscript{82,87}.

Engulfment of apoptotic cells has traditionally been attributed to professional phagocytes, such as macrophages, microglia, neutrophils and dendritic cells (DCs), but neighboring cells are now considered important in the removal of cell corpses\textsuperscript{88}. The knowledge of the molecular mechanisms utilized by nonprofessional, tissue-resident cells in the clearance of apoptotic cells initially came from studies of \textit{Caenorhabditis elegans}, which lacks professional phagocytes. Two evolutionary conserved signaling pathways of recognition and internalization of dead cells, named cell death abnormal (ced), have been identified in the nematode\textsuperscript{81,89-92}. The first mammalian homologous pathway consists of Crk, Dock180 and Elmo (ced-2, -5 and -12) and the other consists of Megf10, Gulp1 and Abca1 (ced-1, -6 and -7). The pathways converge at Rac1 (ced-10), which is necessary for the cytoskeleton rearrangement required for the phagocytic cup formation and subsequent engulfment\textsuperscript{83,90} (Figure 1 and 2). A high expression of both ced-pathways in astrocytes was shown by Cahoy and colleagues\textsuperscript{37}, and astrocytes also express the dead cell receptor, brain-specific angiogenesis inhibitor 1\textsuperscript{93-94}. This supports the notion that astrocytes are phagocytic cells, which has been corroborated by several studies\textsuperscript{88,95-96} including our own (Paper I)\textsuperscript{97}. 
Figure 1. Two different engulfment mechanisms are utilized in dead cell ingestion. Depending on whether the membrane on the dead cells remain intact, the pathway can change from the traditional phagocytic, zipper-like up-take (left) to macropinocytosis (right). Macropinocytosis leads to a spacious phagosome that also contains extracellular liquid, whereas traditional phagocytosis creates a phagosome where the membranes of the phagocyte and the dead cells are closely associated.

Degradation of ingested, dead cells

After engulfment, the ingested material needs to be degraded. Diseases in which the degradation is inhibited or delayed, often presents with early onset neurodegeneration and astrogliosis.

After the ingestion, a multi-step, internal process is initiated, in which the phagosome matures. In each maturation step the pH is lowered and more and more degrading enzymes are added until the phagosome finally fuses with the lysosome (Figure 2). Several components are required for proper maturation of the phagosome, and although many proteins have been identified, much is still uncertain about the required maturation proteins and in what order the different markers appear.
Two of the most well studied maturation proteins are Rab5 and Rab7, which are attributed to early and late endosomes, respectively\textsuperscript{98-100}. However, Kinchen \textit{et al.} showed that Rab5 precedes acidification\textsuperscript{101} and early endosomes are slightly acidic (pH around 6)\textsuperscript{102}, which may point to it being a marker for endosomes/phagosomes rather than a degradation marker (Figure 2). Two other well-studied proteins are the lysosomal membrane-associated proteins 1 and 2 (Lamp1 and Lamp2). These highly glycosylated proteins are present in early and late endosomes, the latter at higher concentrations\textsuperscript{99} (Figure 2). Mutations in either Rab5 or Rab7 result in arrest in the phagosome maturation\textsuperscript{101}. Cells lacking both Lamp1 and 2 fail to acidify the engulfed material and double Lamp-KO mice are not viable at all\textsuperscript{103}. Single Lamp-mutations have no apparent effect on macrophage acidification\textsuperscript{104} and animals with mutated Lamp1 remain largely unaffected, although they show an upregulation of Lamp2\textsuperscript{105}. However, Lamp2 KO is fatal in 50 % of the animals and reduce the size and life span of the surviving mice\textsuperscript{106}. This implies a certain redundancy in the functions of the respective Lamps that may depend on cell type.

\textbf{Figure 2.} The ingested material is degraded in a multi-step maturation process where the phagosome becomes acidified and loaded with degrading enzymes. Several proteins are required for this maturation process, for example Rab5 and Rab7 as well as Lamp1 and Lamp2.
The lowering of the pH is facilitated by V-type ATPases located in the phagosomal membrane, which in an energy consuming process, pumps in protons into the lumen. The acidification of the phagosomic lumen is crucial since the degrading enzymes functionality is optimal at a pH less than 5\textsuperscript{102}.

The acidification must sometimes be limited in order to preserve antigen from total destruction. Rab27a is a protein that has been shown to alkalize the lysosomes in a NADPH oxidase (Nox2) dependent manner and is expressed by DCs, which are professional antigen-presenting cells (APCs)\textsuperscript{107-108}. It has also been shown that, dependent of the ingestion receptors or intracellular signals they evoke, cells can change the rate of acidification\textsuperscript{109-110}. Other limiting steps include the disassembly of the F-actin coating on the phagosome and the concentration of degrading enzymes. By prolonging the actin-coating stage, the phagosome is physically inhibited from fusing with the endosomes and thereby delaying the degradation\textsuperscript{111}. Rab27a has also been implicated in this step by lengthening the time of actin-coating around the phagosome\textsuperscript{112}.

Cathepsins are degrading proteases in the endosomes and lysosomes, but their distributions vary between different compartments and cell types\textsuperscript{113-115}. Their activity is dependent on the pH where most are inactive at neutral pH\textsuperscript{102,114}. It has been shown that alkalized lysosomes induce a redirection of the cathepsin-containing vesicles to the cell surface where they are secreted, which lead to additional inhibition of degradation by lowering the concentration of degradative enzymes\textsuperscript{113}. The release of cathepsins could also have a detrimental impact on surrounding cells due to their toxicity\textsuperscript{114}, inducing a feed-back loop where more cells die that needs to be engulfed and degraded, ultimately overloading the phagocytic cells, extending the actin-coating and delaying the degradation\textsuperscript{111}.

**Inflammation in TBI, a double-edged sword**

Inflammatory responses are now considered to be an important component of TBI\textsuperscript{12,116}. Although it is still not clarified whether the immune activation is good or bad it seems likely that it serves both adverse and necessary repairing properties, and that this in large is dependent on the onset and duration as well as the magnitude of the inflammatory response.

Microglia is the primary immune cell in the brain and damage caused by TBI lead not only to activation of astrocytes, but also microglia. Activated microglia lose their ramified morphology to become rounded, highly mobile and indistinguishable from the blood-borne macrophages\textsuperscript{13,116-117}. Activation of microglia has proven detrimental effects on neuronal survival in vitro even in absence of injury, but activated microglia/macrophages also become professional phagocytes, which has reparative effects on the tissue\textsuperscript{60,118}. Disruption of the BBB can also contribute to the inflammatory response by
permitting leukocytes to migrate into the otherwise tightly controlled brain parenchyma and, together with astrocytes and activated microglia, start to secrete pro-inflammatory molecules. This leads to an exacerbation of the immune response by attracting more and more immune cells\textsuperscript{13} that can persist for years after the initial insult\textsuperscript{15,119}.

Several inflammatory molecules are secreted from reactive astrocytes, for example chemokines, cytokines, tumor necrosis factor (TNF), interferons (IFN), heat-chock proteins and ATP (Table 1)\textsuperscript{13,46,116-117,120-122}. In addition to the activation of the immune response, inflammatory agents promote neuronal survival, induce synaptogenesis and upregulate proliferation\textsuperscript{121,123} through the down-stream release of trophic factors (Table 2).

