Biochimica et Biophysica Acta 1862 (2016) 339–351

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Signal transduction and epigenetic mechanisms in the control of microglia activation during neuroinflammation☆

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ARTICLE INFO

Article history:
Received 30 July 2015
Received in revised form 12 October 2015
Accepted 28 October 2015
Available online 30 October 2015

Keywords:
Neuroinflammation
Microglia
Signaling pathways
Transcription regulation
Epigenetics
HDAC inhibitors

ABSTRACT

Activation of microglia is a common denominator and a pathophysiological hallmark of the central nervous system (CNS) disorders. Damage or CNS disorders can trigger inflammatory responses in resident microglia and initiate a systemic immune system response. Although a repertoire of inflammatory responses differs in those diseases, there is a spectrum of transcriptionally activated genes that encode various mediators such as growth factors, inflammatory cytokines, chemokines, matrix metalloproteinases, enzymes producing lipid mediators, toxic molocules, all of which contribute to neuroinflammation. The initiation, progression and termination of inflammation requires global activation of gene expression, postranscriptional regulation, epigenetic modifications, changes in chromatin structure and these processes are tightly regulated by specific signaling pathways. This review focuses on the function of "master regulators" and epigenetic mechanisms in microglia activation during neuroinflammation. We review studies showing impact of epigenetic enzyme inhibitors on microglia activation *in vitro* and *in vivo*, and critically discuss potential of such molecules to prevent/moderate pathological events mediated by microglia under brain pathologies. This article is part of a Special Issue entitled: Neuro Inflammation edited by Helga E. de Vries and Markus Schwaninger.

1. Overview of major signaling pathways involved in microglia activation

1.1. Microglia are instigators of neuroinflammation

Microglia are the innate immune cells in the central nervous system (CNS). Microglia actively survey the CNS microenvironment [118] and maintain homeostasis [47] under normal physiological conditions and participate in the inflammatory response [93]. Microglia contribute to normal CNS function by mechanisms such as fine tuning of neural circuits [115] and phagocytosis of apoptotic debris [116]. Microglia are highly reactive to insult or injury. The classical M1 type activation is associated with cytotoxicity and inflammatory responses, whilst the alternative activation M2 type is regarded as being beneficial, and can be further subdivided into M2a, involved in repair and regeneration, the immunoregulatory M2b, and an acquired-deactivation M2c type [23].

Activation of microglia is a common denominator and a pathophysiological hallmark of virtually all neurodegenerative disorders. CNS infections, massive trauma, post-ischemic or toxicity-related necrosis, hemorrhage or accumulation of neurotoxic fibers can trigger inflammatory responses in resident microglia. Inflammation is a feature of several neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and multiple

Abbreviations: AP-1, activator protein 1; APC, antigen-presenting cells; ARE, AU-rich element; ASK, Apoptosis Signal regulating Kinase; ATF-2, Activating Transcription Factor 2; CRE, Cyclic AMP Responsive Element; CBP, CREB binding protein; CsA, ciclosporin A; ERK, Extracellular signal Regulated Kinase; iNOS, inducible Nitric Oxide Synthase; ISRE, interferon-stimulated response element; JNK, c-Jun N-terminal Kinase; LPS, lipopolysaccharide; MCAO, middle cerebral artery occlusion; MCP-1, monocyte chemoattractant protein 1; MAPKAP-K2, MAP kinase Activated Protein Kinase 2/3; MEK, MAP/ERK kinase; MKK, MAP Kinase Kinase; MAP3K, MAP Kinase Kinase Kinase; MLK, Mixed Lineage Kinase; MMPs, metalloproteinases; MyD88, myeloid differentiation factor 88; NIK, NF-κB-inducing kinase; PAK, p21 activated kinase; STAT, Signal Transducers and Activators of Transcription; TAK1, Transforming growth factor Activated Kinase 1; TBP, TATA box binding protein; TGFβ, Transforming growth factor; TLR, Toll-like receptors; TNF, Tumor Necrosis Factor; TRAF, TNF receptor associated factor; TRIF, Toll/IL-1R domain-containing adaptor-inducing IFN-beta.

