Characterisation of inflammation in the central nervous system induced by lipoteichoic acid from *Staphylococcus aureus*: role of glia in the mechanisms of neuronal cell death

Dissertation

zur Erlangung des akademischen Grades des Doktors der Naturwissenschaften

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  (submitted to Glia)

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  Agnieszka Kinsner, Valentina Pilotto, Susanne Deininger, Guy C. Brown, Sandra Coecke, Thomas Hartung and Anna Bal-Price  

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  *Clinical Orthodontical Research, 2001, 4; 161-171*

**Poster presentations relevant for this thesis:**

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  Agnieszka Kinsner, Thomas Hartung, Sandra Coecke, Anna Bal-Price.
  *Poster presented at the 7th International Congress of Neuroimmunology, 27 September - 02 October 2004, Venice, Italy.*

• Activated glia as liaisons between immune and central nervous system
  Agnieszka Kinsner, Anna Bal-Price, Thomas Hartung, Sandra Coecke.
ABBREVIATIONS

AD   Alzheimer’s Disease
AIDS  Acquired Immunodeficiency Syndrome
AIF   Apoptosis Inducing Factor
ALS   Amyotrophic Lateral Sclerosis
APC   Antigen Presenting Cell
Ara-C Cytosine-D-Arabinoside
ATP   Adenosine Triphosphate
BBB   Blood-Brain Barrier
BSA   Bovine Serum Albumine
BSE   Bovine Spongiform Encephalopathy
BrdU  Bromodeoxyuridine
CGC   Cerebellar Granule Cells
CJD   Creutzfeldt-Jacob Disease
CNS   Central Nervous System
COX   Cyclooxygenase
CpG DNA deoxy-Cytidylate-phosphate-deoxy-Guanylate DNA
CSF   Cerebrospinal Fluid
DA    Dopamine
DIV   Day In Vitro
DMEM  Dulbecco’s Modified Eagle’s Medium
DNA   Deoxyribonucleic Acid
DNA-se Deoxyribonuclease
dNTP  deoxy-Nucleotide Triphosphate
ELISA Enzyme-Linked Immunosorbent Assay
ERK1/2 Extracellular Signal-Regulated Kinase1/2
FBS   Foetal Bovine Serum
FeTPPS 5,10,15,20-Tetrakis (4-Sulfonatophenyl) Porphyrinato iron (III)
FITC  Fluorescein-5-Isothiocyanate
G-CSF Granulocyte Colony-Stimulating Factor
GFAP  Glial Fibrillar Acidic Protein
GM-CSF Granulocyte Macrophage Colony-Stimulating Factor
GSH   Glutathion (reduced)
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSSG</td>
<td>Glutathion (oxydised)</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular Adhesion Molecule-1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-Gamma</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitory κB</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitory κB Kinase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>Interleukin-1 Receptor Antagonist</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
</tr>
<tr>
<td>IP-10</td>
<td>Interferon-gamma-inducible protein-10</td>
</tr>
<tr>
<td>IRAK</td>
<td>Interleukin-1 Receptor Associated Kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal Kinase</td>
</tr>
<tr>
<td>LBP</td>
<td>Lipopolysaccharide-Binding Protein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic Acid</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Macrophage Chemoattractant Protein-1</td>
</tr>
<tr>
<td>MDP</td>
<td>Muramyl Dipeptide</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MIP-1</td>
<td>Macrophage Inflammatory Protein-1</td>
</tr>
<tr>
<td>MK-801</td>
<td>5R,10S-5(+)-Methyl-10,11-dihydro-5H-dibenzo-cyclohepten-5,10-imine hydrogen maleate</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MnTBAP</td>
<td>Manganese (III) tetrakis (4-benzoic acid) porphyrin chloride</td>
</tr>
<tr>
<td>MPTP</td>
<td>Mitochondrial Permeabililty Transition Pore</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid Differentiation primary response gene (88)</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor-Kappa B</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-Asparate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NRU</td>
<td>Neutral Red Uptake</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-Steroidal Anti-Inflammatory Drugs</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>$O_2^-$</td>
<td>Superoxide</td>
</tr>
<tr>
<td>ONOO$^-$</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet Activating Factor</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly-(ADP-ribose) Polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s Disease</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PGN</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon Activation, Normal T-cell Expressed and Secreted</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RNAse</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive Nitrogen Species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase- Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
</tr>
<tr>
<td>TA</td>
<td>Teichoic Acid</td>
</tr>
<tr>
<td>TGF-$\beta$</td>
<td>Transforming Growth Factor-Beta</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/Interleukin Receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF-$\alpha$</td>
<td>Tumour Necrosis Factor-Alpha</td>
</tr>
<tr>
<td>TNF-R</td>
<td>Tumour Necrosis Factor-Receptor</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF Receptor-Associated Factor</td>
</tr>
<tr>
<td>z-DEVD-fmk</td>
<td>Z-Asp-Glu-Val-Asp-fluoromethyl ketone</td>
</tr>
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<td>Z-Ile-Glu-Thr-Asp-fluoromethyl ketone</td>
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<td>Z-Leu-Glu-His-Asp-fluoromethyl ketone</td>
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<tr>
<td>z-VAD-fmk</td>
<td>Z-Val-Ala-Asp-fluoromethyl ketone</td>
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1. INTRODUCTION

1.1 Inflammation in the CNS

Interest in Central Nervous System (CNS) inflammation has grown rapidly over the past decade driven by the increasing evidence for a role of neuroinflammation in the development of several important neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, stroke, traumatic brain injury, demyelinating disorders, as well as pathology associated with CNS infections.

In peripheral tissues inflammation generally has a protective role, limiting the survival and proliferation of invading pathogens, promoting tissue repair and recovery; it can be characterised by the cardinal signs described already by Celsus in the first century A.D., namely calor, rubor, tumor and dolor, i.e. increase in temperature, redness, swelling and pain which result from increased blood flow, increased vascular permeability, fluid accumulation and infiltration of blood-derived mediators of inflammation. In peripheral tissues it is directly associated with adherence and invasion of leukocytes (neutrophils, macrophages, lymphocytes) into injured or infected tissues. This innate, or immunologically non-specific, response normally resolves over a few weeks, with accompanying tissue repair aided by macrophages recruited to the site. If the stimulus is sufficiently great or persistent the inflammatory response may become chronic and is characterised by the presence of large numbers of macrophages and T-lymphocytes, and fibrosis. In the CNS a number of physiological and immunological processes appear to be differentially regulated.

1.1.1 Specific aspects of CNS inflammation

The CNS has evolved both anatomically and physiologically to protect its vital functions from damaging immune-mediated inflammation and traditionally has been regarded as immunologically privileged. The brain has several distinctive features and the response to inflammatory insult differs from other tissues. The inflamed brain does not show pain, redness and swelling (typical features of inflammation in peripheral tissues). This is due to the lack of sensory nerves endings and lymphatic vessels, and to specialized mechanisms regulating tissue fluid composition. The blood-brain barrier is important for the maintenance of physiological and immunological homeostasis and restricts the entry of pathogens, plasma proteins and immune cells. The nature and number of cellular mediators of inflammation have been shown to be quite distinct in brain tissue compared to the periphery. During brain inflammation there is little neutrophil recruitment and the major resident inflammatory cells are microglia, which represent the brain’s macrophages, and astrocytes, lately shown to
release many proinflammatory mediators (cytokines, nitric oxide and reactive oxygen species).

The CNS appears to show marked resistance to inflammation in response to a number of stimuli as compared with peripheral tissues. These differences reflect the tight regulatory environment of the brain and a balance between inflammation-induced tissue repair and tissue damage. As an example the acute response of brain parenchyma to a classical pro-inflammatory agent such as bacterial lipopolysaccharide (LPS) differs markedly from peripheral tissues (Montero-Menei et al. 1994). Experimental studies in rodents demonstrate that LPS induces a rapid and massive invasion of neutrophils in the skin, but a limited and delayed response in the brain with little signs of vascular response and no neutrophil recruitment at any time (Perry and Andersson 1992). The main cells that become activated and dominate the cellular response upon LPS treatment are microglia, i.e. brain macrophages.

Other mechanisms that have been proposed to contribute to CNS immune privilege include local productions of anti-inflammatory mediators, e.g., transforming growth factor beta (TGF-β) (Dhandapani and Brann 2003), α-melanocyte-stimulating hormone (α-MSH) (Gispen and Adan 1999; Strand 1999; Ter Laak et al. 2003) and vasoactive intestinal peptide (VIP) (Gozes et al. 2003), and constitutive expression of Fas ligand (FasL) responsible for Fas-mediated apoptosis of CNS infiltrating immune cells (Bechmann et al. 1999; Flugel et al. 2000).

Although the features described above may limit immune-mediated events within CNS parenchyma, it is now accepted that the brain responds to peripheral inflammatory stimuli, integrates and regulates many aspects of the acute phase response and exhibits local inflammatory responses. These responses are crucial for the elimination of pathogens, removal of death cells and tissue repair. Nevertheless, a proper balance between pro- and anti-inflammatory processes is important, as extensive and prolonged inflammation can contribute to both acute and chronic CNS diseases (reviewed extensively in section 1.4).

1.1.2 Immunocompetent cells in the CNS

The CNS is constantly surveyed by a well-developed network of innate immune cells that control all portals of entry for blood-derived pathogens into the CNS parenchyma. Macrophages and dendritic cells have been identified in the meninges and choroid plexus, in strategic locations to guard the ventricular/subarachnoid compartment (McMenamin 1999). Perivascular macrophages, which surround small and medium size cerebral vessels, ensure protection of the CNS at the level of the blood-brain barrier displaying phagocytic and
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immune regulatory functions (Thomas 1999; Williams et al. 2001). However, if the inflammatory stimulus (e.g. pathogen or its components) crosses the blood-brain barrier, the CNS parenchyma itself contains other cells that vigorously react to any immunological stimuli and to neuronal injury, and play an active role in the development of inflammation – the glial cells.

Glia cells are generally classified into two groups: 1) macroglia, which include astrocytes and oligodendrocytes, of ectodermal origin, and 2) microglia, of mesodermal origin, that invade the CNS during embryonic development at the time of vascularization (Raine 1999). Glial cells differ from neurons as they possess no synaptic contacts and retain the ability to divide throughout life. The main roles of glial cells include 1) maintaining the ionic milieu of nerve cells, 2) modulating the rate of nerve signal propagation, 3) modulating synaptic action by controlling the uptake of neurotransmitters, 4) providing a scaffold for neural development, and 5) aiding in (or preventing, in some instances) the recovery from neural injury (Raine 1999).

Oligodendrocytes are restricted to the central nervous system; these cells produce a laminated, lipid-rich wrapping called myelin around some axons; myelin plays an important role in the conduction of action potential in neurons (increases the speed of potential propagation).

Due to the particular importance for this thesis, astrocytes and microglia will be discussed more in detail in the following sections.

1.1.2.1 Microglia

The major resident inflammatory cells in the CNS are microglia. These are macrophage-like cells, derived from bone marrow stem cells that populate the CNS early during the development and remain within the CNS as the resident macrophage population. Microglia comprise up to 20% of the total non-neuronal cell population (Davis et al. 1994). These cells are especially important to guard the integrity and homeostasis of the brain. In normal conditions they are quiescent, but become activated by injury or infection and have been suggested to represent the first line of defence for the CNS, which normally lacks professional antigen presenting cells (APCs) and intraparenchymal leukocytes, until they are recruited to the CNS by proinflammatory stimuli (Kreutzberg 1996).

Microglia have several morphological forms depending on their functional and developmental state (Fig. 1). During embryonic development monocytes migrate to the CNS and convert into an intermediate form, amoeboid cells, with flat morphology and
pseudopodia. It is a transient population present during the late prenatal to early postnatal period. Microglia present in adult brain are called ramified microglia. These cells have a small (5-10 µm) oval cell body with large nucleus and only a little amount of cytoplasm, as well as numerous long, branched processes. Ramified microglia have been characterised as down-regulated (or inactive) macrophages, as they lack most of the characteristic markers and activities of this group (lack of phagocytic and endocytic activity, low expression of leukocyte common antigen (CD45), low levels of membrane ligands and receptors that are essential for mediating or inducing typical macrophage functions) (Davis et al. 1994).

One of the most remarkable properties of microglia is to react to a stress signal from the inside (e.g. stressed or damaged cells, cytokines) as well as from outside (e.g. pathogens) and to direct their reaction for the purpose of tissue repair and for further induction of protective immune responses. Following the stimulus (e.g. neuronal injury) microglia migrate to the damaged sites of the CNS where they proliferate and become activated. During this process microglia undergo maturation, leading to the acquisition of macrophage differentiation markers and effector properties. They can assume two distinct forms – activated and reactive microglia (Davis et al. 1994). Activated microglia appear like swollen ramified cells and are characterised by a larger cell body with shorter processes. They are practically active macrophages, as they express CR3 complement receptors and class I major histocompatibility complex (MHC) antigens. Reactive microglia are typically small, spherical cells and lack ramified processes. They are fully active macrophages with increased CR3 receptor and class I MHC, as well as expression of class II MHC, thus the ability to present antigens to T-cells. The different forms of brain microglia are schematically presented in Fig. 1.

Fig. 1 Cellular forms of brain microglia (adopted from Davis et al. 1994)
The activation of microglia is an important host defence mechanism. Activated microglia release various pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) (Thomas 1992; Kreutzberg 1996; El Khoury et al. 1998; Aloisi 2001), chemokines (IL-8, MIP-1α, MIP-1β, MCP-1) (Peterson et al. 1997; Ehrlich et al. 1998), proteases (Nakanishi 2003), as well as oxidative and nitrosative free radicals (superoxide, nitric oxide, peroxynitrite). Microglia can produce also anti-inflammatory mediators, such as TGF-β, PGE₂ and IL-1 receptor antagonist (IL-1ra) (Benveniste et al. 2001). Due to their ability of phagocytosis microglia play also the role of scavengers for macromolecules and apoptotic or damaged brain cells as well as pathogens.

Upon activation microglia can perform several innate immune functions, including the induction and regulation of T-cell responses. They are able to function as antigen presenting cells (APCs) and to influence the intracerebral balance of T-helper Th1 and Th2 cell-mediated immune responses (Aloisi 2001). In the normal CNS parenchyma the expression of major histocompatibility complex (MHC) antigens is generally very low (Hayes et al. 1987) and resting microglia behave as poor APC, but in virtually all inflammatory and neurodegenerative conditions they become activated and express MHC class II (Kreutzberg 1996).

A constitutive and inducible expression of a variety of other surface receptors is indispensable for microglia to exhibit a proper response to a number of infectious and inflammatory stimuli. The receptors include: 1) pattern recognition receptors (Toll-like receptors, CD14) implicated in the recognition of pathogen associated molecules (such as Gram-positive and Gram-negative bacterial components, viral RNA and proteins, pathogen DNA, etc.); 2) complement receptors (called also opsonic receptors), which mediate or enhance phagocytosis; 3) cytokine and chemokine receptors (for both pro- and anti-inflammatory cytokines); these receptors regulate immune functions of microglia (Aloisi 2001).

Although microglia represent the first line of defence in the brain, the activation of these cells can also have negative effects. The inflammatory mediators released from activated microglia can contribute to CNS damage as neurotoxins (Aloisi 2001), and enhance the onset and progression of CNS diseases. Zhang and Fedoroff (1996) showed that in co-culture with neurons, microglial cells at a low degree of activation supported the neuronal survival, but when these microglia were pre-treated with lipopolysaccharide (LPS), a known strong activator of immune cells, intensive neurotoxicity was found. The harmful effects of LPS-activated microglia on neurons in vitro were also shown by other authors (Bal-Price and...
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Brown 2001). Therefore, microglial activation can influence the extent of brain injury following an inflammatory stimulus and it is important to control the degree and duration of inflammation in the CNS. Excessive or chronic microglial activation has been implicated in a number of neurodegenerative diseases, such as Alzheimer’s disease, Parkinson’s disease (McGeer and McGeer 1998; Teismann and Schulz 2004; McGeer and McGeer 2004; Blasko et al. 2004), as well as trauma, ischemia, brain tumours and infectious diseases (Neumann 2003). Microglia are also involved in several other immune response processes including rejection of transplanted tissue, autoimmune CNS diseases (e.g. multiple sclerosis) and AIDS-associated dementia complex. The role of microglia in the mentioned CNS diseases is discussed more in detail in section 1.4.

1.1.2.2 Astrocytes

Astrocytes make up a substantial proportion of the CNS and participate in a variety of physiological and pathological processes. In the adult, astrocytes constitute about 70% of the total population of brain cells. Their primary function is to provide structural, metabolic and trophic support to other cells (Raine 1999). Astrocytes act as a bridge to supply nutrients from blood capillaries to neurons and provide the major site of glycogen storage in the brain. Moreover, they are also able to synthesise and secrete a variety of neurotrophic and growth factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) and insulin-like growth factor-1 (IGF-1), which may be beneficial for neuronal survival (Chernausek 1993; Moretto et al. 1994; Rudge et al. 1994).

Astrocytes are also responsible for maintaining a homeostatic environment in the brain by: 1) “buffering” or clearance of K+ released from electrically active neurons (Walz 2000); 2) detoxification of synaptically released glutamate by uptake and metabolic mechanisms (Rothstein et al. 1996; Bezzi et al. 1999); 3) regulation of extracellular ionic gradients and pH (Lascola and Kraig 1997); 4) clearance and metabolism of arachidonic acid (Staub et al. 1995). Astrocytes may also provide glutathione precursors to neurons as they protect neurons against the toxicity of ROS in a co-culture system (Dringen et al. 2000).

Astrocytes not only support neuronal survival, they may also modulate neuronal signalling (Chvatal and Sykova 2000). Studies of Murphy et al. (1993) and Nedergaard (1994) showed that there is a direct communication between astrocytes and neurons in cultures of brain cells, probably through gap-junction channels (Froes et al. 1999). Moreover, there is growing evidence that astrocytes play an active role in synaptic transmission, not directly forming synaptic contacts, but contributing to the physiological functioning of neurons by the
integration of neuronal inputs, exhibition of calcium excitability and modulation of neighbouring neuronal responses (Araque et al. 1999; Duffy and MacVicar 1999; Araque et al. 2001).

Apart from being involved in a variety of physiologic processes, astrocytes rapidly react to different neurological insults. A series of changes that occur in astrocytes upon activation has a common name of astrocytosis. The main feature of astrocytosis is the increase in the number and size of glial fibrillary acidic protein (GFAP) expressing cells. GFAP is an intermediate filament cytoskeletal protein expressed primarily by astrocytes and it is considered as the marker of astrocytes (Raine 1999). The precise function of GFAP molecule is still not clear, as well as it has not yet been established whether the increase in GFAP levels is a result of enhanced production of this protein by the cells or an increase in the number of astrocytes either due to proliferation or migration. Studies using double labelling with GFAP antibodies and bromodeoxyuridine failed to show, at least in acute lesions, mitotic divisions of GFAP-expressing cells (Norton et al. 1992). Furthermore there is no convincing evidence that GFAP-positive cells of adult brain are able to migrate. Therefore it is likely that the appearance of GFAP-positive astrocytes in regions of acute neuronal injury is primarily due to a change in phenotype. Astroglial proliferation, however, cannot be excluded in chronic astrocytosis. Reactive astrocytes form a glial scar in areas of tissue necrosis, excluding the non-neuronal cells from parenchyma and filling in the space which results from neuronal loss (McGraw et al. 2001). They also produce proteases and protease inhibitors (e.g. matrix metalloproteinases), which allow them to remodel the extracellular matrix at sites of neuronal injury and to clear the debris of degenerating cells (Gardner and Ghorpade 2003; Wu et al. 2004).

One of the important functions of activated astrocytes is the involvement in the immune functions in the CNS. Several studies performed both in vivo (where the expression of a particular molecule or its mRNA was co-localized to reactive astrocytes) as well as in vitro (using primary cultures of astrocytes), have demonstrated that activated astrocytes produce a large variety of molecules, which are involved in the initiation and regulation of the inflammatory response. These include several pro- and anti-inflammatory cytokines (IL-1, TNF-α, IL-6, TGF-α, TGF-β), chemokines (IL-8) and eicosanoids (leukotriens B4 and C4, prostaglandin E, thromboxanes A2 and B2) (Chao et al. 1995; Mollace et al. 1997; De Groot et al. 1999; Dong and Benveniste 2001; De Groot and Woodroffe 2001; Xu et al. 2003; Mrak and Griffin 2005). Activation of astrocytes leads also to the expression of inducible nitric oxide synthase (iNOS) (Galea et al. 1994; Brown and Bal-Price 2003). Activated astrocytes
express molecules involved in immune responses such as major histocompatibility complex antigens (MHC) (Girvin et al. 2002; Jarosinski and Massa 2002; Hofbberger et al. 2004) and are able to present antigens to T lymphocytes (Cornet et al. 2000). Moreover, they up-regulate the expression of several adhesion molecules (selectins, integrins, adherins), which play a role in the migration of leukocytes through the blood-brain barrier into the CNS parenchyma (Prat et al. 2001; Dietrich 2002).

Prominent reactive astrocytosis was found in AIDS dementia complex, a variety of viral infections, prion-associated spongiform encephalopaties, inflammatory demyelinating diseases, acute traumatic brain injury, ischemia (Swanson et al. 2004) and neurodegenerative diseases (e.g. Alzheimer’s disease) (Mrak and Griffin 2005).

Astrocytes are also essential for the morphological and physiological formation of a functional blood brain barrier in the CNS. The concept of the existence of a blood-brain barrier (BBB), which separates the brain from the rest of the body, was developed by Paul Ehlrich in 1906, when in his experiment he has shown that some dyes administered intravenously to rats stain all the organs except the brain (Sukriti 2003). The BBB is a physical barrier between blood vessels in the CNS and the brain tissue that plays an important role in the protection of the CNS. It is formed by non-fenestrated endothelial cells that develop tight junctions among adjacent cells providing an increased resistance to passage of solutes between cells. *In vitro* studies have demonstrated that endothelial cells alone cannot provide a tight barrier without the presence of astrocytes (Deli and Joo 1996; Risau et al. 1998). Several studies suggest that astrocytes secrete soluble factors essential for the development of specific BBB properties (Sobue et al. 1999). The presence of a continuous sheet of astrocyte end feet around the capillaries results in a tight interaction between cerebral astrocytes and endothelial cells. This interaction results in a change in morphology and function of the cerebrovascular endothelium, and is especially important for the maturation and differentiation of tight junctions between endothelial cells (Prat et al. 2001; Abbott 2002), induction of specific metabolic properties of the endothelial cells, such as the expression of alkaline phosphatase and $\gamma$-glutamyltransferase (Roux et al. 1994).

The blood-brain barrier limits access of almost all molecules apart the smallest - oxygen, carbon dioxide and sugars - which pass with no difficulty. Most drugs and hormones are too large to pass the barrier. In addition, the blood-brain barrier is an excellent way to protect the brain from common infections, as most microbes do not cross the BBB. Also the components of the immune system (monocytes, macrophages, lymphocytes, antibodies) do not penetrate across the BBB under normal conditions.
The nature of the BBB can vary depending on the location; in certain areas of the brain, usually associated with ventricular organs and areas of endocrine regulation, commonly known as circumventricular system (consisting of area postrema, hypothalamus, pituitary and pineal gland, choroid plexus) (Ganong 2000), endothelial cells do not form tight junctions. Also some regions of the brain meninges have no functional barrier.

The integrity of the BBB can be also altered in some pathological conditions such as infection (bacterial, viral, parasitic), inflammation, brain injury, tumours and hypertensive encephalopathy, as well as by some drugs and hyperosmotic agents (e.g. mannitol). In these conditions, the increase in BBB permeability may allow access of leukocytes into the brain parenchyma where they can release neurotoxins, activate endogenous inflammatory processes or, in the case of macrophages, phagocytose cell debris.

1.2 Mediators of inflammation in the CNS
Most of the key mediators of inflammation identified in the peripheral tissues are also expressed in the inflamed brain. The primary mediators of inflammation include cytokines, chemokines, eicosanoids, platelet activating factor (PAF), reactive oxygen species (ROS) and nitric oxide (NO). All these factors have been reported to be expressed and/or released by glial cells in the response to insult to the CNS in vivo and in vitro.

1.2.1 Cytokines
Cytokines are signalling molecules (secreted, cell-membrane associated or stored in the extracellular matrix) that transmit signals from the extracellular environment to the nucleus through specific receptors and intracellular signal transduction or second messenger molecular pathways. They are key regulators of innate and adaptive immune responses.
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In the brain a number of cytokines are induced in response to cerebral ischemia, injury, infectious and autoimmune diseases; they include the proinflammatory IL-1α, IL-1β, IL-2, IL-6, IL-8 and TNF-α, as well as some anti-inflammatory cytokines such as TGF-β, IL-10 (Aloisi 2001). The sources of cytokines in the CNS are mainly microglia and astrocytes, but also tissue infiltrating immune cells and CNS-associated macrophages (Raivich and Banati 2004).

The receptors for most cytokines have been described in the CNS (Szelenyi 2001), some of them at very low level, although rapid up-regulation can occur after injury. Cytokines have a multitude of actions that are important in the process of neurodegeneration. The effects of cytokines may depend on which cell type they act upon and whether it is a direct or indirect effect (Allan and Rothwell 2001).

1.2.1.1 Pro-inflammatory cytokines

The two main pro-inflammatory cytokines with pleiotropic and largely overlapping functions are IL-1 and TNF-α. Intracerebrally produced TNF-α can be involved both in initiating CNS tissue destruction and inflammation (Akassoglou et al. 1998) as well as maintaining autoimmune inflammation (Taupin et al. 1997).

Two different TNF-α receptors have been characterised: TNF-RI (or p55), mediating most actions of soluble TNF-α and known to induce apoptosis, and TNF-RII (p75), more sensitive to transmembranous TNF-α and shown to have anti-apoptotic effects (Pan et al. 1997a; Pan et al. 1997b). Moreover, members of the TNF-receptor family activate several transcription factors, including NF-κB, which themselves induce the transcription of mediators of inflammation (O’Neill and Kaltschmidt 1997; Koedel et al. 2000).

TNF-α is reported to have neurotoxic effects in vivo, as mice overexpressing TNF-α developed neuropathological symptoms (Probert et al. 1995). In vitro, TNF-α has been shown to stimulate secretion of glutamate from glial cells (Piani and Fontana 1994) and to potentiate glutamate neurotoxicity in human foetal brain cells (Chao and Hu 1994).

IL-1β induces inflammatory effects similar to TNF-α. In many CNS diseases IL-1β concentrations have been shown to correlate significantly with TNF-α concentrations and with neurological complications (McCracken et al. 1989; van Deuren 1994; Jain et al. 2000). Convincing evidence suggests a prominent role of IL-1β in acute neuronal injury. Increased expression of IL-1β is seen in the CNS after a variety of brain insults and administration of exogenous IL-1β to animals undergoing ischemic or excitotoxic challenges leads to a dramatic increase in cell death. On the other hand, the administration of a selective IL-1 receptor antagonist (IL-1ra) markedly reduces the extent of cell death induced by ischemic or
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excitotoxic injury in rats (Mulcahy et al. 2003; Hailer et al. 2005). Similarly, inhibition of caspase-1 (which is required for the release of active IL-1β) and the administration of anti-IL-1β antibodies, have been also shown to decrease neuronal injury (Rothwell and Luheshi 2000; Touzani et al. 2002). Recent studies have employed transgenic animals with specific modifications of selected genes to investigate the contribution of inflammation to neurodegeneration. Mice deficient in pro-inflammatory cytokines (Boutin et al. 2001; Ohtaki et al. 2003) show a reduction in cell death in response to different insults when compared to their wild-type counterparts, whereas in mice deficient in anti-inflammatory cytokines neuronal injury is increased (Grilli et al. 2000).

TNF-α and IL-1β are known to cause blood-brain barrier breakdown, mainly through their ability to induce in cerebrovascular endothelial cells and astrocytes the expression of adhesion molecules and of chemokines, which facilitate leukocyte extravasation and recruitment into the CNS (Lee and Benveniste 1999; Sedgwick et al. 2000). Intrathecal injection of TNF-α or IL-1β leads to blood-brain barrier injury, influx of leukocytes and serum proteins across the BBB into the CSF, brain oedema, increase in intracranial pressure and CSF lactate levels, reduction in cerebral oxygen uptake and in cerebral blood flow, and neuronal apoptosis (Allan 2000; Allan and Rothwell 2001). They also up-regulate endothelial derived adhesion molecules (P-selectin, E-selecin) in cerebral vasculature, which promotes the recruitment of granulocytes to the site of inflammation (Tang et al. 1996). TNF-α and IL-1β have been shown to induce the expression of iNOS and trigger the release of NO from glial cells, as well as vascular endothelium (Bonmann et al. 1997). TNF-α and IL-1β are also known to induce glial reactions and to promote astrogliosis and microgliosis (Selma et al. 1990; Balasingam et al. 1994; Probert et al. 1995; Herx and Yong 2001; Basu et al. 2002).

Another cytokine produced in the CNS by microglia and astrocytes is interleukin-6 (IL-6), a multifunctional mediator, which plays an important role in cell-cell signalling under normal and pathophysiological conditions (Chao et al. 1995; Gadjient and Otten 1995; Gruol and Nelson 1997). IL-6 can exert both pro-and anti-inflammatory effects (Gadjient and Otten 1997). The IL-6 levels, which are low in the normal brain, are strongly up-regulated following brain injury or inflammation in vivo (Clark et al. 1999), and in response to LPS or TNF-α treatment in vitro (Gruol and Nelson 1997). The main role of IL-6 in the brain is its neurotrophic and neuroprotective effect (Chao et al. 1995). Both in vitro and in vivo studies demonstrated that IL-6 promotes the survival of neurons against several neurotoxic agents (glutamate, MPP+, NMMA), presumably through inhibition of apoptosis (Hama et al. 1991; Yamada and Hatanaka 1994; Hirota et al. 1996; Umegaki et al. 1996). Apart from its
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protective effects on neurons, IL-6 can also mediate glial activation. Mice overexpressing IL-6 have showed severe astrogliosis and increased microglial reactions. IL-6 had also mitogenic effects in astrocytes cultured in vitro (Selmaj et al. 1990).

Glial cells are also considered the major CNS sources of cytokines that stimulate the humoral and cell-mediated immune responses such as IL-12 (Becher et al. 1996; Aloisi et al. 1997), and IL-18 (Prinz and Hanisch 1999). All these cytokines have been shown to be produced during infections and autoimmune diseases and to be critically involved in the development of Experimental Allergic Encephalomyelitis (EAE, an animal model of multiple sclerosis) (Segal et al. 1998; Shevach et al. 1999).

1.2.1.2 Anti-inflammatory cytokines

Although most studies concentrate on glia-derived pro-inflammatory cytokines, recently more attention has been devoted to the role of CNS cells in the anti-inflammatory processes that down-modulate inflammation and immunity. Evidence has provided that microglia produce anti-inflammatory cytokines, such as TGF-β, IL-10, and IL-1 receptor antagonist (IL-1ra), whereas astrocytes secrete TGF-β and IL-10 (Jander et al. 1998; Kiefer et al. 1998; Liu et al. 1998; De Groot et al. 1999; Aloisi et al. 1999). IL-1ra has a major role in counteracting the biological effects of IL-1, thanks to its ability to bind to IL-1 receptor I (IL-1RI) without initiating signal transduction, and thus blocking the IL-1 receptor. IL-10 is found in large amounts in the CSF during bacterial meningitis (van Furth at al. 1995). It inhibits the production of IL-1β, IL-6, IL-8 and TNF-α by monocytes and the release of reactive oxygen species (ROS) by macrophages (Fiorentino et al. 1991; Bogdan et al. 1991; Cunha et al. 1992). TGF-β is also an anti-inflammatory cytokine that can deactivate microglia by suppressing the hydrogen peroxide release (Tsunawaki et al. 1988), as well as release of nitric oxide (Matsuno et al. 2001). It also inhibits endothelial granulocyte adhesion and the production of several cytokines including IL-1, TNF-α, IL-6 and IFN-γ. In addition, TGF-β protects neurons from N-methyl-D-aspartate (NMDA) induced calcium overload and thus from excitotoxic death (Hailer et al. 2001) as well as from β-amyloid induced neurotoxicity (Chao et al. 1994). In microglial cells TGF-β, IL-4 and IL-10 are known to inhibit microglia activation, down-regulate the expression of molecules associated with antigen presentation on these cells, and inhibit the production of pro-inflammatory cytokines, chemokines, nitrogen and oxygen radicals (Aloisi et al. 1999; O'Keefe et al. 1999).
1.2.2 Chemokines

Chemokines are a group of cytokines that play a major role in the recruitment of leukocytes from the blood into the CNS and the migration of some cells, including microglia. Chemokines are a superfamily of small peptides, which based on the cysteine motif are divided into four families (CXC, CC, C, and CX3C). They interact with receptors that have seven G protein coupled transmembrane domains expressed on a wide variety of immune and non-immune cells (Zlotnik and Yoshie 2000). The potential sources of chemokines produced intracerebrally during CNS inflammation are endothelial cells, astrocytes and microglia (Hesselgesser and Horuk 1999). Chemokines of the CXC family (IL-8, IP-10) and of the CC family (MIP-1α, MIP-1β, MCP-1 and RANTES) are produced by adult microglia and may contribute to the recruitment of T-cells, macrophages and dendritic cells to the CNS parenchyma (De Groot and Woodroofe 2001). Also human embryonic microglia release MIP-1α, MIP-1β, and MCP-1 upon LPS stimulation (Lee et al. 2002).