Table 1

<table>
<thead>
<tr>
<th>Inflammatory factors released by astrocytes</th>
<th>Abbreviation</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>Chemokine (C-C) ligand 2</td>
<td>CCL2</td>
<td>122</td>
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<tr>
<td>Chemokine (C-C) ligand 5</td>
<td>CCL5</td>
<td>122</td>
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<tr>
<td>Chemokine (C-X-C) ligand 12</td>
<td>CXCL12</td>
<td>122</td>
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<td>Granulocyte-Macrophage colony-stimulating factor</td>
<td>GM-CSF</td>
<td>121-122,124</td>
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<td>Granulocyte colony-stimulating factor</td>
<td>G-CSF</td>
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<tr>
<td>Interferon-α</td>
<td>INF-α</td>
<td>121</td>
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<tr>
<td>Interferon-β</td>
<td>INF-β</td>
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<tr>
<td>Interferon-γ</td>
<td>INF-γ</td>
<td>122,125</td>
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<td>Interleukin-1</td>
<td>IL-1</td>
<td>122,125-126</td>
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<tr>
<td>Interleukin-6</td>
<td>IL-6</td>
<td>121-122,124-126</td>
</tr>
<tr>
<td>Interleukin-10</td>
<td>IL-10</td>
<td>121</td>
</tr>
<tr>
<td>Macrophage colony-stimulating factor</td>
<td>M-CSF</td>
<td>121</td>
</tr>
<tr>
<td>Monocyte chemoattractant protein-1</td>
<td>MCP-1</td>
<td>121</td>
</tr>
<tr>
<td>Transforming growth factor-β</td>
<td>TGF-β</td>
<td>46,121</td>
</tr>
<tr>
<td>Tumor necrosis factor</td>
<td>TNF</td>
<td>46,121-122,125-126</td>
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Interleukin (IL)-1β and IL-6 are two well studied cytokines that are expressed acutely in TBI. Both adverse and advantageous effects have been attributed these cytokines as they result in activation of astrocytes and microglia, but also in the production of trophic factors. For example, KO of IL-1β or IL-6 show increased BBB leakage in brain injured mice, which has been attributed to a delay in gliosis\textsuperscript{122}. Contrary to the findings in KO mice, the administration of IL-1β antibody directly after injury reduced the number of immune cells and increased outcome after TBI\textsuperscript{127}. The induction of one cytokine can induce another\textsuperscript{124} and provoke a feed-back mechanism that
makes it difficult to determine the exact effect of individual inflammatory agents.

There is a clear connection between clearance of dead cells and inflammation as receptors not only trigger engulfment but also define whether the phagocyte should trigger a pro-inflammatory or an anti-inflammatory response\textsuperscript{63,66}. In the case of apoptotic cell engulfment it is vital for a multi-cell organism that the response remain anti-inflammatory and stimulate cell renewal, by for example releasing growth factors such as vascular endothelial growth factor (VEGF) and transforming growth factor-\(\beta\) (TGF-\(\beta\))\textsuperscript{84}. Necrotic cells have been suggested as pro-inflammatory, but phagocytosis of the cells by macrophages do not induce cytokine production\textsuperscript{69,87} and lymphocytes have been shown to produce anti-inflammatory cytokine IL-10 in response to apoptotic cells\textsuperscript{128}, indicating that dead cells themselves can suppress inflammation.

APCs, like DCs, can under certain conditions present dead-cell associated molecules on major histocompatibility complex (MHC) class I or II, which can elicit T cell responses. The CD8\(\alpha^+\) subclass of DCs are particularly efficient at presenting self-antigens T cells\textsuperscript{129}, which could be detrimental or beneficial dependent on how the T cells respond. The activation of T cells happens in a three step model where step one includes the T cell receptors that interact with the presented antigen. Next step includes the role of co-stimulatory receptors on the presenting cells which initiates proliferation and differentiation of the T cell into an effector cell. The last step is determined by what inflammatory molecules are being secreted. TGF-\(\beta\)\textsuperscript{130} and IL-10\textsuperscript{131} induce regulatory T cells (T\(_{reg}\)) that reduce autoimmune responses and are important for keeping the immune system in check. Natural killer cells (NK) or cytotoxic T cells are in turn induced by for example INF-\(\gamma\)\textsuperscript{130} and IL-2\textsuperscript{132} and can, as their names imply, induce cell death.

Table 2.

<table>
<thead>
<tr>
<th>Trophic factors released by astrocytes</th>
<th>Abbreviation</th>
<th>References</th>
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<tbody>
<tr>
<td>Basic fibroblast growth factor</td>
<td>bFGF, FGF2</td>
<td>46,123,133</td>
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<tr>
<td>Brain-derived neurotrophic factor</td>
<td>BDNF</td>
<td>46,123,133</td>
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<tr>
<td>Ciliary neurotrophic factor</td>
<td>CNTF</td>
<td>122-123,133</td>
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<tr>
<td>Epidermal growth factor</td>
<td>EGF</td>
<td>46,123</td>
</tr>
<tr>
<td>Glial cell-line derived neurotrophic factor</td>
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<tr>
<td>Nerve growth factor</td>
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<td>122,126</td>
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<td>Platelet-derived growth factor</td>
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<td>46,133</td>
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<tr>
<td>Transforming growth factor-(\beta)</td>
<td>TGF-(\beta)</td>
<td>122,126,133</td>
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<tr>
<td>Vascular endothelial growth factor</td>
<td>VEGF</td>
<td>122-123,133</td>
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Astrocytes ability to express MHC class II in conditions of prolonged inflammation or in presence of IFN-γ has been shown in several studies. However, whether or not astrocytes express the vital co-stimulatory receptors or are able to induce T cell responses still remain controversial. Dead cells are a possible source of auto-antigens and it is therefore vital that self-antigen presenting cells do not activate T cells. Due to the release of IL-10 by the phagocytes, the auto-immune response can be subdued, but the possibility still remain that the pro-inflammatory agents may overcome the anti-inflammatory thereby inducing, not only prolonged inflammation, but autoimmune disease.

Biomarkers as future treatment tools

According to the Oxford English dictionary a biomarker is “a diagnostic indicator of (predisposition to) a medical condition”. In a disease as complex as TBI, where to this day no pharmacological treatments are available to reduce secondary injuries, the search for biomarkers as a tool to evaluate patient progression and possible outcome are highly appealing and sought after. Identification of injury-specific markers can also lead to the discovery of future treatment targets and a better understanding of TBI.

The most promising biomarkers for TBI can be found extracellularly in whole-blood or derivatives (serum, plasma), cerebrospinal fluid (CSF) or directly from the brain parenchyma with microdialysis. Blood is more easily obtained, but many TBI patients have multiple traumas affecting several regions of the body, which could compromise the biomarker profile in blood. CSF may be the better option due to the proximity to the brain and the injury itself and is a way to circumvent the BBB.