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http://dx.doi.org/10.1016/j.bbadis.2015.10.026
0925-4439/

sclerosis (MS) [47]. Neuroinflammation can be divided into acute and chronic phases [13]. Inflammatory stimuli activate microglia, which release inflammatory cytokines and phagocyte debris and dead cells, in an attempt to initiate tissue repair and, thus, resolve the inflammatory process. However, if the resolution mechanisms fail or the inflammatory stimulus persists, there is a self-propagating and persistent stage of chronic inflammation, which leads to neurotoxicity and neuronal death [13,22]. Although a repertoire of inflammatory responses differs in various diseases, there is a spectrum of transcriptionally activated genes which encode various endogenous mediators such as growth factors, inflammatory cytokines interleukin 1 (IL-1β), tumor necrosis factor (TNF)-α, interleukin (IL)-6, chemokines Fractalkine (CX3CL1), Macrophage Inflammatory Factor (MIP)-1/CCL3, IL-8, matrix metalloproteinases (MMPs), enzymes producing lipid mediators, nitric oxide and free radicals, all of which contribute to neuroinflammation [1,39,71,98].

Despite different etiology of brain diseases associated to neuroinflammation there are several primary triggers which are recognized by an array of receptors on microglia. After an ischemic insult, damaged neurons release different molecules: ATP, heat shock protein (HSP), adenine dinucleotide (NAD), hyaluronic acid and fibronectin produced by extracellular matrix degradation, nucleic acids, mannose residues proteolytic enzymes and high-mobility group box 1 protein (HMGB1) [84,106]. DAMPs activate immune cells, including microglia [65] which release pro-inflammatory cytokines toxic to already vulnerable neurons [136]. Excessive neuronal release of glutamate, which directly contributes to neuronal death, activates microglia expressing metabotropic glutamate receptors [154,155,112]. Damaged or overactive neurons release or leak purines, including ATP and UTP that activate corresponding receptors. Surveying microglia show a constitutive expression of most receptors (P1 adenosine, P2 ionotropic P2X and metabotropic P2Y receptors) but alter their set of purinergic receptors upon activation. Microglial activation and morphological transformation into amoeboid phagocytic cells after damage require mostly $P2Y_{12}$ and A_3 receptors; A_2A receptors induce amoeboid transformation, $P2Y_{12}$, $P2X_4$, and A_1 receptors interact to induce recruitment to lesion site [90]. The damage signals released from injured cells are collectively called danger-associated molecular patterns (DAMPs) and are recognized and bind by pattern recognition receptors (PRRs).

1.2. TLRs and cytokine signaling in microglia activation upon neuroinflammation

1.2.1. Signaling via Toll-like receptors (TLR) in inflammatory microglia

Toll like receptors (TLRs) recognize a wide variety of danger signals and consequently activate inflammatory cascades [43]. TLRs (with the exception of TLR3) initiate intracellular signaling via recruitment of the intracellular adaptor proteins containing intracellular Toll-IL-1 receptor (TIR) domain. These adaptors include: MyD88 (myeloid differentiation factor 88); TIRAP (TIR-domain-containing adaptor protein), TRIF (TIR-domain-containing adaptor protein inducing IFN-β), and TRAM (TRIF-related adaptor molecule), and can be differentially recruited to the TLR or IL-1 receptors and determine the specificity of signaling (Fig. 1A).

Toll-IL-1 receptors recruit an intracellular adaptor protein MyD88 (myeloid differentiation factor 88) containing TIR domain. When MyD88 or other adaptors are recruited to activated TLRs, either directly (TLRs 5 and 11) or indirectly (TLRs 1, 2, 4, 6), MyD88 engages members of the IRAK (IL-1R-associated kinase) family, to perform auto- and cross-phosphorylation. Phosphorylated IRAKs dissociate from MyD88, and bind TRAF6 (TNF receptor-associated factor 6) and ubiquitin E3 ligases. TRAF6 activates TAK1 (transforming growth factor β-activated kinase) which activates the IKK complex and MAPKK (mitogen activated kinase kinases) as shown in the Fig. 1. The IKK complex phosphorylates IκB proteins which is necessary for the ubiquitination and proteosomal degradation of IκBs and the subsequent nuclear translocation of the transcription factor NF-κB [76,77]. Members of the MAPK family phosphorylate and activate components of the transcription factor AP-1. The endosomal receptors TLR7 and TLR9 can recruit MyD88 which further activates members of the IRAK family that bind TRAF3. Activation of TRAF3 leads to phosphorylation and activation of the interferon responsive factors: IRF3, IRF5, and IRF7. The endosomal TLR3 recruits the Toll-interleukin 1 receptor domain-containing adaptor inducing interferon β (TRIF) which binds kinases TBK1 and IKKε, which activate IRF3. Further, TRIF recruits TRAF6 and RIP-1, which results in activation of MAPK and IKKα/β [76,77].