The panel of chemokines released by astrocytes is slightly different (Hua and Lee 2000). Astrocytes were shown to release IP-10, IL-8, MCP-1 and RANTES, but not MIP-1 and MIP-2, upon stimulation with LPS, viruses, pro-inflammatory cytokines (IL-1β and TNF-α) (Hua and Lee 2000) and complement derived anaphylatoxins (Jauneau et al. 2003). Intracisternal injection of MIP-1 and MIP-2 causes an increase in CSF protein levels and the appearance of leukocytes, indicating an important role of these chemokines in bacterial meningitis (Lahrtz et al. 1997; Lahrtz et al. 1998; Zwijnenburg et al. 2003). Increasing evidence suggests that chemokines could also stimulate the migration of microglia to injured or inflammatory sites (Rappert et al. 2004; Marella and Chabry 2004). Expression of chemokine receptors on microglia has been demonstrated in vitro as well as in vivo in the course of infectious and autoimmune diseases (Harrison et al. 1998; McManus et al. 1998; McManus et al. 2000).

1.2.3 Eicosanoids and leukotriens

Activation of phospholipase A2 induces the hydrolysis of cell membrane phospholipids and the release of arachidonic acid and platelet-activating factor (PAF). Prostaglandins (PG) and thromboxanes, collectively called eicosanoids, are synthesized from arachidonic acid through cyclooxygenase (COX) pathway and are important regulators of inflammation and immune responses. The activation of lipooxygenase leads to the release of leukotriens. Increased levels of eicosanoids and inducible COX-2 isoforms have been demonstrated in several CNS inflammatory pathologies and evidence has been provided that microglia and astrocytes produce large amounts of PGE2 in vitro (Xu et al. 2003; Ajmone-Cat et al. 2003; Teather et al,
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Cultured microglia produce also PGD₂ and thromboxane B₂ (Giulian et al. 1996; Mohri et al. 2003). PGE₂ is thought to have a protective role in inflammation due to its ability to inhibit macrophage pro-inflammatory functions and to down-regulate Th1 responses (Goodwin and Ceuppens 1983). It also inhibits microglial production of pro-inflammatory cytokines and nitric oxide as well as expression of MHC class II molecules (Minghetti et al. 1997; Minghetti and Levi 1998; Zhang and Rivest 2001). These findings suggest that PGE₂ is a factor that locally down-regulates the immune and inflammatory responses in CNS.

Platelet-activating factor is a potent inflammatory mediator derived from endothelial cells, macrophages and platelets. PAF plays an important role in normal CNS functions but also in brain damage and cerebral inflammation. In the rat brain PAF is intensely expressed by microglia. It is interesting that both pneumococcal cell wall components and PAF share a common phosphocholine unit. Pneumococci can bind to the PAF receptor and via this “gate” invade the CNS (Ring et al. 1998). Inhibition of signalling from the PAF receptor attenuates inflammation, particularly leukocytosis, during pneumococcal meningitis.

1.2.4 Metalloproteinases

Metalloproteinases (MMPs) are zinc-dependent endoproteinases that can degrade components of the extracellular matrix. They play a physiological role in embryonic development and tissue morphogenesis. Over the past few years clinical and experimental studies provided evidence for a role of matrix MMPs in neuroinflammation and brain damage involving blood brain barrier breakdown, neuroinflammation, glial reactivity, neuronal death (Leib et al. 2000; Kieseier et al. 2001; Rosenberg 2002). In particular, MMPs have been implicated in the pathogenesis of gliomas (tumours of glial origin), viral infections, multiple sclerosis, Alzheimer’s disease, brain trauma and ischemia (Kaczmarek et al. 2002). MMPs can be produced by glial cells, both astrocytes (Wells et al. 1996) and microglia (Toft-Hansen et al. 2004). In normal conditions they are expressed at low levels in the brain (Kaczmarek et al. 2002), but their expression is up-regulated in diseased tissue (e.g. within CNS lesions in animal models of multiple sclerosis (MS) and in tissue from patients with this disease). The expression of MMPs contributes to tissue destruction and inflammation in MS (Leppert et al. 2001). In bacterial meningitis MMPs were shown to contribute to blood-brain barrier breakdown and to facilitate leukocyte extravasation (Azeh et al. 1998; Paul et al. 1998; Kieseier et al. 1999). The two main MMPs involved in meningitis are MMP-8 and -9. CSF levels of MMP-9 in bacterial meningitis were 10 - 1000 folds higher than in viral meningitis (Kolb et al. 1998). The increase of MMP-8 was found to be a specific feature of bacterial
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meningitis. Inhibition of MMPs is one of the potential therapeutic alternatives in meningitis. Indeed, treatment with a broad-spectrum inhibitor of MMPs decreases MMP-9 and TNF-α concentrations in CSF and reduces the neocortical neuronal damage (Leib et al. 2001).

1.3 Mechanism of inflammatory-induced neuronal cell death

1.3.1 Oxidative and nitrosative stress

Oxidative stress was shown to mediate neuronal damage in wide range of neurological disorders including cerebral ischemia, cerebral trauma, primary neurodegenerative disorders, Alzheimer's and Parkinson's disease, and infectious diseases (e.g. meningitis) (Brown and Bal-Price 2003). Oxidative stress occurs when oxygen and nitrogen free radicals are generated in excess and the endogenous antioxidant systems are no more capable to remove the overload of these radicals. The biologically relevant free radicals are superoxide anion (O$_2^-$), perhydroxyl radical (protonated superoxide anion (HO$_2^-$)), the hydroxyl radical (HO') and free radical nitric oxide (NO'). The production of ROS occurs at the site of inflammation and contributes to tissue damage. Several features of the brain suggest that it is highly sensitive to oxidative stress. The brain is known to have the highest oxygen metabolic rate of any organ in the body, consuming approximately 20% of the total amount of oxygen yet representing only 2 - 5% of the total body weight (Dringen et al. 2000). This increases the probability that excessive levels of reactive oxygen species will be produced. Neurons are very vulnerable to ROS because of the high local oxygen tension, high levels of polyunsaturated fatty acids that can be oxidized by ROS, and high concentration of iron, which has been associated with free radical injury (Dringen et al. 2000). Also, local levels of endogenous antioxidants in the CNS are relatively low (Bolanos 1995).

1.3.1.1 Reactive oxygen species (ROS)

Superoxide anion (O$_2^-$) is the most commonly occurring oxygen free radical. Tissue toxicity from extracellular superoxide is based on its direct reaction with biological targets, such as lipids, catecholamines, DNA, RNA, steroids, as well as is a result of the secondary formation of other oxygen radicals (Cuzzocrea et al. 2001). Nature has evolved a class of superoxide dismutase enzymes (SOD) to remove the deleterious free radical by-product of oxygen metabolism. These enzymes react rapidly with superoxide and dismutate the radical to non-radical products, H$_2$O$_2$ and oxygen, faster than superoxide can react with potential biological targets. Dismutation leads to the production of H$_2$O$_2$, which can concomitantly reduce ferric
ion to ferrous ion. Reduction of ferric iron in the presence of hydrogen peroxide (also described as Haber-Weiss reaction) leads to the formation of the most active free oxygen radical - the hydroxyl radical (HO\textsuperscript{•}) that may cleave covalent bonds in proteins and carbohydrates, cause lipid peroxidation, and destroy cell membranes.

Oxidative stress represents a significant pathway that leads to the destruction of both neurons and vascular cells in the CNS. It occurs as a consequence of an alteration in the equilibrium between the production of ROS and the antioxidative processes (in favour of the formation of ROS). The production of ROS can lead to neuronal injury through: 1) destruction of cellular membrane lipids by their peroxidation (Cuzzocrea et al. 2001); 2) cleavage of DNA during the hydroxylation of guanine and methylation of cytosine (Hemmani and Parihar 1998; Vincent and Maiese 1999); 3) oxidation of proteins that yield protein carbonyl derivatives and nitrotyrosine (Cuzzocrea et al. 2001).

ROS are able to activate or suppress the activities of protein tyrosine kinases, serine/threonine kinases and phosphatases (Wang et al. 2003), and to induce the activation of several transcription factors including activator protein-1 (AP-1), nuclear factor κB (NF-κB), and p53, thereby modulating cellular responses (Kamata and Hirata 1999). However, the possibility that mild ROS generation may also provoke protective mechanisms through activation of pro-survival factors cannot be ruled out. In addition to the detrimental effects on cellular integrity, reactive oxygen species can inhibit complex enzymes in the electron transport chain of the mitochondria resulting in blockage of mitochondrial respiration (Yamamoto et al. 2002; Huang and Manton 2004).

1.3.1.2 Reactive nitrogen species

Nitric oxide (NO) influences many aspects of the normal physiology or pathophysiology of the CNS being either beneficial or detrimental to the nervous tissue. It is produced from oxidation of L-arginine to L-citrulline via an enzymatically catalyzed process (Bredt 1999). The enzymes that produce NO have been divided into two classes depending on their special features: constitutive and inducible NO synthases (Wang and Marsden 1995). The brain expresses all three identified nitric oxide synthases (NOS) isoforms. Moreover NOS activity in the brain is higher than in any other tissue of the body (Duncan and Heales 2005).

The constitutively expressed isoforms of nitric oxide synthase (cNOS) include neuronal NOS (nNOS or type 1) and endothelial NOS (eNOS or type 3). Their function is dependent on the changes in intracellular Ca\textsuperscript{2+} levels and binding to calmoduline (Moncada et al. 1995). These enzymes can produce relatively small amounts of NO, mainly under physiological
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Nitric oxide has several physiological functions in the CNS. It can act as a neurotransmitter, as well as co-transmitter. A subset of neurons termed as “nitrergic nerves” were shown to release NO (Moncada et al. 1995) and a large body of evidence suggests that these neurons play a role in the control of neuroendocrine secretion in the hypothalamus, behavioural changes, as well as in learning and memory (Holscher 1997). NO’s role as a neurotransmitter in the hippocampus appears to be important in the phenomenon of “long term potentiation”, which is a form of synaptic plasticity associated with learning and memory (Arancio et al. 1996). NO produced by eNOS also functions as a vasodilator in the brain, acting on smooth muscle cells of the arteries and arterioles. It regulates the local blood flow and is believed to help to preserve the cerebral blood flow in cases of brain ischemia (Alderton et al. 2001). The role in maintaining uninterrupted circulation is vital, since the brain (and especially neurons) have high metabolic demand and do not tolerate reduced blood flow (Sims and Anderson 2002).

In contrary to the constitutive nNOS and eNOS, the expression of the inducible form of the enzyme (iNOS or type 2) is evoked only by appropriate stimuli such as bacterial lipopolysaccharide (LPS), pro-inflammatory cytokines (IFN-γ, IL-1β, TNF-α, IL-6) (Forstermann and Kleinert 1995; MacMicking et al. 1997). iNOS, first identified in macrophages, is expressed in many brain cells, however, not under normal, physiological conditions. Astrocytes in vitro can be stimulated to express iNOS and generate up to 1 µM NO in the extracellular medium within few hours (Brown 1995). Human microglial cells have also been shown to express iNOS both in vivo and in vitro (Kitamura et al. 1998). Some neurons can express iNOS and release NO at least under in vitro conditions (Moro et al. 1998).

Unlike the constitutive forms of nitric oxide synthases, iNOS can produce high amounts of NO for relatively long periods. Induction of iNOS may have either toxic or protective effects, depending on the type of the insult, the tissue type, the level and duration of iNOS expression. In high local concentration NO can be cytostatic and cytotoxic for fungal, bacterial and protozoal organisms, as well as for tumour cells (Xu et al. 2002; Colasanti et al. 2002; Ascenzi et al. 2003). On the other hand, prolonged exposure to high concentration of NO may be also cytotoxic for the host cells (Abramson et al. 2001; Wink et al. 2001).

The iNOS is regulated at the expression level by transcriptional and post-transcriptional mechanisms (Kleinert et al. 2003). The main regulator of iNOS expression at transcriptional level seems to be nuclear factor-κB (NF-κB). Pharmacological inhibition of NF-κB significantly attenuates iNOS mRNA expression and NO production in cytokine stimulated
cells (de Vera et al. 1996; Salzman et al. 1996; Taylor et al. 1998). The important role for NF-κB binding sites in the induction of iNOS has been shown in murine (Blanchette et al. 2003), rat (Eberhardt et al. 1998) and human cells (Chu et al. 1998; Marks-Konczalik et al. 1998). Several other transcription factors were demonstrated to have an important function in the regulation of iNOS at the promoter level, among which are the octamer factor (Oct), interferon regulatory factor-1 (IRF-1), signal transducer and activator of transcription-1α (STAT-1α), cAMP-induced transcription factors CREB and C/EBP, activating protein-1 (AP-1), peroxisome proliferator-activated receptors (PPAR) (extensively reviewed by Kleinert et al. 2003). Along with the transcriptional control, post-transcriptional mechanisms play an important role in regulation of iNOS expression. Several RNA binding proteins seem to destabilize iNOS mRNA and inhibit translational efficiency (reviewed by Taylor and Geller 2000). Also transforming growth factor β1 (TGF-β1) was shown to destabilise iNOS mRNA, retard the synthesis of iNOS protein and accelerate its degradation (Vodovotz et al. 1996).

Once produced, NO has a short half-life, and it decomposes rapidly to nitrite (NO₂⁻) and nitrate (NO₃⁻) (Singh and Evans 1997). These two NO-metabolites can be detected using a colorimetric assay – Griess reaction (Green et al. 1982). However, NO can also combine rapidly with the superoxide anion (O₂⁻) and form peroxynitrite (OONO⁻), which can damage DNA and proteins, cause lipid peroxidation as well as inhibition of cellular respiration. Peroxynitrite can diffuse much faster through the cell membrane and exerts more toxic effects than hydroxyl radicals. Many of the toxic effects previously attributed to superoxide and nitric oxide alone may be in fact due to peroxynitrite. Peroxynitrite was described to cause cell death and tissue damage in number of neurodegenerative diseases such as Parkinson’s disease, Alzheimer’s disease, amyotrophic lateral sclerosis (ALS) and brain ischemia (Torreilles et al. 1999; Ebadi and Sharma 2003), as well as in toxic shock, arthritis, acute reperfusion injury (Bauerova and Bezek 1999; Boveris et al. 2002; Wang et al. 2003).

There are in vitro observations showing that NO and peroxynitrite can trigger DNA damage as a result of nitrosylation and deamination of nucleic acids leading to single strand and double strand breaks in DNA. In addition, damage to DNA can cause activation of the poly-(ADP-ribose) polymerase (PARP). PARP is an enzyme that is activated during DNA damage and is suggested to regulate gene expression and gene amplification, cellular differentiation, cellular division and malignant transformation as well as apoptotic cell death. Upon binding to DNA PARP becomes activated and cleaves NAD⁺ in an ATP dependent manner. Therefore, excessive activation of PARP leads to the depletion of cellular NAD⁺ and ATP pools, and contributes to cellular energy depletion (Cosi and Marien 1999). It was shown
that ROS and RNS activate PARP not only via DNA damage but also directly (Hasko et al. 2002). Evidence shows that PARP activation resulting from oxidative/nitrosative stress is involved in the pathogenesis of neurodegenerative diseases (Virag and Szabo 2002).

Another mechanism leading to neuronal damage induced by NO and peroxynitrite is the inhibition of mitochondrial respiration compromising cellular energy metabolism. RNS are known to interact and inhibit irreversibly components of the electron transport chain in mitochondria and in particular complex I, complex II and complex IV (Duncan and Heales 2005).

There are differences in the susceptibility of brain cells to NO. Upon \textit{in vitro} exposure to NO astrocytes appear more resistant to the effects of RNS, when compared to neurons. This may be explained by the astrocytes’ ability to up-regulate glycolysis, as following NO-mediated inhibition of respiration these cells rapidly increase the activity of key regulatory enzymes of glycolysis (Almeida et al. 2004). Another factor influencing the difference in susceptibility to RNS between astrocytes and neurons appears to be related to the cellular availability of anti-oxidative reduced glutathione (GSH). In astrocytic cultures the levels of GSH are approximately double than in neuronal cultures (Bolanos et al. 1995). Moreover astrocytes were shown to up-regulate quickly the biosynthesis of GSH upon exposure to NO (Heales et al. 2004).

Although astrocytes may have a neuroprotective role, increasing the GSH availability for neuronal cells, prolonged co-culture of neurons with NO generating astrocytes leads to mitochondrial dysfunction and to neuronal cell death (Stewart et al. 2000) probably due to the formation of more potent oxidizing species such as peroxynitrite.

\textit{1.3.1.3 Anti-oxidative mechanisms}

Reactive oxygen species are generated in high amounts during normal oxidative metabolism in the brain. Since neuronal cells are highly susceptible to oxidative and nitrosative stress, in the brain there are several antioxidative mechanisms, which protect neurons by inactivating the excess of ROS and RNS. Antioxidants may be classified according to their chemical nature and mode of function into: 1) enzymes that act on specific radicals after they are formed and degrade them to less harmful products (e.g. superoxide dismutase, catalase); 2) preventive antioxidants that act by binding to and sequestering oxidation promoters and transition metal ions (iron, copper), which strongly accelerate free radical formation; 3) scavenging antioxidants (with different mechanisms of action).
Reduced glutathione (GSH) is a tripeptide (Glu-Cys-Gly) considered to act as a major defence mechanism against ROS/RNS toxicity in the brain (Dringen et al. 2000). GSH oxidation occurs in the presence of GSH peroxidases, which catalyse the conversion of H$_2$O$_2$ to H$_2$O and O$_2$ in the cytoplasm, forming GSH disulphide (GSSG). The oxidation product can subsequently be regenerated to GSH by the NADPH dependent enzyme GSH reductase (Dringen et al. 2000). GSH directly reacts with RNS and ROS (Heales and Bolanos 2002). Intact reduced glutathione pools are of extreme importance for the cells as they provide protection from oxidant induced injury.

Another important mechanism that protects brain cells against excessive ROS production is dysmutation of O$_2^-$ by superoxide dismutase (SOD). There are three forms of SOD: mitochondrial (Mn, SOD2), cytosolic (Cu/Zn, SOD1), and linked with extracellular surfaces (SOD3). SODs catalyze the formation of oxygen and H$_2$O$_2$ from two O$_2^-$. Hydrogen peroxide that is formed in this reaction is subsequently transformed by another enzyme - catalase - into oxygen and water. Under normal conditions SOD keeps under control the formation of superoxide; however in acute and chronic inflammation the formation of O$_2^-$ overwhelms the capacity of SOD to remove it.

The brain contains also some antioxidant proteins, which bind metal ions that are co-factors in reactions of ROS formation (lactoferrin, transferrin – bind ferric ions; ceruloplasmin binds copper). Other antioxidant substances that are important for the CNS are scavengers of ROS/RNS such as $\alpha$-tocoferol (vitamin E), ascorbic acid (vitamin C), carotene (pro-vitamin A) and coenzyme Q. Also melatonin, an endogenous substance produced in the pineal gland, is a powerful antioxidant of particular interest for the CNS (Gupta et al. 2003).

In the brain astrocytes are considered to play an important role in the defence against oxidative stress and have a higher capacity to detoxify ROS than other CNS cells (Dringen et al. 2000). They maintain high intracellular concentrations of certain antioxidants, which makes them more resistant to oxidative stress compared to oligodendrocytes and neurons (Wilson 1997). Astrocytes are the main source of reduced glutathione and contain the highest quantities of GSH from all CNS cells. Neurons have significantly lower GSH content, which is considered to be one of the reasons for higher susceptibility of this cell type to oxidative stress (Dringen et al. 2000). Following reactive gliosis, the neuroprotective role of astrocytes may be accentuated because of increases in a number of activities: 1) expression of antioxidant enzymes; 2) transport and metabolism of glucose that yields reducing equivalents for antioxidant regeneration and lactate for neuronal metabolism; 3) synthesis of glutathione; 4) recycling of vitamin C (Wilson 1997).
Attempts for new therapies in many inflammatory CNS diseases are based on the control of oxidative stress. There are three strategies to protect the tissues from locally produced oxygen radicals: 1) deliver SOD or SOD mimetics; 2) deliver catalase or related peroxynitrite scavenger; 3) chelate and thereby inactivate the trace iron that catalyses the reaction. Until now, pharmacological interventions to reduce ROS generation in shock, inflammation and ischemia/reperfusion include: 1) vitamin E-like antioxidants; 2) N-acetylcysteine; 3) SOD mimetics (e.g. MnTBAP) (Szabo 1996); 4) peroxynitrite decomposition catalysts (e.g. FeTPPS) (Crow 2000); and 5) iNOS inhibitors (Southan and Szabo 1996).

1.3.2 Excitotoxicity

Excitotoxicity is a process, in which excessive amounts of excitatory amino acids, in particular glutamate, induce neuronal toxicity. Under normal conditions glutamate is cleared from the neuronal synapses by astrocytes via glutamate transporters and is converted into glutamine, which is released and in turn taken up by neurons (Simard and Nedergaard, 2004). This prevents that glutamate reaches excitotoxic levels in the extracellular space. Astrocytes can also themselves release glutamate that modulates the activity of both excitatory and inhibitory synapses.

However, when released in excess, glutamate induces neurotoxicity interfering with the NMDA and/or AMPA receptors, which results in the overload of neurons with Ca\(^{2+}\) and Na\(^{+}\). Increased levels of Ca\(^{2+}\) can stimulate the production of ROS and nitric oxide (via activation of nNOS) and cause mitochondrial dysfunction, which leads to the opening of the mitochondrial permeability transition pore (MPTP), release of apoptosis inducing factor (AIF) and cytochrome c to the cytoplasm, and finally to apoptotic death (discussed in detail in the next section). On the other hand, nitric oxide itself can be a trigger of excitotoxicity, as recently it has been shown that NO, at concentrations that inhibit respiration, cause release of glutamate from neurons (McNaught and Brown 1998), microglia (Barger and Basile 2001) and astrocytes (Bal-Price et al. 2002). It has been shown that excitotoxicity contributes to the pathogenesis of several neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis, ischemia, brain injury (Leigh and Meldrum 1996; Rodriguez et al. 1998; Arundine and Tymianski 2004; Hynd et al. 2004).
1.3.3 Mitochondrial dysfunction

Mitochondria are vital organelles that maintain the cellular energy reserves by production of ATP in the respiratory electron transfer chain. Besides, they are known to be a significant source of superoxide radicals and other ROS that are associated with oxidative stress (Nohl et al. 2005). Impairment of the electron transfer chain at the flavin mononucleotide group of complex I (NADPH ubiquinone oxidoreductase) or at the ubiquinone site of complex III (ubiquinone-cytochrome c reductase) results in active generation of ROS. Once generated ROS further impair mitochondrial electron transport and enhance ROS production (Nohl et al. 2005).

It has been widely shown that mitochondrial dysfunction resulting from oxidative stress is the cause of neuronal cell death (Kirkland et al. 2002). The permeability of the mitochondrial membrane is dependent upon the mitochondrial permeability transition pore (MPTP) located at the contact sites between the inner and outer mitochondrial membrane (Crompton 1999; Bernardi et al. 2001). The opening of the MPTP, regulated by change in the mitochondrial membrane potential, results in the release of cytochrome c and AIF (apoptosis inducing factor) from the mitochondria to the cytosol and the activation of caspases cascade that leads the cell to apoptotic death (Petronilli et al. 2001). In animal studies in vivo cytochrome c released by mitochondria following cerebral ischemic-reperfusion injury promotes delayed neurotoxicity (Namura et al. 2001). Additionally, it has been demonstrated that nitric oxide generated following anoxia (Lin and Maiese 2001; Parikh et al. 2003; Mander et al. 2005) or released from glial cells activated with pro-inflammatory stimuli (e.g. LPS) (Bal-Price and Brown 1999; Bal-Price and Brown 2001) causes depolarization of mitochondrial membrane, subsequent release of cytochrome c and activation of caspases.

1.3.4 Caspase activation

Caspase activation, identified as the hallmark of apoptosis, has been closely associated with the pathogenesis of many neurodegenerative disorders. Caspases are a family of cysteine aspartic acid-specific proteases, which contain a cysteine residue at their active site and cleave substrates after an aspartate residue (reviewed by Zimmermann et al. 2001). Caspases are expressed as inactive pro-enzymes. Upon apoptotic stimulation, pro-caspases are cleaved at aspartate residues at the maturation sites by upstream caspases, other proteases or autocatalytic maturation, thereby becoming active caspases (reviewed by Kumar 1999a).
Currently, at least 14 caspase family members have been identified in mammalian cells, but it is unclear whether all of these proteases participate in apoptosis (Stennicke and Salvesen 1999; Kumar 1999a; Kumar 1999b). Based on their potential cellular functions, these caspases can be divided into two major subfamilies: 1) caspases involved in cytokine maturation including caspase-1, -4, -5, and -13 (Rano et al. 1997); 2) caspases involved in execution of apoptosis including caspase-2, -3, -6, -7, -8, -9, and -10 (Thornberry 1997). Among the caspase activating cascades, caspase-8, -9 and -10 usually serve as initiators that respond to apoptotic stimuli and become activated. These activated caspases then in turn activate caspase-2, -3, -6 and -7 which largely perform the subsequent proteolytic cleavage of their substrates, thereby executing apoptotic death (Thornberry 1997).

One of the mechanisms of caspase activation involves the formation of membrane channels in the mitochondrial membrane or opening of mitochondrial permeability transition pore (MPTP), that results in release of apoptosis inducing factor (AIF) (Susin et al. 1999a), cytochrome c (Saelens et al. 2004), caspase-2 and caspase-9 (Susin et al. 1999b) to the cytoplasm. AIF is a flavoprotein; it is normally confined to mitochondria but translocates to the nucleus when apoptosis is induced. An in vitro study showed that recombinant AIF in isolated nuclei causes chromatin condensation and DNA fragmentation and induces the release cytochrome c in purified mitochondria and caspase-9 activation (Susin et al. 1999a). Cytochrome c is an essential cofactor in the activation of caspase-9 along with Apaf-1 (Zou et al. 1999; Zou et al. 2003). Diverse stimuli, such as ROS (Atlante et al. 2000) and excitotoxicity, lead to cytochrome c release from mitochondria and initiate the cascade of caspases activation (caspase-9 and subsequently the effector caspase-3).

Additionally, the initiation of apoptosis in neuronal cells can also be triggered by cytokines, such as TNF-α acting on the p55 TNF receptor, which contains intracellular death domain (DD). Binding of TNF-α to its receptor facilitates the recruitment of adaptor proteins such as FADD and TADD, which in turn recruit pro-caspases (mainly pro-caspase-8) (Baker and Reddy 1998; Haviv and Stein 1998; Schneider and Tschopp 2000; Denecker et al. 2001). Caspase-8 can subsequently activate caspase-3 (Budihardjo et al. 1999). In addition, caspase-8 activation may also result in the cleavage of Bid (a pro-apoptotic member of the Bcl-2 family), allowing the truncated bid (tBid) to translocate to the mitochondria, which leads to the release of cytochrome c and activation of executioner caspases (Yin 2000).

The activation of executioner caspases evokes activation of proteolytic and nucleolytic enzymes and breakdown of a wide range of cellular substrates including structural proteins, nuclear proteins and DNases, cytoplasmic proteins and repair enzymes, thereby promoting...
apoptotic cell death. Poly-(ADP-ribose) polymerase (PARP) is a DNA repair enzyme whose cleavage by caspases increases the sensitivity to DNA damage during apoptosis (Tewari et al. 1995). Cleavage of a 45 kDa subunit of DNA fragmentation factor-45 (DFF-45) by caspase-3 is known to release a DNase (CAD) leading to fragmentation of genomic DNA into internucleosomal fragments (Widlak 2000). Cleavage of lamins may facilitate nuclear breakdown (Rao et al. 1996) while cleavage of cytoskeletal proteins such as actin and gelsolin disorganises the cellular structure and promotes morphological changes during apoptosis (Fujita et al. 1999). Cleavage of cell adhesion components such as β-catenin and focal adhesion kinase (FAK) dismantles cell-cell contacts, thereby facilitating dissociation of dying apoptotic cells from tissues (Brancolini and Schneider 1997; Levkau et al. 1998). The above events result in the typical morphology of apoptotic cells that is DNA condensation and nuclear fragmentation, cell shrinkage, and formation of apoptotic bodies.

1.4 Evidence for inflammation in CNS pathology
The role of neuroinflammation as the potential pathogenic factor in a number of CNS diseases has been recognised only recently. The concept of “neuroinflammation” implies that specific innate immune responses in the brain are mediated mainly by activated microglia and astrocytes, which precedes and causes neuronal degeneration. Before “neuroinflammation” became a commonly used term, neuroscientists recognised “reactive gliosis” as the endogenous CNS tissue response to acute brain injury.

Acute pathological conditions such as cerebral ischemia and traumatic brain injury are characterised by rapid and usually severe insults to the brain, which lead to substantial loss of nerve cells and subsequent functional deficits. Several processes have been implicated in the neuronal damage including increased glutamate release (excitotoxicity), oxidative stress and disturbances in ionic homeostasis (Morimoto et al. 2002; Chavarria and Alcocer-Varela 2004). There are also data suggesting the active role of inflammatory processes in these diseases - the activation of inflammatory cells (microglia, astrocytes) and increased expression of inflammatory mediators (cytokines, complement) (McGeer and McGeer 2001).

Although such specific responses in acute brain injury might be included in the term “neuroinflammation”, it is more commonly applied to chronic CNS diseases. Any chronic inflammatory process can damage healthy tissue and the brain may be particularly vulnerable, since neurons are post-mitotic cells and once lost cannot be replaced. There is vast evidence indicating that chronic inflammation in the brain may play an important role in the
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progressive neuronal cell death in many chronic CNS diseases such as Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis or multiple sclerosis.

1.4.1 Alzheimer’s disease

The greatest evidence of the role of inflammation in chronic CNS disorders comes from studies on Alzheimer’s disease (AD). Alzheimer’s disease is the most common neurodegenerative disease - approximately 10% of all people over the age of 65 and as many as 50% of those over the age of 85 are diagnosed with the condition. It is an irreversible, progressive disorder characterised by a loss of neurons predominantly in the cerebral cortex and hippocampus (Poulin and Zakzanis 2002). Evidence for the importance of inflammation in AD comes from two directions. First, extensive immunohistochemical and molecular biology studies of brain tissues collected from patients who suffered from AD revealed the presence of many hallmarks of inflammation, including microglial activation, expression of cytokines, activation of the complement system, invasion of immune cells (McGeer and McGeer 2001). Secondly, there is an ongoing debate on the value of non-steroidal anti-inflammatory drugs (NSAIDs, e.g. aspirin, ibuprofen) in AD treatment. Epidemiological studies suggest that patients on long term anti-inflammatory treatment (mainly because of arthritis) have a significantly reduced risk of developing AD (Szekely et al. 2004).

As in the case of many neurodegenerative diseases, the aetiology of AD is not clear, however, evidences suggest that accumulation of β-amyloid in neuritic plaques and remnants of a cytoskeletal protein tau (neurofibrillary tangles) act as irritants, causing activation of complement system, the initiation of reactive changes in microglia, the release cytokines (IL-1β, IL-6, IL-8) (McGeer and McGeer 1997) and potentially neurotoxic products such as reactive oxygen species, nitric oxide, excessive extracellular glutamate (Klegeris et al. 1997). All these toxic inflammatory products contribute to neuronal cell death, which further activates the immune reactions and leads to a chronic and progressive neurodegeneration.

1.4.2 Parkinson’s disease

Similar processes are observed in Parkinson’s disease (PD), a chronic, progressive neurodegenerative disorder characterised by degeneration of the nigrostriatal dopamine (DA) neurons in the substantia nigra. According to a common hypothesis, neurodegeneration in Parkinson's lays in abnormal accumulation of the protein α-synuclein in neurons, that similarly to β-amyloid in AD, aggregates and triggers activation of glial cells and the progression of inflammation. Indeed, activated microglia, and to a lesser extent reactive
astrocytes, are found in the area associated with neuronal cell loss (Teismann and Schulz 2004), possibly contributing to the inflammatory process by the release of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 (Nagatsu and Sawada 2005), prostaglandins (PGE₂, PGD₂), reactive oxygen and nitrosative species (NO). The anti-inflammatory treatment with NSAID or dexamethasone has demonstrated to have a beneficial role also in PD, although it is much less evident than in the case of AD (Teismann et al. 2003; Minghetti 2004; Kurkowska-Jastrzebska et al. 2004).

1.4.3 Multiple sclerosis

Multiple sclerosis is another example of a disease involving inflammation of the brain and associated neurological impairment. The key feature of MS is demyelination of axons in peripheral nerves and brain areas that leads to their subsequent degeneration and formation of plaques (Bitsch et al. 2000). The aetiology of MS is still not fully understood. Considerable evidence suggests the main role of autoimmune reactions to an unknown antigen and the activation of T lymphocytes (or deactivation of suppressor T cells) that, together with macrophages, invade the CNS. There are suggestions that an initial infection – viral or bacterial – can trigger inflammation and activation of T cells cross-reacting with autoantigens present in the CNS. Many studies demonstrate that the cytokines TNF-α and IFN-γ mediate some aspects of the disease (Bettelli and Nicholson 2000). They are both toxic to oligodendrocytes and can stimulate local inflammatory cytokines production (Benveniste and Benos 1995; Liu et al. 1998). Also clinical studies report the increased expression of cytokines in the brain parenchyma and CSF (Olsson et al. 1990; Rieckmann et al. 1995), but the therapeutic strategies targeted to block TNF-α and IFN-γ have not yet been successful. The current treatments of MS use immunosuppressive drugs, anti-inflammatory approaches and the treatment with interferon-β. The mechanism of action of IFN-β is not fully known, but in in vitro studies it was shown to suppress many actions of IFN-γ, inhibit the expression and release of IL-1 and TNF-α, and induce the release of IL-1ra (Coclet-Ninin et al. 1997; Jungo et al. 2001).