Brain-specific proteins that are found extracellularly after TBI indicate that destruction of cells has taken place and that the waste ends up in the blood or CSF. Therefore, these intracellular proteins can function as biomarkers of outcome and/or severity and serve as important predictors of TBI progression. Several markers have been proposed as possible biomarkers for TBI, for example GFAP, S100β, and neuron-specific enolase (NSE). S100β have been positively correlated with outcome and high levels of S100β and GFAP are predictors of an increased fatality risk in TBI patients. NSE did not correlated with outcome, but has shown a strong relationship with IL-6 levels indicating that it may be a predictor of severe inflammation. S100β, NSE, and GFAP in serum have also been shown to correlate with imminent onset of cerebral hypoxia (CH). It would be extremely valuable to have predictors of harmful, secondary injuries before they occur in the patient and factors predictive of for example CH, with a high morbidity, could lead to early intervention and may significantly impact the outcome.
Information of injury progression could also have the benefit of a more personalized care and interventions could be planned accordingly.

**Actin and actin interacting proteins**

Actin is a highly conserved and abundantly expressed protein which is encoded by six genes in humans. Different isoforms are expressed in different tissues; α-actin isoforms are predominant in muscle cells, whereas β and γ are located to non-muscle cells. Actin filaments are constructed by globular subunits (G-actin) that bind together in a thread-like shape. Cell motility is driven by polymerizing F-actin which can shape protruding filopodia and lamellipodia, but actin is also important in phagocytosis (see above). Many actin-interacting proteins are involved in the so called treadmilling, a term that describes the modulation of F-actin structures. These include for example Arp2/3, ADF/cofilin, profilin and capping proteins.

Since the filaments are straight, branching proteins are needed to create wider, yet adherent, structures for example at the leading edge of neuronal growth cones. Arp 2/3 is such a branching protein complex that attaches to the F-actin from which polymerization can continue in a different direction. Capping proteins can bind to either side of F-actin where they prevent both the elongation and the disassembly of the structure. ADF stands for actin depolymerizing factor and cofilin belong to the same family of actin severing proteins. Cofilin is the most common severing factor in the brain and can cause neurodegeneration in AD and ischemia. The activity of cofilin is in part regulated by phosphorylation where the dephosphorylated cofilin has greater severing capacity. ATP is the substrate of the phosphate required to phosphorylate cofilin, *i.e.* inactivate it. In pathologies that presents with energy depletion, the ATP levels are low and cofilin phosphorylation is therefore inhibited which leads to continuous severing of actin. Additionally to the reduced F-actin in these cells, the dephosphorylated cofilin can form rod-like aggregates that can induce neurodegeneration.

Profilin acts by binding to G-actin, which thereby enhances the speed of assembly into filaments. Thymosin-β4 (Tβ4) is a so called sequestering protein that binds the monomers and inhibits the formation of filaments, but Tβ4 also ensure that monomers are readily available to form filaments. In times of high demand, the Tβ4 will be exchanged with profilin and the filaments elongated quickly. Interestingly, Tβ4 have many functions separate from their actin-interaction, for example can it protect cells from apoptosis and reduce inflammation and recruit progenitor cells. Tβ4 is readily released to promote wound healing and has anti-microbial properties. Moreover, Tβ4 has successfully been used to treat rats after TBI by improving outcome, reduce inflammation and lesion volume and enhance proliferation and angiogenesis.
The actin binding ezrin/radixin/moesin proteins

Ezrin/radixin/moesin (ERM) are proteins that are known to bridge the actin cytoskeleton to the cell membrane and have a role in most actin-dependent mechanisms, for example motility, phagocytosis and phagosome maturation, cell signaling, secretion and relocation of membrane proteins^{109,149-151}. The ERM proteins are highly homologous proteins that belong to the ERM protein family^{151-152}. Their activity is controlled by phosphorylation, particularly to a conserved threonine residue that is present in all the proteins in the family^{153}. The phosphorylation is preceded by ERM interaction with phosphatidylinositol 4,5-bisphosphate (PIP2) located in the cell membrane^{154-155}, which leads to a conformational change that allows kinases to phosphorylate the ERM proteins^{154,156}. The unphosphorylated proteins are bound in an autoinhibited form in which the C-terminal interacts with the N-terminal and phosphorylation causes the proteins to open up so that the activated proteins can interact with F-actin and membrane-bound proteins^{154-157}. Ezrin has previously been depicted as interacting with F-actin via its C-terminal^{151}, although data show that that part of ezrin actually bind actin poorly^{158}. Newer data indicate that ezrin collapses around the filament and interact with actin at multiple points encompassing approximately 10 actin subunits^{154}. This could explain the approximate 1:10 ezrin to actin binding ratio seen also in other studies^{158-159}.

The individual proteins have been proposed to be redundant, but the respective ERM proteins are localized to different tissues or cell structures; ezrin in epithelial cells and moesin primarily in endothelial cells^{160}, and radixin in adherens junctions^{151}. Taken together, the specific distributions and the fact that ezrin KO mice do not survive past weaning^{161}, indicate that the individual proteins have specific functions that cannot be rescued by expression of the other homologs.

The ERM proteins have been connected to a number of pathologies for example has an increased expression been associated with poor out-come and cancer metastasis^{162}. A clear up-regulation of the ERM genes has also been shown after cryogenic TBI^{163} and an increased activation of ERM has been implicated in allodynia following peripheral nerve injury^{164}. All the aforementioned conditions regard intracellular expression of the ERM proteins, but moesin has also been found exposed extracellularly on the cell surface of T cells, NK and monocytes^{165-166}. It has been shown that antimoesin antibodies are produced in a subgroup of patients with acquired aplastic anemia (AA)^{167}, which can bind to the cells that exhibit surface moesin and induce the secretion of TNF-α and INF-γ in patients with AA^{166,168}. Altogether, this indicates that extracellular ERM proteins found in our culture (Paper III and Paper IV) could have similar effects after TBI and induce autoimmune responses that could have detrimental effects on patient outcome.
Aims

The overall aim of this study was to gain better understanding of the cellular and molecular processes following traumatic brain injury. To be able to study the effect of dead cells and/or injury to the three main cell types in the brain; astrocytes, neurons and oligodendrocytes, without the confounding effects of inflammatory cells we used primary cell cultures. The focus was mainly how astrocytes affect the immediate environment after trauma by engulfment of dead cells and the release of various agents. Moreover, the aim was to elucidate whether the observed responses in vitro were also involved in whole organisms. Therefore, the findings were confirmed in animal models and/or in patients with TBI.

More specifically the aims of the papers were:

Paper I: To identify engulfing astrocytes in a cell culture model of TBI and to gain better understanding of the mechanisms responsible for the ingestion of dead cells. We also sought to understand how the clearance of dead cells by non-professional phagocytes can impact the immediate environment after TBI.

Paper II: To identify the factors responsible for the slow degradation rate observed in astrocytes and possible approaches to enhance the astrocytic digestion. We also aimed to understand the reason for the delayed degradation, such as a possible role for astrocytes in antigen presentation.

Paper III: To identify proteins uniquely expressed after in vitro injury in order to find new, possible biomarkers for TBI. The ambition was also to link the identified injury-specific proteins to secondary responses observed after injury.