TLRs and their downstream signaling molecules control microglial behavior during neurodegeneration. TLR4 defects reduced levels of TNFα, IL-1β, IL-10 and IL-17 in the brains of APP (APPswe/PSEN1)-mice [68], diminished amyloid β (Aβ)-induced IL-1β, CCL3, and CCL4 expression in monocytes, reduced microglia activation and increased Aβ deposits which was associated with impairment in cognitive functions [145]. TLR2 deficiency (TLR2-KO) in APPswe/PSEN1 mice increased soluble Aβ in the brain and exacerbated cognitive impairments [132]. MyD88 deficiency reduced brain amyloid β pathology and microglial activation [103,145], although a recent observation using same transgenic mice showed that MyD88 signaling does not significantly affect Aβ-induced microglial activation and cerebral Aβ-deposits [168]. Reconstitution of the immune system of irradiated APP(swe)/PSEN1 mice with MyD88-deficient cells significantly accelerated memory deficits [113]. In a cellular model of PD, microglia cultured from TLR2 KO mice or cells treated with blocking antibody against TLR2a showed reduced production of inflammatory mediators after the exposure to the conditioned media from SH-SY5Y cells overexpressing human α-synuclein. These data implicate TLR2 as a receptor for α-synuclein released from damaged neurons, responsible for microglial activation observed in PD [89].

A growing body of evidence shows that TLRs and their downstream signaling molecules modulate microglial responses during acute neuroinflammation induced by nerve transection injury, intracerebral ischemia and hemorrhage, traumatic brain injury, and hippocampal excitotoxicity [45]. TLR-4 deficiency protected mice against ischemia and retinal ganglion cell axotomy-induced degeneration [86]. TLR4-deficient mice had smaller cerebral ischemia–reperfusion injury and reduced TNF-α and IL-6 levels [45]. Further studies revealed that TLR4-KO (but not TLR3- or TLR9-KO) mice had reduced infarct area in a middle cerebral artery occlusion (MCAo) model. TLR4 was expressed in $CD11b^+$ microglial cells in the ischemic striatum and $CD11b^+$ cell accumulation was reduced in TLR4-KO mice [64]. In ischemic brains, TLR2 expression was induced in lesion-associated microglia and TLR2-deficient mice had decreased brain injury after focal cerebral ischemia [97]. Systemically administered TLR ligands induce tolerance to subsequent ischemic injury and it has been proposed that stimulation of TLRs prior to ischemia reprograms TLR signaling which leads to reduced expression of pro-inflammatory molecules and enhanced expression of anti-inflammatory mediators [108]. Investigation of stroke patients demonstrated that upregulated expression of TLR4 in monocyte subpopulations correlates with severity of acute cerebral infarction [160,172]. TLR2 and TLR4 expression on monocytes were independently associated to poor outcome and correlated with higher serum levels of IL1β, IL6, TNFα, and VCAM1 [10].

Findings in animal models and in humans suggest interactions between TLRs and complement. Microglial cells constitutively express the receptors for complement cascade components: C1q and for cleavage products of C3, that mediate phagocytosis and stimulate cytokine production by microglia. CR3 can regulate the signaling activity of TLR2 and TLR4 via an adaptor protein TIRAP, which acts as a sorting adaptor and facilitates the recruitment of the signaling adaptor MyD88 to either TLR2 or TLR4. Microglia also respond to C1q with a pro-inflammatory activation in CNS diseases with blood–brain barrier impairment [52].

1.2.2. Signaling via a TNF receptor family

TNF receptor family includes lymphotoxin (LT) receptor, Fas, CD40, the low affinity nerve growth factor receptor, TRAIL receptors, RANK