A separate group of diseases that are classically recognised as inflammatory in nature are infectious diseases. The main difference between them and the previously described chronic neurodegenerative diseases is that the pathogenic factor is usually well known (bacteria, viruses, fungi etc.). Infectious diseases of the CNS will be discussed in detail in the next section.
1.4 CNS infectious diseases

Infections of the central nervous system are rare but life-threatening complications of systemic infections. Due to its anatomy, the CNS represents a special milieu for bacterial, fungal, viral and parasitic infections. The brain and spinal cord are protected by bone and meningeal coverings that compartmentalise infection; they are separated from the systemic circulation by the blood-brain barrier, which largely prevents macromolecules from entering the brain parenchyma. The CNS lacks an intrinsic immune system, therefore immunoglobulins and immune-competent cells are scarce, except at foci of inflammation. Moreover, the brain and the spinal cord have a unique compact structure and space between cells in the parenchyma is too small to permit passage even of a virus (some viruses travel through the CNS by axoplasmic flow).

The infections in the CNS comprise several forms. The most common one is meningitis - an inflammation of the pia-arachnoid meninges, caused by a variety of different agents (bacteria, fungi, or parasites) growing within the subarachnoidal space. The most common, but also quite benign, is viral meningitis, primarily caused by echoviruses, coxackie viruses, mumps. Bacteria are the second cause of meningitis however, the clinical symptoms and the complications of this disease are much more severe.

Fig. 3 Major causes of acute meningitis (all ages, worldwide). “Other” viruses include herpes simplex virus type 2, arthropod-borne viruses, Epstein-Barr virus, influenza virus, and measles virus, as well as infections caused by *Mycoplasma pneumoniae*, *Leptospira*, fungi and rickettsiae (from Baron 1996).
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Another form is the brain abscess - a focus of purulent infection of the brain parenchyma, usually caused by bacteria. Brain abscesses develop from either a contiguous focus of infection (such as the ears, the sinuses or the teeth) or hematogenous spread from a distant focus (such as the lungs or heart, particularly with chronic purulent pulmonary disease, subacute bacterial endocarditis or cyanotic congenital heart disease). Many brain abscesses have a mixed flora of aerobic and anaerobic bacteria. Approximately 60 to 70% contain streptococci; *Staphylococcus aureus*, enterobacteria and *Bacteroides* are frequently present. Fungi cause fewer than 10% of brain abscesses. Generally, the brain abscess is a condition very difficult to cure, requiring multiple antibiotic treatment and sometimes surgical interventions.

Unlike an abscess, which is a localized area of bacterial or fungal growth, encephalitis (often defined as inflammation of the brain) is usually due to viruses that produce more widespread intracellular infections. Many viruses, including enteroviruses, mumps and lymphocytic choriomeningitis viruses, cause mild forms of encephalitis. Life-threatening viral encephalitis is due primarily to herpes simplex viruses (HSV-1 and HSV-2) and arboviruses (mainly togaviruses, flaviviruses, and bunyaviruses).

Encephalitis can result either from the entry of a new virus from the blood (e.g. arboviruses), reactivation of latent virus in the trigeminal ganglia (in case of HSV or varicella zoster virus) or spreading of the virus by axonal transport from other parts of the body (e.g. rabies). Once in the brain parenchyma, the virus spreads from cell to cell over a contiguous localized area, infecting neurons and glial cells.

Some infections in the CNS can develop into a chronic form. Today, the most important example of a chronic brain infection is HIV encephalitis, in which the HIV virus enters and persists in the CNS (in monocytes, perivascular cells and microglia) (Garden 2002). HIV infection is uniquely different from all other CNS infections in that the virus targets and disables these cells that are key players in neuroinflammation – the microglia in the brain and T lymphocytes in the periphery. Chronic infection can occur also in the course of syphilis (caused by the bacteria *Treponema pallidum*), which persist over many years with the unpredictable appearance of varied neurological complications. Chronic brain inflammation has been implicated also in the pathogenesis of prion infectious diseases, including transmissible spongiform encephalopathies in animals (e.g. scrapie, bovine spongiform encephalopathy - BSE) and humans (Creutzfeld-Jakob disease - CJD) (Coe et al 2001; van Everbroeck et al. 2002).
1.4.1 Bacterial Meningitis

As already mentioned, the most common infectious disease is meningitis. Before the discovery of antibiotics bacterial meningitis was a fatal disease in 95% of patients. Those who survived carried always severe neurological deficiencies. However, despite the broad use of antibiotics the morbidity and mortality rate in bacterial meningitis is still very high (5% to 40% patients with bacterial meningitis will die) (Kastenbauer and Pfister 2003).

1.4.1.1 The aetiology of bacterial meningitis

The aetiology of meningitis differs with age. Infants are particularly susceptible to bacterial infections, possibly due to lower integrity of barriers and immature defence mechanisms. In neonates meningitis is usually due to enteric bacilli (especially *Escherichia coli*), group B streptococci, or *Listeria*. In the postnatal period, *Haemophilus influenzae* was the most common cause of bacterial meningitis; however, significant reductions in some countries are occurring due to wide use of capsular polysaccharide-protein conjugate vaccines during infancy. Adult bacterial meningitis is predominantly due to *Streptococcus pneumoniae* and *Neisseria meningitidis*, except in cases where there had been a penetrating wound to the skull, surgery, or immunosuppression in the host, often complicated with *Staphylococcus aureus* infection. Meningitis caused by *Neisseria meningitidis* is relatively benign and the majority of patients can be treated with a short course of antibiotics and recover completely. The most difficult to cure and the most devastating in terms of neurological sequelae is meningitis caused by Gram-positive *Streptococcus pneumoniae*. The overall mortality in pneumococcal meningitis occurs in 5% to 40% of cases, and 30 to 50% of survivors have a broad spectrum of neurological sequelae including motor handicaps, seizures, mental retardation, impairment of hearing and loss of cognitive functions (Kastenbauer and Pfister 2003).

1.4.1.2 The pathogenesis of bacterial meningitis

Most organisms causing meningitis colonise the mucosal membranes of the nasopharynx or the gastrointestinal tract. Pneumococci bind to polymeric immunoglobulin A (IgA) receptors to cross the nasopharyngeal epithelium via their major adhesion molecule – CpbA (Zhang et al. 2000). Meningococcal pili adhere to the CD46 and CD66 receptors in the host, often complicated with *Staphylococcus aureus* infection. Meningitis caused by *Neisseria meningitidis* is relatively benign and the majority of patients can be treated with a short course of antibiotics and recover completely. The most difficult to cure and the most devastating in terms of neurological sequelae is meningitis caused by Gram-positive *Streptococcus pneumoniae*. The overall mortality in pneumococcal meningitis occurs in 5% to 40% of cases, and 30 to 50% of survivors have a broad spectrum of neurological sequelae including motor handicaps, seizures, mental retardation, impairment of hearing and loss of cognitive functions (Kastenbauer and Pfister 2003).

Bacteria enter the CNS via the bloodstream or focal infections in the vicinity of the CNS. *Escherichia coli* enters brain endothelial cells by interaction of bacterial molecules with endothelial receptors (Kim 2000). *Streptococcus pneumoniae* binds to the receptor for platelet
activating factor (PAF) and can cross the cerebral microvasculature endothelia by transcytosis in a manner dependent on the presence of pneumococcal choline binding protein A (Ring et al. 1998). As host defence mechanisms are limited, once present in the cerebrospinal fluid (CSF) pneumococci can multiply and reach titres up to $10^9$ colony-forming units/ml (Nau and Bruck 2002).

Although meningitis is an inflammatory disease of the meninges, usually it is not restricted to these membranes, but brain cells are also implicated, both as effectors and as objects of inflammatory injury. Therefore, neurological injury in bacterial meningitis is not only due to meningeal inflammation but also to cerebral vasculitis, cerebral oedema, cerebral necrosis, intracranial haemorrhage (Nau and Bruck 2002). The presence of live bacteria in the CSF does not necessary cause injury or even symptoms of inflammation. The induction of symptoms requires initiation of the inflammatory response, which is triggered by bacterial components such as the cell wall (e.g. lipopolysaccharide in Gram-negative bacteria, lipoteichoic acid and peptidoglycan in Gram-positive bacteria). Within the CSF bacteria multiply, lyse spontaneously and release pro-inflammatory and toxic compounds by autolysis or secretion. The bacterial products can traffic easily from the CSF to the brain parenchyma via the CNS paravascular fluid circulation. In humans with meningitis, high concentrations of LPS or lipoteichoic acid in the CSF are associated with poor outcome (Waage et al. 1989; Schneider et al. 1999). Bacterial components can directly induce some neuronal toxicity (e.g. the $S. pneumoniae$ toxin pneumolysin has been shown to directly damage neurons (Braun et al. 2002), but most evidence suggests that they stimulate glial cells (astrocytes and microglia) and resident macrophages to release several host-derived inflammatory mediators (cytokines, nitric oxide, ROS), which play a dominant role in neuronal damage. A rapid increase of pro-inflammatory cytokines (TNF-$\alpha$, IL-1$\beta$, IL-6) and chemokines (IL-8, MIP-1, MIP-2) is observed in the CSF in response to bacterial cell wall components, and their levels correlate with bacterial counts, severity of disease and occurrence of complications.

Endothelial cells of the blood-brain barrier also participate in the inflammatory response to bacterial components by serving as a platform for activating the complement pathway, procoagulant activity and PAF release into the CSF. Initial inflammation in the CNS stimulates the immune cells to cross the blood brain barrier. Blood derived leukocytes are attracted into the subarachnoidal space by soluble mediators (e.g. chemokines) and their trafficking through the blood vessels is facilitated by the up-regulation of selectins and adhesins (e.g. ICAM-1) (Freyer et al. 1999; Lopez et al. 1999). Leukocytes accumulate in the CNS and further promote inflammation through the release of cytokines, complement and
arachidonic acid metabolites (prostaglandins, leukotriens). Increase in blood-CSF and blood-brain permeability, as well as vasculitis lead to the evolvement of brain oedema, with such consequences as brain herniation and large secondary necrotic lesions – the major cause of death and severe neurological deficits in meningitis. In conclusion, brain damage in meningitis is induced both by bacterial components and host inflammatory mediators derived mainly by activated glial cells. The cascade of pathophysiological events in bacterial meningitis is presented schematically in Fig. 4.
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1.4.1.3 Neuronal injury in bacterial meningitis

Neuronal injury in meningitis is not a single event and several mechanisms are interacting with each other ultimately leading to the death of neurons. As previously discussed, cells functionally involved during meningitis include glial cells (astrocytes and microglia), meningeal macrophages, endothelial cells of the intracerebral vessels, and later also immunocompetent cells (monocytes, lymphocytes) recruited from the systemic circulation. The main factors triggering inflammation are the bacterial components released into the CSF, that are recognised by glial cells and involve innate immune functions stimulating Toll-like receptors (TLRs) (Kielian et al. 2002; Bowman et al. 2003; Esen et al. 2004) and inducing translocation of nuclear factor \( \kappa B \) (NF-\( \kappa B \)), the activation of mitogen activated protein kinases (MAPK) and the transcription of genes encoding inflammatory mediators (Akira and Hemmi 2003) (discussed in detail in further sections). This initial inflammatory response from the brain parenchyma - and in particular astrocytes and microglia - seems to be an important factor contributing in neuronal damage. As demonstrated in some \textit{in vitro} studies, heat-inactivated pneumococci injure both neurons co-cultured with glial cells (Kim and Tauber 1996) and neurons in organotypic hippocampal cultures (Schmidt et al. 2001; von Mering et al. 2001). Neuronal damage can be induced by pneumococcal lipoteichoic acid, peptidoglycan and bacterial DNA (Schmidt et al. 2001; Kim and Tauber 1996).

Evidence from \textit{in vivo} studies shows that the cortical brain damage in bacterial meningitis has morphological features of necrosis. The principal elements of necrosis are mitochondrial energy depletion and non-caspase proteolytic cascades (serine proteases, calpains, cathepsins). Several clinical and experimental studies indicated that ischemia is an important contributor to this injury (Forderreuther et al. 1992; Koedel et al. 1995; Nau and Bruck 2002). The mechanisms of ischemic or hypoxic lesions in meningitis are 1) vasculitis, vasospasm and obstruction of cerebral arteries, 2) generalised cerebral oedema, 3) impairment of cerebral autoregulation, leading to hypoxic lesions during hypotension. The pro-inflammatory mediators released by activated glial cells are largely responsible for these events.

On the other hand there are evidences of apoptotic cell death in several regions of the brain in the course of meningitis (Braun et al. 2002; Mitchell et al. 2004). Apoptosis can involve both the caspase-dependent and the caspase-independent pathway, and both pathways have been implicated in neuronal cell death induced by \textit{Streptococcus pneumoniae}. Several caspases were demonstrated to be involved in experimental pneumococcal meningitis (von Mering et al. 2001; Gianinazzi et al. 2003).
One of the mechanisms involved in neuronal damage during meningitis is oxidative and nitrosative stress. Reactive oxygen species (superoxide, hydrogen peroxide, hydroxyl radical) and nitrogen species (nitric oxide, peroxynitrite) are produced by stimulated glial cells, macrophages and granulocytes: 1) after challenge with bacterial components and cytokines, 2) as a consequence of hypoxia and ischemia, or produced directly by bacteria (Hirst et al. 2000).

During bacterial meningitis, superoxide generation has been detected cytochemically in meningeal and ventricular inflammatory cells, as well as along penetrating cortical vessels (Leib et al. 1996a). The participation of ROS in injury is supported by the ability of a wide variety of inhibitors to ameliorate the course of inflammatory damage. Antioxidants such as superoxide dismutase, N-acetyl-L-cysteine and catalase reduce brain oedema, intracranial pressure and CSF leukocytosis, and attenuate the increase in regional cerebral blood flow in early pneumococcal meningitis (Pfister et al. 1992; Koedel and Pfister 1997).

Moreover, nitric oxide can be induced in most resident brain cells and invading immune cells in response to bacterial products. The release of NO can be stimulated directly by the bacterial components or by the pro-inflammatory mediators (e.g. TNF-α, IFN-γ, IL-1β) (Kong et al. 2000). Nitric oxide (NO) and superoxide radicals (O₂⁻) react rapidly to form the peroxynitrite anion (ONOO⁻), which decomposes and forms the strong oxidants, hydroxyl radical and nitrogen dioxide. The significant role of NO in the pathophysiology of bacterial meningitis has been shown for group B streptococci, S. pneumoniae, H. influenzae and E. Coli (Bernatowicz et al. 1995; Koedel and Pfister 1999). The CSF concentration of nitrite, a stable end product of NO metabolism and indicator of NO production, is increased in bacterial meningitis (Uysal et al. 1999). N-nitro-L-arginine, an inhibitor of NO synthase, inhibits the increase of regional cerebral blood flow and intracranial pressure in the early phase of experimental pneumococcal meningitis (Koedel et al. 1995). Nevertheless, despite the indications of the damaging role of NO, the clinical application of NO inhibitors remains to be established.

Another mechanism that triggers neuronal cell death is excitotoxicity. The excessive release of glutamate causes membrane depolarisation, Ca²⁺ influx and energy failure, which leads to the release of cytochrome c from mitochondria. There are evidences that excitotoxicity plays a role in meningitis. Glutamate concentrations in the interstitial space of the brain and in the CSF are increased in animals and humans suffering from meningitis (Guerra-Romero et al. 1993; Spranger et al. 1996). In the infant rat model of bacterial meningitis the glutamate antagonist kynurenic acid was moderately neuroprotective (Leib et
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It has been shown that the pro-inflammatory TNF-α, which is rapidly produced by microglia or astrocytes during experimental meningitis or after injection of bacterial cell wall components, can promote the release of glutamate from microglia that subsequently leads to excitotoxicity (Chao and Hu 1994).

TNF-α can also induce apoptosis through caspase activation. As mentioned previously, the binding of TNF-α to the TNF-R (p55) leads to the cleavage and activation of caspase-8 and subsequently caspase-3. Von Mering et al. (2001) in a model of experimental meningitis demonstrated that the activation of caspases was impaired in TNF-α deficient mice, suggesting the role of this pathway in neuronal apoptosis. TNF-α was also shown to be in part responsible for hippocampal damage in meningitis (Bogdan et al. 1997).

The above-mentioned mechanisms that lead to neuronal cell death in bacterial meningitis are summarized in Fig. 5.

Fig. 5. Hypothetical cascade of events leading to neuronal cell death in bacterial meningitis (adopted from Nau and Bruck 2002)
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1.4.1.4 Therapy of bacterial infections

Treatment of a bacterial infection with cell wall-active antibiotics causes a rapid release of pro-inflammatory or toxic bacterial compounds. For many years beta-lactams (penicillin, cefalosporins) were the drugs of first choice in meningitis. These antibiotics induce rapid bacterial lysis and therefore cause a quick and strong release of endotoxin (in Gram-negative infections) or lipoteichoic acid and peptidoglycan (in Gram-positive infections) into the cerebrospinal fluid (CSF). The initial total endotoxin concentration in CSF correlates with the severity of Gram-negative bacterial meningitis (Waage et al. 1989). In adults with \textit{S. pneumoniae} meningitis the clinical outcome is related to the lipoteichoic acid concentration in the CSF (Schneider et al. 1999). \textit{In vitro} studies, in which mononuclear cells were exposed to supernatants of bacterial cultures treated with different antimicrobial agents, indicate that the release of inflammatory cytokines (TNF-\(\alpha\), IL-1) is smaller when drugs induced less release of endotoxin or LTA/TA (Purswani et al. 2000). Therefore, the use of antibacterial drugs that cause reduced release of bacterial compounds may be advantageous. New trends in the treatment of CNS bacterial infections include the use of protein synthesis inhibitors, (e.g. rifampin, clindamycin, quinupristin, linesolid and ketolides), which kill the bacteria without lysing them immediately (Trostdorf et al. 1999; Stuertz et al. 1999; Nau and Eiffert 2002; Gerber et al. 2003).

Nevertheless, improvements in antibiotic therapy are unlikely to change the outcome of the disease substantially and current therapeutic approaches are insufficient to prevent severe disability in the majority of patients. The development of new drugs, which control cerebral inflammation and oedema, is therefore the aim for novel effective meningitis therapies. In order to attenuate inflammation in the subarachnoidal space, adjunctive anti-inflammatory drugs, such as dexamethasone, have been widely used, but their value in the adjuvant therapy of meningitis is still under discussion.

Recent studies suggest beneficial effects of drugs that inhibit the production or action of cytokines, matrix metalloproteinases and reactive oxygen species (e.g. acetylcysteine, tirilazad) (Auer et al. 2000; Leib et al. 2001; Pomar et al. 2004). However, the development of newer therapies requires a greater knowledge of the molecular and pathological events implicated in the evolution of inflammation, especially in the case of those caused by Gram-positive bacterial components, which are still poorly studied. Therefore, a better understanding of how the events involved in bacterial meningitis may be interrupted in the patient receiving antibiotic therapy is crucial.
1.5 Pathogen associated molecular patterns (PAMPs)

The first line of defence against pathogens in mammals is the innate immune system. In contrast to the adaptive immunity, which is mediated by T and B lymphocytes clonally selected and characterised by a high specificity and memory to antigens, innate immunity is non-antigen specific and is mediated mainly by macrophages, monocytes and neutrophils, which upon activation in the presence of infection release several pro-inflammatory mediators (cytokines, proteins of the complement system, chemokines, enzymes (iNOS, COX-2)), that collectively engage and control the innate immune response essential for eliminating the pathogen and mobilise the more specific acquired immune response. The innate immune system exists also in the CNS, and its main responses are mediated by the glial cells (microglia and astrocytes), which recognise the pathogens and become activated leading to the evolution of inflammation.

The innate immune cells recognise the presence of infection through receptors for specific elements called pathogen-associated molecular patterns (PAMPs) from microorganisms such as bacteria, viruses, fungi (Medzhitov and Janeway 2000). The PAMPs can have different chemical structures. They can be fragments of bacterial cell walls (lipopolysaccharide, lipoteichoic acid, and peptidoglycan), bacterial and viral DNA fragments, flagellin (a protein derived from the bacterial flagella), viral RNA or fungal proteins (zymosan). The recognition of PAMPs by specific Toll-like receptors is the first step of a complex inflammatory reaction that characterises the innate immune responses.

The main PAMPs derived from Gram-negative and Gram-positive bacteria will be discussed below.

1.5.1 Lipopolysaccharide (LPS)

The first, most widely studied bacterial component that stimulates the innate immunity is the Gram-negative bacterial lipopolysaccharide. More than a hundred years ago Richard Pfeiffer discovered that lysates of heat killed bacteria (*Vibrio cholerae*) caused toxic shock in guinea pigs; from this experiment he postulated that the toxic principle is localized inside the bacterial wall and named it endotoxin (from Greek *endo* = inside) (Alexander and Rietschel 2001). Today we know that the cell wall of Gram-negative bacteria is characterised by the presence of two lipid bilayers – the outer and the inner (cytoplasmic) membrane, separated by the periplasmic space containing a network of peptidoglycan. The substance responsible for the biological effects is mainly lipopolysaccharide (LPS, also commonly known as endotoxin), a constituent of the outer membrane of the cell wall in Gram-negative bacteria.
LPS is also one of the best studied bacterial products; almost 50,000 scientific articles have been published on LPS since 1966 (www.pubmed.com).

Lipopolysaccharides are present in almost all Gram-negative bacteria, amongst which several important commensal or human pathogenic species (Escherichia coli, Salmonella ssp., Neisseria meningitidis, Haemophilus influenzae, Klebsiella pneumoniae, Chlamydia trachomatis, Helicobacter pylori etc.) (Caroff et al. 2002). Within the last decades highly purified preparations of LPS from a large number of bacteria have been characterised chemically, physically and biologically and on base of these studies LPS have been shown to be among the most powerful classes of immunostimulators known to physiologically function as specific indicators for infection by Gram-negative bacteria (Alexander and Rietschel 2001).

1.5.1.1 Structure

Lipopolysaccharides are a class of heat-stable amphiphilic glycolipid molecules composed of a hydrophilic poly- or oligosaccharide core and a hydrophobic region known as lipid A (Caroff and Karibian 2003). The polysaccharide region of LPS is subdivided into the terminal O-specific chain and the core region most proximal to lipid A (Fig. 6). The O-specific chain consists of 50 repeating oligosaccharide units formed of 2-8 monosaccharide moieties in a highly species- and strain-specific manner (Brandenburg and Wiese 2004). The core region can be divided into two parts – inner and outer core – differing in monosaccharide composition (Fig. 6). The inner core shows the least variability within the polysaccharide region of LPS and in most bacteria is composed of 2-keto-3-deoxy-D-manno-octulosonic acid (Kdo - a characteristic and essential component considered also as a diagnostic marker of LPS) and L- or D-glycero-D-manno-heptose. The outer core mainly consists of hexoses, such as D-glucose, D-galactose, D-glucosamine, N-acetylglucosamine or N-acetylgalactosamine (Rietschel et al. 1994).

The conserved lipid A structure has been identified as the immunostimulatory principle of LPS (Raetz and Whitfield 2002; Brandenburg and Wiese 2004). The lipid A structure is quite homogenous within different Gram-negative bacterial species (Kusumoto et al. 2003). The backbone consists of a central β-(1-6)-linked disaccharide units composed of D-glucosamine (D-GlcN) or D-2,3-diamino-2,3-dideoxyglucose (D-GlcN3N; DAG) (Caroff et al. 2002). Comparative studies of lipids A from several bacterial species have shown that there is a considerable diversity in the specific acylation patterns (number, position and chemical nature of the acyl residues) and it is the acylation pattern that plays the major role in determining the immunostimulatory potential of LPS (Rietschel et al. 1993).
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1.5.1.2 Biological effects of LPS

LPS is released by bacteria during their growth or bacteriolysis. Recognition of LPS (or lipid A) by the professional phagocytes of the innate immune system - peripheral monocytes, macrophages and neutrophils - is a key event in host microbial defence reactions. Also microglia and astrocytes in the CNS have been shown to respond to LPS (Heine et al. 2001; Lee and Lee 2002).

In humans, especially mononuclear cells react with extreme sensitivity to LPS preparations - the threshold values for the activation of isolated human monocytes or monocytes in whole blood are about 1-10 pg/ml. The activation of mononuclear cells by LPS or free lipid A in vitro leads to the secretion of a wide spectrum of endogenous mediators among which: 1) the pro-inflammatory cytokines - TNF-α, IL-1β, IL-6, IL-8, IL-12, IL-15 and IL-18; 2) colony stimulating factors M-CSF, G-CSF and GM-CSF; 3) arachidonic acid derivatives such as PAF, PGE2, tromboxane A2, leukotriens; 4) reactive oxygen species (superoxide, hydroxyl radicals) and 5) nitric oxide. In addition, LPS may cause activation of the complement system - both via the classical and the alternative pathways.

By diverse mechanisms the autocrine and paracrine mediators released in the early phases of LPS-induced activation may initiate a complex network of secondary reactions, which include the stimulation of acute phase protein secretion by the liver, activation of blood cells from all lineages (trombocytes, basophilis, mast cells, eosinophils) (extensively reviewed by Alexander and Rietschel 2001). The early activation of innate immune cells subsequently induces the recruitment of adaptive highly specific immune responses via the selection and clonal expansion of pathogen specific T and B lymphocytes (Ulmer et al. 2000). According to current knowledge IL-1β and IL-6 are the primary mediators in the induction of fever by LPS (Dinarello 2004).
1.5.1.3 LPS in CNS diseases

The involvement of LPS in the pathogenesis of CNS diseases, and in particular meningitis, is well studied. In a rabbit model of meningitis, activities of pro-inflammatory TNF-α, IL-1, and IL-6 were rapidly detected in the CSF after injection of meningococcal LPS into the subarachnoidal space (Waage et al. 1989). Intracysternal administration of LPS in rats induced NO synthesis from the lateral and third ventricle choroid plexi and surface meninges (Korytko and Boje 1996). Several in vitro studies show that LPS activates glial cells – microglia and astrocytes – and induces production of inflammatory cytokines such as TNF-α (Chung and Benveniste 1990; Appel et al. 1995), IL-1 (Corsini et al. 1996; Hur et al. 2001) and IL-6 (Sebire et al. 1993), chemokines (Thibeault et al. 2001), nitric oxide (Possel et al. 2000), prostaglandins (Pistritto et al. 2000), adhesion molecules and matrix metalloproteinases (MMPs) (Lee et al. 2003).

LPS can increase the permeability of the blood-brain barrier by the induction of cytokines and several adhesion molecules on the cerebral endothelium, which facilitate the infiltration of leukocytes in the CSF (Burroughs et al. 1992; Wong and Dorovini-Zis 1992). Also, LPS-induced NO, PGD₂ and MMPs were proposed to disrupt the BBB integrity in an experimental rat model of bacterial meningitis (Jaworowicz et al. 1998).

Finally, the LPS-induced neuroinflammation contributes to neuronal cell death. Recently it has been shown that inflammatory neurodegeneration is mediated by several factors released by LPS-stimulated microglia and astrocytes, such as cytokines TNF-α and IL-1β (Cai et al. 2003), nitric oxide (Bal-Price and Brown 2001) and NADPH-oxidase activation (Qin et al. 2004).

1.5.2 Lipoteichoic acid (LTA)

Although Gram-positive bacterial infections represent an important problem worldwide, it is still under discussion what is their main component responsible for the stimulation of the innate immune response. However, increasing evidence suggests that lipoteichoic acid (LTA) derived from Gram-positive bacteria is the analogue of Gram-negative bacterial LPS and shares many of its biochemical and physiological properties (Ginsburg 2002).

The immunostimulatory potential of LTA is controversial, mainly because the commercially available preparations of LTA used in early studies were of poor quality. LTA from S. aureus, obtained by phenol extraction, was biologically inactive and not able to induce cytokine release (a measure of immunostimulatory activity) (Kusunoki et al. 1995; Suda et al. 1995). On the other hand, it was demonstrated that commercial preparations of
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LTA contained large and variable amounts of LPS contaminations (Gao et al. 2001; Morath et al. 2002a). Only recently, thanks to a novel butanol-extraction procedure, it was possible to obtain essentially homogenous, pure and biologically active LTA (Morath et al. 2001), which was shown to efficiently stimulate monocytes to produce several pro-inflammatory cytokines (Hermann et al. 2002; Morath et al. 2002b). These studies re-established LTA as an important immunostimulatory principle of Gram-positive bacteria.

1.5.2.1 Structure

Similarly to LPS, LTA is a member of a structurally related group of macroamphiphiles, which consist of a hydrophobic diacylglycerol membrane anchor and a hydrophilic head group exposed on the outer bacterial surface. Lipoteichoic acids derived from various types of bacteria have a common molecular architecture consisting of a diacylglycerol-containing glycolipid anchor and a covalently coupled polymeric backbone structure. However, LTA isolated from different species of Gram-positive bacteria differ in the composition of the so-called “repeating units” of the polymeric backbone (Greenberg et al. 1996). Staphylococcal LTA, like other common LTAs, is composed of 20 to 50 small repeating units that contain D-alanine and N-acetylglucosamine linked to a central linear 1-3-linked polyglycerolphosphate chain (Fischer et al. 1981) (Fig. 7). These D-alanine chains have been shown to be important for the immunostimulatory properties of LTA (Morath et al. 2001; Deininger et al. 2003). A similar motif is found also in the backbone of Enterococcus sp, Bacillus subtilis and some Streptococci. However in S. pneumoniae the polymeric chain has a markedly different structure, consisting of 6 to 8 large tetrasaccharide repeating units that contain phosphorylcholine and are linked to each other by ribitol phosphate (Behr et al. 1992; Fischer 1997). There are evidences that these differences in chemical structure influence the biological activity of different LTAs. Han et al. (2003) have shown that LTA from S. pneumoniae is an about 1000-fold less potent stimulator of human peripheral blood mononuclear cells than LTA from S. aureus. In contrast Schröder et al. (2003) did not find such differences and their pneumococcal preparation was as potent as staphylococcal LTA in stimulating cytokine production. These discrepancies could be due to the different procedures of LTA-isolation and purification, resulting in LTAs of different chemical structures (Han et al. 2003).
1.5.2.2 Biological effects

In the last decade, increasing experimental evidence has proven that LTA is the major immunostimulatory component of Gram-positive bacteria. LTA was shown to be a potent immune stimulator capable, during severe infection, of inducing haemodynamic, haematological and metabolic changes of a magnitude similar to those induced by lipopolysaccharide from Gram-negative bacteria.

Several groups reported that LTA stimulates human monocytes and human whole blood to produce high levels of endogenous inflammatory mediators, such as TNF-α, IL-6, IL-1β, IL-10, which are capable of sustaining an inflammatory state (Bhakdi et al. 1991; Morath et al. 2001; Ellingsen et al. 2002; Hermann et al. 2002; Jones et al. 2005). LTA was also shown to be a strong inducer of chemokines: MIP-1α (Danforth et al. 1995), IL-8, MCP-1 (Standiford et al. 1994; von Aulock et al. 2003). Although sharing many similarities with its counterpart LPS, the spectrum of cytokines release induced by LTA is slightly different. Indeed, relative to LPS, LTA is a more potent inducer of chemokines and less potent inducer of pro-inflammatory cytokines (von Aulock et al. 2003; Moller et al. 2003). It has been demonstrated that LTA, in comparison to LPS, is a weak inducer of IL-12 and subsequent IFN-γ formation (Hermann et al. 2002). These discrepancies are most probably due to the fact that different receptors, with slightly diverse intracellular signalling pathways, are involved in the recognition of LPS and LTA (Kawai et al. 2001; Albrecht et al. 2004). Unlike in the case of LPS, where its role in inducing oxidative stress in many different cell types and organs is very well documented (Feng et al. 1995; Carbonell et al. 2000; Victor and De la Fuente 2002), there are no evidences so far that LTA is capable of triggering the production of reactive oxygen species. However, LTA is well known to stimulate iNOS expression and NO release.
both *in vivo* (Chatterjee et al. 2002), and *in vitro* in macrophages (Korhonen et al. 2002; Kuo et al. 2003) and vascular smooth muscle cells (Auguet et al. 1992; Lonchampt et al. 1992; Hattori et al. 2003). Interestingly, LTA and peptidoglycan (another component of bacterial cell wall) from *S. aureus*, administered simultaneously synergise to induce iNOS expression, nitric oxide production and lethal shock in rats (De Kimpe et al. 1995; Kengatharan et al. 1998). Thus, the combination of two components could substitute for whole bacteria in inducing inflammation, and this phenomenon was addressed also in the present study.

1.5.3.3 Role of LTA in CNS

Although Gram-positive bacteria represent an important cause of CNS infections, most of the studies conducted so far on the mechanisms of these pathologies were performed using whole bacteria as the triggers of inflammation, and there are only few reports, discussed below, showing the important role of isolated LTA in inducing neuroinflammation.

In 1980 Aasjord et al. first reported that rabbits immunized with lipoteichoic acid from *Staphylococcus aureus* developed encephalitis (Aasjord et al. 1980). High amounts of LTA were found in the cerebrospinal fluid of patients diagnosed of *S. pneumoniae* meningitis and treated with antibiotics (Trostdorf et al. 1999), and the concentrations of LTA in CSF were associated with neurological sequelae and mortality (Schneider et al. 1999).