Paper IV: To further study extracellular ezrin and moesin, which were identified as possible biomarkers of TBI in Paper III. We sought to confirm the presence of ERM in cell culture medium and CSF from both rats and humans after TBI and find out if the release was due to active secretion or passive release. Furthermore, the goal was to study how the extracellular levels changed over time in comparison to the intracellular ERM expression.
Materials and Methods

Cell culture models

TBI has been described as the most complicated disease in the most complex organ. Therefore, in vitro models are useful, which simplify the system and enable studies of specific cellular and molecular responses, for example studies of neurons and glia in the absence of inflammatory cells. Cell culture models are easily reproducible and are good when studying interactions between specific cells and has the benefit of reducing the number of animals used.

Our cell culture model is based on differentiated neural stem cells consisting only of neurons, astrocytes and oligodendrocytes, but not microglia. Briefly, cortices from E14 (embryonic day 14) C57/BL6 mouse embryos were dissected, dissociated and grown non-adherently in medium together with the bFGF and EGF. The mitogens kept the cells from losing their stem cell potential and promote proliferation as they grew into neurospheres. The neurospheres were expanded by passage every three to five days for a maximum of three times. The cells from the neurospheres were seeded as single-cell layers on poly-L-ornithine and laminin pre-coated glass coverslips and were then differentiated for seven to eleven days in growth factor-free medium before the initiation of the experiments.

The injury we used was a so called transection model which created a clear border between uninjured and injured cells. Several different tools can be used to lesion the cells for example a pipette tip or, as in our lab, a scalpel. The scalpel was used to cut the cells perpendicularly in two directions, 20 cuts in total. Uninjured cultures served as controls. The cells were thereafter incubated for a set period of time before they were fixed in phosphate buffered paraformaldehyde (PFA).

In addition to the injury model used, we studied astrocytic engulfment by adding dead cells to the differentiated cultures. Undifferentiated, neural stem cells that were killed in a UV chamber by two bursts of light (480 mJ in total) were added to the differentiated neural cultures. For positive engulfment controls we used primary macrophages from mouse spleens. In short, spleens were removed and dissociated mechanically with a plunger, blood cells were lysed with lysis buffer and thereafter washed away. The cells were seeded on poly-L-ornithine and laminin and incubated for 2 h before wash-
ing away unattached cells. The attached macrophages were then incubated for 2 days prior to addition of dead cells.

![Figure 3. Schematic image of the set-up for medium and cell collection for WB analysis. The cells were seeded in 6-well plates, three independent cultures per plate (nX), and injured in three directions or left uninjured to serve as controls. The medium and cells were collected and saved separately two hours or two days after experiment start.]

The collection of medium and cells for protein analysis

For the mass spectrometry (MS) analysis, cultures were injured by 20 cuts or left uninjured as described above. Twenty-four hours after experiment start, the medium was collected from the culture and centrifuged to clear any debris. The adherent cells were mechanically dissociated by a cell lifter, collected and sedimented by centrifugation before careful removal of any remaining medium. The medium and cells were saved in -70 °C until analysis.

For Western blot analysis (WB), we modified the injury model to include 30 cuts, in three directions (Figure 3). After two hours or two days, the medium was collected from parallel, injured and uninjured cultures and any cells or debris was cleared by centrifugation before transferring the supernatants to new tubes. Proteases inhibitors were added to the medium and the samples stored in -70 °C until analysis (Figure 3). Cell lysates were made to study the intracellular protein levels from uninjured and injured cultures (Figure 3) or from cultures that had been treated with dead cells. All medium was carefully removed and lysis buffer with protease inhibitors was added to the adherent cells. The lysates were cleared of any unsolved particles by centrifugation and the supernatant was transferred to new tubes and saved in -70 °C.

Animal model

All animal experiments were preapproved by the Uppsala Animal Ethics Committee and were in line with the Swedish animal welfare legislation.
The animal model used is called controlled cortical impact (CCI) and is one of the most widely used injury models for TBI. It can be used on both mice\textsuperscript{172} and rats\textsuperscript{173} and reproduces the pathology and the behavioral disturbances associated with acute brain trauma in humans\textsuperscript{14}. The procedure was identical for mice and rats in all but the size of the craniotomy (6*6 mm in rats and 4*4 mm in mice), the impactor diameter (4.5 mm in rats and 3 mm in mice) and the depth of the compression (2 mm in rat and 0.5 mm in mice).

In short, male C57/BL6 mice or Sprague-Dawley rats were anaesthetized and placed in a stereotaxic frame under continued anesthesia (isoflurane in nitrous oxide and oxygen) through a nose cone. Core body temperature was controlled by a heating pad connected by a rectal probe. A midline incision was made after a subcutaneous injection of bupivacaine (Marcain\textsuperscript{®},) and a craniotomy was made over the parietal cortex. The stereotaxic frame with the animal was then moved to the CCI device (VCU Biomedical Engineering Facility) and injury produced by compression of the brain (Figure 4). The wound was closed up with interrupted sutures and the animals were put under a heating lamp while waking before they were returned to their home cage. Animals used for immunohistochemistry were euthanized by a pentobarbital sodium overdose followed by transcardial perfusion with PBS followed by fixation in PFA. As controls we used naïve animals.

\textit{Figure 4.} Schematic image of the experimental TBI method. CCI is one of the most common experimental TBI models used on either rats or mice. The animal is put in the stereotaxic frame under anesthesia and a craniotomy is opened over the parietal lobe. The frame and animal is then moved to the CCI apparatus (X) and the impactor is used to compress the brain, consequently creating a lesion. The bone is then replaced and the wound stitched up.
Collection of cerebrospinal fluid from rats and humans

The experimental injury was produced as described above and naïve and CCI injured rats were given an intraperitoneal overdose of pentobarbital. In contrast to the animals used for immunohistochemistry, the rat were not perfused but placed in a stereotactic frame after the overdose. To get access to the cisterna magna at the back of the neck, the skin and muscles were dissected through with forceps. A very fine syringe was used to pierce through the bone and the CSF was drawn. The samples were centrifuged to clear any cells or debris and the supernatant transferred to new tubes and kept at -70 °C until analysis.

Human ventricular cerebrospinal fluid was obtained from three patients at two separate time-points while they were being admitted to the Neurointensive care unit at Uppsala University Hospital, Uppsala, Sweden. Samples were taken by external ventriculostomy and the collected CSF was centrifuged to remove any cells before being stored at -70 °C. The participants or the next of kin, caretaker, or guardian provided their written, informed consent to participate in this study.

Immunohistochemistry and other stainings

In immunohistochemistry antibodies are produced by immunizing animals with specific peptide sequences found on the target protein. The specific antibodies can then be incubated with the tissue or cells of interest which allows them to bind to the peptide sequence on the protein marker. The next step is to visualize where the protein marker is located, which is done by antibodies against the first antibodies. The secondary antibodies carries a reporter signal as for example a fluorescent tag and by using antibodies that emit light of different wavelengths enables the visualization of several protein targets in the same specimen. This can be used to study the colocalization of proteins or, by using antibodies against a cell type specific marker, determine what cells express a certain protein.