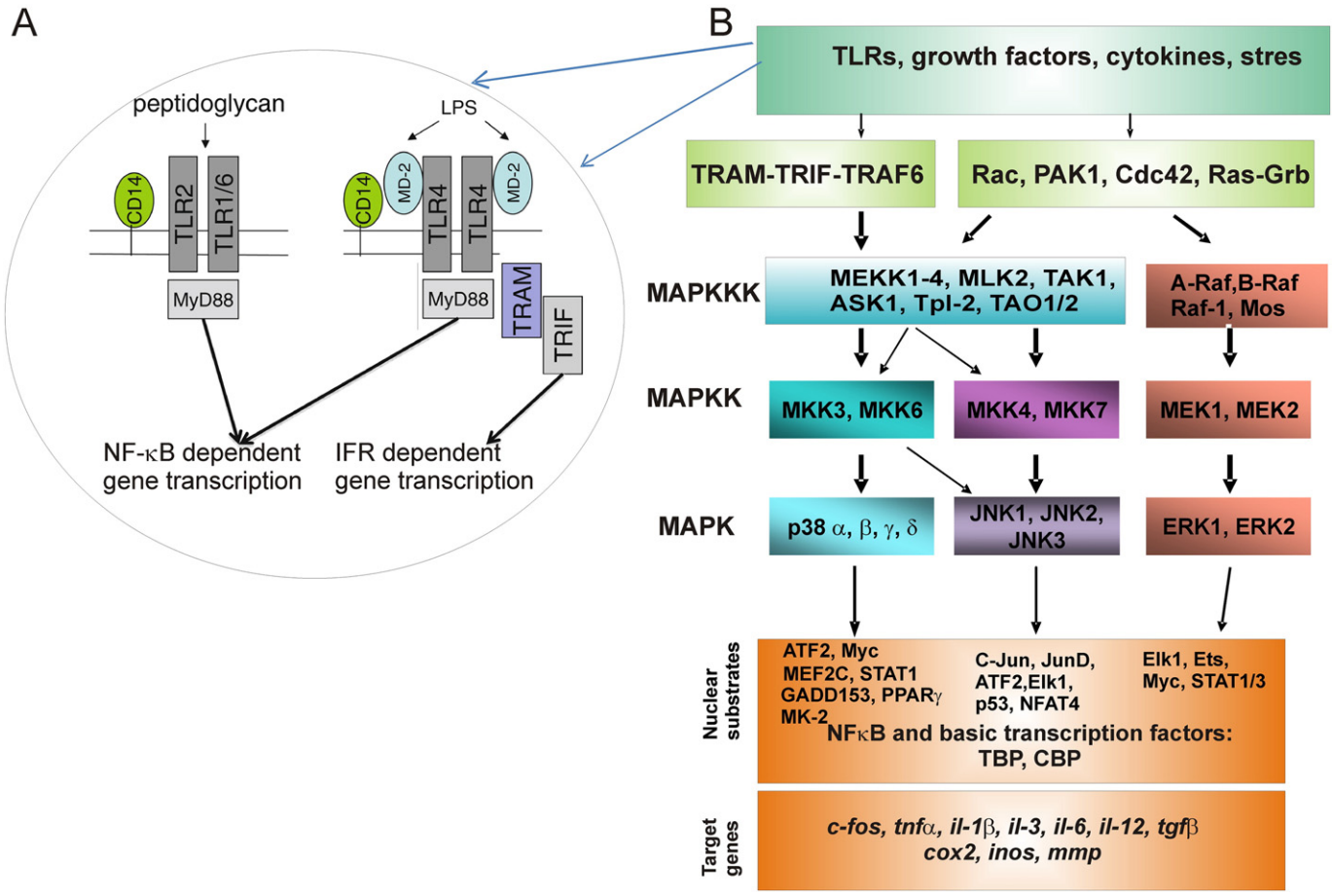

Fig. 1. The TLR activation and MAPK signal transduction pathway A. TLRs in a response to different ligands initiate intracellular signaling via recruitment of the intracellular adaptor proteins: MyD88 (myeloid differentiation factor 88); TIRAP (TIR-domain-containing adaptor protein), TRIF (TIR-domain-containing adaptor protein inducing IFN-β), and TRAM (TRIF-related adaptor molecule). These adaptors can be differentially recruited to the TLR or and determine the specificity of signaling. B. MAPK family is composed of three modules serially activated kinases: a MAPK kinase kinase (MAP3K), a MAPK kinase and ERK, JNK and p38 MAPK. Several substrates or indirect targets of each pathway are indicated. Abbreviations: ATF, activating transcription factor; ASK, Apoptosis Signal-regulating Kinase; CBP, CREB binding protein; CREB, cAMP response element binding protein; Elk-1, Ets-like transcription factor; ERK, extracellular signal-regulated kinase; Ets, transcription factor with v-ets domain (viral-E-twenty-six); GADD 153, growth arrest and DNA damage-inducible protein 153; iNOS, inducible nitric oxide synthase; JNK, c-Jun amino-terminal kinase; MAPKAP-K2, MAP kinase-activated protein kinase 2; MEF, myocyte-enhancing factor; MEK, MAP/ERK kinase; MEKK, MEK kinase; MLK, mixed-lineage protein kinase; MyD88, myeloid differentiation factor 88; NFAT4, Nuclear factor of activated T cells 4; NIK, NF-κB-inducing kinase; PAK, p21 activated kinase; TAK, transforming growth factor-β-activated protein kinase; TAO, one thousand and one amino acids; TGF β, transforming growth factor β; TLR, Toll-like receptors; TBP, TATA box binding proteins; TNF, Tumor Necrosis Factor; Tpl-2, Tumor progression locus 2 kinase; TRAF, TNF receptor associated factor; TRAM, TRIF-related adaptor molecule; TRIF, Toll/IL-1R domain-containing adaptor-inducing IFN-beta.