Recently, using immunohistochemical staining for various markers of glial activation it has been demonstrated that LTA administered intravenously in rats activates both astrocytes and microglia in the pineal gland (an area devoid of BBB) (Jiang-Shieh et al. 2005). LTA was also shown to induce neuronal cell death, mainly described as apoptotic, in an *in vitro* model of organotypic hippocampal slices (Schmidt et al. 2001).

Given the importance of Gram-positive bacteria in the aetiology of CNS infectious diseases, the present study will concentrate in great detail on the potential of LTA from *S. aureus* to induce astrocyte and microglial activation and subsequent neuronal damage.

1.5.3 Peptidoglycan (PGN) and muramyl dipeptide (MDP)

Peptidoglycan (PGN) is present in the cell wall of most Gram-negative and Gram-positive bacteria, however the amount of PGN differs markedly between the two groups of pathogens. Gram-positive bacteria have a thick layer of a peptidoglycan, which determines the organism's shape (e.g. bacilli, cocci). There may be up to 40 layers of this polymer, conferring enormous mechanical strength to the bacterial cell wall. In contrast, Gram-negative organisms have only a very thin layer of peptidoglycan immediately outside their cell membrane (about
one twentieth of the thickness of that found in gram-positive organisms), surrounded by a bilayered membrane composed of phospholipids and bacterial lipopolysaccharide. The main role of peptidoglycan is to provide certain rigidity and mechanical strength to the cell wall, and therefore to protect bacteria against osmotic lysis.

1.5.3.1 Structure

Peptidoglycan of all bacterial species is composed of long chains of two alternating sugar residues, N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), which are highly crosslinked by peptide bridges. The peptide subunits consist of alternating L- and D-amino acids and are connected with the carboxyl groups of MurNAc. Among different bacterial species the structure of sugar chains is highly preserved, whereas the composition of the peptide subunits varies. For example, the peptides in *S. aureus* contain a sequence L-alanine-D-isoglutamine-L-lysine-D-alanine-D-alanine; additionally, PGN from *S. aureus* contains pentaglycine bridges. The complete peptidoglycan network is a highly complex and ordered network, the detailed structure of which is still under debate (de Jonge et al. 1992; Dmitriev et al. 2003; Dmitriev et al. 2004). PGN can be enzymatically cleaved into smaller components by two enzymes – N-acetyl-L-alanine amidase and muramidase. The smallest element formed after PGN degradation, which still preserves biological activity, is muramyl dipeptide (MDP) (Ellouz et al. 1974).

1.5.3.2 Biological effects

During Gram-positive bacterial infections PGN is released, together with LTA, and has been shown to co-stimulate the innate immune system. Over the last 30 years a vast number of cellular activities have been assigned to peptidoglycan. Several studies have demonstrated the role of PGN in initiating host cytokine response associated with sepsis and organ injury. PGN was found to induce TNF-α, IL-1β and IL-6 release in human monocytes, with kinetics similar to that induced by LPS (Verhoef and Kalter 1985; Mattsson et al. 1993; Timmerman et al. 1993; Mattsson et al. 1996). Also in a human whole blood model PGN induced the release of TNF-α, IL-6 and IL-10, which coincided with the accumulation of mRNAs for these cytokines in both monocytes and T-cells (Wang et al. 2000). PGN is also known to activate the complement system, as well as to induce procoagulant activity (Mattsson et al. 2002; Mattsson et al. 2004).

The ability of PGN alone to induce nitric oxide synthase (iNOS) has not been proven. However, evidence from *in vitro* and *in vivo* studies suggests that PGN can synergise with
LTA in the induction of iNOS and production of nitric oxide (De Kimpe et al. 1995; Kengatharan et al. 1996; Kengatharan et al. 1998). Similar synergism was also demonstrated in case of PGN and LPS (Flak et al. 2000; Wray et al. 2001). Recently, PGN from *Listeria monocytogenes* was also shown to induce oxidative stress and the production of superoxide anion in macrophages (Remer et al. 2005).

It has been argued that PGN is not an important initiator of inflammatory responses because the amounts of this cell wall component needed to induce cellular response are typically high (1-10 µg/ml), several log orders higher than the concentration of LPS. One of the explanations is that only a small part of the PGN structure is essential for its pro-inflammatory activities. PGN is insoluble in its native form and must be enzymatically cleaved into smaller components. Indeed, this smallest active part of PGN has been identified to be the muramyl dipeptide (Ellouz et al. 1974). Only recently, the specific intracellular Nod2 protein has been identified as the receptor for MDP and it was suggested that Nod2 is a general sensor of peptidoglycan through the recognition of MDP (Girardin et al. 2003). MDP itself was shown to activate macrophages (Bahr et al. 1987), monocytes (Kalyuzhin et al. 2002) as well as glial cells (Cottagnoud et al. 2003). On the other hand, in recent studies Traub et al. (2004) demonstrated that the pro-inflammatory activity of MDP could be due to its contamination with LPS, as recombinant MDP not contaminated with endotoxin was not able to stimulate whole blood cells and isolated human monocytes alone. However, it still strongly synergised with LPS to induce cytokine production (Cottagnoud et al. 2003; Traub et al. 2004).

The role of PGN and MDP in the mechanisms of CNS infections has been scarcely studies so far. Nevertheless, the existing evidence suggests that they may play a role in the initiation of the inflammatory response in CNS. In a rabbit model of meningitis, intrathecal injection of MDP triggered TNF-α release and subsequent infiltration of leukocytes (Burroughs et al. 1992; Cottagnoud et al. 2003). In glial cells, MDP was shown to induce PGD₂ production (Yamamoto et al. 1988) and to potentiate the cytokine (IFN-γ, IL-1β)-induced iNOS activation and NO production in primary rat astrocytes (Trajkovic et al. 2000).

In the present study we investigated the role of MDP in glia activation. Especially, we were interested whether MDP could act in synergy with LTA in glia and what would be the intra- and extracellular mechanisms involved.
1.6 Toll-like receptors

The essential role in the activation of early immune responses to invading pathogens is played by a group of specialized receptors called Toll-like receptors (TLRs), which recognise specific pathogen associated molecular patterns (PAMPs). These evolutionary conserved receptors are homologues of the *toll* gene that controls dorsoventral pattern formation during the early embryonic development of *Drosophila*. Interestingly, Toll protein participates also in antimicrobial immune responses upon infection in adult *Drosophila*.

TLRs are transmembrane proteins with extracellular leucine-rich repeat domains (LRR), and cytoplasmic signalling domains that are similar to the cytoplasmic domain of the interleukin-1 receptor (IL-1R), commonly termed as the Toll/IL-1 receptor homology domain (TIR). Both IL-1R and TLRs induce signal transduction pathways leading predominantly to activation of the transcription factor NF-κB, a key regulator of inflammatory responses.

Recently 11 members of the Toll-like receptor (TLR) family have been identified (TLR1-11) in mammals and associated to their specific ligands (see Table 1) (Takeda et al. 2003; Heil et al. 2003; Zhang et al. 2004; Heil et al. 2004). The ligands for TLRs are mostly PAMPs, such as fragments of bacterial cell walls, bacterial and viral DNA, viral double stranded RNA, fungal proteins. Surprisingly, for some TLR the ligands can be also chemical substances (e.g. the only ligands identified so far for TLR7 are drugs of potent antitumour and antiviral properties, TLR4 also recognises taxol - a plant derived antitumour drug) (Byrd-Leifer et al. 2001). It was proposed that, apart from the involvement in infectious diseases, TLR may play a role in the development of autoimmune diseases since some TLRs were shown to recognise also host-derived elements (e.g. TLR4 recognises fibronectin and HSP60) (Beutler 2004).

The expression of TLRs has been mainly analysed in immune cells, such as macrophages, neutrophils, monocytes, dendritic cells, and it is different depending on the cell type and cell differentiation state (Muzio et al. 2000; Hornung et al. 2002). TLRs have also been found in parenchymal cells; for example, TLR5 is expressed at the basolateral surface of intestinal epithelial cells (Gewirtz 2003); renal epithelial cells express TLR2 and TLR4 upon IFN-γ stimulation (Wolfs et al. 2002). TLR4 and TLR2 have been also detected in cultured human dermal endothelial cells (Faure et al. 2000) and corneal epithelial cells (Johnson et al. 2005). In the CNS several TLRs have been identified to be expressed in glial cells (microglia, astrocytes and oligodendrocytes) and involved in pathogen recognition and glia activation (discussed in section 1.6.4).
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<table>
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<tr>
<th>type of TLR</th>
<th>ligand</th>
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<tr>
<td>TLR2</td>
<td>Lipoteichoic acid</td>
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<td>Peptidoglycan</td>
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<td>Glycophosphatidylinositol anchors</td>
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<td>Lipoarabinomannan</td>
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<td>Porin</td>
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<td>TLR1/TLR6</td>
<td>Interacts with TLR2 forming heterodimers, thus enhancing the recognition of some PAMPs</td>
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<td>TLR3</td>
<td>Double stranded RNA Poly(I:C)</td>
<td>Viruses</td>
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<td>Lipopolysaccharide</td>
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<td>Fibronectin</td>
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<td>HSP60, HSP70</td>
<td>Bacterial flagella</td>
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<td>Flagellin</td>
<td>Chemicals</td>
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<td>TLR7</td>
<td>Imidazoquinolines</td>
<td>Chemicals, substances with potent antiviral and antitumour properties</td>
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<td>Loxoribine</td>
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<td>Bropirimine</td>
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<tr>
<td>TLR10</td>
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<tr>
<td>TLR11</td>
<td>Uropathogenic bacteria</td>
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Table 1. Mammalian Toll-like receptors and their ligands (adopted from Akira and Hemmi (2003), with some modifications).

1.6.1 Signalling through Toll-like receptors

As mentioned before there are profound similarities between the TLR-dependent signalling pathways triggered by PAMPs and the signalling cascades that are activated by endogenous cytokines of the IL-1/IL-18 family. Binding of the ligand to TLR involves the primary adapter protein myeloid differentiation factor (MyD88), a protein consisting of an aminoterminal death domain (DD) and the carboxyterminal TIR domain – a specific and essential adapter protein for TIR domain receptors. Evidence for the essential role of MyD88 comes from studies on MyD88 deficient mice. MyD88 deficient cells showed to be extremely resistant to various bacterial components, including LPS, peptidoglycan, CpG DNA, mycobacterial lysates (Takeuchi et al. 2000; Akira et al. 2000). Recruitment of MyD88 to the cytoplasmic TIR domain of TLRs triggers an additional association and activation (phosphorylation) of the IRAK (IL-1R associated kinase) (Suzuki et al. 2002). Phosphorylated IRAK-1 dissociates
from the complex and binds to TRAF6 (TNF-receptor activation factor-6). The central role of TRAF6 is activation of the major types of intracellular signalling pathways – the NF-κB pathway and the MAPK pathway (Jefferies et al. 2001). So far several members of the MAP kinase kinase kinase (MAP3K) such as MAPK/ERK kinase kinase (MEKK), TGF-β activating kinase (TAK1) and NF-κB-inducing kinase (NIK) have been found to be activated by TRAF6. Activation of the transcription factor NF-κB is a pivotal element in signalling induced by various pathogen-derived stimuli (Li et al. 2003). It proceeds via the phosphorylation inhibitory IκB protein by specific IκB kinases (IKK). The MAPK3K-like kinase NIK and TAK1 have been identified as the central TRAF6-binding activators of the IKK (extensively reviewed in Hatada et al., 2000). Phosphorylated IκB is subsequent ubiquitinylated and degraded the proteasomes; liberated NF-κB is therefore free to translocate into the nucleus where it activates several genes involved in the immune response to pathogens.

1.6.1.1 MAPK

Along with NF-kB, several protein kinases are also activated by bacterial components in the TLR signalling cascade. These include the family of mitogen activated protein kinase (MAPK) (Guha and Mackman 2002; An et al. 2002). MAPK family members are defined as proline-directed serine/threonine protein kinases. The activity of MAPK is mediated by a dual phosphorylation of threonine/serine and tyrosine residues within a signature sequence Thr-X-Tyr by an upstream kinase called MAPK kinase (MAPKK). The MAPKK are in turn activated by MAP3K - of which 30 different kinases have been described. To date 12 different MAPKs have been identified in mammals and homologs are found in all eukaryotic cells. The most studied cascades in mammalian cells are the classical extracellular signal regulated kinase (ERK1/2), p38 kinase and Jun N-terminal kinase (JNK). The activation of MAPK leads to the transactivation of numerous transcription factors that include the AP-1 family, the ATF/CREB family and NF-AT, which in turns regulate the expression of an array of immune response genes (e.g. for cytokines, iNOS, cyclooxygenase, lipooxygenase). The role of all three MAPK pathways in LPS stimulation of immune cells is very well documented.

Although for many years ERK1/2 was considered to be mainly activated by mitogens and growth factors, and to play an important role in the control of cell growth and differentiation, recently it has become clear that ERK1/2 activation has a pivotal role in stress and inflammatory responses. Many studies have shown that ERK1/2 activation is involved in
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LPS-induced cellular responses, such as the increased production of TNF-α, inducible nitric oxide synthase (iNOS) and nitric oxide (NO) and IL-6 (Bhat et al. 1998; Carter et al. 1999; Ajizian et al. 1999). Inhibition of MEK (the upstream kinase that phosphorylates ERK1/2) with the selective inhibitor PD98059 reduced LPS induction of several inflammatory cytokines, including IL-1, IL-8 and TNF-α and PGE₂ in monocytes (Scherle et al. 1998; van der Bruggen et al. 1999; Guha et al. 2001), as well as iNOS expression in murine macrophages (Lahti et al. 2000). One of the downstream targets of the MEK/ERK1/2 pathway is the transcription factor Elk-1. The stimulation of THP-1 cells ( monocytic cell line) by LPS induced transient phosphorylation of Elk-1, which was inhibited by PD98059.

The second MAPK pathway includes isoforms of the p38 kinase that were originally identified in LPS-stimulated murine macrophages (Han et al. 1994). The p38 signal transduction pathway is present ubiquitously in mammalian tissues and involved in promoting cytokine expression as well as neuronal apoptosis induced by excitotoxins or growth factor deprivation (Ono and Han 2000). Activation of p38 by phosphorylation can be initiated by several stimuli, including cellular stress (e.g. oxidative stress), bacterial infection and pro-inflammatory cytokines IL-1β, TNF-α (Ono and Han 2000). In monocytes and macrophages the p38 kinase appears to be necessary for LPS-induced release of pro-inflammatory TNF-α (Manthey et al. 1998; Ropert et al. 2001), nitric oxide (Chen and Wang 1999; Chan and Riches 2001; Watters et al. 2002), GM-CSF (Meja et al. 2000) and cyclooxygenase-2 (Hwang et al. 1997). The specific inhibitor of p38 – SB203580 – reduced the LPS-induced expression of IL-1, IL-6 and TNF-α (Baldassare et al. 1999; Carter et al. 1999) as well as COX-2 (Dean et al. 1999) in human monocytes and macrophages. Phosphorylation of p38 MAPK was also shown to be critical for the maturation of immature dendritic cells in response to LPS (Arrighi et al. 2001).

The c-Jun N-terminal kinase (JNK) is another MAPK activated, similarly to p38, in response to cellular stress factors, such as inflammatory cytokines (IL-1β and TNF-α), ischemia, reversible ATP depletion, heat shock, and genotoxic stress, as well as lipopolysaccharide. LPS stimulation rapidly activates JNK in RAW 263.7 cells and THP-1 cells (Hambleton et al. 1996). In mouse macrophages the LPS-induced activation of JNK, together with p38 and ERK, was shown to play a crucial role in the induction of iNOS (Chan and Riches 2001), TNF-α (Jarvis et al. 2002) and COX-2 (Cho et al. 2003).

Also in glial cells many of the pro-inflammatory effects induced by LPS are mediated by MAPK. LPS was shown to activate ERK and p38 in cultures of astrocytes (Schumann et al. 1998). In astrocytes and microglia, p38 was involved in the release of TNF-α (Bhat et al. 1999).
expression of inducible nitric oxide synthase (iNOS) (Da Silva et al. 1997; Han et al. 2002) and induction of caspase 11 (required for the maturation of IL-1 and IL-18) (Hur et al. 2001). Watters et al. (2002) showed that in murine BV-2 microglial cells LPS stimulates IL-1 and NO production, which requires p38 and JNK phosphorylation. In LPS-stimulated astrocytes, ERK1/2 mediated the induction of MMP-9 (Lee et al. 2003).

In contrast to LPS, evidence for the role of Gram-positive derived components in the activation of MAPK is limited. Schröder et al (2001) have shown that exposure of myelomonocytic cells to LTA-like compounds extracted from Treponema pallidum induced an LBP-dependent phosphorylation of mitogen-activated protein kinases ERK1/2, p38 and JNK, which mediated TNF-α release. Tumour necrosis factor-α levels were significantly reduced by addition of inhibitors of ERK1/2 (PD98059) or p38 (SB203580) (Schröder et al. 2001). Activation of p38 plays a role in the induction of TNF-α, IL-6 and iNOS in RAW 264 macrophages, peritoneal macrophages and dendritic cell by S. aureus derived LTA (Dalpke et al. 2002). Finally, lipoteichoic acid was shown to activate ERK1/2 kinase in smooth muscle cells via TLR2, as inhibition of the Toll-receptor with monoclonal antibodies completely inhibited ERK1/2 phosphorylation (Lin et al. 2002). The role of MAPK in the LTA-mediated induction of cytokines and nitric oxide in glial cells was, to my knowledge, addressed for the first time in the present work.

1.6.2 Toll-like receptor 4

The first mammalian Toll-receptor discovered was TLR4. It has been identified as the specific receptor for Gram-negative bacterial lipopolysaccharide (LPS). The most important evidence for the essential role of TLR4 in LPS-induced signalling was provided by the finding that the lps gene responsible for profound hyporesponsiveness to LPS in the classical C3H/HeJ and C57BL/10ScCr strains of mice is identical to the gene of murine TLR4 (Poltorak et al. 1998; Hoshino et al. 1999; Vogel et al. 1999; Qureshi et al. 1999).

According to current knowledge LPS released from bacteria is present in body fluids as free-floating aggregates, which can be monomerized by the LPS-binding protein (LBP). LBP is a 58-kD serum glycoprotein that binds to the lipid A moiety of LPS and facilitates the extraction of single LPS molecules from LPS-aggregates (Su et al. 1995; Tobias et al. 1997). In humans, LBP is present in plasma at 3-10 µg/ml but levels rise dramatically after acute phase response to bacteria (Prucha et al. 2003). Immunodepletion of LBP from whole blood lowers the sensitivity of monocytes to LPS by at least two orders of magnitude (Martin et al. 1994). LBP catalyses the transfer of monomerised LPS to CD14, a 55-kD glycoprotein
present either in GPI-anchored form on the cell surface (mCD14), or in soluble form in the extracellular space (sCD14) (Kitchens et al. 2000). The CD14 molecule is important for the activation of cells by LPS. It is constitutively expressed at high levels in monocytes ($10^5$ molecules/cell), tissue macrophages, neutrophils (Diks et al. 2001) as well as microglia and astrocytes (Galea et al. 1996; Cosenza et al. 2002). CD14 is a co-factor for the LPS-induced cellular response in cultured cells, as addition of CD14 results in a 1000-folds increase in LPS-sensitivity (Haziot et al. 1995). Antibodies to CD14 block the LBP-dependent activation of macrophages by LPS (Yang et al. 1995). The CD14 binds LPS with high affinity and is involved in mediating LPS responses. Since CD14 is a glycosylphosphatidylinositol (GPI)-anchored membrane protein devoid of a cytoplasmic domain, it does not elicit intracellular signalling events directly, but needs to bind to the TLR4 receptor (Triantafilou and Triantafilou 2002).

CD14 seems to have a role in amplifying the LPS responses, which was documented in numerous studies (Diks et al. 2001). Transfection of a CD14 negative cell line with CD14 expression vector induced strong sensitization to LPS. Monoclonal antibodies against CD14 had profound inhibitory effects on LPS-induced activation. Finally, transgenic mice lacking CD14 were hyporesponsive to LPS at lower concentrations and resistant to the lethal effects of LPS. However at higher concentrations of LPS, CD14 (-/-) mice responded normally to LPS (Haziot et al. 1995).

LBP and CD14 have been also shown to mediate rapid cellular internalisation of LPS aggregates and even phagocytosis of intact Gram-negative bacteria \textit{in vitro} (Poussin et al. 1998; Dunzendorfer et al. 2004a). This function is maintained in mononuclear cells from TLR4 deficient mice, showing that clearance of LPS aggregates and Gram-negative bacteria from the circulation or infected tissues does not depend on signal transduction via TLR4 (Dunzendorfer et al. 2004b). TLR4 represents the central transmembrane signal transducer in the activation of mammalian cells by LPS.

The main function of TLR4 in endotoxin signal activation of human and murine mononuclear cells has been demonstrated to require tight complex formation of the TLR with MD-2, an accessory protein ligand that specifically binds to the extracellular domain of TLR4. Studies from MD-2 knockout mice have demonstrated that MD-2 is indispensable primarily for TLR4 signalling (Shimazu et al. 1999; Miyake 2004). Indeed, MD-2 does not affect the response to peptidoglycan and lipoteichoic acid (TLR2 ligands) or to DNA-containing CpG dinucleotides (TLR9 ligand) (Nagai et al. 2002; Schröder et al. 2003).
Another characteristic and unique feature of TLR4 signalling is the presence of a MyD88-independent pathway, along with the MyD88-dependent pathway typical for all TLRs. Both pathways lead to the activation of MAP kinases and NF-κB. However, the MyD88-independent pathway seems to be responsible also for the activation of IFN regulatory factor 3 (IRF-3) and the subsequent induction of IFN-β and IFN-inducible genes, such as IP-10 (Kawai et al. 2001; Toshchakov et al. 2002; Toshchakov et al. 2003).

1.6.3 Toll-like receptor 2

Unlike TLR4, which plays a major role in the recognition of Gram-negative bacterial infections, the main Gram-positive bacterial components, lipoteichoic acid and peptidoglycan, are recognised by the TLR2. Other ligands for this receptor include lipoproteins and lipopeptides from different bacteria, glycolipidanchor from *Trypanosoma cruzi*, lipoarabinomannan from *Mycobacterium tuberculosis* and the yeast component zymosan (Akira and Hemmi 2003). Interestingly, TLR2 can also mediate responses to necrotic cells (Beg 2002).

The recognition of LTA by TLR2 was for a long time controversial due to different degrees of purity and endotoxin contamination of the LTA preparations. Clear evidences of the role of TLR2 in LTA and PGN recognition came from experiments performed on HEK293 cells transfected with TLR2 (Schwandner et al. 1999) and TLR2-deficient mice (Takeuchi et al. 1999; Lehner et al. 2001), where peritoneal macrophages from TLR2-deficient mice were unable to produce TNF-α in response to *S. aureus* PGN and LTA.

A recent study has demonstrated that TLR2 forms heterodimers with TLR6 and TLR1, and can cooperate with these TLRs to mediate responses to certain stimuli in macrophages (Ozinsky et al. 2000; Hajjar et al. 2001; Tapping and Tobias 2003; Sandor et al. 2003). Thanks to a differential expression and cooperation (heterodimerisation) between different TLRs a wider number of different microbial structures can be recognised.

Additionally, also CD14 has been demonstrated to have a potentiating role in TLR2 signalling. Co-expression of CD14 and TLR2 was shown to enhance the activation of HEK293 cells by peptidoglycan, suggesting that CD14 functions and a binding protein for PGN (Schwandner et al. 1999). CD14, together with LBP have been found to bind LTA, but with lower affinity that LPS (Schröder et al. 2003).

As mentioned in the previous section an important difference between the recognition of Gram-positive and Gram-negative bacteria is the presence of an additional and unique MyD88-independent pathway in the TLR4 signalling (Kawai et al. 2001). This distinction
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may lay upon the differences between genes induced by TLR4 and TLR2 ligands. When compared with TLR2 ligands, TLR4 ligands preferentially induce the production of IL-1β, IFN-γ, IL-12, p40 and MCP-2, as well as the release of nitric oxide. On the other hand cells stimulated with TLR2 ligands release more IL-4, IL-5, G-CSF, IL-8, MIP-1α and less TNF-α, IL-1β, IL-12 (Hermann et al. 2002; Supajatura et al. 2002; von Aulock et al. 2003).

1.6.4 Toll-like receptors in the CNS

There is growing evidence that Toll-like receptors are also present in the nervous tissue and play an important role in the recognition of pathogens associated with CNS infections. In vivo experiments performed on mice deficient in Toll-like receptor 2 have shown that after intracerebral injection of live S. pneumoniae these mice had more severe clinical symptoms, reduced bacterial clearing and enhanced inflammatory reactions within the brain parenchyma and meninges, compared to the wild-type animals (Echchannaoui et al. 2002).

Primary cultured murine microglia were recently shown to constitutively express low levels of mRNA for various TLRs (TLR1, TLR2, TLR6). The expression of these TLRs was significantly increased following S. aureus exposure (Kielian et al. 2002; Kielian et al. 2005). Olson et al. (2004) have demonstrated that murine microglia express several Toll-like receptors (TLR1-9) and activation of cultured microglia in vitro with several TLR agonists (LPS, peptidoglycan, poly(I:C), CpG DNA, Theiler's murine encephalomyelitis virus) led to up-regulation of cytokines and chemokines at the mRNA and protein levels.

The mRNA encoding Toll-like receptors-2, -4, -5 and -9 was also detected in murine astrocytes and the expression was up-regulated in the presence of several PAMPs, such as LPS, flagellin, CpG DNA and peptidoglycan (Bowman et al. 2003). TLR2 has been shown to mediate astrocytes activation in response to S. aureus, as exposure of primary murine astrocytes in vitro to heat inactivated S. aureus and PGN derived from these bacteria stimulated the production of TNF-α, IL-1β, IL-6, MCP-1, MIP-1β, MIP-2, and nitric oxide (Esen et al. 2004).

Using human post-mortem brain tissues as sources of astrocytes, microglia, and oligodendrocytes, Bsibsi et al. (2002) have demonstrated, at the level of mRNA as well as by immunohistochemical staining, that also human glial cells express TLRs. In human microglia all TLRs, except from TLR9, could be detected, whereas oligodendrocytes and astrocytes showed only expression of TLR2 and TLR3. An in vitro study on human embryonic astrocytes confirmed that these cells preferentially express TLR3 both constitutively and after activation with its agonist poly-(I:C) (Farina et al. 2005). The immunohistochemical analysis
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of TLR3 and TLR4 expression in brain and spinal cord sections from control and multiple sclerosis patients revealed that both receptors were enhanced in inflamed CNS tissues (Bsibsi et al. 2002).

Interestingly, it has been shown that unlike glial cells, neurons do not express TLRs. Using immunohistochemical staining, Lehnardt et al. (2003) were unable to show TLR4 expression on neurons, and LPS did not have a neurotoxic effect in pure neuronal cultures, but extensive neuronal death in vitro that is detected in the presence of LPS-activated microglia expressing TLR4. The crucial role of TLR4 in LPS-mediated neurodegeneration was confirmed using TLR4 mutant mice, as mixed glial-neuronal cultures form mice with the mutated, inactive form of TLR4 were resistant to LPS-induced neuronal injury (Lehnardt et al. 2003). In another study, Iliev et al (2004) have demonstrated that mRNA for TLR9 is expressed in microglial cells but not in cultured hippocampal neurons.

Therefore, the expression of TLRs was detected in all types of glial cells (astrocytes, microglia and oligodendrocytes) and has been shown to play an important role in the activation of glia by several pathogen derived components. However, none of the studies conducted so far addressed the role of Toll-like receptors in the response of astrocytes and microglia to the main stimulatory molecule derived from Gram-positive bacteria - lipoteichoic acid. The determination and characterisation of TLRs involved in LTA-induced activation of rat glial cell will be of particular interest of the present study.
2. AIMS OF THE STUDY

Bacterial cell wall components are important stimuli that in the course of infectious diseases trigger inflammation in the CNS. The mechanisms of glia activation and subsequent neuronal damage induced by LPS, the main component of Gram-negative bacterial cell walls, are well characterised. However, as discussed in the introduction, the pro-inflammatory potential in the CNS of the main components of Gram-positive bacterial cell walls is still poorly understood. In this Ph.D. thesis I studied the inflammatory response of astrocytes and microglia to LTA (in the presence or absence of MDP) and the mechanisms of LTA-induced neuroinflammation leading to neuronal cell death.

The aims of this study were:

1. To characterise the inflammatory responses of rat primary cortical astrocytes and microglia to lipoteichoic acid (LTA) +/- muramyl dipeptide (MDP), in terms of:
   • release of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β).
   • induction of nitric oxide synthase (iNOS) and nitric oxide production.

2. To identify which Toll-like receptors (TLR2 or TLR4) are involved in the LTA-induced activation of microglia and astrocytes by:
   • characterisation of the expression of TLR2 and TLR4 in astrocytes and microglia, under control conditions and upon stimulation with LTA and LPS.
   • characterisation of the inflammatory response to LTA of glial cells isolated from TLR2 and TLR4 deficient mice (by measurement of the release of pro-inflammatory cytokines and nitric oxide).

3. To determine which intracellular pathways are involved in the LTA (+/- MDP)- mediated inflammatory response of astrocytes and microglia, and in particular the role of mitogen-activated protein kinases (MAPK) p38 and ERK1/2.

4. To investigate the mechanisms of neuronal cell death induced by LTA-induced glial cells by:
   • Assessment of necrotic and apoptotic neuronal cell death in cerebellar granule cells cultures (CGCs) treated with LTA (+/- MDP) in the presence and absence of glial cells.
AIMS OF THE STUDY

• Identification of the role of oxidative and nitrosative stress in the mechanisms of LTA-induced neuronal cell death with emphasis to the role of:
  - Superoxide
  - Peroxynitrite
  - Nitric oxide

• Determination of the contribution of caspase-3, caspase-8 and caspase-9 activation in LTA-induced neurotoxicity.
3. Lipoteichoic acid-induced pro-inflammatory signalling in cultured rat microglia and astrocytes: roles of Toll-like receptor 2, MAPK ERK1/2 and p38 kinase.

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submitted to Glia

3.1 Abstract

In contrast to lipopolysaccharide (LPS) from Gram-negative bacteria, the role of Gram-positive bacterial components in inducing inflammation in the CNS remains controversial. In the present study we analysed the potency of highly-purified lipoteichoic acid (LTA) isolated from Staphylococcus aureus to activate primary cultures of rat astrocytes and microglia. Exposure of either type of glial cells to LTA triggered a significant time- and dose-dependent production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and nitric oxide (NO). Muramyl dipeptide, the smallest active fragment of peptidoglycan, strongly potentiated LTA-induced iNOS expression and NO production; however, it did not have any significant influence on the production of pro-inflammatory cytokines. As bacterial components are recognised by the innate immunity through Toll-like receptors (TLRs), we analysed the expression and function of TLR2 and TLR4 in cultured glial cells before and after exposure to LTA. Real-time-RT PCR showed a basal expression of mRNA for both TLR2 and TLR4 in rat astrocytes and microglia. Using cells isolated from TLR2 and TLR4 knockout mice we showed that LTA was recognised in astrocytes and microglia by the TLR2, while LPS by the TLR4, as cells isolated from mice lacking TLR2 or TLR4 did not produce pro-inflammatory cytokines and nitric oxide upon LTA or LPS stimulation respectively. Furthermore, we showed that LTA-induced glia activation was mediated by p38 and ERK1/2 MAP kinases, since LTA caused transient activatory phosphorylations of these kinases, and pre-treatment with SB203580 (inhibitor of p38) and PD98059 (inhibitor of ERK1/2) decreased the LTA-induced cytokine release, iNOS mRNA expression and NO production.
3.2 Introduction

Inflammation in the Central Nervous System (CNS) is mediated by microglia and/or astrocytes that become activated by inflammatory stimuli in a wide range of CNS neurodegenerative diseases such as Parkinson’s, Alzheimer’s disease, and multiple sclerosis (Aloisi 2001; Eddleston and Mucke 1993; Minagar et al. 2002), as well as in ischemia (Weiner and Selkoe 2002; Allan and Rothwell, 2001), trauma and brain infections such as AIDS dementia, malaria and meningitis (Nau and Bruck 2002; Schmidt et al. 2001). During the progression of inflammation glia change from their normal resting to an activated status as indicated by morphological changes as well as the release of pro-inflammatory cytokines including IL-1β, TNF-α, IL-6, IL-8, chemokines, cell adhesion molecules, proteins of the complement system and expression of enzymes such as iNOS or COX-2. These molecules trigger the more specific acquired immune response that is essential for eliminating pathogens. However, excess activation of both microglia and astrocytes can also cause neuronal cell death (Bal-Price and Brown 2001; McGeer and McGeer 1998; Swanson et al. 2004).