Antibodies and their targets

In this thesis we used antibodies against GFAP to visualize astrocytes, which successfully stained the astrocytes in culture although some more than others (Figure 5). Nestin antibodies, which is a marker for reactive astrocytes and cells with stem cell potential, also stained the astrocytes in the culture (Figure 5). Antibodies against tubulin beta III isoform (βIII) were used to identify neurons and 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase) antibodies were used to study oligodendrocytes (Figure 5). Activated microglia and macrophages were not present in the cell cultures, but was visua-
lized by antibodies against Mac-2 in brain sections. Both CNPase and Mac-2 are surface markers, whereas the others are part of intracellular filaments found specifically in the respective cells. Ki67 antibodies were used to investigate proliferation and labels cell nuclei only once the cell has left the G0 phase. Antibodies against ERM react with ezrin, moesin and radixin, and the activated ERM proteins (pERM) were detected with antibodies that were specific to the phosphorylated forms of the proteins. We also used antibodies that detected the individual proteins, *i.e.* ezrin, moesin and radixin. Phagosome maturation was studied by Lamp1 and Lamp2 antibodies, respectively, and to study possible proteins involved in lysosome alkalization we used antibodies against Nox2 and Rab27a.

*Figure 5.* Representative images from uninjured cultures stained with antibodies against GFAP, nestin, βIII and CNPase (see inlays in the respective images). Observe that the same field is visualized in both the GFAP and nestin stainings and show that astrocytes that stain more robustly with GFAP antibodies often stain less intensively with nestin.
Other staining methods

Cell nuclei were detected by mounting the coverslips with hard set Vectashield with DAPI (4',6-diamidino-2-phenylindole). For actin visualization we used the fungal toxin from Amanita phalloides called phalloidin that was conjugated to fluorescein, a green fluorescent protein. To study apoptotic cells (or necrotic cells), we used terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). The hallmark of apoptosis is DNA degradation, which yields an abundant amount of free 3'-ends. The free 3'-ends of DNA were labeled in an enzymatic reaction by attaching dUTP with a fluorochrome that could be visualized and counted. Initially TUNEL labeling was considered to be a strict marker for apoptosis, but also necrotic cells can display degraded DNA, and TUNEL is now considered a marker for programmed cell death.

The thymidine analog, 5-bromo-2-deoxyuridine (BrdU) is a common marker for proliferation as it incorporates into the DNA of the dividing cell during the DNA replicating phase. In contrast to Ki67, BrdU can be detected also after the cells die, but has the disadvantage that it needs to be added to the culture (or injected into the animal) beforehand. The BrdU detection, however, is immunohistochemical where antibodies bind to the BrdU in the cells that have incorporated it. In this study it was used to discriminate between dead cells added during our experiments from cells that died during the differentiation.

To evaluate acidification of ingested material, a modified protocol of the one developed by Miksa et al. was used. The dead cells were prestained with pHrodo™ Red succinimidyl ester (pHrodo), which is a dye that binds to amines on the dead cells and is non-fluorescent at neutral pH, but fluoresce more brightly red the more acidic the environment becomes. The pKa of the die changes dependent after the ligation to amine, but is around 6.5. The dead cells (see “Cell culture”) were incubated in medium with pHrodo at room temperature for 45 minutes. The cells were then washed once and resolved in new medium and added to the differentiated neural or macrophage cultures for a maximum of 3 days.

Lysosomes were studied by LysoTracker® Red DND-99 dye (LysoT) and LysoSensor™ Green DND-153 (LysoS). These probes accumulate selectivity in endosomes and lysosomes, and are used to label live cells although the dye remains in the cells also after fixation. The LysoT dye is a weakly basic amine selective for acidic cellular compartments, but cannot be used to determine the organellar pH. The LysoS dye has a pKa of 7.5, which means that it fluoresce green in mildly basic compartments, whereas the more acidic the organelles become, the less bright the dye will be. Therefore, LysoS can be used to determine a relative pH of the labeled lysosomes and can be used to detect basic organelles that will not be labeled by LysoT. In short, the optimal incubation times and concentrations for LysoT and LysoS labe-
ling were determined and set to 0.5 µM of LysoT and 1 µM of LysoS for two hours. After two hours the dyes were removed and the cells fixed in PFA, washed three times and mounted directly with hard set Vectashield with DAPI.

Gene and protein expression analysis
Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) was used to measure the relative change in mRNA expression of several genes. In short, 10 µg of mRNA was added of each sample to individual wells together with primers designed against GFAP, Megf10, Crk, Rac10 or Mfge8. The primers bind to any corresponding mRNA in the sample and during each cycle a reverse transcript is formed. During the annealing phase of the cycle, a green fluorescent dye that is added to the reaction mix, binds to the created double-stranded cDNA. The amplification continues and when the threshold of the green signal is reached, the number of cycles is recorded. A lot of mRNA of the gene of interest meant that fewer cycles are needed to reach the threshold, hence, a relative measurement of gene expression is given. Due to the relativity of the method, a minimum of two samples to compare against each other, e.g. uninjured versus injured, are required.

In WB analyses, specific antibodies are used to measure the relative levels of protein expression. The same amount of protein form the different samples were loaded into separate wells in a gel. The gel was run and the proteins were separated by size. However, the predicted size of a protein does not always comply with how the proteins are separated due to post-translational modifications, structure of the protein or the type of gel used. After the proteins had been separated they were transferred to a membrane. To limit unspecific binding of antibodies, the membrane was blocked with bovine serum albumin. After the block, the membrane was incubated with primary antibodies that bound to the protein of interest. We used secondary antibodies that had been conjugated with biotin, which emits light when incubated with ECL-solution. The proteins signals were thereafter detected by exposing light-sensitive film to the membrane. The film was then developed and the detected bands (relative proteins levels) were measured with Image J software.

In collaboration with Magnus Wetterhall and Ganna Shevchenko from the department of Physical and Analytical Chemistry at Uppsala University, we performed detailed analyses of proteins in cell culture media and cells by mass spectrometry. This method is great for screening as no antibodies or primers against a specific protein are needed, but analyze the amino acid sequence of the proteins in the sample. In short, 35 µg of protein from medium and cells of uninjured and injured cultures were analyzed. The proteins were first digested with trypsin under reducing conditions before loading the
samples into the MS apparatus. The identified amino acid residues in the peptides were then analyzed against MASCOT search engine that has all the known proteins and can then tell us with what certainty the peptide belongs to a specific protein. A choice had to be made of what certainty would be considered to be positively matched. In our case we chose to be stringent and therefore chose a MudPIT MASCOT ionscoring of $p \leq 0.05$ and at least one peptide passed the required bold red criteria. The program also scored the hits, in which a protein with many identified peptides as well as a high copy number, gained a higher score. Only proteins that scored $\geq 26$, was included in the study.