and death or decoy receptors. TNF-α acts through two receptors: TNFR1 and TNFR2. TNFR1 contains a death domain (DD) in the cytoplasmic tail that is not present in TNFR2. Activation of TNFR1 leads to the recruitment of the adaptor protein TRADD (TNFR-associated DD), which can further recruit receptor-interacting protein kinase 1 (RIP1), TNFR-associated factors (TRAFs) and Fas-associated death domain (FADD) [29].

The downstream signaling pathways diverge: association of FADD with pro-caspase-8 leads to apoptosis, whereas association of TRAF-2 with the cytoplasmic inhibitor of apoptosis protein signals survival by transcriptional activation of NF-κB and cFos/cJun. TRADD can also interact with the TLR4 complex after lipopolysaccharide (LPS) stimulation. In TRADD deficient mice, TNFα induced apoptosis and TNFα-stimulated NF-κB, JNK, and ERK activation was defective. TRADD-deficient macrophages show impaired cytokine production in response to LPS and poly (I:C) *in vitro*. TRAF6 is a common mediator of the signals from different receptors, including the TNF receptors, IL-1R/TLR superfamily, TGFβR, IL-17R and IL-25R, and the NOD-like pattern recognition receptors [29,105]. The molecular switches and changes in the expression of pathway components contribute to the multi-faceted roles of TNF-α in stroke progression and rehabilitation.

Mice lacking TNF-α receptors exhibited enhanced neuronal damage after kainate-induced seizures and focal cerebral ischemia [14], or traumatic injury [146]. These data were corroborated by observations of the increased infarction in TNFR1 (TNF-p55 receptor, TNF-p55R)-KO mice [92]. Further studies demonstrated that the TNFR1, but not TNFR2-KO, mice have significant reduction in JNK activation, reduced microglial activation and blood–brain barrier breakdown which resulted in a smaller cortical and white matter injury after LPS-sensitized hypoxic–ischemic injury in the immature brain [163].

1.2.3. IκB kinase (IKK) complex in neuroinflammation

The pro-inflammatory cytokines, TLRs and other stress-like stimuli activate NF-κB via the IκB kinase (IKK) complex, which contains kinases IKKα, IKKβ and the regulatory subunit IKKγ/NEMO (NF-κB essential modulator) [57]. Canonical signaling strictly depends on IKKγ, while the catalytic subunits seem to be more redundant. IKKγ forms a functional IKK complex with IKKα in response to IL-1β, but TNFα requires IKKβ to form a signaling unit [51,144]. The non-canonical pathway is activated by a narrow group of stimuli, such as lymphotoxin-α/β, BAFF or CD40L, which trigger posttranslational stabilization of NF-κB interacting kinase (NIK). The substrate spectrum of the IKK complex is not restricted to IκBs, but includes other proteins such as RelA and p53. Therefore, the IKK complex besides its role as a signaling hub for NF-κB activation

is also a platform for crosstalk between NF-κB activating pathways and other physiological processes [57].

Deficiency of IKKβ in myeloid cells resulting from the LysM-Cre-Lox knock out of the *ikbkb* gene in the myeloid cell lineage (LysM-Cre/Ikkbeta mice) reduced neuronal cell death after kainate (KA)-induced seizures which was associated with decreased KA-induced microglia and astrocyte activation, and reduced expression of *tnf-α* and *il-1β* [24]. Deficiency of IKKβ in myeloid cells in APP-transgenic (TgCRND8) mice reduced inflammatory activation and Aβ accumulation in the brain which was associated with reduction of cognitive deficits and protection of synaptic proteins. IKKβ deficiency enhanced microglial recruitment and facilitated Aβ internalization [104].