A model molecule widely used to study glia activation is lipopolysaccharide (LPS), which is the main component of the Gram-negative bacterial cell walls. The response and the mechanism involved in LPS-induced glia activation are well characterised. However, the pro-inflammatory potential of lipoteichoic acid (LTA), peptidoglycan (PGN) and muramyl dipeptide (MDP), the main components of Gram-positive bacterial cell walls in CNS and the extra- and intracellular pathways involved in glial activation are still poorly understood. There is increasing evidence that in peripheral systems LTA has similar pathogenic properties as LPS. The immunostimulatory potential of LTA has been described in human whole blood, peripheral blood monocytes and macrophages where significant production of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, IL-8) (Morath et al. 2002b; Hermann et al. 2002) and nitric oxide (Kengatharan et al. 1996) were observed. In vitro and in vivo studies show that LTA is involved in the pathogenesis of CNS infectious diseases such as encephalomyelitis, meningitis (Nau and Bruck 2002) and brain abscesses (Aaron and Kielian 2004). Brain abscesses are mainly caused by Streptococcal strains and *Staphylococcus aureus* (Aaron and Kielian 2004), whereas the principal cause of meningitis in adults and children is *Streptococcus pneumoniae* (Schuchat et al. 1997). Despite effective antibiotic therapy these diseases are still associated with high mortality and cause severe neurological defects (Kastenbauer and Pfister 2003; Mathisen and Johnson 1997; Townsend and Scheld 1998). The conventional antibiotic therapy in meningitis or brain abscesses leads to bacteriolysis and massive release of pro-inflammatory and toxic bacterial cell
LTA-induced glial inflammatory response

wall components (Gerber et al. 2003). However it is still not clear which of these components mediate the inflammatory response to Gram-positive bacteria and which cell types and receptors are involved.

Usually pathogen-derived molecules (LPS, LTA, lipopeptides, bacterial DNA and RNA) are recognised by the systemic innate immune system through a family of receptors called Toll-like receptors (TLRs). TLR2 is essential for the recognition of a variety of molecules, especially derived from Gram-positive bacteria, including lipoproteins, peptidoglycan and teichoic and lipoteichoic acids. TLR4 and CD14 are mainly activated by Gram-negative bacterial lipopolysaccharide. TLRs are predominantly expressed in tissues involved in immune functions, but recently have also been described in glial cells (microglia and astrocytes) (Bsibsi et al. 2002; Kielian et al. 2002; Bowman et al. 2003).

In this study we characterised the inflammatory response of primary cultures of rat glial cells (astrocytes and microglia) using LTA purified according to a novel procedure to ensure high purity (>99%) and specific bioactivity (Morath et al. 2001) that it is not guaranteed by commercially available LTA, often contaminated with LPS (Gao et al. 2001; Morath et al. 2002a). The response of glia to LTA activation was compared with LPS in terms of cytokine release and nitric oxide production. Of particular interest was a strong synergistic role played by MDP (minimal active component of peptidoglycan) in LTA-induced iNOS expression followed by NO production.

To characterise further which Toll-receptors (TLR4 or TLR2) were involved in LTA- or LPS-induced glia activation, cultures of astrocytes and microglia prepared from knockout TLR4 or TLR2 mice were used. The results clearly demonstrated that TLR2 was essential for full pro-inflammatory cytokine and nitric oxide production induced by LTA (+/- MDP) in contrast to TLR4 that was necessary for LPS- induced signal transduction.

As signalling via TLRs depends on the intracellular signal adapter protein MyD88 and activation of mitogen protein kinases (MAPK), we studied whether glia activation induced by LTA (+/- MDP) was mediated by p38 and ERK1/2. Our results indicated that ERK1/2 and p38 MAPK play an important role in this process as the release of cytokines and nitric oxide was significantly blocked in the presence of SB203580 (inhibitor of p38 MAPK) and PD98059 (inhibitor of ERK1/2).

Presented results demonstrate that in the CNS both type of glial cells, microglia and astrocytes, become activated in the presence of LTA (+/- MDP) as induction of pro-inflammatory cytokines and nitric oxide production was observed. LTA-induced glia activation is mainly
mediated by TLR2, and ERK1/2 and p38 are the main MAPK kinase involved in the signal transduction pathway. These studies suggest that glia could play a key role in the immune response of CNS inflammatory disorders induced by the Gram-positive bacteria such as encephalomyelitis or meningitis.

3.3 Material and Methods

3.3.1 Animals

Rats. The 7-day-old rat pups (OFA/SPF strain) were purchased from Charles River Laboratories (Les Oncins, France) and hosted at the animal research facilities of the University of Insubria, Varese, Italy.

Mice. C3H/HeN and C3H/HeJ were purchased from Charles River Laboratories (Suzfeld, Germany). TLR2 deficient mice on a Sv129xC57BL/6 background and wild-types were kindly provided by Tularik (South San Francisco, CA, USA). The mice were bred in the animal research facilities of the University of Konstanz, Germany.

All animals received humane care in accordance with the National Institutes of Health and legal requirements in Europe.

3.3.2 Primary culture of astrocytes and microglia

Primary, mixed glial cultures were prepared from the cerebral cortex of 7-day-old rats as described previously (Bal et al. 1994). Briefly, the cells isolated from cerebral hemispheres were dissociated in Hank’s balanced salt solution (HBSS) containing 0.25% trypsin (Sigma-Aldrich, Milano Italy), 0.02 mg/ml deoxyribonuclease I (DNA-se I) (Sigma-Aldrich) and 1% bovine serum albumin (BSA) (Sigma-Aldrich). Cells were plated at a cell density of 1 x 10^4 cells/cm^2 in 75-cm^2 culture flasks (Costar) for microglia isolation, 6-well plates (Costar) for RNA isolation or 12-well plates for induction of cytokines and nitric oxide (Costar). All flasks and plates were coated with poly-L-lysine (Sigma-Aldrich). The DMEM medium (Invitrogen, Milano, Italy) contained 10% foetal bovine serum (FBS) (Invitrogen) and 1% antibiotics (penicillin/streptomycin) (Invitrogen). Cells were grown in a humidified incubator at 37°C in 5% CO2 in air. Medium was changed every 3 days. At confluence (14 -16 days in vitro (DIV)) the primary mixed glial cultures were used to isolate the microglial cells. The mixed glial cultures were shaken for 2 hours to dislodge microglia that were loosely attached to the astrocytes. Microglia were seeded into 6-well or 12-well plates at a density of 2.0 x 10^5 cells/cm^2 and maintained in astrocyte-conditioned medium (medium collected from astrocytic cultures after 2
days and spun down) mixed 1:1 v/v with fresh DMEM medium (containing 10% of foetal bovine serum). The purity of the microglial and astrocytic cultures (after isolation of microglia) was determined immunocytochemically with anti-GFAP antibody (astrocytic marker; Santa Cruz Biotech., Santa Cruz, California, USA) and anti-OX-42 antibody (microglial marker, an anti-CR3 complement receptor antibody; Serotec, Cergy Saint-Christophe, France). The cells were fixed with 4% paraformaldehyde and permeabilized with 3% Triton X-100 (Sigma-Aldrich) in PBS, followed by incubation with anti-GFAP or anti-OX42 antibody overnight at 4°C. After extensive washing with 1% Triton X-100 in PBS, cells were incubated with secondary antibodies conjugated with FITC or Cy3 (Chemicon, Hampshire, UK) for 2 hours at room temperature. Cells were extensively washed followed by a 10-min staining with 5 µg/ml Hoechst 33324 (Molecular Probes Europe, Leiden, The Netherlands). The staining was analysed using confocal laser scanning microscope (BioRad Radiance MP2000, USA).

3.3.3 Activation of astrocytes and microglia in culture

Cultures of astrocytes and microglia (24 hours after isolation from mixed glial cultures) were activated by exposure to 10 µg/ml or 30 µg/ml lipoteichoic acid (LTA) from *Staphylococcus aureus* or 10 µg/ml or 30 µg/ml lipopolysaccharide (LPS) from *Salmonella abortus equi* (Sigma-Aldrich). Some cultures were pre-treated with MDP 100 ng/ml (Bachem, Bubendorf, Switzerland) for 30 minutes before exposure to LTA. It was obtained according to a novel butanol extraction procedure (Morath et al. 2001) to ensure biological activity and high purity (lack of LPS contamination).

For the assessment of cytokine release the medium was collected at 1, 6, 12 and 24 hours, and for the measurement of nitric oxide (NO) production the medium was collected at 24, 48 and 72 hours after the treatment with LTA (+/- MDP). At the end of the experiments astrocytes were gently trypsinised (0.1% trypsin, Sigma-Aldrich) for 2-3 minutes and the number of cells was counted using a haemocytometer.

In some experiments the cultures were pre-treated with selective inhibitors of p38 (25 µM SB203580, Calbiochem, Darmstadt, Germany) or ERK1/2 (25 µM PD98059, Calbiochem, Darmstadt, Germany), before exposure to LTA (+/- MDP).

Studies were performed also using primary cell cultures of astrocytes and microglia from TLR4-deficient C3H/HeJ and C3H/HeJ (control) mice and TLR2 knockout (-/-) and wild-type (+/+), prepared as described above. After exposure to stimulus (LTA or LPS +/- IFN-γ) the
medium was collected at 6 hours for TNF-α, 24 hours for IL-6, and 48 hours for nitric oxide measurement.

### 3.3.4 Measurement of cytokine content in medium

Quantification of cytokines level in medium collected from LTA (+/- MDP)- and LPS-activated rat or murine glial cells was performed by a sandwich ELISA, using commercially available antibody pairs. The Nunc Maxisorp ELISA plates (Nunc) were coated overnight with anti-TNF-α, anti IL-1β or anti-IL-6 antibodies diluted in 100 mM NaHCO₃, pH 8.3 at 4°C. All antibodies against rat cytokines and recombinant proteins (used as standards) were from Biotrend (Köln, Germany). Antibodies against murine TNF-α and IL-6 and recombinant cytokine proteins were purchased from R&D (Wiesbaden, Germany). After blocking with 3% BSA/PBS for 2 hours, samples and standard solutions (prepared in 3% BSA/PBS) were added for 3 hours followed by incubation for 45 minutes with biotinylated anti-TNF-α, anti IL-1β or anti-IL-6 antibodies and incubation with streptavidin-peroxidase (Biosource, Nivelles, Belgium) for 30 minutes. Detection of bound cytokines was carried out using TMB (3,3',5,5'-tetramethylbenzidine, Sigma-Aldrich). The reaction was stopped using 1M H₂SO₄ and the absorption was measured at 450 nm in a multiwell spectrophotometer (Spectra Max, Molecular Devices, Sunnyvale, CA, USA). The cytokines concentrations were calculated using standard solutions of recombinant TNF-α, IL-1β or IL-6 and expressed in pg/ml/10⁶ cells.

### 3.3.5 Measurement of nitric oxide production

The accumulation of nitrite in the medium, as indicator of NO synthesis, was assessed using Griess reaction. Sulfanilamide (1 mM), hydrochloric acid (6 mM) and N-1(1-naphtyl) -ethylenediamine (1 mM) (Sigma-Aldrich) were added to the medium. After 30 minutes incubation at room temperature the absorbance was measured at 548 nm in a multiwell spectrophotometer (Spectra Max, Molecular Devices, Sunnyvale, CA, USA). The nitrite concentrations were calculated using standard solutions of sodium nitrite prepared in culture medium. The nitrite production is expressed in µM/10⁶ cells.

### 3.3.6 Staining of mixed glial cells with rhodamine-labelled LTA

To evaluate whether LTA binds to the cell surface receptors on both types of glial cells (microglia and astrocytes), we used LTA conjugated with a fluorescent dye – rhodamine red
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(Kindly provided by S. Deininger, University of Konstanz, Germany). The labelling was performed as follows: 3 mg LTA from S. aureus, 4.5 mg sulforhodamine Q 5-acidfluoride (Fluka, Buchs, Switzerland), 2.5 ml dimethyl sulfoxide (Wak-Chemie-Medical GmbH, Steinbach, Germany) and 25 µl trimethylamine (Acros Organics, Leicestershire, UK) were sonified for 10 min. The mixture was shaken overnight at 37°C, and then spun 4 times for 90 min at 7000 g in pyrogen-free centrifugal ultrafiltration tubes (Microsep 3K Centricons, Pall, MI, USA). To remove remaining unbound dye, the labelled LTA was filtered through a pyrogen-free sepharose column (PD-10 desalting column, Amersham Biosciences, Freiburg, Germany). Labelling efficiency, calculated as fluorescence (560 nm/620 nm) per phosphate content, was 1-2 rhodamine molecules per LTA. Phosphate determination was performed by adding a mixture of H_2SO_4 : HClO_4 : H_2O (556 : 105 : 3339, v/v/v) to 50 µl LTA solution and incubating for 2 h at 145°C. Then 1 ml of ascorbic acid : ammonium heptamolybdenum - Na-acetate (1 : 9, v/v) was added and incubated for 2 h at 50°C. Absorption was measured at 700 nm. Rhodamine-LTA was negative in the LAL (i.e., <0.05 EU/ mg LTA).

To assess whether fluorescently-labelled LTA binds to both types of glial cells we used cultures of pure microglia or mixed glial cells seeded on 4-well chamber slides (Lab-tek Chamber Slide System, Nunc) and exposed them to rhodamine-labelled LTA. Additionally, to visualize microglia, the cultures were co-stained with a microglial marker – isolectin B4 (Sigma-Aldrich). Rhodamine-labelled LTA (30 µg/ml) and FITC-labelled isolectin B4 (10 ng/ml) were added directly to the culture medium. After incubation at 37°C for 30 minutes the cells were washed three times with PBS and the staining was analysed under confocal laser scanning microscope (BioRad Radiance MP2000, USA).

3.3.7 Real-time RT-PCR

RNA was isolated with an RNasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Possible contamination with DNA was removed by digestion using an RNase-free DNAse set (Qiagen). RNA concentration and protein contamination were assessed spectrophotometrically (Biophotometer; Eppendorf, Milano, Italy). Reverse transcription was performed as follows: 500 ng RNA was incubated with dNTP (0.5 mM each; Promega, Madison, USA) and random hexamer (12.5 µg/ml; Promega) for 5 min at 65°C using a Perkin-Elmer Geneamp PCR system 9600. Subsequently RNAse inhibitor (RNaseOut, 40 U; Invitrogen), reverse transcriptase (Superscript™ II RNAse H-, 200 U;
Invitrogen), DDT (10 mM; Invitrogen) and first-strand buffer (Invitrogen) were added and incubated for 10 min at 25°C, for 50 min at 42°C and for 15 min at 70°C.

An AbiPrism 7000 sequence detector system in conjunction with TaqMan® Universal PCR Master Mix and TaqMan® Real-Time PCR Assays-on-Demand (all Applied Biosystems, Monza, Italy) was used for 18S rRNA, TLR4 and iNOS PCR according to the manufacturer’s protocol. For TLR2 PCR, an Assay-by-Design (Applied Biosystems) was used, consisting of the forward primer 5'-GCCACAGGACTCAAGAGCAT-3’, the probe 5’-FAM-TCTCCAGGTCAAATCT-NFQ-3’ and 5’-GCCAAAGAGCTTGAGCAT-3’ as the reverse primer. Relative quantification was performed via the comparative CT method, based on 18S rRNA quantities.

3.3.8 Detection of phosphorylated kinases using Luminex 100

Gliarial cells cultured in 25 cm² flasks, non-treated (control) or exposed to LTA (10 µg/ml) +/- MDP (100 ng/ml) for 10, 30 and 60 minutes were placed on ice and lysed using a commercially available Bio-Plex cell lysis kit (BioRad, Hercules, CA, USA). Cells were scraped and the flasks were agitated on a microplate shaker at 300 rpm for 20 minutes at 4°C. The cell lysates were collected to Eppendorf 1.5 ml tubes and centrifuged at 4500 rpm for 20 minutes at 4°C. The supernatant was collected and the protein content in lysates was determined using Bradford assay. Samples were stored at -80°C for further analysis.

The analysis of the samples was performed according to the instructions specified in the Bio-Plex phosphoprotein assay (BioRad, Hercules, CA, USA). The assay was performed on 96-well filter plate (Millipore, Bedford, MA). Briefly, the samples and the provided controls were incubated with capturing beads coupled with total-p38, total-ERK1/2, phospho-p38 (Thr180/Tyr182) and phospho-ERK1/2 (Thr202/Tyr204, Thr185/Tyr187) antibodies overnight, at room temperature, agitated on a microplate shaker at 300 rpm. As the beads are light sensitive, the plates were covered with aluminium foil. On the next day the wells were washed 3 times with 100 µl wash buffer, using a filter plate vacuum manifold at 2 psi (Millipore, Bedford, MA) and 25 µl of detection antibodies were added. The plate was shaken for 30 minutes at room temperature (300 rpm) and subsequently washed 3 times with 100 µl wash buffer, followed by incubation with 50 µl streptavidin-PE for 10 minutes at room temperature. After washing the beds were resuspended in 125 µl resuspension buffer for analysis. Immediately before analysis, the plates were shaken again to ensure complete resuspension of beads. The fluorescence intensity readings for 100 beads/kinase were determined and analysed using a Luminex 100
system (Luminex Corp., Austin, TX, USA) and the results were calculated as the mean
fluorescence intensity.

3.3.9 Statistical analysis
Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad software, San Diego,
USA). All data given are means of three independent experiments ± SEM. Differences
between two groups were assessed by Student’s t-test. Unpaired samples were assessed by
one-way analysis of variance followed by Bonferroni’s or Dunnet’s Multiple Comparison test
(where indicated). Data were log-transformed to achieve Gaussian distribution. In the figures
p values are as follow *p<0.05, **p<0.01 and ***p<0.001.

3.4 Results
3.4.1 LTA induces pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and nitric oxide
production in primary culture of rat microglia and astrocytes
To determine whether LTA could activate both types of glial cells, we generated pure microglial
cultures (99.0% of OX-42 positive cells, less than 1.0% of GFAP positive cells) and enriched
astrocytic cultures (93.7 ± 4.3 % of GFAP positive cells, 6.3 ± 4.7 % of OX-42 positive cells)
and measured the production of pro-cytokines and nitric oxide after treatment with LTA or LPS
(10 or 30 µg/ml). Exposure of either type of glial culture (enriched astrocytes or microglia) to 10
µg/ml or 30 µg/ml of lipoteichoic acid (LTA, S. aureus) or lipopolysaccharide (LPS, S. abortus
equi) resulted in a dose-dependent release of TNF-α, IL-6 and IL-1β. The levels of TNF-α
production by astrocytes activated with LTA (10 µg/ml) after 6 hrs were slightly lower then after
LPS (10 µg/ml) stimulation (Fig. 8 A). The highest TNF-α levels were detected between 6 - 8
hours after LTA treatment (1930 ± 96 and 2403 ± 50 pg/ml/ 10^6 cells after 10 and 30 µg/ml of
LTA respectively), followed then by a decrease at 24 hrs (1015 ± 108 and 1191 ± 116 pg/ml/ 10^6
cells after 10 and 30 µg/ml of LTA, respectively). After treatment with LPS the highest TNF-α
levels were also detected after 6 - 8 hrs of exposure (2182 ± 343 and 2406 ± 360 pg/ml/ 10^6 cells
after 10 and 30 µg/ml of LPS respectively), followed by a decrease at 24 hours (975 ± 146 and
1226 ± 208 pg/ml/ 10^6 cells after 10 and 30 µg/ml of LPS respectively). Similar kinetics and
levels of TNF-α production were obtained in LTA- and LPS-stimulated primary culture of
microglia (Fig. 9 A).

The kinetics of IL-1β and IL-6 release differed from the one of TNF-α. Both cytokines (IL-1β
and IL-6) were detected in astrocyte culture medium after 6 hours of exposure to LTA or LPS,
but the levels were steadily increasing and did not reach a plateau even after 24 hours (Fig. 8 B and C). Interestingly, in pure cultures of microglia the kinetics of IL-1β and IL-6 release was comparable with astrocytes cultures, however the values of IL-1β are slightly higher (Fig. 9 B and C).

Exposure of cultured astrocytes to LTA or LPS led to a significant production of nitric oxide, measured by nitrite accumulation (Griess reaction). Cultured astrocytes exposed to 30 µg/ml of LTA produced comparable level of nitric oxide to astrocytes activated with 10 µg/ml of LPS suggesting that LTA was a slightly weaker immunostimulant than LPS (Fig. 8 D). In a pure culture of microglia, NO (measured by nitrite accumulation) was produced with similar kinetics as in the culture of astrocytes, but at lower levels (Fig. 9 D). Perhaps the interaction between two types of glial cells is necessary for full iNOS activation.

These results indicate that both type of glial cells (astrocytes and microglia) responded to LTA and LPS activation in a comparable way, and it seems that 10% of microglia present in astrocyte culture could not count for the total release of cytokines and nitric oxide. Non-stimulated glial cells did not produce detectable amounts of cytokines or nitric oxide. Treatment of glial cells with LTA or LPS at the doses used in this study did not induce any cell death (data not shown) as assessed by trypan blue (0.4%) or propidium iodide (20 µg/ml) staining. These results indicate that both types of glial cells, microglia and astrocytes, are capable of recognising LTA (the component of Gram-positive bacteria) and respond to this stimulus by production of pro-cytokines (TNF-α, IL-1β, IL-6) and nitric oxide in a time- and concentration-dependent way.
Fig. 8 LTA (S. aureus) and LPS (S. abortus equi) stimulate TNF-α (A), IL-1β (B), IL-6 (C) release and nitric oxide production (D) in rat primary culture of astrocytes in a dose- and time-dependent manner. Cells were exposed to 10 or 30 µg/ml LTA or LPS for up to 72 hours. Culture supernatants were collected and analysed using ELISA (TNF-α, IL-1β, IL-6) and Griess reaction (nitrite level) as described in Materials and Methods. Results are presented as the amount of cytokines in pg/ml calculated per 10^6 cells or nitrite in µM/10^6 cells. Data from at least three independent experiments performed in duplicates are presented as means ± SEM.
Fig. 9 Rat primary microglia culture stimulated with LTA (S. aureus) and LPS (S. abortus equi) release TNF-α (A), IL-1β (B), IL-6 (C) and nitric oxide (D) in a concentration- and time-dependent manner. Cells were stimulated with 10 or 30 µg/ml LTA or LPS for up to 72 hours. Culture supernatants were collected and analysed using ELISA (TNF-α, IL-1β, IL-6) and Griess reaction (nitrite level) as described in Materials and Methods. Results are presented as the amount of cytokines in pg/ml calculated per 10⁶ cells or nitrite in µM/10⁶ cells. Data from at least three independent experiments performed in duplicates are presented as means ± SEM.
3.4.2 TLR2 is essential for mediating LTA-induced astrocytes and microglia activation

Since in previous studies it has been shown that bacterial components (LPS, CpG DNA, heat inactivated bacteria etc.) can regulate the expression of TLRs in astrocytes and microglia (Bowman et al., 2003; Esen et al., 2004; Kielian et al., 2002) we investigated whether rat astrocytes and microglia cultured under our conditions express Toll-like receptor 2 and -4 and whether their expression is changed after the exposure to LTA or LPS (Real-time RT-PCR studies). After treatment with LTA (30 µg/ml) or LPS (100 ng/ml) for 1 hour, 6 hours, 10 hours and 24 hours the expression of both TLR2 and TLR4 did not statistically differ from the untreated control culture (see Table 2). Similar results were observed in pure culture of microglia (data not shown) suggesting that both stimuli (LTA or LPS) did not significantly change the basal expression of Toll-like receptor 2 or -4 in our system. In further studies, using LTA conjugated with a fluorescent dye (rhodamine red), we confirmed that LTA binds to the cell surface of either type of glial cells. Both microglia and astrocytes in cultures were stained intensively with rhodamine-labelled LTA (Fig. 12). Co-staining with isolectin B4 (microglial marker) enabled us to distinguish between astrocytes and microglia in mixed glial cultures. The specificity of the LTA binding was confirmed, as Balb-3T3 cells (a mouse fibroblast cell line) did not bind rhodamine-labelled LTA (a negative control, data not shown). To study the role of Toll-like receptor 2 in LTA-induced activation of glial cells we compared the response to LTA (1, 10 and 30 µg/ml) or LPS (10, 100 ng/ml) treatment of primary astrocytes and microglia isolated from TLR2 (-/-) knockout mice and wild-type (+/+ ) mice by measuring the production of TNF-α (at 6 hours), IL-6 (at 24 hours), and NO (at 48 hours). Peripheral macrophages isolated from TLR2 knockout mice were previously shown to be unresponsive to LTA (Lehner et al., 2001). In line, astrocytes isolated from TLR2 (-/-) mice did not release any IL-6 or TNF-α after treatment with LTA (1, 10 or 30 µg/ml), while LPS-treated astrocytes from TLR2 (-/-) mice responded as the astrocytes isolated from wild-type mice, producing comparable amounts of both cytokines (Fig. 10 A and B). Similar results were obtained in cultured microglia isolated from TLR2 (-/-) mice. These results indicate that TLR2 is essential for mediating astrocyte and microglia activation in response to LTA, but does not play any major role in LPS-induced glia activation.

To determine the involvement of TLR4 in LTA-signalling in glia we compared TNF-α, IL-6 and NO production in astrocytes and microglia isolated from C3H/HeJ mouse (lacking functional TLR4) and wild-type C3H/HeN mice. In contrast to glial cells from TLR2 (-/-) knockout mice, astrocytes from TLR4 deficient mice did not respond to LPS (1, 10 or 100 ng/ml) treatment confirming the well-known status of LPS-hyporesponsiveness of the C3H/HeJ strain. However,
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exposure to LTA induced the release of comparable amounts of TNF-α and IL-6 in glial cells from both C3H/HeN and C3H/HeJ strains (Fig. 11 A and B, respectively) confirming that LTA signalling in astrocytes and microglia is mediated through the Toll-like receptor 2, while LPS signalling is mediated by Toll-like receptor 4.

To determine the role of Toll-like receptors 2 and -4 in NO production we activated mice primary glial cells cultures (astrocytes and microglia) with LTA or LPS in the presence of IFN-γ (10 ng/ml). The addition of IFN-γ was necessary since LTA or LPS alone did not induce NO production (contrary to rat glial cells). TLR2 was clearly involved in LTA-induced signalling, leading to iNOS expression and NO release in glial cells, as cells from TLR2 (-/-) mice stimulated with LTA + IFN-γ did not produce significant amount of nitric oxide (Fig. 10 C). On the other hand LPS-induced NO production was mediated by the TLR4, as the glial cells from TLR4 deficient mice did not release NO upon LPS + IFN-γ stimulation (Fig. 11 C). Astrocytes and microglia from TLR2 and TLR4 wild-type mice produced comparable, high amounts of NO upon stimulation with LTA (+ IFN-γ) or LPS (+ IFN-γ) (Fig. 10 C and 11 C).

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<tr>
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<th>TLR2 (relative quantity of TLR2 mRNA vs 18 rRNA)</th>
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<td>1 h</td>
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<td>LPS</td>
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Table 2. The expression TLR2 and TLR4 mRNA in rat primary astrocyte culture after stimulation with LTA and LPS. Total mRNA was extracted at 1 hour, 6 hours, 10 hours and 24 hours from control astrocyte cultures (untreated) or cultures treated with LTA (30 µg/ml) or LPS (100 ng/ml). The expression of TLR2 and TLR4 mRNA was measured using Real-Time RT PCR. The values represent relative quantity ± SEM of TLRs mRNA calculated versus 18s rRNA from three independent experiments.
Levels of cytokines are expressed in pg/ml/10^6 cells or nitrite in µM/10^6 cells. The results represent data (mean ± SEM) from three independent experiments performed in triplicates. Statistical analysis was performed using Student’s t-test and indicated statistical differences are comparisons of TLR2 (-/-) with TLR2 (+/+), *p<0.05, **p<0.01, ***p<0.001.

Fig. 10 LTA- and LPS-induced cytokine release and NO production in astrocytes isolated from TLR2 deficient mice. Cells isolated from TLR2 (-/-) (■) and TLR2 (+/+)(□) cultured on 12-well plates were stimulated with LTA (10 or 30 µg/ml) or LPS (10 ng/ml or 100 ng/ml). Culture supernatants were collected at indicated time points and analysed by ELISA for TNF-α (6 hrs), IL-6 (24 hrs) and by Griess reaction for nitrite level (48 hrs). TNF-α (A), IL-6 (B) and nitrite level (C) are significantly inhibited in the culture medium of astrocytes isolated from TLR2 (-/-) mice whereas the response to LPS is not altered. Note unchanged TNF-α, IL-6 and nitrite level in the medium of astrocytes isolated from TLR2 (+/+) wild-type mice exposed to either LTA or LPS.

Levels of cytokines are expressed in pg/ml/10^6 cells or nitrite in µM/10^6 cells. The results represent data (mean ± SEM) from three independent experiments performed in triplicates. Statistical analysis was performed using Student’s t-test and indicated statistical differences are comparisons of TLR2 (-/-) with TLR2 (+/+), *p<0.05, **p<0.01, ***p<0.001.

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Fig. 11 LTA- and LPS-induced stimulation of cytokine release and NO production in astrocytes from isolated TLR4 deficient C3H/HeJ mice (TLR4 (-/-) ) and CH3/HeN control mice (TLR4 (+/+) ). Cells were stimulated with LTA (10 or 30 µg/ml) or LPS (10 ng/ml or 100 ng/ml) and culture supernatants were collected at indicated time points and analysed by ELISA for TNF-α (6 hrs), IL-6 (24 hrs) and by Griess reaction for nitrite level (48 hrs). The LPS-induced release of TNF-α (A), IL-6 (B) and NO production measured by nitrite accumulation (C) are significantly attenuated in the culture medium of astrocytes isolated from C3H/HeJ mice. The response to LTA is not altered. Control C3H/HeN mice exhibit unaltered TNF-α, IL-6 and NO release in response to both LTA and LPS. Levels of cytokines are expressed in pg/ml/10⁶ cells or nitrite in µM/10⁶ cells. The data represent mean ± SEM from three independent experiments performed in triplicates. Statistical analysis was performed using Student’s t-test and indicated statistical differences are comparisons of C3H/HeJ with C3H/HeN, *p<0.05, **p<0.01, ***p<0.001.
Fig. 12 Binding of rhodamine-labelled LTA to microglia and astrocytes in mixed glial cultures. All pictures represent the same microscopic field co-stained with: FITC-isolectin B4 (10 ng/ml, microglial marker) (green) (A); rhodamine-LTA (30 µg/ml) (red) (B) and merged: FITC-isolectin B4 and rhodamine-LTA (C) and phase-contrast image (D). Note that rhodamine-LTA bound to both microglia and astrocytes. Glial cell types are indicated by the following abbreviations (placed on the right of the cell): a, astrocyte; m, microglia; Scale bar - 40 µm.
3.4.3 *Muramyl dipeptide (MDP) significantly potentiates iNOS mRNA expression and nitric oxide production in LTA-activated rat astrocytes and microglia*

Muramyl dipeptide is the smallest, active fragment of peptidoglycan, a component of bacterial cell wall. In previous studies it has been shown that PGN and MDP can activate immune cells as well as glial cells. However, recent findings suggest that this activation could be due to contamination of MDP by LPS (Traub et al. 2004).

We tested whether MDP could activate glial cells by measuring nitrite concentration in culture medium (NO release) and cytokines production (ELISA) by both types of glial cells (astrocytes and microglia) exposed to recombinant MDP. MDP used in this study was tested to be LPS-free to ensure that its eventual activity is not due to LPS contamination. In our system MDP alone did not induce significant production of cytokines or nitric oxide by neither astrocytes nor microglia. Interestingly, MDP significantly potentiated LTA-induced NO production (~ 3 folds) in both types of glial cells (microglia and astrocytes) (Fig. 13 A).

In primary culture of astrocytes exposed to lower concentration of LTA (10 µg/ml) in the presence of MDP (100 ng/ml) the production of NO was as high as in the parallel astrocytic culture exposed to LTA alone at higher concentration (30 µg/ml) (Fig. 13 A). MDP and LTA acted also in synergy in the culture of microglia as after 72 hrs of exposure to LTA (10 µg/ml) + MDP (100 ng/ml) the level of nitrite was 3-fold higher (12.2 µM ± 4.1 µM) when compared with exposure to LTA (10 µg/ml) alone (4.1 µM ± 1.2 µM).

The synergism between LTA and MDP was only observed in the case of nitric oxide production as MDP did not affect LTA-induced cytokine (TNF-α and IL-1β) release in astrocyte or microglia cultures. Cells treated with LTA (10 µg/ml) alone or in the presence of MDP (100 ng/ml) released comparable levels of TNF-α and IL-1β (data not shown).