Visualization techniques

Several microscopy techniques were used, such as fluorescence and confocal microscopy. Regular fluorescence microscopy was used for cell countings. Confocal microscopy was used to gain three-dimensional images by taking several micrographs from different focal planes that was then composed into one image by the Zen microscopy software from Zeiss. Transmission electron microscopy (TEM) can be used to study cellular structures down to nanometers, which are too small to see by regular light microscopy. The method uses a high energy beam that passes through a dehydrated sample in a vacuum with a detector that records the “transparency” of the samples. Dark parts let fewer electrons pass whereas lighter allows for more to pass, which creates a black and white image. In this thesis, TEM was used to confirm results from confocal microscopy and to gain better resolution images. Live cell cultures were also studied using time-lapse microscopy were images were taken at different intervals and then composed into films. The BioStation apparatus can take both fluorescence and bright-field images and was used herein to study gathering of dead cells and debris by astrocytes, migration and bystander cell death of neurons etc.

Cell counting and statistics

Cell counting was performed manually or in collaboration with Martin Simonsson from Centre for Image Analysis (CBA) and SciLifeLab at Uppsala University by CellProfiler and Ilastik programs. The statistical analyses performed depended on hypothesis and parameters tested and were made using the GraphPad Prism or Statistica software.
Results

Paper I

In Paper I, we studied astrocytes’ role after TBI in cell cultures and in adult mice. We found that most cells in the culture survived the injury if not cut directly through or close to their nuclei. The cells that actually died from the cut were quickly gathered and engulfed by the astrocytes. Confocal microscopy and TEM imaging showed that the ingested, dead cells were surrounded by liquid in spacious vesicles encircled by F-actin. Our in vivo studies showed that ingesting astrocytes could also be found after CCI injury in mice.

By time-lapse microscopy of injured cell cultures we found that neurons migrated towards and along the laceration, but we also found that healthy neurons were sensitive to contact with dead cells. When the highly mobile neurons came in direct contact with dead cells through their cell bodies, they quickly became apoptotic. By taking up the dead cells and thereby remove them from the immediate environment, astrocytes could save the neurons from the contact-induced apoptosis.

Although macropinocytic engulfment may be independent of receptors, TEM imaging indicated receptor-interaction between the phagocytic membrane and the dead cell. Therefore, qRT-PCR of Megf10 (ced-1), Crk (ced-2), Rac1 (ced-10), and Mfge8, a bridging protein, was performed to test whether the expression of known engulfment genes changed in response to injury. No significant changes were found after injury, but the genes were expressed in the cultures pointing to their possible involvement in astrocyte-ingestion.

To study the digestion, astrocytes and control macrophages were fed dead cells prestained with pHrodo. The macrophages acidified the phagocytosed dead cells within hours and had completely degraded the cells by day three. Astrocytes on the other hand failed to acidify the dead cells even at day three, but continued to accumulate dead cells over time.

Paper II

In this paper the focus lay in identifying factors responsible for the extremely slow degradation of dead cells by astrocytes, observed in Paper I. By Ilas-
tik and CellProfiler analysis we measured the total area comprised of dead cells over time. The results show that astrocytes do degrade the dead cells although it takes twelve days compared to the less than three days observed in macrophages. The acidification of dead cells was investigated by pHrodo and showed that the maturation was delayed in astrocytes and that the lysosomal pH was higher in astrocytes than in the macrophages.

WB analysis of Lamp1 and Lamp2 showed a high expression of both proteins throughout the experiment. Moreover, immunostainings for Lamp1 and Lamp2 showed that both Lamps were located to the ingested, dead cells. We found that F-actin surrounded the dead cells for long periods of time which could inhibit degradation. Latrunculin B, an actin severing toxin was added to the cell culture, which had a significant inhibitory effect on the number of actin rings, but showed no effect on the rate of degradation.

To investigate possible reasons for the delay in degradation we stained for MHC II, and found it expressed by the astrocytes. This finding raised the possibility that other alkalizing proteins were expressed that could prevent antigen destruction. WB analysis showed expression of Rab27a and Nox2 and immunostainings showed that they partly localized to the lysosomes. Inhibition of the alkalizing Nox2 with Apocynin, had however no impact on the degradation.

Next, we added acidic nanoparticles (NPs) to the cells which were taken up and fused with the lysosomes. The three different NPs had different acid capacity, which correlated with LysoT incorporation. The acidification of lysosomes enhanced the degradation, especially the most acidic of the NPs, demonstrating that the alkalized lysosomes negatively affect the degradation in astrocytes.

**Paper III**

In this paper we used our cell culture model of TBI to identify new, possible biomarkers. By MS analysis we screened for proteins that were uniquely found in medium or expressed specifically after injury in the cells. We found 46 proteins in the cells and 53 proteins in medium that were not present in uninjured cultures. The proteins were separated into subgroups based on previously published function for the respective proteins. We also studied the cells in the cultures to try and link the functions of injury-specific proteins to the cells responses to injury. Many of the observed reactions after injury were connected to actin remodulation, for example did the injury attract neurons and induced proliferation of neuronal cells and increased the size for the growth cones. Surprisingly, many of the medium proteins had actin-interacting properties, for example the highly scored ezrin and moesin. Stainings against the ERM proteins and pERM showed that astrocytes were the most likely source of these proteins which was confirmed in vivo. WB
analysis of whole-cell lysates from naïve mice and seven days post-CCI, showed a 25-fold increase in pERM compared to naïve animals, whereas the total ERM proteins levels remained unchanged.

Although no immune cells were present in the cultures many of the identified proteins had previously been linked to inflammation, and may work as immunoattractants after injury. Several of the proteins had previously also been connected to neurodegenerative disorders or TBI, indicating that they may be useful as biomarkers.

**Paper IV**

In Paper III we identified ezrin and moesin in the cell culture medium after injury by MS. In this study we further investigated their possible route of release and confirmed the presence of ERM proteins in medium and CSF from rats and humans by WB analysis. Cell culture medium was analyzed for ezrin content at two hours and two days after injury and the levels were compared to uninjured controls. The result showed that ezrin was released acutely after two hours, but did not differ significantly from uninjured levels until two days after injury. The secretion of ezrin was most likely an active process since moesin, pERM and β-actin levels in medium did not change in response to injury. High levels of all ERM, pERM and β-actin were expressed intracellularly in the cell cultures, but no changes were found in any of protein levels in response to injury or over time. In contrast, intracellular levels of radixin, moesin and pERM increased in expression after an initial decrease *in vivo*. Surprisingly, the intracellular levels of ezrin were the most stable of all the ERM proteins and showed a decreased expression at three days and 30 days post-CCI. Controversially, at times when the activity of the ERM proteins peaked, *i.e.* the ability to bind actin, the β-actin levels decreased the most. We confirmed that the ERM proteins and pERM were expressed by astrocytes *in vivo*, but we also showed that activated microglia/macrophages expressed the proteins in vesicular-like structures.

We tested the CSF from CCI injured rats, three and seven days post-trauma, and compared the levels of ezrin, moesin, pERM and β-actin to those observed in naïve animals. Similarly to the medium, ezrin increased significantly after injury at both time-points, whereas moesin, pERM and actin did not.