1.3. A MAP kinase cascade—a hub for inflammatory signaling in microglia

TLRs, receptors of TNFα, IL-1β and many other cytokines employ a common signaling hub—MAP kinases—to transduce extracellular signals to the nucleus. Three major groups of distinctly regulated MAPK cascades leading to altered gene expression: ERK1/2, JNK, and p38 MAP kinase are known in humans. ERK1/2 is activated by MKK1 and MKK2, JNK by MKK4 and MKK7, and p38 MAP kinase by MKK3, MKK4, and MKK6, respectively (Fig. 1). Upon activation of the MAP kinases, transcription factors present in the cytoplasm or nucleus are phosphorylated and activated, which results in transcription of target genes. The multiple interactions between the different MAP kinase cascades serve to integrate the responses and activate specific sets of genes [75]. TAK1, which belongs to the family of mitogen-activated protein kinase kinase kinases (MAP3Ks), is activated by cytokines such as TNF-α, TGF-β as well as by LPS, and is upstream of p38MAPKK, JNK and NF-κB in various cells. Recent advances in genetic targeting of microglia enable to address specifically questions of signal transduction in microglia activation [169]. Conditional deletion of TAK1 (transforming growth factor (TGF)-β-activated kinase 1/mitogen-associated protein kinase kinase kinase, MAP3K) in microglia during autoimmune inflammation reduced the NF-κB, JNK and ERK1/2 activation, lessened CNS inflammation, and diminished axonal and myelin damage suppressing a disease [48]

The p38 MAPK family consists of four major isoforms encoded by p38α (*MAPK14*), p38β (*MAPK11*), p38γ (*MAPK12*), and p38δ (*MAPK13*) genes [3]. The expression of p38 MAPK isoforms differs: p38α was expressed predominantly in monocytes, whereas p38δ expression was low; in macrophages p38α and p38δ were abundant. Studies of knock-in mice (the drug-resistant, p38αT106M mice) demonstrated that inhibition of the p38α isoform *in vivo* is sufficient for suppression of increased pro-inflammatory cytokine production in macrophages after a systemic LPS challenge. Deletion of p38α (p38αΔM) only in the myeloid lineage cells protected mice from the lethal effects of LPS or cecal ligation and puncture induced sepsis [73]. The p38α MAPK inhibitor (069A) reduced IL-1β and TNFα production induced by TLR agonists or Aβ in the BV-2 microglial cells and primary microglial cultures. Microglia from p38α-KO mice showed the reduced cytokine production in response to LPS and oral administration of the inhibitor blocked the increase of IL-1β in the cerebral cortex of mice injected with LPS [4]. p38β MAPK deficiency did not impair the pro-inflammatory cytokine production and neurotoxicity after administration of LPS by intraperitoneal or intracerebroventricular injection [170].

Major effects of p38 MAPK on the synthesis of inflammation mediators depend on MAPK-activated protein kinase 2 (MK2). The nuclear MK2 facilitates translocation to the cytoplasm and phosphorylation of tristetraprolin (TTP). TTP regulates mRNA turnover by binding to adenine/uridine-rich element (ARE)-dependent and recruiting mRNA-degrading enzymes which reduces a stability of mRNA [12]. The p38 pathway has been implicated in the regulation of mRNA stability of *cyclooxygenase 2*, *tnf-α*, *il-3*, *il-6*, *il-8*, *cmp-1α*, *granulocyte-macrophage colony-stimulating factor* (*GM-CSF*), *vascular endothelial growth factor*, *urokinase-type plasminogen activator*, and *inos* genes. MK2-KO mice were resistant to the LPS injury and did not induce TNFα or NO production [91]. The expression of p38α and MK2 in microglia is several fold higher than in neurons or astrocytes, and MK2 expression is further increased in LPS and interferon γ-stimulated microglial cells. Elevated activation and expression of MK2 correlated with Aβ deposition, microglial activation, in double-mutant APPswe/PSEN1.M146V mice ([26]. Cultured microglia from MK2-KO mice had reduced expression of *tnfα*, *ccl3*, *kc* (mouse chemokine with highest sequence identity to human GROs and interleukin-8) when challenged with LPS and IFNγ or $A\beta_{1-42}$ [26]. Increased expression and phosphorylation of MK2 in microglia/macrophages, neurons and astrocytes was detected after spinal cord injury. MK2-KO mice showed better locomotor recovery, reduced neuron and myelin loss, and increased sparing of serotonergic fibers in the injury site. This was associated with the reduced expression of pro-inflammatory cytokines and protein nitrosylation [44]. Studies of immortalized bone marrow macrophages from MK2-KO mice demonstrated that activation of the p38 MAPK/MK2 pathway is critical for imidazoquinoline S28463- (TLR7 ligand) and CpG DNA (TLR9 ligand)-induced cytokine and chemokine production [158]. The myeloid-specific TTP deficiency resulted in an early-onset, severe inflammatory phenotype, myeloid hyperplasia and autoimmunity [130].