To determine whether MDP regulates the LTA-induced NO production at the transcriptional level (iNOS mRNA expression) we analysed the iNOS mRNA expression in cultured astrocytes after 1 - 24 hrs exposure to LTA (10 µg/ml) alone and in the presence of MDP (100 ng/ml) (Real time RT-PCR studies). In mixed glial cultures exposed to LTA alone the iNOS mRNA reached the maximal level at 6 hours of induction and was stable up to 24 hours (data not shown). MDP (100 ng/ml) significantly potentiated the LTA-induced expression of iNOS mRNA at 6 hours (Fig. 14) whereas MDP alone did not induce any iNOS mRNA expression. The observed potentiation of LTA-induced iNOS mRNA expression by MDP correlated well with higher nitrite levels (NO production) detected in the medium of mixed glia exposed to both LTA and MDP (Fig. 14). These results clearly indicate that MDP up-regulates LTA-induced iNOS mRNA expression that results in the increased NO production.
Fig. 13 Potentiation of LTA-induced NO production by muramyl dipeptide (MDP). Primary cultures of rat astrocytes were treated with 10 µg/ml LTA (+/- 100 ng/ml MDP), LTA (30 µg/ml) or MDP (100 ng/ml) alone (A) or pre-treated with p38 MAPK inhibitor SB203580 (25 µM) or ERK1/2 inhibitor PD98059 (25 µM) for 30 min before addition of 10 µg/ml LTA (+/- MDP 100 ng/ml) (B). The culture supernatant was collected after 24, 48 and 72 hours and nitrite levels (µM) were determined using Griess reaction (see Materials and Methods). PD98059 (25 µM) and SB203580 (25 µM) alone did not alter the nitrite level. Results are expressed as nitrite (µM) per 10^6 cells and represent the mean ± SEM of three independent experiments performed in triplicates. Statistical analysis was performed using one-way ANOVA followed by Bonferroni’s post hoc test; *p<0.05, **p<0.01, ***p<0.001 from control cultures; +++p<0.001 from LTA (10 µg/ml) + MDP (100 ng/ml) treated.
3.4.4 LTA (+/- MDP)-induced nitric oxide production is blocked by p38 and ERK1/2 inhibitors

To investigate whether MAPK-pathways were involved in LTA-induced iNOS mRNA expression and subsequent NO production, we used specific inhibitors to block p38 kinase (SB203580) or ERK1/2 (PD98059) activity. SB203580 is pyridinyl imidazole, which acts as a selective inhibitor of p38 MAP kinase and does not inhibit the other MAP kinases (JNK and ERK1/2 MAP kinase). Since LTA-induced NO production was found to be significantly potentiated by MDP we studied the iNOS mRNA expression and NO production in both types of glia cultures treated with LTA in the presence and absence of MDP. The pre-treatment of mixed glial cells with SB203580 (25 µM) for 30 minutes and than exposure to LTA (10 µg/ml) +/- MDP (100 ng/ml) for 6 hours reduced significantly iNOS mRNA expression (Real-time PCR studies) when compared with the cultures not treated with the kinase inhibitor (58.7 ± 18.2% and 70.0 ± 5.1% decrease in LTA alone and LTA + MDP treated cultures, respectively) (Fig 14). In the same experiment we found that SB203580 almost completely prevented NO production measured by nitrite level (80.3 +/- 11.1 %) (Fig. 13 B). The inhibition of iNOS mRNA expression and decreased nitrite levels in the medium were also observed in the presence of PD98059 (ERK1/2 inhibitor) (Fig. 13 B and 14). The concentrations of both inhibitors used in these studies (25 µM SB203580 or PD98059) did not cause any cell death of glial cells as determined by propidium iodide and Hoechst staining (data not shown) and did not affect basal expression of iNOS mRNA or NO production of control glial cultures (data not shown).

![Fig. 14 Potentiation of LTA-induced iNOS mRNA expression by MDP. Total RNA was isolated from astrocytes cultured on 6-well plates, stimulated with LTA (10 µg/ml) +/- MDP (100 ng/ml) for 6 hours in the presence of p38 MAPK inhibitor SB203580 (25 µM) or ERK1/2 inhibitor PD98059 (25 µM). Real-time RT-PCR was performed for the target gene (iNOS) and the housekeeping gene (18S). The level of gene expression was calculated after normalizing iNOS mRNA versus (vs) 18S rRNA signal and is presented in relative mRNA expression units (mean of three experiments ± SEM). Statistical analysis was performed using one-way ANOVA followed by Dunnet’s post hoc test; *p<0.05, **p<0.01, from control cultures.](image-url)
To confirm the involvement of p38 and ERK1/2 in LTA (+/- MDP)-induced glia activation we have studied whether there is increased level of phosphorylated p38 and ERK1/2 in LTA treated glial cultures. To detect phosphorylated p38 and ERK1/2 MAPK we used commercially available beads (Bio-Plex, BioRad) coupled with antibodies against total and phosphorylated forms of these kinases and analysed the samples using a Luminex 100 system. The results revealed that phosphorylation of both p38 (Fig. 15 A) and ERK1/2 kinase (Fig. 15 B) was significantly increased especially after a short time of exposure to LTA (max. phosphorylation detected at 10 min and 30 min, respectively). MDP alone did not have any effect on the levels of LTA-induced phosphorylation of p38 or ERK1/2 (Fig. 15 A and B).

These results suggest that both p38 and ERK1/2 are involved in the signalling pathway of the iNOS mRNA expression and subsequent NO production induced by LTA in cultured glial cells. However, both p38 and ERK1/2 are not involved in the MDP-mediated potentiation of LTA-induced NO production since the levels of phosphorylation observed in LTA alone and LTA + MDP treated cells were comparable.

Fig. 15 LTA (+/- MDP) induced phosphorylation of p38 (A) and ERK1/2 (B) MAPK kinase in primary culture of astrocytes. Glial cells were treated with LTA10 µg/ml +/- MDP (100 ng/ml) for 10 min, 30 min and 60 min and the cell lysates were analysed in a Luminex™ platform using the Bio-Plex phosphoprotein assay kit as described in Materials and Methods.

Note the most significant phosphorylation of p38 was after 30 min and ERK1/2 after 10 min of exposure to LTA (+/- MDP). Results are expressed as the mean ± SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni post hoc test; **p<0.01, ***p<0.001 from control cultures.
3.4.5 LTA-induced TNF-α release is blocked by a p38 inhibitor, whereas IL-1β production is prevented by an ERK1/2 inhibitor

To investigate whether MAPK p38 and ERK1/2 are also involved in LTA-induced cytokine production we studied the release of TNF-α and IL-1β by astrocytes and microglial in primary culture in the presence of SB203580 (inhibitor of p38 MAPK) or PD98059 (inhibitor of ERK1/2). Microglia and astrocytes cultured on 12-well plates were pre-treated for 30 min with SB203580 (25 µM) or PD98059 (25 µM) before the addition of LTA (10 µg/ml) and MDP (100 ng/ml). The medium was collected after 6 hours and the levels of cytokines (TNF-α and IL-1β) were measured in the medium using ELISA. Very low basal level of TNF-α and IL-1β in control culture medium were not affected by any of the inhibitors at tested concentrations (data not shown).

TNF-α release induced by LTA in the presence of MDP in astrocytes (Fig. 16 A) was significantly prevented in the presence of SB203580 (83.1 ± 5.1%), whereas in the presence of PD98059 only slight inhibition was detected (20.8 ± 11.6%). In contrast, the production of IL-1β by astrocytic or microglial cultures exposed to LTA in the presence of MDP was blocked in the presence of ERK1/2 inhibitor PD98059 (46.0 ± 24.4% and 78.6 ± 8.4% decrease of IL-1β levels in astrocytic and in microglial cultures, respectively) (Fig. 16 B) suggesting that p38 was involved in LTA-induced TNF-α production but ERK1/2 mainly mediated IL-1β induction.

**Fig. 16** Effects of p38 and ERK1/2 MAPK kinase inhibitors LTA (+/- MDP) induced TNF-α (A) and IL-1β (B) release by primary culture of astrocytes. Astrocytes were pre-treated with 25 µM SB203580 (p38 inhibitor) or 25 µM PD98059 (ERK1/2 inhibitor) for 30 min before addition of LTA (10 µg/ml) and MDP (100 ng/ml). The culture supernatant was collected after 6 hours and analysed for TNF-α and IL-1β release by ELISA. Results are expressed as percentage of TNF-α or IL-1β release after the treatment with LTA and MDP (100%) as mean ± SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Dunnet’s post hoc test; *p<0.05, **p<0.01 from control cultures.
3.5 Discussion

In the present studies we have shown that lipoteichoic acid from *Staphylococcus aureus* could directly induce an inflammatory response in the CNS, as exposure of both types of rat primary glial cells (astrocytes and microglia) to LTA caused a time- and concentration-dependent release of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6) and nitric oxide. Moreover, using primary culture of glial cells isolated from TLR4 and TLR2 knockout mice we demonstrated that TLR2 is essential for mediating astrocyte and microglia response to LTA, whereas LPS-induced glia activation is mediated by TLR4. Additionally, we have shown that MDP (the smallest active fragment of peptidoglycan from the bacterial cell wall) alone does not stimulate glia to release pro-inflammatory cytokines or NO, but selectively and significantly synergises with lipoteichoic acid to potentiate iNOS mRNA expression followed by NO production.

Lipoteichoic acid, a molecule of Gram-positive bacterial cell walls, is considered to be the counterpart of lipopolysaccharide (LPS) derived from Gram-negative bacteria. *In vitro* and *in vivo* studies show that LTA could be involved in the pathogenesis of CNS infectious diseases (e.g. meningitis and encephalomyelitis) and can induce neuronal cell death (Schmidt et al. 2001; Nau and Bruck 2002). However the components of Gram-positive bacterial cell wall that trigger the inflammatory response during the infection are still not fully characterised, especially in CNS. It is still poorly understood which type of cells in the brain and what specific pathways are involved in LTA-induced inflammatory response. In these studies we have used LTA that was purified using butanol extraction to preserve D-alanine constituents that are important to maintain the LTA pro-inflammatory activity (Morath et al. 2001). Exposure of microglia or astrocytes in primary culture to such highly purified LTA from *Staphylococcus aureus* “activated” both type of glial cells in a comparable manner to LPS as assessed by the release of pro-inflammatory cytokines and nitric oxide. The concentrations of LTA (10 and 30 µg/ml) used in these experiments were chosen based on human blood studies (Lehner et al. 2001; Ellingsen et al. 2002) and on the level of LTA (6.0 µg/ml) found in cerebrospinal fluid of patients suffering from meningitis (Schneider et al. 1999). Both types of glial cells, microglia and astrocytes, responded to LTA by robust production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and nitric oxide in a time and concentration-dependent way. These results suggest that equally astrocytes and microglia could be involved in the inflammatory response to bacterial components in the CNS. Although the levels of TNF-α and NO production by astrocytes activated with LTA 10 µg/ml after 6 hrs were slightly lower then after LPS (10 µg/ml) activation, generally the response to both stimuli was comparable. Moreover the kinetics of cytokine release was similar to the observed in systemic models (human whole blood, isolated human monocytes) (Hermann
et al. 2002; Schröder et al. 2003). LTA was a poor inducer of IFN-\(\gamma\) (data not shown). This is also in line with previous studies where systemic models were used (Hermann et al. 2002), suggesting that LTA is not an important trigger of IFN-\(\gamma\) production upon infection, also in CNS.

Bacterial products are recognised by the innate immune system through a specific group of pattern recognition receptors: the Toll-like receptors. Recent data indicate that the Toll-like receptor-2 recognises Gram-positive bacterial teichoic acids, lipoteichoic acid and lipoproteins, while Toll-like receptor-4 is involved in Gram-negative bacterial LPS signalling pathways (Takeuchi et al. 1999; Beutler 2000). Recently TLRs have been described to be expressed also on glial cells (Kielian et al. 2002; Bowman et al. 2003; Esen et al. 2004; Farina et al. 2005) but their role in LTA-mediated glial activation has not yet been studied. In our \textit{in vitro} model rat astrocytes and microglia showed the basal constitutive expression of TLR2 and TLR4 mRNA that was not significantly altered after exposure to LTA (+/- MDP) or LPS as shown by Real-time-RT PCR studies. Although there are some studies indicating that stimulation of astrocytes and microglia with bacterial components could lead to elevation of Toll-like receptor expression (Kielian et al. 2002; Bowman et al. 2003; Esen et al. 2004), they were not able to confirm it at the protein level (Esen et al. 2004). We have also found that both microglia and astrocytes express mRNA for TLRs suggesting that astrocytes could be actively involved in response to bacteria induced inflammation. The fact that LTA binds to both types of glial cells was confirmed using a rhodamine-labelled LTA as only microglia and astrocytes (not neurons) were intensively stained. The specificity of this staining was confirmed, using Balb-3T3 cells (a mouse fibroblast cell line) that did not show any binding of fluorescent LTA, as these cells do not express TLR receptors (negative control). The functional expression of TLRs, responsible for the recognition of LTA and LPS in our model was confirmed using culture of glial cells isolated from TLR2 knockout and TLR4 deficient mice. Our results clearly show that LTA is recognised by glial cells (both astrocytes and microglia) through the TLR2, while LPS through TLR4, as astrocytes and microglia isolated from mice lacking TLR2 did not release pro-inflammatory cytokines and nitric oxide after exposure to LTA, and glial cells lacking TLR4 did not respond to LPS stimulation. The hyporesponsiveness to LPS of TLR4 deficient C3H/HeN mice, which carry a mutation in the \(Lps\) locus, is described in previous studies (Ryan et al. 1979; Poltorak et al. 1998). On the contrary, peritoneal macrophages isolated from TLR2 knockout mice did not respond to LTA (Takeuchi et al. 2000; Lehner et al. 2001).

Apart from LTA, bacterial cell walls contain also peptidoglycan that could play a role in the process of glia activation since during the course of infection or antibiotic therapy both fragments of bacterial cell wall are released. Muramyl dipeptide, essential for the biological activity of PGN
(Ellouz et al. 1974), is present in both Gram-positive and Gram-negative bacteria. Some studies indicate that MDP (and peptidoglycan) itself can activate macrophages (Bahr et al. 1987; Kalyuzhin et al. 2002), monocytes (Suzuki et al. 1994) as well as glial cells (Cottagnoud et al. 2003). In the recent studies Traub et al. (2004) have shown that the pro-inflammatory activity of MDP was due to its contamination with LPS, and MDP itself was not able to stimulate whole blood cells and isolated human monocytes. However, it strongly synergised with LPS to induce cytokine production (Traub et al. 2004) in contrast to our studies, as we did not observe any synergy between LTA and MDP in the case of TNF-α or IL-1β release. Also MDP alone was not able to induce any cytokine or nitric oxide release by both microglia and astrocytes. On the other hand, co-stimulation of glial cells (both astrocytes and microglial cultures) with LTA in the presence of MDP resulted in a significant (~ 3 folds) increase of iNOS mRNA expression and subsequent NO production as compared with LTA treatment. A synergy between LTA and peptidoglycan derived from *S. aureus* to potentiate iNOS induction and NO production has been previously described in macrophages by Kengatharan et al. (1998). They suggest that the ability of Gram-positive bacteria to induce iNOS activity is mainly triggered by LTA and that PGN only amplifies a biological response of LTA as PGN alone does not induce any response. Peptidoglycan and LTA have also been shown to synergise in vivo in inducing inflammation, septic shock and multiple organ failure (De Kimpe et al. 1995). Moreover MDP has been demonstrated to potentiate the cytokine (IFN-γ, IL-1β)-induced iNOS activation and NO production in primary rat astrocytes (Trajkovic et al. 2000).

It is interesting that in our model MDP potentiated selectively the LTA-induced NO production in glial cells but not pro-inflammatory cytokines. The mechanism behind this phenomenon could be mediated by MAPK kinases, since TLR signalling involves both p38 and ERK1/2 MAPK kinases activation (Beutler 2000). The role of these kinases in LPS-induced cytokine and NO production has been largely studied in both innate immune cells (Chen and Wang 1999; Lahti et al. 2000) and glial cells (Da Silva et al. 1997; Bhat et al. 1998; Hua et al. 2002). Recent studies have demonstrated that lipoteichoic acid can also activate ERK and p38 MAPK in murine macrophage cell line (Schröder et al. 2001) and human epithelial pulmonary carcinoma cell line (Lin et al. 2002). In the present study we have found, to our knowledge for the first time, that mitogen activated kinase (MAPK) p38 is responsible for LTA (+ MDP)-induced TNF-α production in glial cells, since in the presence of SB203580 (p38 inhibitor) the TNF-α release was almost completely blocked. ERK1/2 was only partly involved in LTA (+ MDP)-induced TNF-α release (20.8 ± 11.6% decrease in the presence of SB203580). The release of IL-1β by astrocytic or microglial cultures exposed to LTA (+ MDP) was blocked in the
LTA-induced glial inflammatory response

The presence of ERK1/2 inhibitor PD98059 (46.0 ± 24.4% and 78.6 ± 8.4% decrease of IL-1β release, respectively), while the p38 inhibitor had no effect. The involvement of MAPK kinases in LTA (+/- MDP)-induced glia activation was confirmed by the presence of phosphorylated p38 and ERK1/2 when compared with non-treated glial cultures.

At the same time, an inhibitor of p38 (SB203580) almost completely, and an ERK1/2 inhibitor (PD98059) partially decreased the LTA (+/- MDP)-induced nitric oxide production and iNOS mRNA expression indicating that activation of both kinases was involved in the transcription and translation regulation of LTA-induced nitric oxide production. However the relative level of suppression of iNOS mRNA expression and NO release was similar in both, LTA alone and LTA + MDP treated cells, suggesting that the mechanism through which MDP potentiates LTA-induced NO production does not involve p38 or ERK1/2 activation, and thus the mechanism needs further investigation to clarify it.

Since nitric oxide and pro-inflammatory cytokines (TNF-α, IL-1β) produced by LTA-activated glia mediate neuronal cell death (Kinsner et al. 2005a), pharmacological control of MAPK signalling pathway as well as suppression of iNOS induction and TLR2 activity could be beneficial during inflammation in CNS induced by Gram-positive bacteria.

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4. Inflammatory neurodegeneration induced by lipoteichoic acid from *Staphylococcus aureus* is mediated by glia activation, nitrosative and oxidative stress, and caspase activation.

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4.1 Abstract

In this study we investigated the mechanisms of neuronal cell death induced by lipoteichoic acid (LTA) and muramyl dipeptide (MDP) from Gram-positive bacterial cell walls using primary cultures of rat cerebellar granule cells (CGCs) and rat cortical glial cells (astrocytes and microglia). LTA (+/- MDP) from *Staphylococcus aureus* induced strong inflammatory response of both types of glial cells (release of IL-1 beta, TNF-alpha and nitric oxide). The death of CGCs was caused by activated glia as in the absence of glia (treatment with 7.5 µM cytosine-D-arabinoside to inhibit non-neuronal cell proliferation) LTA + MDP did not cause significant cell death (less than 20%). Additionally, staining with rhodamine-labelled LTA confirmed that LTA was bound only to microglia and astrocytes (not neurons). Neuronal cell death induced by LTA (+/- MDP)-activated glia was partially blocked by an iNOS inhibitor (1400W, 100 µM), and completely blocked by a superoxide dismutase mimetic (MnTBAP, 50 µM) and a peroxynitrite scavenger (FeTPPS, 100 µM) suggesting that nitric oxide and peroxynitrite contributed to LTA-induced cell death. Moreover, neuronal cell death was inhibited by selective inhibitors of caspase-3 (z-DEVD-fmk, 50 µM) and caspase-8 (z-IETD-fmk, 50 µM) indicating that they were involved in LTA-induced neuronal cell death.
4.2 Introduction

Since the successful elimination of *Haemophilus influenzae* type b disease due to vaccination, *Streptococcus pneumoniae*, a Gram-positive bacterium, is the main pathogen responsible for bacterial meningitis in adults and children (Schuchat et al. 1997). Inflammation-induced brain injury frequently complicates bacterial meningitis despite rapid identification of disease and administration of antibiotics. The mortality and morbidity rate in bacterial meningitis is still high and up to 50% of survivors have permanent neurological damage including seizures, mental retardation, impairment of hearing and loss of cognitive functions (Davis and Greenlee 2003; Kastenbauer and Pfister 2003).

In meningitis, the presence of live bacteria in the cerebrospinal fluid (CSF) does not necessary cause injury or even symptoms of inflammation. The induction of symptoms requires initiation of the inflammatory response, which is triggered by bacterial components such as lipopolysaccharide (LPS) in Gram-negative bacteria, lipoteichoic acid (LTA) and peptidoglycan (PGN) in Gram-positive bacteria. They are released into the CSF during bacteriolysis resulting from host defence mechanism or administration of antibiotics (Braun and Tuomanen 1999; Nau and Bruck 2002). In animal models of meningitis, bacterial components, when injected into the cerebrospinal space, induce a rapid inflammatory response (Tuomanen et al. 1985). Although meningitis is an inflammatory disease of the meninges, usually it is not limited only to these membranes, but brain glial cells (astrocytes and microglia) and neurons are also implicated. One of the first hallmarks of neuroinflammation in CNS is glia activation by different inflammatory stimuli (Eddleston and Mucke 1993; Lukiw and Bazan 2000; Swanson et al. 2004). On one hand, glia activation is thought to be protective via destruction of pathogens, removal of debris and promotion of tissue repair. However, if it is excessive and long-lasting it could lead to neurotoxicity. Microglial- and astrocytic-derived pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and related products (nitric oxide, reactive oxygen species, arachidonic acid metabolites or quinolinic acid) can kill neurons in co-culture, and this may also occur in vivo during neurodegenerative diseases, brain trauma, inflammation and infections (Aloisi 2001; Eddleston and Mucke 1993; Tuomanen et al. 1985).

Most studies characterise the mechanism of glia activation and subsequent neuronal damage induced by LPS (the main immunostimulatory component of Gram-negative bacterial cell walls) (Bal-Price and Brown 2001; Cai et al. 2003; Kim et al. 2000; Qin et al. 2004). However, the pro-inflammatory potential in CNS of lipoteichoic acid (LTA) and peptidoglycan (PGN) (the main components of Gram-positive bacterial cell walls) are still
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poorly understood. Relatively little is known whether in bacterial meningitis microglia and/or astrocytes become activated in the presence of Gram-positive bacterial products and if so, whether it could induce neuroinflammation and subsequent neurodegeneration.

In this study we investigated whether lipoteichoic acid and muramyl dipeptide (MDP, the smallest bioactive fragment of peptidoglycan) could cause neuronal cell death of cerebellar granule cells (CGCs) in culture by direct actions or indirectly, through glia activation. The production of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and nitric oxide (NO) was measured after the exposure of pure glial cultures (microglial or astrocytic) or mixed glial-neuronal cultures of CGCs to LTA alone or in the presence of MDP.

Here, we demonstrate that LTA and MDP from *Staphylococcus aureus* induced neuronal cell death indirectly, through glia activation since in the absence of glia LTA (+/- MDP) did not cause any significant cell death. Moreover, we show that the main mechanisms involved in LTA-induced CGCs toxicity were mediated by reactive oxygen and nitrogen species production (nitric oxide, superoxide and peroxynitrite) and caspase-3 and -8 activation.

4.3 Material and Methods

4.3.1 Chemicals and reagents

Reagents for cell culture were purchased from Gibco Invitrogen (Milano, Italy); poly-L-lysine, trypsin, deoxyribonuclease I (DNAse I), bovine serum albumin (BSA), cytosine-D-arabinoside (Ara-C), isolectin B4, triton X-100, (5R,10S)-(+)5-Methyl-10,11-dihydro-5H-dibenzo- [a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801), 3,3′,5,5′-tetramethylbenzidine (TMB), sulfanilamide, hydrochloric acid, N-1(1-naphthyl)-ethylenediamine from Sigma-Aldrich (Milano, Italy); 1400W dihydrochloride, Z-Val-Ala-Asp fluoromethyl ketone (z-VAD-fmk), z-DEVD-fmk, Z-Ile-Glu(O-ME)-Thr-Asp(O-Me) fluoromethyl ketone (z-IETD-fmk), Z-Leu-Glu(O-ME)-His-Asp(O-Me) fluoromethyl ketone (z-LEHD-fmk) from Alexis Biochemicals (Lausanne, Switzerland); Manganese (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP), 5,10,15,20-tetrakis (4-sulfonatophenyl) porphyrinato iron (III) (FeTPPS) from Calbiochem (Nottingham, UK); Antibodies: anti-GFAP (glial fibrillar acidic protein) antibody from Santa Cruz Biotech. (Santa Cruz, California, USA); anti-OX-42 antibody from Serotec (Cergy Saint-Christophe, France); secondary antibodies conjugated with FITC or Cy3 from Chemicon (Hampshire, UK); anti-TNF-α, IL-1β or anti-IL-6 antibodies from Biotrend (Köln, Germany). Hoechst 33324 and Propidium Iodide (PI) were purchased from Molecular Probes Europe (Leiden, The
Neuronal injury induced by lipoteichoic acid

Netherlands). MDP was purchased from Bachem (Bubendorf, Switzerland); LTA was kindly provided by S. Morath from the University of Konstanz, Germany.

4.3.2 Neuronal cell culture
The primary culture of cerebellar granule cells (CGCs) were prepared from 7-day-postnatal rat pups (OFA/SPF rat strain) as described previously (Cambray-Deakin 1995) with some modifications. The cerebella were dissociated in Versene solution (1:5000) and plated at 0.25 x 10^6 cells/cm² in 24-well coated with poly-L-lysine. Cultures were maintained in DMEM supplemented with heat inactivated horse serum (5%), foetal calf serum (5%), 13 mM glucose, 0.5 mM HEPES buffer, 2 mM L-glutamine, 25 mM KCl and 10 µg/ml gentamicin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂/ 95% air.

In some experiments (where indicated) cytosine-D-arabinoside (7.5 µM, Ara-C) was added to the medium 48 hours after plating to inhibit proliferation of non-neuronal cells (astrocytes, microglia). In all the experiments the cells were used at 8 DIV, to ensure morphological and physiological maturity.

4.3.3 Astrocyte and microglial cultures
Primary, mixed glial cultures were prepared from the cerebral cortex of 7-day-old rat pups (OFA/SPF rat strain) as described previously (Bal et al. 1994). Briefly, the cells isolated from cerebral hemispheres were dissociated in Hank’s balanced salt solution (HBSS) containing 0.25% trypsin, 0.02 mg/ml deoxyribonuclease I (DNA-se I) and 1% bovine serum albumin (BSA). Cells were plated at a cell density of 1.0 x 10⁴ cells/cm² in 75-cm² culture flasks (Costar), 6-well plates or 12-well plates (Costar) coated with poly-L-lysine. The DMEM medium contained 10% foetal bovine serum (FBS) and antibiotics (1% penicillin/streptomycin). Medium was changed every 3 days. At confluence (14-16 days in vitro (DIV)) the primary mixed glial cultures were used to isolate microglial cells and astrocytes. Briefly, mixed glial cultures were shaken to dislodge microglia that were loosely attached to the astrocytes. Microglia were seeded into 6-well or 12-well plates at a density of 2.0 x 10⁶ or 1.0 x 10⁶ cells/well respectively (2.0 x 10⁵ cells/cm²) and maintained in astrocyte-conditioned medium (medium collected from astrocytic cultures after 2 days and spun down) mixed 1:2 with fresh DMEM (containing 10% FBS). The cultures were characterised using immunocytochemical staining with anti-GFAP antibody (astrocytic marker) and anti-OX-42 antibody (microglial marker, an anti-CR3 complement receptor antibody). Briefly, the cells were fixed with 4% paraformaldehyde and permeabilized with 3% Triton X-100 in PBS,
followed by incubation with anti-GFAP or anti-OX-42 antibody overnight at 4°C. After washing with 1% Triton X-100 in PBS, cells were incubated with secondary antibodies conjugated with FITC or Cy3 for 2 hours at room temperature. Cells were extensively washed followed by a 10-min staining with 5 µg/ml Hoechst 33324. The staining was analysed under confocal laser scanning microscope (BioRad Radiance MP2000, USA).

4.3.4 Treatment of the mixed neuronal-glial cultures
Cultures of cerebellar granule cells (CGCs, 8 DIV) were exposed to LTA (10 or 30 µg/ml) +/- MDP (100 ng/ml) for 24, 48 and 72 hours in the presence or absence of: selective inhibitor of inducible nitric oxide synthase (1400W, 25 µM), superoxide dismutase mimetic (MnTBAP, 50 µM), peroxynitrite decomposition catalyst (FeTPPS, 25 µM), anti-TNF-α antibodies (anti-TNF-α, 10 ng/ml), interleukin-1 receptor antagonist (IL-1ra, 10 ng/ml), pan-caspase inhibitor (z-VAD-fmk, 50 µM), inhibitors of caspase-3 (z-DEVD-fmk, 50 µM), caspase-8 (z-IETD-fmk, 50 µM) and caspase-9 (z-LEHD-fmk, 50 µM).

4.3.5 Assessment of neuronal cell morphology and viability
The viability of CGCs and glial cells was estimated by a fluorescent staining with two dyes - propidium iodide (PI, 20 µg/ml) and Hoechst 33342 (10 µg/ml) using a fluorescent microscope (Olympus IX70, Hamburg, Germany). Propidium iodide positive cells were considered to be necrotic cells since PI stains only cells with disrupted cell membrane. The nuclear morphology of the cell (chromatin condensation and fragmentation) was studied using the cell-permeable dye Hoechst 33342 to assess whether apoptotic cells were present. Cells with homogenously stained blue nuclei were considered as viable, but with condensed, fragmented blue nuclei were counted as apoptotic. The characteristic shape and size of the nuclei of three types of cells present in CGCs culture (neurons, microglia and astrocytes) together with a typical morphology (contrast phase microscopy image) and cell specific staining (GFAP and OX-42) permitted to distinguish and quantify the different cell types. Alive and dead (necrotic and apoptotic) neurons were counted in five microscopic fields in each well (two wells per treatment) and expressed as a percentage of the total number of neurons. Each experiment was repeated at least 3 times. To estimate the number of cells that disappeared (due to phagocytosis by activated microglia), the total number of dead and live cells was quantified and compared with the total number of cells in non-treated control cultures.
4.3.6 Staining of CGCs cultures with rhodamine-labelled LTA

The labelling of LTA was performed as follows: 3 mg LTA from S. aureus, 4.5 mg sulforhodamine Q 5-acidfluoride (Fluka, Buchs, Switzerland), 2.5 ml dimethyl sulfoxide (Wak-Chemie-Medical GmbH, Steinbach, Germany) and 25 µl trimethylamine (Acros Organics, Leicestershire, UK) were sonified for 10 min. The mixture was shaken overnight at 37°C, then spun 4 times for 90 min at 7000 g in pyrogen-free centrifugal ultrafiltration tubes (Microsep 3K Centricons, Pall, MI, USA). To remove remaining unbound dye, the labelled LTA was filtered through a pyrogen-free sepharose column (PD-10 desalting column, Amersham Biosciences, Freiburg, Germany). Labelling efficiency, calculated as fluorescence (560 nm/620 nm) per phosphate content, was 1-2 rhodamine molecules per LTA. Phosphate determination was performed by adding a mixture of H2SO4 : HClO4 : H2O (556 : 105 : 3339, v/v/v) to 50 µl LTA solution and incubating for 2 h at 145°C. Then 1 ml of ascorbic acid : ammoniumheptamolybdenum-Na-acetate (1 : 9, v/v) was added and incubated for 2 h at 50°C. Absorption was measured at 700 nm. Rhodamine-LTA was negative in the LAL (i.e., <0.05 EU /mg LTA).

To assess whether LTA binds glial cells and/ or neurons in CGCs cultures we exposed the cells to rhodamine-labelled LTA. Additionally, to visualize microglia, the cultures were co-stained with a microglial marker – isolecin B4. Rhodamine-labelled LTA (30 µg/ml) and isolectin B4 (10 ng/ml) were added directly to the culture medium. After incubation at 37°C for 30 minutes the cells were washed three times with PBS and the staining was analysed using confocal laser scanning microscope (BioRad Radiance MP2000, USA).

4.3.7 Activation of astrocytes and microglia in culture

Primary cultures of astrocytes (17 DIV) and microglia (24 hours after isolation from mixed glial cultures) were activated by exposure to 10 µg/ml or 30 µg/ml lipoteichoic acid (LTA) from Staphylococcus aureus. Some cultures, before exposure to LTA, were pre-treated with MDP (100 ng/ml) for 30 minutes. LTA was obtained according to a novel butanol extraction procedure to ensure biological activity, high purity and lack of LPS contamination (determined using LAL assay) (Morath et al. 2001). For the assessment of cytokine release the medium was collected at 1, 6, 12 and 24 h and for the measurement of nitric oxide (NO) production at 24, 48 and 72 h after the treatment with LTA (+/- MDP). At the end of the experiments astrocytes were gently trypsinised (0.1% trypsin) for 2-3 minutes and the number of cells was counted using a haemocytometer.
4.3.8 Assessment of microglia and astrocytes proliferation

Proliferation of astrocytes and microglia was assessed after exposure to LTA (+/- MDP) in mixed neuronal-glial cultures by counting (as described in neuronal culture) or in pure glial cultures. Microglia were isolated as described above and seeded into 12-well plates or 96-well plates at a density of $1.5 \times 10^5$ cells/cm$^2$. 24 hours after seeding microglia were treated for 72 hours with 30 µg/ml LTA in the presence or absence of MDP (100 ng/ml).

Astrocytes cultured on 75 cm$^2$ flasks were shaken to dislodge microgla, the cells were then trypsinised and seeded on 12-well plates or 96-well plates at a density of $1.0 \times 10^4$ cells/cm$^2$. Astrocytes or microgla in the proliferation phase were treated for 72 hours with LTA 30 µg/ml (+/- MDP). The cell proliferation of both types of glial cells (control and after LTA (+/- MDP) treatment) was assessed using Cell Proliferation (BrdU) ELISA kit (Roche Applied Science, Monza, Italy) or by cell counting after HS and PI staining as described before.

4.3.9 Measurement of cytokine release

Quantification of cytokines in medium collected from LTA (+/- MDP) activated microgla or astrocytes in culture was performed using a sandwich ELISA. The Nunc Maxisorp ELISA plates (Nunc) were coated with anti-TNF-α, anti-IL-1β or anti-IL-6 antibodies diluted in 100 mM NaHCO$_3$, pH 8.3 at 4°C, overnight. After blocking with 3% BSA/PBS for 2 hours at room temperature, samples and standard solution were added. Recombinant TNF-α, IL-1β or IL-6 standard solutions were prepared in 3% BSA/PBS. After 3 hours incubation, biotinylated anti-TNF-α, anti-IL-1β or anti-IL-6 antibodies were added and incubated for 45 minutes at room temperature, followed by incubation with streptavidin-peroxidase (Biosource, Nivelles, France) for 30 min. Detection of bound cytokines was carried out using TMB. The reaction was stopped using 1M H$_2$SO$_4$ and the absorption was measured at 450 nm in a multiwell spectrophotometer (Spectra Max, Molecular Devices, Sunnyvale, CA, USA). The cytokine concentrations were calculated per $10^6$ cells using standard solutions of recombinant TNF-α, IL-1β or IL-6 and expressed in pg/ml.