To confirm that ezrin could be a possible biomarker for TBI, we tested CSF from two different time-points from three individual TBI patients. Ezrin was present in all the samples, in contrast to moesin, pERM and β-actin, which were not. The levels of extracellular ezrin differed between the individual patients and changed over time.
In the soluble fraction, we observed high molecular bands with all the antibodies tested that could mean that F-actin and the ERM proteins remain bound to each other.
Discussion

It is becoming generally acknowledged that astrocytes are phagocytic cells capable of ingesting both dead cells or debris and pathological proteins such as α-synuclein and Aβ. Although many study engulfment itself, there is still much that remains unexplored in regards to the pathological consequences of astrocytic ingestion. We showed in Paper I that neurons could be saved from contact-induced apoptosis by the removal of dead cells by astrocytes. Since the injury induced proliferation and migration of neurons into the laceration area and the new neurons are vulnerable to dead cells, the removal of the dead cells may have similar effects in vivo. TBI also induce proliferation of stem cells in the adult mammalian brain, which migrate to the injured part of the brain. However, most cells mature into glial cells as neuronal cells are vulnerable to the hostile environment created by the injury. Transplantation of stem cells has been shown to improve outcome after TBI, but similarly to the endogenous stem cells, most transplanted cells die. Hence, increased removal of dead cells by astrocytes from the immediate environment could have a beneficial effect on neuronal survival also in vivo.

Our findings, that astrocytes accumulate more and more dead cells (Paper I and II), but degrade the ingested cells very slowly could account for the occurrence of multinuclear astrocytes that have been detected in epilepsy, Alexander Disease, multiple sclerosis and Creutzfeld-Jakob disease. How the digestive delay impact the progression of TBI is still not clear, but impaired degradation is present in many neurodegenerative diseases. TBI lead to an up-regulation of proteins involved in AD and has been linked to acquired dementia and although astrocytes are able to clear Aβ, AD patients are less efficient at degrading it. The delayed or inhibited degradation may induce a propagation of undegraded proteins through secretion from the astrocytes or lead to development of autoimmune disease. The development of chronic arthritis has been linked to the escape of undegraded DNA into serum, which indicates that unsuccessful degradation can propagate disease or inflammation.

We did not find any apparent signs of astrocyte distress in response to the phagocytosed dead cells, but it is possible that the ingestion leads to disruption of the regular functions of the cells such as neurotransmitter buffering, BBB integrity or modulation of neuronal signaling. This could in turn have detrimental effects on the survival of neurons and create a feed-back
loop in which more cells needs to be engulfed and then lead to additional deficiency.

Astrocytes have previously been shown to express MHC II\textsuperscript{121,126}, but their ability to express the necessary co-stimulatory receptors\textsuperscript{135-137} and elicit T cell responses remain controversial\textsuperscript{134,137,190}. In Paper II, we found that astrocytes express MHC II and, although the expression did not change in response to addition of dead cells, dead-cell associated antigen presentation could lead to either development of autoimmune disease or diminished inflammation and suppress autoreactivity, dependent on the type of effector T cell. The activation of T cells is not enough to induce antibody production, which is produced by B cells. B cells are activated after CNS injury, but how and where is still unclear\textsuperscript{191}. The autoreactive antibodies against for example β-III\textsuperscript{192} and basal lamina\textsuperscript{193} that have been found after brain injury, show that TBI can have autoimmune characteristics.

Autoantibodies have been found against moesin in patients with AA\textsuperscript{167} which can bind to various immune cells and induce secretion of pro-inflammatory agents\textsuperscript{166,168}. Although we did not find an upregulation of extracellular moesin in response to brain injury, extracellular ezrin was upregulated and the release of ezrin may culminate in the production of antibodies similarly to the ones seen in AA. The ERM proteins’ ability to bind F-actin could in itself lead to the production of antibodies as F-actin is the agonist to the DNGR-1 (CLEC9A) receptor located on DCs\textsuperscript{194}. Ezrin has been found to collapse around F-actin\textsuperscript{154} and we hypothesized that the high molecular bands that we found in medium and CSF could represent a complex of actin and ERM that could prevent the F-actin from collapsing. The ERM stabilization could remain until the F-actin/ezrin complex is released into the extracellular space where dephosphorylation by for example ceramide\textsuperscript{195-196}, could free the two. Dephosphorylation inactivates the ERM proteins\textsuperscript{157} and the F-actin is free to interact with the DNGR-1 receptor. DNGR-1 activation by F-actin amplifies cross-presentation of necrotic cell material by CD8α+ DCs\textsuperscript{197-198} and if ezrin is the antigen presented, could induce antibody production.

Damaged cells can release DAMPs that can act as immunoattractants through Toll-like receptors (TLRs)\textsuperscript{199-200}. TLRs are expressed by all major cell types in the brain and induce cell activation and cytokine production in response to injury\textsuperscript{200}. The ligands of the TLRs vary from bacterial pathogens to endogenous proteins and nucleic acids. The injury-specific proteins that usually are situated intracellularly, could be considered DAMPs and possibly induce inflammation if they are secreted or released passively. The proteins could also be used as antigens for cross-presentation and lead to autoimmunity.
Conclusions

In Paper I we found that astrocytes are efficient phagocytes of dead cells after TBI both in cell cultures and *in vivo*. The removal of dead cells by astrocytes could save the sensitive neurons from contact-induced apoptosis. The dead cells accumulated in the astrocytes due to a delayed degradation, which was studied in Paper II. Compared to the professional phagocytes, astrocytes did not acidify the ingested material over the first three days, but over time the material was slightly acidified although much less than in macrophages. The degradation was not back to base-line until after twelve days after the removal of the dead cells, but the administration of acidic nanoparticles speeded the degradation which indicates that the low acidity of astrocytic lysosomes delay the digestion. Several factors that could alkalize the lysosomes were found to be expressed by astrocytes, for example Rab27a and Nox2. Since astrocytes expressed MHC II molecules, on which antigens are loaded, engulfing astrocytes may elicit T cell responses.

In paper III we found that injury in cell cultures results in the release or secretion of proteins that normally are situated intracellularly. Many of these proteins interact with actin and several have previously been linked to inflammation, proliferation or cell death or neuropathological diseases etc. Ezrin, one of the actin-interacting proteins, is actively secreted in response to injury, and were found in cell culture medium and CSF from both rats and humans after TBI. The other ERM homologs or actin do not increase extracellularly after injury, which indicates that ezrin has a function that is separate from its actin binding properties and may be used as a biomarker for TBI.
Future perspectives

Identification of possible subclasses of astrocytes

In our *in vitro* studies, we found that some astrocytes were full with dead cells, whereas others are not, indicating that only certain subtypes of astrocytes are capable of engulfment or are faster at degrading the ingested cells. It has been shown previously that multinucleated astrocytes co-express other markers than GFAP, such as 14-3-3σ or 14-3-3ε or GFAPδ. To identify specific markers for engulfing astrocytes, we will primarily focus on markers that are known to be expressed differently in various astrocytes such as Vim, S100β, nestin, 14-3-3 isoforms and GFAPδ together with engulfment proteins and TUNEL labeling. It would be helpful to be able to identify specific genes that are expressed by the different subclasses. By FACS cell sorting, the astrocytes with ingested dead cells can be sorted from the non-phagocytic cells and gene array analyses of the two groups can be used to identify differences in gene expression between the subclasses and perhaps point to degradation proteins that differ between the two populations.