MAPK are also involved in the regulation of the protein translation. ERK and p38 MAPK phosphorylate MAPK signal-integrating kinase 1—Mnk1, which phosphorylates an eukaryotic initiation factor 4E (eIF4E), crucial for initiation of protein synthesis [167]. Stimulation of primary microglial cultures with fibronectin resulted in the activation of the translational factor eIF4E via MEK-ERK-MNK1-dependent manner. An MNK1 inhibitor attenuated the increase of P2X (4)R in microglia under inflammatory or neurodegenerative conditions [159].

JNKs regulate various physiological processes such as brain development, repair, and memory formation but are potent effectors of neuroinflammation and neuronal death [56,161]. LPS, TNF-α and thrombin induced a robust activation of JNKs in primary microglial cultures. Inhibition of JNKs with an inhibitor SP600125 reduced the LPS-induced metabolic activity and the AP-1 inducible genes such as cyclooxygenase-2 (*cox-2*), *tnf-α*, *mcp-1* and *il*-6. Inhibition of ERK1/2 and p38 α affected LPS-induced cell enlargement [161]. Ciclosporin A or tacrolimus (FK506), potent immunosuppressants and neuroprotectants inhibited ERK, p38 MAPK and JNKs activation in primary microglial cultures stimulated with LPS. The treatment blocked many microglial functions: amoeboid transformation, migration, cytokine gene expression (*il-1β*, *il-6*, *tnfα*) and NO production [177]. The mechanism of inhibitory effects of immunosuppressants on phosphorylation of ERK, p38, and JNKs is unknown. The inhibition does not fully rely on inhibition of calcineurin activity as both drugs act at doses higher than required for calcineurin inhibition in glial cells. FK506 is more effective, therefore, the inhibition of FK506-binding proteins (FKBP) and downstream signaling molecules is likely.

Termination of MAPK signal transduction is carried out by dual-specificity phosphatases (DUSP/MKP), dephosphorylate the regulatory threonine and tyrosine residues on MAPKs [138]. DUSP3 is expressed in human and mouse monocytes and macrophages and its deficiency in mice led to tolerance to LPS-induced septic and cecal ligation and puncture shock. Resistance to LPS was macrophage-dependent and associated with reduced TNFα production, impaired ERK1/2 activation and increase of M2-like macrophages in Dusp3-KO mice [142].

MKP-1 (MAPK phosphatase-1), which preferentially dephosphorylates p38 MAPK and JNK, is present in the nucleus. Mkp-$1^{-/-}$ peritoneal macrophages exhibited prolonged p38 and JNK activation as well as enhanced TNF-α and IL-6 production upon LPS challenge [179]. MKP-1 deletion exacerbated stroke outcome in a MCAo model and consistently the MKP-1 pharmacological inhibitor worsened stroke outcome of wild type mice. MKP-1 deletion increased microglial JNK and p38 MAPK activation, IL-6 and TNF-α production, and apoptotic responses after MCAo [104]. Several compounds with an anti-inflammatory activity such as endocannabinoid anandamide [33] or JWH015 (a CBR type II

agonist) act by upregulation of MKP-1 or MPK-3 expression in microglial cells, reducing p-ERK1/2 level and cell migration in LPS-stimulated primary microglial cultures [134]. Dexamethasone (Dex) had the inhibitory effect on the *mcp-1* mRNA and protein expression, and suppressed ROS and NO release, migration and inflammatory responses of activated microglial cells in MKP-1 dependent manner [185]. Overexpression of MKP-1 or inhibition of JNK and p38 MAPKs with specific inhibitors down-regulated the expression of *nox-2* and *inos* in activated BV-2 cells in MKP-1 dependent manner [62].