4.3.10 Measurement of nitric oxide production

The accumulation of nitrite in the medium was assessed using Griess reaction (Green et al. 1982). Sulfanilamide (1 mM), hydrochloric acid (6 mM) and N-1(1-naphtyl) ethylenediamine (1 mM) were added to the medium. After 30 minutes incubation at room temperature the absorbance was measured at 548 nm in a multiwell spectrophotometer (Spectra Max,
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Molecular Devices, Sunnyvale, CA, USA). The nitrite concentrations (μM) were calculated using standard solutions of sodium nitrite prepared in culture medium.

4.3.11 Statistical analysis

Statistical analysis was performed using GraphPad Prism 4.1 (GraphPad software, San Diego, USA). All data given are means ± SD. Significance of differences was assessed by one-way ANOVA followed by Bonferroni’s post-test. Statistical significance is marked as follows p<0.05; ** p<0.01; *** p<0.001.

4.4. Results

4.4.1 LTA- induced neuronal cell death in CGCs primary cultures is mediated through glia activation

To determine whether LTA could induce neuronal cell death, we used mixed glial-neuronal primary cultures of cerebellar granule cells (CGCs, 8 DIV) and exposed them for 24, 48 or 72 h to LTA (10 or 30 µg/ml). The chosen concentrations of LTA were based on the previous studies performed in vitro in systemic models (human monocytes or human whole blood) (Lehner et al. 2001), as well as based on the clinical data, which describe the concentrations of LTA found in the cerebrospinal fluid of patients suffering from meningitis (Schneider et al. 1999).

CGCs were exposed to LTA alone or in the presence of MDP (100 ng/ml). MDP is the minimal active part of PGN and in our previous studies we showed that it significantly potentiates LTA-induced glia activation (Kinsner et al. 2005b). Control cultures of CGCs (83% ± 3% neuronal, 11% ± 2% astrocytic and 4% ± 1% microglial) showed well-differentiated neurons with an extensive neuritic network (Fig. 17 A) and very low necrotic cell death (Fig. 17 A and B). After exposure of CGCs to LTA (10 or 30 µg/ml) concentration-dependent neuronal cell death was observed that was only slightly potentiated by MDP as assessed by propidium iodide (necrosis) and Hoechst 33342 (apoptosis) staining (Fig. 17 B). After 24 h of treatment with LTA (10 or 30 µg/ml) only 11.4% ± 4% of neurons were PI-positive (necrosis) or had disappeared (11.2% ± 5%) as compared with the number of neurons in control, sister cultures (Fig. 17 C), presumably because they were phagocytosed by activated microglia. After 48 h exposure to LTA (10 or 30 µg/ml) in the presence or absence of MDP, the neuronal death did not significantly change as assessed by PI and Hoechst staining. However, the prolonged treatment (72 h) with LTA (+/- MDP) increased significantly the cell death (50.0% ± 3.1% of phagocytosis, 6.9% ± 3.2% of necrotic, 1.3% ±
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1.1% of apoptotic cells) (Fig. 17 C). During 72 h of treatment with LTA (+/- MDP), the number of apoptotic cells (bright nuclei with condensed or fragmented chromatin) was always low suggesting that neurons died mainly by necrosis. However, it is possible that apoptotic cells were rapidly phagocytosed by activated microglia, so they were not seen.

Fig. 17 Cell death of CGCs (mixed neuronal-glial cultures, 11 DIV) induced by exposure to lipoteichoic acid (LTA, 10 or 30 µg/ml) and muramyl dipeptide (MDP, 100 ng/ml). (A) In the control cultures of CGCs note the phase-contrast bright cell bodies with dense neuritic network and low necrotic cell death (propidium iodide (PI) positive cells). Viable cells showed round blue nuclei with weak fluorescence (Hoechst 33342 staining). (B) Cell death of CGCs induced after 72 hours of exposure to LTA (10 or 30 µg/ml) in the presence and absence of MDP (100 ng/ml) in a dose-dependent manner. (C) The kinetics of LTA-induced neurotoxicity after treatment with LTA 30 µg/ml (+ MDP) for 24, 48 and 72 hours. Values represent the means ± SD of three independent experiments. In each experiment two wells (n = 2) per treatment were analysed and the cells in five fields per well were counted (~ 80 ± 25 cells per field). In the last column (LTA 30 + MDP) the error bars are very low (SD ± 0.07). **p<0.01, ***p<0.001 from control cultures. In (A) particular cell type is indicated by the following abbreviations (placed on the right of the cell): a, astrocyte; am, activated microglia; dn, dead neurons; n, healthy neurons. Scale bar (shown in A), 40 µm.
To determine whether neuronal cell death was induced directly by LTA or mediated indirectly by LTA-activated glial cells, the proliferation of glia in mixed neuronal-glial cultures of CGCs was inhibited by the addition of 7.5 µM cytosine arabinoside (Ara-C) at 24 - 48 hours after the isolation of CGCs. This concentration of Ara-C (7.5 µM) blocked almost completely glia proliferation (less than 2% of non-neuronal cells were present) causing only slight increase of neuronal cell death as determined by propidium iodide and Hoechst 33342 staining (Fig. 18 B).

CGCs pre-treated at 1 - 2 DIV with Ara-C (7.5 µM) were exposed to LTA (10 or 30 µg/ml) in the presence or absence of MDP (100 ng/ml) at 8 DIV for 72 hours. In the absence of glial cells (after Ara-C pre-treatment), LTA-induced neuronal cell death was significantly prevented (43.3% +/- 6.2%) when compared with cell death observed in CGCs cultured in the presence of glial cells (non-treated with Ara-C) (82.1% +/- 8.0%) (Fig. 18 A and B). These results clearly indicate that neuronal cell death in our model was mainly induced by LTA-activated glial cells since in the absence of glia the neuronal cell death was largely prevented. In further studies using LTA labelled with rhodamine we confirmed that LTA did not have a direct action on neurons since CGCs did not show any binding of rhodamine-labelled LTA. In contrast, microglia and astrocytes were stained intensively with rhodamine-labelled LTA in both pure and mixed glial-neuronal cultures (Fig. 19).
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**Fig. 18** Inhibition of neuronal cell death in the absence of glia (pre-treatment with Arabinoside Cytosine, Ara-C).

(A) Note significantly decreased neuronal cell death in the presence of Ara-C (7.5 µM) after 72-hours exposure to LTA (30 µg/ml) and MDP (100 ng/ml). (B) Necrotic cell death of CGCs (PI-positive cells) and phagocytosed cells were quantified (see Material and Methods) after exposure of CGCs to LTA 30 µg/ml + MDP 100 ng/ml. In each experiment percentages of necrotic neurons (PI-positive cells) and phagocytosed neurons were calculated in two separate wells (n = 2) per treatment (five fields per well), and the values represent the means ± SD of at least three independent experiments. ***p<0.01 from control cultures; *p<0.05 from LTA + MDP treated cultures; #p<0.05 from Ara-C treated cultures. Particular cell types are indicated by the following abbreviations (placed on the right of the cell): a, astrocyte; am, activated microglia; dn, dead neurons; n, healthy neurons; Scale bar (shown in A), 40 µm.

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**A**

control (with Ara-C)  
without Ara-C  
with Ara-C  
LTA 30 + MDP

**B**

- **phagocytosis**
- **PI-positive cells**

neuronal cell death (%)  
ctrl  
ctrl (+ Ara-C)  
LTA + MDP  
LTA + MDP (+ Ara-C)
**Fig. 19** Distribution of rhodamine-labelled LTA in mixed neuronal-glial cultures of CGCs. Note binding of LTA to glial cells (both astrocytes and microglia), but not to neurons. CGCs cultures were co-stained with rhodamine-LTA (30 µg/ml) (red) and microglial marker FITC-isolectin B₄ (10 ng/ml) (green). Note that rhodamine-LTA bound to microglia and astrocytes, but not to neurons. Particular cell types are indicated by the following abbreviations (placed on the right of the cell): *a*, astrocyte; *m*, microglia; *n*, neurons; Scale bar - 40 µm.
4.4.2 LTA induces proliferation of astrocytes and microglia

To assess whether LTA induced changes in the number of astrocytes and/or microglia, the number of both types of glial cells was quantified in CGCs cultures after 24, 48 and 72 h of exposure to LTA (30 µg/ml) + MDP (100 ng/ml) and in pure cultures of astrocytes or microglia exposed to LTA (+/- MDP) for 72 hours. In CGCs cultures not only was there no glial cell death observed, but on the contrary LTA induced a significant increase of cell proliferation of both astrocytes and microglia (79% +/- 21% and 134% +/- 45%, respectively) as assessed by GFAP (astrocyte marker) and isolectin B4 (microglial marker) staining (Fig. 20 A and B). Since after 72 h exposure to LTA a high rate of neuronal cell death and phagocytosis was observed, in many microscopic fields astrocytes and microglia were the only cell type that survived (Fig. 17 A). The increase in glia proliferation was partly due to the direct LTA effect on glial cells since in the absence of neurons (pure culture of microglia or astrocytes) an augmentation in the number of glia was still observed (astrocytes: 18.4% ± 7.1% and 22.4% ± 7.2%; microglia: 37.2% ± 17.9% and 39.1% ± 25.7% after LTA or LTA + MDP treatment respectively, in comparison with control). However this effect observed in pure glial cultures was less pronounced in comparison with mixed neuronal-glial cultures possibly due to the absence of communication between glia and neurons. The observed (after 72 h) increase in the quantity of astrocytes and microglia could explain the fact why significant cell death was observed only after prolonged (72 h) exposure to LTA. The increased quantity of microglia and astrocytes could potentiate neuronal cell death since it was mainly mediated by LTA-activated glia.

![Fig. 20](image1)

**Fig. 20** Proliferation of astrocytes (A) and microglia (B) in mixed CGCs cultures (11 DIV) exposed to LTA (30 µg/ml) +/- MDP (100 ng/ml) for 24, 48 or 72 hours. GFAP-positive cells (astrocytes) and isolectin B4-positive cells (microglia) were quantified in each independent experiment, two wells per treatment (n = 2, in five fields per well) and compared to the untreated control cultures of CGCs. Values represent the means ± SD of three or more independent experiments. *p<0.05, **p<0.01 from control cultures.
4.4.3 LTA-activated astrocytes and microglia release pro-inflammatory cytokines: TNF-α, IL-1β and IL-6

Since neuronal cell death in CGCs cultures was mainly mediated by LTA-activated glia we characterised the response of both, cultured astrocytes and microglia, to LTA (10 µg/ml and 30 µg/ml) +/- MDP (100 ng/ml) stimulation by measuring the time and dose dependence of the release of key pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α). Cultures of microglia were pure (>99% of OX-42 positive cells), however, astrocytic cultures were contaminated with microglia (8% ± 2%). Both types of glial cells (astrocytes and microglia) responded to LTA (30 µg/ml) activation by production of comparable levels of IL-1β (max. levels observed at 24 hours: 3458 ± 342 and 4750 ± 196 pg/ml/10⁶ cells in microglia and astrocytes cultures, respectively), IL-6 (max. levels observed at 24 hours: 3639 ± 120 and 4389 ± 145 pg/ml/10⁶ in microglia and astrocytes cultures, respectively) and TNF-α (max. levels observed at 6 hours: 1930 ± 96 and 2403 ± 50 pg/ml/10⁶ in microglia and astrocytes cultures, respectively) (Fig. 21). MDP alone did not induce the production of cytokines (IL-1β, IL-6, TNF-α) and did not have any significant effect on LTA-induced cytokine release (data not shown).

![Fig. 21](image-url)

**Fig. 21** LTA stimulated TNF-α, IL-1β, IL-6 release in rat primary enriched astrocyte cultures in a dose dependent manner. Cells on 12-well plates were stimulated with 10 or 30 µg/ml LTA. Culture supernatants were collected at 6 hours (for TNF-α) and 24 hours (for IL-1β and IL-6) and analysed using ELISA (see Material and Methods). Results are presented as the amount of cytokines in pg/ml. Data from at least three independent experiments performed in duplicates (n = 2) are presented as means ± SD. ***p<0.001 from control cultures.
Since TNF-α and IL-1β produced by LTA-activated glia could contribute to the observed neuronal cell death, we tested whether pre-treatment with an antibody against TNF-α or with the IL-1 receptor antagonist (IL-1ra) would prevent neuronal cell death induced by LTA-activated glial cells. However, neither pre-treatment with antibodies against TNF-α nor IL-1ra caused any significant protection against neurotoxicity (Fig. 22). These results suggest that IL-1β and TNF-α alone were not involved in the mechanisms of LTA-induced neuronal cell death.

**Fig. 22** Cell death of CGCs (mixed neuronal-glial cultures, 11 DIV) induced by exposure to LTA (30 µg/ml) and MDP (100 ng/ml) for 72 hours is not prevented by anti-TNF-α (10 ng/ml) and IL-1ra (10 ng/ml) pre-treatment as assessed by quantification of necrotic and apoptotic cells (PI- and Hoechst 33342-positive cells, respectively). In each experiment two wells (n = 2) per treatment were analysed and the cells in five fields per well were counted. The data represent the mean ± SD from three independent experiments. ***p<0.001 from control cultures.

**4.4.4 Nitric oxide partially contributes to LTA-induced neuronal cell death in CGCs cultures**

Microglia and astrocytes in culture exposed to LTA produced nitric oxide in a concentration-dependent manner (astrocytes: 10.2 ± 6.7 and 25.1 ± 4.3 µM nitrite/ 10⁶ cells; microglia: 5.3 ± 0.7 and 12.2 ± 3.2 µM nitrite/ 10⁶ cells; levels after 72 hours exposure to 10 and 30 µg/ml of LTA, respectively). As expected, the NO production was delayed in comparison to cytokine release since it requires inducible nitric oxide synthase (iNOS) induction that is induced in the presence of pro-inflammatory cytokines. Interestingly, MDP (100 ng/ml) synergised with LTA as comparable levels of NO were produced after exposure of astrocytes to higher concentration of LTA (30 µg/ml) alone and to a 3-fold lower dose of LTA (10 µg/ml) but in presence of MDP (100 ng/ml) (25.1 ± 4.6 µM and 26.0 ± 2.6 µM nitrite, respectively). MDP
alone did not induce NO production. As mentioned before, this potentiation did not occur in the case of LTA-induced cytokine release.

To test whether nitric oxide contributed to neuronal cell death, CGCs cultures were exposed for 72 h to LTA (30 µg/ml) + MDP (100 ng/ml) in the presence of 1400W (25 µM), a selective inhibitor of iNOS. The production of NO in neuronal-glial CGCs cultures pre-treated with 1400W was almost completely blocked (Fig. 23 A). In the presence of the iNOS inhibitor neuronal cell death was also partially prevented (decreased by 25% +/- 8%) as assessed by propidium iodide and Hoechst 33342 staining (Fig. 23 B). These results suggest that nitric oxide contributed to neuronal cell death of CGCs induced by LTA and MDP-activated glial cells.

**Fig. 23** Nitrite measurements (Griess reaction, µM) in the medium of CGCs cultures exposed to LTA (30 µg/ml) and MDP (100 ng/ml) for 72 hours in the presence or absence of a selective iNOS inhibitor - 1400 W (50 µM) (A). In the same experiment neuronal cell death was assessed by counting propidium iodide (necrosis) and Hoechst 33342 (condensed or/and fragmented DNA, apoptosis) positive cells (B). Note that 1400W almost completely blocked NO production (A) and partly prevented neuronal cell death (B). The data represent mean ± SD from three independent experiments performed in duplicates (five fields per well). ***p<0.001 from control cultures; *p<0.05 and +++p<0.001 from LTA + MDP treated cultures.

**4.4.5 LTA-induced neuronal cell death is prevented by MnTBAP (superoxide dismutase mimic) and FeTPPS (peroxynitrite scavenger)**

To determine whether possible ROS production from both glial and neuronal cells upon LTA-treatment could be involved in the mechanism of neuronal cell death, we studied whether MnTBAP (cell-permeable superoxide dismutase mimic) and FeTPPS (peroxynitrite decomposition catalyst) protected CGCs against LTA-induced cell death. The cells were pre-treated with MnTBAP (100 µM) or FeTPPS (25 µM), 30 min before exposure to LTA (30
µg/ml) + MDP (100 ng/ml) for 72 hours. The concentrations of both inhibitors at the above concentrations were not cytotoxic in CGCs cultures. As evaluated using propidium iodide/ Hoechst 33342 staining, neuronal cell death induced by LTA was entirely prevented in the presence of superoxide dismutase mimetic, MnTBAP (Fig. 24) and almost completely blocked by the peroxynitrite scavenger, FeTPPS (Fig. 24), suggesting that both peroxynitrite (ONOO⁻) and superoxide (O₂⁻) play a major role in the mechanism of LTA-induced cell death of CGCs in culture.

Fig. 24 Cell death of CGCs (mixed neuronal-glial cultures, 11 DIV) induced by exposure to LTA (30 µg/ml) and MDP (100 ng/ml) for 72 hours was completely prevented by SOD mimetic (MnTBAP, 100 µM) and peroxynitrite scavenger (FeTPPS, 25 µM) as assessed by quantification of necrotic and apoptotic cells (PI- and Hoechst 33342-positive cells, respectively). The data present the mean ± SD from three independent experiments (n = 2 per experiment). ***p<0.001 from control cultures; +++p<0.001 from LTA + MDP treated cultures.

4.4.6 Caspase-3 and -8 activation is involved in LTA-induced neuronal cell death
LTA-induced neuronal cell death of CGCs in cultures assessed after 24, 48 or 72 h was mainly necrotic as revealed by propidium iodide (PI) staining and only a very few Hoechst 33342 positive cells with condensed or fragmented chromatin (apoptotic) were observed. However, it cannot be excluded that what we observed was secondary necrosis and the possible, initial mode of cell death was apoptotic. To test this we used a broad spectrum, non-specific caspase inhibitor, z-VAD-fmk as apoptosis is mainly mediated by caspase-activation. The cells were pre-treated with 50 µM z-VAD-fmk for 30 minutes before LTA (30 µg/ml) + MDP (100 ng/ml) addition. The inhibition of caspase activation by z-VAD-fmk decreased the percentage of LTA (+ MDP)-induced neuronal cell death by 35% +/- 3% in comparison with
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cultures not pre-treated with z-VAD-fmk, but exposed to LTA + MDP (Fig. 25), suggesting that caspase activation contributed to LTA-induced neuronal cell death.

To determine which were the caspases involved in this process, we pre-treated CGCs cultures with specific inhibitors of caspase-3 (z-DEVD-fmk, 50 µM), caspase-8 (z-IETD-fmk, 50 µM) and caspase-9 (z-LEHD-fmk, 50 µM) and then exposed the cells to LTA (30 µg/ml) + MDP (100 ng/ml) for up to 72 hours. The cell death (apoptotic and necrotic) was assessed after 24, 48 and 72 h using propidium iodide and Hoechst 33324 staining. The results suggest that the activation of caspase-3 and caspase-8 (but not caspase-9) were involved in the mechanism of LTA-induced neuronal cell death, as in the presence of specific inhibitors (z-DEVD-fmk and z-IETD-fmk respectively) we observed a significant decrease in cell death (Fig. 25). The concentrations of the inhibitors at above concentrations were not cytotoxic in CGCs cultures.

Fig. 25 Cell death of CGCs induced by exposure to LTA (30 µg/ml) and MDP (100 ng/ml) for 72 hours was partly prevented by a non-specific caspase inhibitor z-VAD-fmk (50 µM) and almost completely prevented by selective caspase-3 (z-DEVD-fmk) or -8 (IETD-fmk) inhibitors, but not by selective caspase-9 inhibitor (LEHD-fmk). The data present the mean ± SD from three independent experiments (n = 2 per experiment). **p<0.01 from control cultures and ***p<0.001 from LTA + MDP treated cultures.
4.5 Discussion

In the present study, using in vitro mixed glial-neuronal cultures, we have demonstrated for the first time that lipoteichoic acid (LTA) derived from Staphylococcus aureus induces neurotoxicity indirectly, through glia activation, since exposure of neuronal cultures with only a low amount of glia (< 2%) to LTA did not cause significant cell death.

Lipoteichoic acid and peptidoglycan (PGN) are the major components of the Gram-positive bacterial cell walls, and LTA is considered to be the counterpart of lipopolysaccharide from Gram-negative bacteria (Morath et al. 2001; Morath et al. 2002). LTA-induced neuronal cell death could have important implications for pathology since both in vitro and in vivo studies have shown that Gram-positive bacterial cell wall components are implicated in the pathogenesis of many infectious diseases in CNS, such as encephalomyelitis and meningitis (Nau and Bruck 2002; Schmidt et al. 2001). However, it is still not clear which is the main component of the Gram-positive bacterial cell wall and what is the mechanism by which neuronal cell death was induced. In our experiments we used highly purified LTA from Staphylococcus aureus prepared according to the recently described butanol extraction procedure (Morath et al. 2001). LTA was tested by Limulus assay to rule out the presence of LPS and ensure that the effects we observed are not due to the contamination with LPS, especially with regard to a synergy with MDP (Traub et al. 2004).

Exposure of mixed neuronal-glial culture of cerebellar granule cells (CGCs) to LTA (10 or 30 µg/ml) caused concentration- and time-dependent neuronal cell death that was most prominent after a prolonged incubation (72 h). The observed cell death was mainly necrotic as the number of apoptotic cells with bright nuclei and condensed or fragmented chromatin (Hoechst positive and propidium iodide negative) was always low. However, the possibility that apoptotic cells once present were rapidly phagocytosed by activated microglia cannot be excluded, since a high proportion of neurons after LTA treatment disappeared. Indeed, from the changes of microglial phenotype (spherical cells and vacuoles packed with particles) (see Fig. 17 A) it was obvious that in the presence of LTA microglia had phagocytosed dead cells.

A similar phenomenon, high phagocytosis of dead neurons by activated microglia, was observed in the culture of CGCs exposed to LPS (Bal-Price et al., 2001).

During the course of Gram-positive bacterial infection, neurons and glial cells are likely to be exposed not only to LTA but also to MDP (the smallest bioactive fragment of PGN). However, the addition of MDP only slightly potentiated LTA-induced neurotoxicity, suggesting that LTA was the main component of Gram-positive bacterial cell wall responsible for neuronal cell death.
Increasing evidence suggests that in many neurological disorders, such as trauma, infection or inflammation, neuronal injury is mediated by inflammatory molecules released by activated astrocytes and microglia (Eddleston and Mucke 1993; Ruffolo et al. 1999; Lukiw and Bazan 2000; Nau and Bruck 2002). To determine whether in our in vitro model LTA-induced neuronal cell death was mediated through glia activation (indirectly), CGCs cultures were pre-treated with Ara-C to inhibit glial proliferation. In these cultures (containing less than 2% of glial cells) neuronal cell death was significantly decreased, even if Ara-C itself slightly increased neuronal cell death (by 13.1% ± 1.3% in comparison without Ara-C), probably due to the neuroprotective role of glia (Kirchhoff et al. 2001; Srebro and Dziobek 2001) suggesting that indeed neurotoxicity was mediated by LTA-activated glial cells. Moreover, using rhodamine-labelled LTA, we have confirmed that LTA binds only to glial cells (both to microglia and astrocytes) but not to neurons (see Fig. 19).

In mixed neuronal-glial cultures treated with LTA there was a marked increase in the number of both microglia (134 ± 45% of control) and astrocytes (79 ± 21% of control) especially after prolonged exposure (72 h) to LTA. It is a known fact from in vivo studies that astrocytes proliferate in the area of a neurodegenerative lesion (gliosis), often stimulated by cytokines released from activated microglia. Gliosis is a prominent feature of many neurodegenerative diseases, including multiple sclerosis, trauma or ischemia (Dietrich et al. 2003; Liberto et al. 2004). Since in pure glial cultures the proliferation of astrocytes and microglia was only slightly increased (but still statistically significant) this could suggest that the communication between neurons and glia is important to fully trigger glia proliferation.

The increased proliferation of astrocytes and microglia could contribute to LTA-induced neuronal death since it was mediated by glia activation. Indeed microglia and astrocytes recognise and respond to LTA, becoming fully activated, releasing nitric oxide and several pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) (see Fig. 21). These factors could subsequently initiate a cascade of events leading to neuronal cell death. TNF-α has been suggested to be an important mediator of LPS-induced inflammation (Waage et al. 1989) and the levels of TNF-α in the CSF of patients with bacterial meningitis correlated well with concentrations of bacterial endotoxin in the brain (Arditi et al. 1990). However, in our model, pre-treatment of CGCs cultures with anti-TNF-α antibodies or with IL-1β receptor antagonist (IL-1ra) did not protect against neurotoxicity suggesting that other mediators were involved and it was not enough to block only the effects of IL-1β or TNF-α to prevent LTA-induced neuronal cell death.

The mechanisms by which LTA-activated glia induce neurotoxicity could also involve the
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release of nitric oxide and reactive oxygen species (ROS), since both are key mediators of the pathophysiological changes during bacterial meningitis (Koedel et al. 1995; Koedel and Pfister 1999; Christen et al. 2001). Under physiological conditions, NO plays an important role in intra- and intercellular signalling (Garthwaite 1991), however, when produced in excessive levels it can induce neuronal cell death (Boje and Arora 1992; Bal-Price and Brown 2001). Interestingly in our model, MDP significantly potentiated (~ 3-fold) LTA-induced glial nitric oxide production (Kinsner et al. 2005b) but did not have significant effect on LTA-induced neuronal cell death. These results suggest that nitric oxide directly was not the major mediator of LTA-induced neurotoxicity. Indeed, in the presence of 1400W (selective iNOS inhibitor) that blocked completely NO release (measured by nitrite levels in the medium) only partial prevention (25.0% ± 8%) of neuronal cell death was observed. These results are in contrast to in vitro studies on neuronal cell death induced by LPS-activated glia. In this case neuronal cell death was predominantly mediated by nitric oxide since the pre-treatment with iNOS inhibitors almost entirely prevented neuronal cell death (Bronstein et al. 1995; Bal-Price and Brown 2001).

Nitric oxide produced in high amounts by LTA-activated glial cells could rapidly react with the superoxide anion (O$_2^-$) and form a toxic oxidant, peroxynitrite (ONOO$^-$) (Cuzzocrea et al. 2001). Recent evidence showed that peroxynitrite contributes to neuronal damage, once attributed entirely to nitric oxide (Xie et al. 2002; Brown and Bal-Price 2003; Stewart and Heales 2003). Under normal conditions superoxide formation is tightly controlled by the superoxide dismutase (SOD, cytoplasmic and mitochondrial). However, in acute and chronic inflammation the production of O$_2^-$ is increased at a rate that could exceed the capacity of SOD to remove it. Indeed, the neuronal cell death was almost completely blocked in the presence of MnTBAP (cell permeable SOD mimic) (Szabo et al. 1996), suggesting that O$_2^-$ contributes to LTA-induced neurodegeneration directly or indirectly, through peroxynitrite (ONOO$^-$) formation. The contribution of peroxynitrite in the mechanism of LTA-induced neuronal cell death was confirmed in our model, since strong protection was observed in the presence of FeTPPS (peroxynitrite decomposition catalyst) (Crow 2000). Similar neuroprotective role of FeTPPS was also shown in LPS- (Misko et al. 1998) and amyloid-β peptide-induced cell death (Xie et al. 2002). Taken together, these results suggest that nitric oxide plays an important role in LTA-induced neurotoxicity, but indirectly, through peroxynitrite production. One of the mechanisms by which NO and peroxynitrite kill neurons is inactivation of key metabolic enzymes such as succinate dehydrogenase and cytochrome oxidase of the mitochondrial electron transport chain causing inhibition of mitochondrial
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respiration and ATP depletion (Bal-Price and Brown 2000; Brown 1999). We were unable to
detect any significant changes in ATP and mitochondrial membrane potential levels after
exposure of CGCs to LTA (+/- MDP) (data not shown). It is possible that cell death assessed
by non-specific neuronal endpoints (ATP level or mitochondrial membrane potential) was
masked by LTA-induced glia proliferation.

Although in the present study LTA-induced neurotoxicity was mainly necrotic (propidium
iodide positive cells) and only a low amount of cells presented typical apoptotic features
(condensed or fragmented chromatin; propidium iodide negative/Hoechst 33342 positive
staining), it could not be excluded that the initial mode of cell death was apoptotic and what
we observed was secondary necrosis. Indeed, in the presence of z-VAD-fmk, neuronal cell
death was significantly decreased (by 35% ± 3%) suggesting that at a certain stage caspase
activation was involved. Mainly caspase-3 an -8 (but not -9) were playing a major role, since
selective inhibitors these two caspasess blocked significantly LTA-induced neuronal cell death.
It is well documented that caspase-8 induces activation of executioner caspase-3 causing cell
death (Stennicke et al. 1998). However, it is conceivable that these caspase inhibitors are
acting on the glia rather than the neurons, as IL-1β production by microglia requires caspasas
(Kim et al. 2003).

In conclusion, we have found that LTA-induced neuronal cell death is mediated indirectly,
through glia activation, as LTA is recognised only by astrocytes and microglia (not neurons)
(see Fig. 26). LTA-activated glial cells release pro-inflammatory cytokines (TNF-α, IL-1β and
IL-6), nitric oxide and superoxide, and presumably peroxynitrite was produced. Nitrosative
and oxidative stress and caspase activation lead to cell death of neurons, which then were
phagocytosed by activated glia.
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Fig. 26 Proposed scheme of LTA-induced neuronal cell death mediated by glia activation. LTA did not cause directly neurotoxicity, but only in the presence of glia. LTA-activated glial cells (astrocytes and microglia) released pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and other mediators (nitric oxide, superoxide, peroxynitrite) that could cause oxidative stress, mitochondrial dysfunction, caspase activation and finally induced neuronal cell death.

4.6 Acknowledgements

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5. SUMMARIZING DISCUSSION

Gram-positive bacteria represent an important cause of infections in the CNS and *S. pneumoniae* is the most common pathogen in bacterial meningitis in adults and children. Before the discovery of antibiotics almost all patients with bacterial meningitis died. The introduction of antibiotics brought a substantial reduction in the number of fatal cases of pneumococcal meningitis, but the mortality and the percentage of patients with persistent neurological deficit is still high (Kastenbauer and Pfister 2003). It is now clear that antibiotics are essential, but not sufficient, and in depth research on the molecular and pathological events involved in bacterial meningitis is needed to develop better adjunctive therapies.

The pathological events in bacterial infections of the CNS are complex, and neurological injury is caused in part by the pathogens and in part by the host’s own inflammatory response. At the molecular level components of bacterial cell walls have been shown to activate cells of the CNS - microglia, astrocytes and endothelial cells - to release cytokines, chemokines and reactive oxygen species (Eddleston and Mucke 1993; Ruffolo et al. 1999; Lukiw and Bazan 2000; Nau and Bruck 2002). These inflammatory mediators are known to contribute significantly to neuronal cell death by diverse mechanisms (Nau and Bruck 2002; Lee and Lee 2002). So far, most of the knowledge on the mechanisms of neuroinflammation and neuronal cell death induced by bacterial components comes from studies on lipopolysaccharide (LPS) derived from Gram-negative bacterial cell walls, and there is little information on the role of Gram-positive bacterial components. LPS is known to activate glial cells and induce the release of several pro-inflammatory cytokines, among which TNF-α, IL-1β, IL-6 (Chung and Benveniste 1990; Sebire et al. 1993; Appel et al. 1995; Corsini et al. 1996; Hur et al. 2001). LPS is also a strong inducer of iNOS expression and NO production in both astrocytes and microglia (Mayer 1998; Possel et al. 2000). Pro-inflammatory cytokines and nitric oxide derived from LPS-activated glia was shown to mediate neuronal cell death *in vivo* and *in vitro* (Bal-Price and Brown 2001; Perez-Capote et al. 2004). LPS induces also oxidative stress, which significantly contributes to brain injury and neuronal cell death (Bal-Price and Brown 2001; Xie et al. 2002; Qin et al. 2004).

Only recently it became clear that lipoteichoic acid (LTA), a molecule of Gram-positive bacterial cell walls, is the counterpart of lipopolysaccharide (LPS) derived from Gram-negative bacteria. Several studies performed in human whole blood, peripheral blood monocytes and macrophages confirmed the immunostimulatory potential of LTA, as in these models, LTA was shown to induce the production of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, IL-8)
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(Morath et al. 2001; Hermann et al. 2002; Morath et al. 2002b) and nitric oxide (Kengatharan et al. 1998), similarly to LPS. However evidence of the role of LTA in the CNS infections is still controversial. It is known from clinical studies that LTA is present in the cerebrospinal fluid (CSF) of patients with meningitis and its concentration correlates well with the severity of the disease (Schneider et al. 1999; Stuertz et al. 1999; Heer et al. 2000). Moreover, injection of LTA in rabbits induces symptoms of encephalomyelitis in vivo (Aasjord et al. 1980). Finally, Schmidt et al. (2001) demonstrated that LTA could induce neuronal damage in organotypic cultures of hippocampal slices in vitro. Nevertheless, it is still poorly understood which types of cells in the brain respond to LTA and what are the specific intra- and extracellular pathways involved. Therefore, in the present study, we first characterised the inflammatory response of glial cells (astrocytes and microglia) to LTA and then we investigated the mechanisms of LTA-induced neuronal cell death.