Possible mechanisms of ingestion and degradation in astrocytes

In our qRT-PCR studies, we found that not only the ced homologues were expressed in the cell cultures, but also an integrin and the Fc-receptor gamma-1 (unpublished observations). Fc-receptors are usually expressed by professional phagocytes and act by binding to antibodies on the material that is being ingested. By inhibition of different receptors and/or possible receptor targets, we will quantify any changes in ingestion to elucidate the mechanism/s used by astrocytes. Since the receptors and their down-stream intracellular signals can control the rate of degradation, we want to mark the dead cells with antibodies to investigate if we can speed up the degradation by using specific phagocytic receptors. Based on our findings, that Rab27a is highly expressed in the astrocytes, we would try to enhance the degradation speed by inhibiting Rab27a with siRNA or use Rab27a KO mice.
Antigen presentation and T cell activation by astrocytes

It has been shown that astrocytes can express MHC class II under certain conditions\textsuperscript{121,126} and we have shown that the receptors are expressed in our cultured astrocytes. It is however still uncertain whether the astrocytes can activate T cells\textsuperscript{134-137}, so the next question would be to investigate whether phagocytic astrocytes are actually presenting the engulfed material and thereby activate, or inhibit T cells. For these experiments we will perform co-culture studies of murine T cells and astrocytes loaded with either dead cells or ovalbumin. Ovalbumin is known to activate T cells when loaded on DC MHC II and can be added directly to the culture or attached to beads to study whether the engulfment pathway change astrocytes ability to elicit T cell activation. Since astrocytic T cell responses have been linked to the exposure to IFN-\(\gamma\)\textsuperscript{121}, the cells will also be treated with IFN-\(\gamma\) to study whether astrocyte activation of T cells can be enhanced that way. The activation of the T cells will be measured by using a proliferative assay for T cell function as well as ELISA study of inflammatory agent secretion from T cells in response to the loaded astrocytes.

Ezrin secretion pathways

We have shown that ezrin is actively secreted after injury, but it still remains to be determined how the protein is secreted. The ERM proteins have been found in exosomes from many different cells\textsuperscript{201-202} but the function of the proteins in the exosomes is still unclear. However, we propose that ezrin may work in consort with F-actin that is the ligand for the DNGR-1 receptor\textsuperscript{194,203}, a receptor involved in the presentation of dead-cell associated antigens\textsuperscript{197}. Since ezrin collapses around F-actin\textsuperscript{154}, the plan is to investigate whether stabilization of actin enhances the secretion of ezrin and actin into the medium. Studies to determine whether destabilization of actin decreases the amount of high molecular weight actin and increase the amount of free ezrin, will also be performed.

Timing and correlation with secondary injuries in the release of ezrin in patients

Previously, we have shown that ezrin is present in CSF from human TBI patients but not correlated it to the type of injury or any secondary events seen after TBI. The time of release of ezrin into CSF will be studied in samples from more patients and compared the levels with other neurological conditions, for example hematomas and normal pressure hydrocephalus. Whether the ezrin levels correlate to a specific type of injury, e.g. contusive
or diffuse, or if the release could be used to predict secondary events, will also be studied in TBI patients and animal models.
Populärvetenskaplig sammanfattning

Traumatisk hjärnskada (THS) orsakas av att huvudet utsätts för våld av något slag och, beroende på typen av våld, kan ge väldigt olika typer av skador. De allvarligare skadorna uppkommer ofta vid trafikolyckor och kan ha dödliga följder. THS-patienter som överlever får många gånger allvarliga och livslånga handikapp eftersom det i dagsläget inte finns några läkemedel som kan hjälpa dem.

I den här avhandlingen har skadornas effekter studerats på molekylär och celular nivå genom cell kulturer, djurmodeller och material från människor med THS. Framför allt har hjärnans vanligaste celltyp, astrocyten, studerats.

Sedan astrocytens upptäckt år 1856, då man ansåg att cellen endast fungerade som ett stöd för nervceller, har intresset för astrocyter växt sig större och större. Idag vet man att de tar hand om ämnen i hjärnan som annars kunde skada de känsliga nervcellerna och hjälper till att bygga upp den s.k. blod-hjärn-barriären som kontrollerar vad som tar sig in och ut ur hjärnan. Astrocyterna kan även ge energi till nervcellerna när de arbetar hårt och modifierar även nervsignaleringen i hjärnan.

Våra resultat visar att astrocyter är fagocyterande celler, d.v.s. slukande celler som kan ”äta upp” döda celler efter skada. Vidare visar vi att fagocytos av döda celler kan rädda nervceller från celldöd, men att astrocyter som tar upp döda celler bryter ner dessa väldigt långsamt. Professionella fagocytter, såsom makrofager, bryter ofta snabbt ner det fagocyterade materialet, men det finns även celler som gör detta långsammare och mer kontrollerat. Til dessa celler hör dendritiska celler, som bryter ner fagocyterade proteiner till mindre delar, s.k. peptider, som sedan kan visas för T-celler. Visningen av peptiderna för T-cellerna gör att de vet vilka hot som finns och vilka celler eller ämnen som ska attackeras och elimineras. Astrocyter, i likhet med dendritiska celler, kan ha förmågan att visa peptider och rikta immuncellerna mot eventuella hot, men man vet inte säkert i dagens läge. Astrocyter i våra kulturer har receptorer som visar peptiderna för T-celler och två av de proteiner som dendritiska celler uttrycker för att sakta ner nedbrytningen så att peptiderna inte förstörs.

Eftersom THS är en så komplicerad sjukdom och att det fortfarande är mycket man inte vet om den, så har vi undersökt vilka proteiner som utsändras från de skadade cellerna. Dessa kan eventuellt används som biomarkörer för att ta reda på hur allvarlig hjärnskadans är, för att ta reda på vilka skademekanismer som startats eller för att förutsäga vad som kommer att hända.
med patienten. Detta kan leda till att patienter med THS får rätt hjälp i rätt tid, innan skadan blivit större.

Efter skada på celler i kultur såg vi att många proteiner som vanligtvis ska finnas inne i cellerna fanns utanför cellerna, i mediet. Många av de identifierade proteinerna efter skada har tidigare kopplats till degenerativa nervsjukdomar eller THS, celldöd eller cellöverlevnad och inflammation. De mest förvånande av proteinerna som vi hittade i mediet interagerar med aktin, proteiner som tillhör cellskelettet. Två av dessa var ezrin och moesin som har liknande funktion inne i cellerna. Vi studerade dessa proteiner närmare och fann att ezrin, men inte moesin, utsöndras aktivt i cell kulturer och i cerebrospinalvätska både hos råttor och hos människor efter skada. Eftersom både moesin och ezrin finns i cellerna och har liknande funktion och uppbyggnad, tyder en aktiv utsöndring av ezrin på att detta protein har en funktion även utanför cellen och att den skulle kunna användas som en biomarker efter THS.
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References


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