To evaluate the pro-inflammatory potential of LTA in glial cells, we used primary cultures of rat microglia and astrocytes and assessed the release of cytokines (TNF-α, IL-1β, IL-6, IFN-γ) and nitric oxide after exposure to LTA from Staphylococcus aureus and compared it with the glial response to LPS. Interestingly both types of glial cells (microglia and astrocytes) responded to LTA by a time and concentration-dependent production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and nitric oxide. All cytokines were released with kinetics similar to the one observed in LTA-treated human whole blood (Morath et al. 2002b). Generally, the response of glial cells was comparable between LPS and LTA treatment, although at the lower concentrations used (i.e. 10 µg/ml) LPS seemed to be a stronger inducer of TNF-α and NO than LTA. In the same experiments LTA did not induce release IFN-γ in glia. However, this result is in agreement with in human blood studies, where LTA was a found to be a poor inducer of IFN-γ (Hermann et al. 2002). In contrast, LTA was a more potent inducer of several chemokines (MIP-1α, IL-8, MCP-1) in human monocytes and whole blood (Standiford et al. 1994; Danforth et al. 1995; von Aulock et al. 2003). It would be interesting to study whether LTA would induce chemokines release from astrocytes and microglia, since S. aureus infections in the CNS are often associated with the migration of neutrophils mediated by chemokines, and result in complications such as accumulation of pus and formation of brain abscesses (Mathisen and Johnson 1997; Townsend and Scheld 1998; Kiellian et al. 2001). Unfortunately, partly due to the lack of commercially available antibodies for rat chemokines, we could not compare the release of chemokines upon LTA and LPS stimulation of glial cells. Nevertheless, our results clearly demonstrate that both types of glial cells- astrocytes and microglia – are important for the inflammatory response to lipoteichoic acid in the CNS.
During the course of infection and antibiotic therapy bacteria are lysed and not only LTA, but also peptidoglycan (PGN), which constitutes about 50% of the Gram-positive bacterial cell walls, is likely to be released (Moreillon and Majcherczyk 2003). Therefore the simultaneous exposure of glial cells to both LTA and PGN (or MDP) in our experimental *in vitro* model closely mimicked the clinical situation. Several studies have demonstrated that PGN alone can induce the release of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in human monocytes (Verhoef and Kalter 1985; Mattsson et al. 1993; Timmerman et al. 1993) and human whole blood (Wang et al. 2000). Recently, PGN from *Listeria monocytogenes* was also shown to induce oxidative stress mediated by the production of superoxide anion in macrophages (Remer et al. 2005).

Peptidoglycan can be enzymatically cleaved into smaller components, and its smallest element, which still preserves biological activity, was found to be muramyl dipeptide (MDP) (Ellouz et al. 1974). Some studies indicate that MDP itself can activate macrophages (Bahr et al. 1987; Kalyuzhin et al, 2002), monocytes (Suzuki et al. 1994) as well as glial cells (Cottagnoud et al. 2003). However, recently it was demonstrated that pro-inflammatory activity of MDP was probably due to its contamination with LPS, since recombinant MDP (with no endotoxin contamination) itself was not able to stimulate whole blood cells and isolated human monocytes (Traub et al. 2004). Nevertheless, in the same study, MDP was shown to strongly synergise with lipopolysaccharide, but not LTA, to induce cytokine production (Traub et al. 2004).

We were interested whether MDP would have any pro-inflammatory effects on glial cells alone or in combination with LTA. In agreement with the study of Traub et al. (2004) on human whole blood, exposure of primary astrocyte and microglial cultures to MDP alone did not induce any cytokine release. In line with their results, MDP did not potentiate LTA-induced cytokine (TNF-α and IL-1β) release by glia. However, in the case of NO production, significant potentiation of LTA-induced iNOS mRNA expression and subsequent NO release was observed in both microglia and astrocyte cultures. At the same time MDP alone was not able to induce NO production. These results are in concordance with the existing literature data, as the ability of PGN or MDP alone to induce nitric oxide synthase (iNOS) has not been proven yet but there are strong evidences from *in vitro* and *in vivo* studies suggesting that PGN can synergise with LTA in the induction of iNOS and production of nitric oxide (De Kimpe et al. 1995; Kengatharan et al. 1998). Similar synergism was also demonstrated in case of PGN and LPS (Flak et al. 2000; Wray et al. 2001). Kengatharan et al. (1998) in their study have shown that the ability of Gram-positive bacteria to induce iNOS expression in macrophages is mainly triggered by LTA and that PGN only amplifies a biological response of LTA. They also demonstrated that the whole
molecule of PGN is not necessary for the synergistic effect with LTA. It has been have identified that NAG-NAM-L-ala-D-isoglutamine moiety is the smallest fragment of PGN that accounts for the induction of iNOS (attributed previously to the whole PGN polymer) (Kengatharan et al. 1998). Interestingly, MDP was also shown to synergise with IFN-γ- or IL-1β-induced iNOS activation and NO production in primary rat astrocytes culture (Trajkovic et al. 2000). The described capacity of PGN or MDP to potentiate the induction of iNOS has very important clinical implications, as from in vivo studies it is known that peptidoglycan and LTA synergise in inducing inflammation, septic shock and multiple organ failure in rats (De Kimpe et al. 1995).

Since it would be clinically beneficial to control LTA-induced inflammation in the CNS we studied the extra- and intracellular pathways involved in LTA-recognition and signalling in astrocytes and microglia, and compared them with the response of the immune cells in peripheral models (monocytes, macrophages) (Kengatharan et al. 1998; Morath et al. 2001; Hermann et al. 2002). In the innate immune system bacterial products and other pathogen-associated molecular patterns (PAMPs) are recognised by a family of receptors called Toll-like receptors. TLRs were first identified on monocytes and macrophages, and only recently it became clear that they are expressed also on glial cells and play an important role in the activation of glia by several pathogen derived components (LPS, CpG DNA, flagellin, PGN) (Kielian et al. 2002; Bowman et al. 2003; Esen et al. 2004; Farina et al. 2005). However, none of the studies conducted so far addressed the role of Toll-like receptors in the response of astrocytes and microglia to lipoteichoic acid.

In the innate immune system, lipoteichoic acid is recognised by Toll-like receptor-2, while Toll-like receptor-4 is involved in LPS signalling pathways (Takeuchi et al. 1999; Beutler 2000; Ellingsen et al. 2002). Valuable tools in studying the role of TLRs in PAMP recognition are knockout mice which carry a mutation or complete deletion of genes for specific TLR. The first mice described were TLR4 deficient C3H/HeJ mice, which have a mutation in the Lps locus (a sequence that corresponds to the TLR4 gene) that renders them hyporesponsive to LPS (Ryan et al. 1979; Poltorak et al. 1998). TLR2 knockout mice are also available and it was shown that peritoneal macrophages isolated from these mice do not release pro-inflammatory cytokines in response to LTA (Takeuchi et al. 2000; Lehner et al. 2001).

In the present study, using glial cell isolated from TLR2 deficient mice, we have shown for the first time that astrocytes and microglia recognise LTA through the Toll-like receptor 2. Both types of glial cells isolated from mice lacking TLR2 did not release pro-inflammatory cytokines and nitric oxide after exposure to LTA, but responded to LPS stimulation similarly to the control wild-type mice. Additionally, we confirmed that in both types of glial cells LPS is recognised by
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TLR4. Indeed, astrocytes and microglia derived from C3H/HeJ (TLR4 deficient) mice were hyporesponsive to LPS, while released TNF-α, IL-6 and NO upon treatment with LTA, at levels comparable to the glial cells from control C3H/HeN (wild-type) mice.

To verify whether LTA binds to microglia and astrocytes, we co-stained the cultures with rhodamine-labelled LTA and isolectin B4 (a marker of microglia). Due to technical problems (unspecific binding of LTA to paraformaldehyde fixed, GFAP positive cells) we were not able to use also an astrocytic marker (GFAP); however, the characteristic morphology and the fact that isolectin B4 bound only to a small percentage of all cells (microglia) in the mixed glial cultures enabled us to assume that the cells with large flat cell body (typical for astrocytes), intensively stained with rhodamine-LTA were astrocytes, while cells double stained with fluorescent LTA and isolectin B4 were microglia. The specificity of rhodamine-LTA staining was confirmed using Balb-3T3 cells (negative control) that did not show any binding of fluorescent LTA.

Some studies indicate that exposure of astrocytes and microglia to bacterial components leads to an increase of Toll-like receptor expression, therefore enhancing the sensitivity of the cells to the pathogen derived stimuli (Kielian et al. 2002; Bowman et al. 2003; Esen et al. 2004). In our experiments both types of glial cells, microglia and astrocytes, constitutively expressed mRNA for TLR2 and TLR4, however neither exposure to LPS nor LTA at any time point studied (1, 6, 10 or 24 hours) did increase significantly the level of TLR2 or TLR4 mRNA expression as revealed by Real-time RT PCR studies. These discrepancies could be due to the fact that in the three mentioned studies TLR mRNA expression was analysed using semi-quantitative RT PCR – a method less accurate and less sensitive than Real-time RT PCR. Moreover, they were not able to confirm the increased TLR expression at the protein level (Esen et al. 2004).

Binding of a PAMP to the Toll-like receptor initiates several intracellular cascades of events that finally lead to the activation of different transcription factors and genes, involved in the inflammatory response of the cells (cytokines, chemokines, iNOS, COX-2 etc.). TLR signalling involves, among others, the activation of p38, ERK1/2 and JNK MAPK kinases (Beutler 2000). The role of these kinases in LPS-induced cytokine and NO production has been largely studied in both innate immune cells (Chen and Wang 1999; Lahti et al. 2000) and glial cells (Da Silva et al. 1997; Bhat et al. 1998; Hua et al. 2002). Activation of MAPK by LPS is an important signal transduction pathway that contributes to glia-induced neuronal cell death (Nolan et al. 2003; Xie et al. 2004). In contrast to LPS, evidence for the role of Gram-positive derived components in the activation MAPK is limited. Recent studies have demonstrated that in myelo-monocytic cells (Schröder et al. 2001) and in a human epithelial pulmonary carcinoma cell line (Lin et al. 2002) LTA-induced TNF-α release is mediated by activated ERK and p38 MAPK. Activation of p38 by
LTA plays an important role in the induction of TNF-α, IL-6 and NO release also in RAW 264 macrophages, peritoneal macrophages and dendritic cell (Dalpke et al. 2002).

In this study we have demonstrated, to our knowledge for the first time, that LTA activates p38 and ERK1/2 kinases in astrocytes and microglia. Using antibodies against the phosphorylated forms of these kinases we detected transient phosphorylations of p38 and ERK1/2 in glial cells exposed to LTA as compared with non-treated cultures. The maximum levels of phosphorylation after LTA-treatment were at 30 min for p38 and 10 min for ERK1/2 kinase. Using selective inhibitors of p38 (SB203580) or ERK1/2 (PD98059) we assessed the role of each kinase in LTA-induced TNF-α, IL-1β and NO release. Interestingly, p38 kinase was found to be involved in LTA-induced TNF-α and NO (but not for IL-1β) release in glia. Pretreatment of glial cells with SB203580 almost completely blocked the LTA-stimulated TNF-α release, iNOS mRNA expression and NO production, while it had no effect on IL-1β release. On the other hand, the release of IL-1β by astrocytic or microglial cultures exposed to LTA was markedly blocked in the presence of ERK1/2 inhibitor PD98059. The same inhibitor only moderately decreased the LTA-induced TNF-α release, iNOS mRNA expression and NO production in glia.

Since both p38 kinase and ERK1/2 were involved in the regulation of LTA-induced nitric oxide production, we investigated whether these kinases were also responsible for the observed potentiation of NO production by MDP in the presence of LTA. However, the levels of p38 kinase and ERK1/2 phosphorylation measured in the Luminex platform were similar in LTA alone- and LTA (+ MDP)-treated glial cells. Moreover, both SB203580 and PD98059 inhibited LTA (+/- MDP)-induced iNOS mRNA expression and NO production, but the level of suppression of iNOS mRNA expression and NO release was similar in LTA alone- and LTA (+ MDP)-treated cells. These results suggest that the mechanism by which MDP potentiates LTA-induced NO production does not involve p38 kinase or ERK1/2 activation. From literature we know that iNOS is primarily regulated at the transcriptional level, mainly by the transcription factor NF-be, but several other factors are also involved (e.g. Oct, IRF-1, STAT-1α, CREB, C/EBP, AP-1, PPAR) (Kleinert et al. 2003). Along with the transcriptional control, post-transcriptional mechanisms play an important role in regulation of iNOS expression (e.g. the control of mRNA stability and regulation of iNOS mRNA translation) (Taylor and Geller 2000). It would be interesting to study further whether the synergistic effects of LTA and MDP occur at the level of iNOS promoter (and what are the transcription factors implicated), or already at the post-transcriptional level (e.g. if MDP induces an increase in iNOS mRNA stability).
DISCUSSION

The inflammatory response of glial cells to LTA can have several pathological implications including the damage of the blood-brain barrier (BBB). In parallel studies (Boveri et al., 2006) we determined the effects of LTA and MDP on the function of BBB using a co-culture of bovine capillary endothelial cells and rat primary glial cells (an in vitro model of BBB). The exposure of glia-endothelial cell co-cultures to LTA caused a time- and dose-dependent damage of the BBB function, as measured by a decrease in trans-endothelial electrical resistance (TEER) and increase in FITC-insulin permeability. This disruption of BBB was caused indirectly by the activation of glial cells, as exposure of endothelial cells to LTA alone did not induce any BBB damage. Interestingly, the injury of BBB was increased when glial cells in the co-cultures were co-stimulated with LTA and MDP. Moreover, we demonstrated that TNF-α and IL-1β released by LTA (+ MDP)-activated glia significantly contributed to BBB disruption, as direct addition of these cytokines to the endothelial cells increased BBB permeability, whereas pre-treatment of LTA-treated cultures with anti-TNF-α and IL-1 receptor antagonist (IL-ra) significantly attenuated the LTA-mediated damage. The LTA-induced BBB disruption was also partly mediated by NO since the inhibition of iNOS by a specific inhibitor (1400W) prevented the LTA (+/- MDP)-induced BBB damage. These results indicate that LTA induced BBB injury was due to the action of several pro-inflammatory mediators released by LTA-activated glial cells such as pro-inflammatory cytokines (TNF-α, IL-1β) and nitric oxide. It is possible that the loss of BBB integrity observed in bacterial meningitis in vivo could be mediated by similar mechanisms as described in our in vitro studies.

Since LTA can strongly activate glial cells to produce pro-inflammatory cytokines and NO, we evaluated whether this inflammatory response could cause neuronal damage. Exposure of mixed neuronal-glial cells, containing ~85% neurons and ~15% non-neuronal cells (astrocytes and microglia), to LTA induced significant neuronal cell death in a time- and dose-dependent manner, as assessed by propidium iodide/Hoechst 33342 staining. The cell death observed was mainly necrotic (recognised as propidium iodide positive cells) and only few cells with apoptotic phenotype (condensed and/or fragmented chromatin) could be detected. Interestingly, most neuronal cells in the LTA-treated cultures disappeared, suggesting that they were rapidly phagocytosed by activated microglia. In fact, in the LTA treated cultures we could observe many enlarged, spherical microglial cells containing vacuoles and packed with little particles (probably fragments of phagocytosed dead neurons).

To demonstrate that LTA-induced neurotoxicity was mediated by the activated glial cells, we used CGCs cultures pre-treated with Ara-C (inhibitor of glial proliferation) and exposed them to LTA (these cultures contained less then 2% of glial cells). Indeed, in the absence of
astrocytes and microglia LTA-induced neuronal cell death was significantly lower than in mixed neuronal-glia cultures. Additionally, using fluorescently labelled LTA we have demonstrated that in mixed neuronal-glial cultures LTA bound only to astrocytes and microglia, but not to neurons. These findings are in line with the study of Lehnardt et al. (2003) where it was shown that in primary culture of cortical neurons LPS binds only to glial cells but not to neuronal cells. Taken together these results demonstrate that LTA induces neurotoxicity indirectly, through glia activation.

Activation of glial cells in the course of neurodegenerative diseases is commonly called gliosis (Dietrich et al. 2003; Liberto et al. 2004). It involves not only the induction of pro-inflammatory mediators, but also changes of cell morphology and proliferation rate of astrocytes and microglia. From in vivo studies it is known that astrocytes proliferate in the area of a neurodegenerative lesion, and form a glial scar (Kernie et al. 2001; Ciccarelli et al. 2004; Alonso 2005). The activation of microglial cells results in increased proliferation and migration of these cells to the sites of cell damage, often stimulated by cytokines or chemokines present in the injured CNS tissue (Lee et al. 1994; Spleiss et al. 1998). TNF-α and IL-1β are commonly known to induce glial reactions and to promote astrogliosis and microgliosis (Selmaj et al. 1990; Balasingam et al. 1994; Probert et al. 1995; Herx and Yong 2001; Basu et al. 2002). In our experiments, LTA-treatment of CGCs cultures caused neuronal cell death, but had an opposite effect on glial cells as increased proliferation of glial cells was observed. In mixed neuronal-glial CGCs cultures treated with LTA there was a marked increase in the number of both microglia (134% +/- 45%) and astrocytes (79% +/- 21%), especially after long, 72-hours exposure. Also in pure cultures of microglia and astrocytes the numbers of glial cells were increased upon LTA treatment, however, this effect was less pronounced in comparison with CGCs. This might suggest that the communication between neurons and glia is important to fully trigger glia proliferation. It is also possible that the observed higher proliferation of glial cells in mixed neuronal glial cultures was partly induced by the massive neuronal cell death.

Since neurotoxicity was mediated by glia activation, we studied more in detail which factors released by LTA-activated glia contribute to neuronal cell death. The pro-inflammatory cytokines are commonly known to play a role in inflammatory-induced neuronal injury. TNF-α has been suggested to be partly responsible for LPS-induced neurodegeneration (Waage et al. 1989), and the levels of TNF-α in the CSF of patients with bacterial meningitis correlated well with concentrations of bacterial endotoxin in the brain and the severity of the disease (Arditi et al. 1990). The anti-IL-1β antibodies have been shown to
reduce neuronal injury in acute cerebral ischemia and traumatic brain injury (Rothwell and Luheshi 2000; Allan and Rothwell 2001). However, in our model, pre-treatment of CGCs cultures with anti-TNF-α antibodies or with IL-1β receptor antagonist (IL-1ra) did not protect against neurotoxicity suggesting that other mediators were also involved in the mechanisms of LTA-induced neuronal cell death and it was not enough to block only the effects of IL-1β or TNF-α to prevent it. Nevertheless, TNF-α and IL-1β could contribute to neuronal injury, as both cytokines have been shown to induce the expression of iNOS and the release of NO from glial cells, as well as vascular endothelium (Bonmann et al. 1997).

Under normal conditions, NO plays an important role in intra- and intercellular signalling (Garthwaite 1991), however, when produced in excess it is known to cause neurotoxicity. Several in vitro studies have shown that LPS-induced neuronal cell death was predominantly mediated by NO released from activated glia (Bronstein et al. 1995; Bal-Price and Brown 2001). Our results demonstrated that nitric oxide plays an important role also in LTA-induced neurotoxicity. Indeed, in the presence of 1400W (a selective iNOS inhibitor) a significant prevention of neuronal cell death was observed.

As we have shown that in pure astrocytic and microglial cultures MDP significantly synergises with LTA to induce NO production, it was interesting to evaluate whether it potentiates also LTA-induced neurotoxicity. However, neurotoxicity in CGCs cultures co-treated with LTA and MDP was not significantly higher when compared to cultures exposed to LTA alone, suggesting that LTA was the main component of the Gram-positive bacterial cell wall responsible for neuronal cell death.

One of the actions of nitric oxide is the inhibition of mitochondrial respiration that can result in generation of reactive oxygen species (superoxide (O₂⁻), hydrogen peroxide (H₂O₂)). All these free radicals are key mediators of the pathophysiological changes during bacterial meningitis (Koedel et al. 1995; Koedel and Pfister 1999; Christen et al. 2001). In our experiments, we were unable to demonstrate directly that ROS are produced in CGCs exposed to LTA, however, the LTA-induced neuronal cell death was almost completely blocked in the presence of MnTBAP (superoxide dismutase mimetic), suggesting that O₂⁻ contributes to LTA-induced neurodegeneration. Superoxide is toxic to neurons, as it can damage cell membrane lipids, DNA and proteins (Cuzzocrea et al, 2001). However, O₂⁻ can also combine rapidly with nitric oxide, produced in high amounts by LTA-activated glial cells, and form a very toxic radical - peroxynitrite (OONO⁻). The toxicity, previously attributed to superoxide and nitric oxide alone, may be in fact due to the action of peroxynitrite, as it has been shown that peroxynitrite contributes significantly to neuronal damage (Xie et al. 2002; Stewart and
DISCUSSION

Heales 2003; Wang et al. 2003). The role of peroxynitrite in the mechanism of LTA-induced neuronal cell death was confirmed in our model, since strong protection was observed in the presence of FeTPPS (peroxynitrite decomposition catalyst). A similar neuroprotective effect of FeTPPS was also shown in LPS- (Misko et al. 1998) and amyloid-β peptide-induced cell death (Xie et al. 2002). Taken together, these results suggest that nitric oxide, superoxide and peroxynitrite play an important role in LTA-induced neurotoxicity both directly and indirectly.

One of the mechanisms by which NO and peroxynitrite kill neurons is inactivation of key metabolic enzymes such as succinate dehydrogenase and cytochrome oxidase of the mitochondrial electron transport chain causing inhibition of mitochondrial respiration and ATP depletion (Bal-Price et al. 1999). The impairment of mitochondrial respiration causes also decrease in mitochondrial membrane potential (MMP), which in turns can cause the opening of the mitochondrial permeability transition pore, release of cytochrome c in the cytoplasm and activation of caspases leading to apoptotic cell death. We studied whether LTA affects ATP and MMP levels in our CGCs model, but we were not able to detect any significant changes in ATP and MMP levels after exposure of CGCs to LTA. This might be partly due to the fact that our cultures are not pure neuronal but mixed neuronal-glial cultures, with additionally increased number of astrocytes and microglia due to LTA-induced glia proliferation. It is possible that the changes in ATP or mitochondrial membrane potential which occurred in LTA-induced neuronal injury were masked by the increased presence of glial cells.

Recently, it has been shown that nitric oxide can also trigger excitotoxicity, as it causes the release of glutamate from neurons (McNaught and Brown 1998), astrocytes (Bal-Price et al., 2002) and microglia (Barger and Basile 2001). This mechanism is involved in LPS-induced neurotoxicity as neuronal cell death in CGCs exposed to LPS was significantly decreased in the presence of the NMDA receptor antagonist - MK-801 (Bal-Price et al., 2001). Since high amounts of NO are released by LTA-activated glia we investigated whether excitotoxicity could be involved in the mechanism of LTA-induced neuronal injury. However, in our model MK-801 did not inhibit neuronal cell death. It is possible that LTA-induced increase in extracellular glutamate was not high enough to activate NMDA receptors or once release was immediately cleared by astrocytes. It would be interesting to evaluate further if astrocytes and/or microglia release extracellular glutamate upon LTA-activation, especially in the context of a comparison between LPS- and LTA-induced neuronal damage.
Although in the present study LTA-induced neurotoxicity was mainly necrotic, as most dead cells observed were propidium iodide-positive, and only a low number of cells presented typical apoptotic features (condensed or fragmented chromatin; propidium iodide negative/Hoechst 33342 positive staining), it could not be excluded that the initial mode of cell death was apoptotic and what we observed was secondary necrosis. To further evaluate whether neuronal cell death in CGCs cultures exposed to LTA was primarily apoptotic, we evaluated the contribution of caspase activation (one of the main hallmarks of apoptosis) in LTA-induced neurotoxicity.

Using a broad-spectrum caspase inhibitor z-VAD-fmk we demonstrated that at certain stage caspase activation was involved in neuronal cell death as in the presence of z-VAD-fmk, neuronal cell death was significantly decreased (by 35% +/- 3% in comparison with control). Interestingly, using selective inhibitors of caspases-3, -8 and -9 (z-DEVD-fmk, z-IETD-fmk and z-LEHD-fmk, respectively) we have shown that only inhibition of caspase-3 and caspase-8 (but not caspase-9) blocked significantly the LTA-induced neurotoxicity. One of the possible explanations why caspase-3 was involved in LTA-induced neuronal cell death without the participation of caspase-9, could be that it was activated via the caspase-8 pathway. Indeed it is well documented that caspase-8 can induce activation of executioner caspase-3 leading to cell death (Stennicke et al. 1998).

In conclusion our data demonstrate that lipoteichoic acid from Staphylococcus aureus causes neuronal cell death indirectly via the activation of both types of glial cells (astrocytes and microglia) that recognise LTA through the constitutively expressed Toll-like receptor-2. Binding of LTA to TLR2 initiates the activation of several signalling pathways, in which the phosphorylation of p38 and ERK MAPK are involved. LTA-activated glial cells proliferate at higher rate and release pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), nitric oxide, superoxide and peroxynitrite, which cause damage of the blood-brain barrier followed by neuronal cell death. Based on our results the main mechanisms involved in LTA-induced neurotoxicity are caused by nitrosative and oxidative stress and subsequent caspase activation leading to neuronal necrotic or apoptotic cell death. Presumably, apoptotic cells are cleared up by activated microglia (phagocytosis). The proposed cascade of events is summarized in Fig. 27. Because the mechanisms of neuronal cell death during bacterial infections are complex, the therapy should ideally intervene at the several different levels of the cascade of events to increase effectiveness of treatment. In addition to bactericidal therapy there is an urgent need for effective and specific therapeutic interventions to decrease the still high mortality, especially in the case of bacterial meningitis. Bacterial protein synthesis-inhibiting antibiotics
(not acting by lysis of bacteria) and scavengers of ROS and RNS production are already used in clinical practice.

Based on the results obtained in these studies, the pharmacological control of glia activation e.g. by blocking Toll-like receptors 2 and/or 4 to prevent production of pro-inflammatory cytokines or blocking MAPK signalling pathway that is involved in glia inflammatory response, could be beneficial as a novel, adjunctive therapy of Gram-positive bacterial infections in the central nervous system.

**Fig. 27** Proposed cascade of events induced by lipoteichoic in the CNS, leading to glia activation, neuroinflammation and neuronal cell death. Recognition of LTA by TLRs on glial cells leads to the activation of several intracellular pathways (e.g. MAPK p38 and ERK1/2 phosphorylation). Activated glial cells release several pro-inflammatory mediators that subsequently can cause oxidative and nitrosative stress, activation of caspase-3 and -8 and finally lead to neuronal cell death, both necrotic and apoptotic, and the cell debris is cleared by phagocytosing microglia.
6. SUMMARY

In the Central Nervous System (CNS), Gram-positive bacterial infections represent about 50% of the bacterial diseases and are often associated with high mortality and severe permanent neurological consequences. The major immunostimulatory principles of Gram-positive bacteria are the constituents of bacterial cell walls, lipoteichoic acid (LTA) and peptidoglycan (PGN). In contrast to lipopolysaccharide (LPS) from Gram-negative bacteria, the role and the mechanisms of Gram-positive bacterial components in inducing inflammation in the CNS are still poorly understood.

The aim of the present Ph.D. thesis was to characterise which type of cells is involved in the inflammatory response of the CNS induced by LTA isolated from *Staphylococcus aureus* and/or muramyl dipeptide (MDP, the smallest active fragment of peptidoglycan).

Since inflammation in the CNS is mainly mediated by activated glia, in the first part of the thesis, the inflammatory potential of lipoteichoic acid (LTA) (in the presence and absence of MDP) was studied using primary cultures of astrocytes or microglia. Furthermore, the extra- and intracellular pathways involved in LTA-signalling were investigated, with emphasis on the role of Toll-like receptors (-2 and -4) and mitogen-activated protein kinases (MAPK), p38 and ERK1/2. In the second part of the studies, the mechanisms of neuronal cell death induced by lipoteichoic acid (LTA) and muramyl dipeptide (MDP) were investigated using primary culture of rat cerebellar granule cells (CGCs).

The main conclusions of these studies are as follow:

1. LTA induced strong inflammatory response in both types of glial cells (astrocytes and microglia). Exposure of primary cultures of rat astrocytes or microglia to LTA triggered time- and dose-dependent production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and nitric oxide (NO). The kinetics of cytokine and NO release was comparable with the response observed in systemic models, such as human whole blood or isolated primary monocytes.

2. Toll-like receptors were involved in LTA- and LPS-recognition in glial cells. Both astrocytes and microglia constitutively expressed mRNA for Toll-like receptor-2 (TLR2) and Toll-like receptor 4 (TLR4). LTA was recognised by glial cells through the Toll-like receptor 2, while LPS was shown to be recognised by TLR4, as astrocytes and microglia
isolated from TLR2 or TLR4 knockout mice did not produce cytokine and nitric oxide upon LTA- or LPS-stimulation, respectively.

3. LTA-induced glia activation was mediated through p38 and ERK1/2 MAP kinases, since LTA caused transient phosphorylations of these kinases, and pre-treatment with SB203580 (inhibitor of p38) and PD98059 (inhibitor of ERK1/2) decreased the LTA-induced cytokine release, iNOS mRNA expression and NO production.

4. Muramyl dipeptide selectively potentiated the LTA-induced iNOS mRNA expression followed by NO production, but it did not have any significant influence on the production of pro-inflammatory cytokines. The mechanism by which MDP potentiated LTA-induced NO production did not involve p38 kinase or ERK1/2 activation.

5. LTA induced significant neuronal cell death in rat primary cultures of cerebellar granule cells (CGCs). The neuronal cell death was mediated by LTA-activated glia, as in the absence of glia LTA did not cause any significant neurotoxicity. Moreover, LTA was shown to act only through glial cells, i.e. astrocytes and microglia (not to neurons) as confirmed by rhodamine-labelled LTA staining.

6. Oxidative and nitrosative stress (in particular nitric oxide, superoxide and peroxynitrite) contributed to LTA-induced neurotoxicity, since neuronal cell death induced by LTA-activated glia was blocked by an iNOS inhibitor (1400W), a superoxide dismutase mimetic (MnTBAP) and a peroxynitrite scavenger (FeTPPS).

7. Activation of caspase-3 and caspase-8 (not caspase-9) was involved in the mechanism of LTA-induced neurotoxicity, as in the presence of specific caspase-3 or -8 inhibitors significant neuroprotection was observed.

The obtained results indicate that LTA-induced inflammation in the CNS is mediated by activated astrocytes and microglia. LTA-activated glial cell can cause neuronal cell death that is mediated by oxidative and nitrosative stress as well as caspase activation. These results suggest that pharmacological control of glia activation (blockage of Toll-like receptor-2 and the inhibition of MAPK signalling pathway) and the control of oxidative and nitrosative stress or caspase activation could provide the basis of a potential, novel adjunctive therapy of Gram-positive bacterial infections in the CNS.
7. ZUSAMMENFASSUNG


Das Ziel der vorliegenden Doktorarbeit war es, zu charakterisieren, welche Zellentypen in die inflammatorische Antwort des ZNS, verursacht durch LTA isoliert von *Staphylococcus aureus* und/oder Muramyl-Dipeptid (MDP, das kleinste aktive Fragment des Peptidoglykan) involviert sind.


Die Haupterkenntnisse dieser Studien sind folgende:

1. LTA induziert eine starke inflammatorische Antwort in beiden Typen von Gliazellen (Astrozyten und Microglia). Stimulierung der Primärkulturen von Ratten-Astrozyten oder -Microglia mit LTA rief zeit- und dosisabhängige Produktion der proinflammatorischen Zytokine (TNF-α, IL-1β, IL-6) und Stickoxid (NO) hervor. Der Zeitverlauf der Zytokin- und NO-Freisetzung war vergleichbar mit der Freisetzung in einem systemischen Modell, wie dem humanen Vollblutmodell oder isolierten primären Monozyten.

3. LTA-induzierte Gliazell-Aktivierung wurde durch p38 und ERK1/2 MAP-Kinasen vermittelt, da LTA eine transiente Phosphorylierung dieser Kinasen verursachte. Eine Vorstimulierung mit SB203580 (Inhibitor von p38) und PD98059 (Inhibitor von ERK 1/2) verringerte die LTA-induzierte Zytokin-Freisetzung, iNOS-mRNA-Expression und die NO-Produktion.

4. Muramyl-Dipeptid erhöhte selektiv die LTA-induzierte iNOS-mRNA-Expression in Zusammenhang mit einer NO-Produktion, aber hatte keinen signifikanten Einfluss auf die Produktion der pro-inflammatorischen Zytokine. Der Mechanismus, durch den MDP die LTA-induzierte NO-Produktion erhöhte, bezog nicht p38 Kinase oder ERK1/2-Aktivierung mit ein.


6. Oxidativer und nitrosativer Stress (genauer Stickoxid, Superoxid und Peroxynitrit) trugen zur LTA-induzierten Neurotoxizität bei, da neuronaler Zelltod induziert durch LTA-aktivierte Gliazellen durch einen iNOS-Inhibitor (1400W), ein Superoxid-Dismutase-Mimetikum (MnTBAP) und einen Peroxinitrit-Scavenger (FeTPPS) gehemmt werden konnte.


Die erzielten Resultate deuten an, dass LTA-induzierte Entzündung im ZNS durch aktivierte Astrozyten und Mikroglia hervorgerufen wird. Auf Dauer, können LTA-aktivierte Gliazellen neuronalen Zelltod verursachen, der durch oxidativen und nitrosativen Streß und Kaspase-
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