The expression of CD45, a membrane-bound protein-tyrosine phosphatase (PTP) was found elevated in microglia in AD brain compared with controls. Stimulation of microglial CD45 by anti-CD45 antibody inhibited p44/42 MAPK and production of NO and TNFα, suggesting that CD45 is a negative regulator of microglial activation. Cultured microglia from CD45-deficient mice were hyper-responsive to Aβ and when crossed with a transgenic mouse model of AD (APPswe mice) demonstrated markedly increased production of TNF-α compared with CD45KO/APPswe mice [152].

Increased activities of MAPK, in particular p38 MAPK, and their multifaceted involvement in the regulation of the inflammation mediator synthesis, make them potential targets for anti-inflammatory therapeutics. MAPK inhibitors, capable of reducing both the synthesis of pro-inflammatory cytokines and their signaling, emerge as attractive compounds blocking neuroinflammation resulting in many ongoing clinical trials [2].

1.4. Cytokine signaling via JAK/STAT in microglia activation

Interferons (IFNs) are important signal transduction mediators in the innate immune system influencing viral infections, inflammation, and immune surveillance [81]. IFN-α and IFN-β are the predominant Type I IFNs in the CNS, mostly produced in microglia and astrocytes. The signaling pathway for both IFN-α and IFN-β involves IFNAR, a common cell surface receptor. IFNAR associates with STAT1 and STAT2, together with IFN regulatory factor (IRF)9 form a complex that binds to IFN-stimulated response element (ISRE) in promoters of many genes. It stimulates a type I IFN-dependent transcription of *IRF7*, described as a master regulator of type I IFN signaling. IFN-γ is a type II interferon which triggers antiviral and adaptive immune responses through a Jak-STAT signaling pathway [122].

Members of the JAK family include: Jak1, Jak2, Jak3, and Tyk2. Functional and physical association of Jak1 with receptors for the type I (IFN-α/β), type II (IFN-γ) interferons, IL-2, and IL-6 have been reported. Biochemical studies and analyses of Jak2-KO tissues confirmed an important role of Jak2 in the response to receptors binding the single-chain peptides, IL-3, 5 and GM-CSF cytokine families, as well as the IFN-γ receptor. Leukocyte-specific Jak3 associates with the IL-2 receptor γ-chain, which serves as a component for the receptors of cytokines (IL-4, -7, -9, -15, -21). Tyk2 associates with receptors for IFN type I, IL-6, IL-10 and IL-12/23 cytokine families [139] Table 1. summarizes various elements of JAK/STAT signaling employed by cytokines and growth factors.

Cytokine-derived immune signaling is strictly regulated with respect to both magnitude and duration. Inhibitory molecules including the suppressors of cytokine signaling (SOCS1–7), cytokine-induced STAT inhibitor (CIS) and protein inhibitor of activated STAT (PIAS) form the negative feedback regulation of JAK-STAT signaling [46,176]. Those inhibitory proteins inactivate JAKs and block the access of STATs to receptor binding sites. SOCS inactivate JAKs, CIS binds to STAT on the activated receptor and PIAS associates with tyrosine-phosphorylated STAT dimers. The myeloid-specific SOCS3-deficient mice were more vulnerable to myelin oligodendrocyte glycoprotein (MOG)-induced EAE (experimental autoimmune encephalomyelitis), with a severe, non-resolving form of disease, showed enhanced infiltration of inflammatory cells and demyelination in the cerebellum. Myeloid-specific SOCS3-deficient mice had enhanced STAT3 signaling,

Table 1
Components do JAK/STAT signaling.

Ligand	Kinase	STAT protein
IFNα/β	TYK2, JAK1	STAT1, STAT2
IFNγ	JAK1, JAK2	STAT1
IL6, IL11, OSM, LIF, CNTF	JAK1 (JAK2)	STAT3, STAT1
IL12, IL23	JAK2, TYK2	STAT4
EPO, GH, PRL, TPO	JAK2	STAT5
IL3, IL5, GM-CSF	JAK2	STAT5
IL2, IL7, IL9, IL15	JAK1, JAK3	STAT5
IL4, IL13	JAK1, JAK3	STAT6
EGF, FGF, HGF, PDGF	JAK1, JAK2	STAT1, STAT3, STAT5

Abbreviations: CNTF, ciliary neurotrophic factor; IFN, interferon; IL, interleukin; EGF, epidermal growth factor; EPO, erythropoietin; FGF, fibroblast growth factor; GH, growth hormone; GM-CSF, granulocyte macrophage colony stimulating factor; HGF, hepatocyte growth factor; LIF, leukemia inhibitory factor; OSM, oncostatin M; PDGF, platelet derived growth factor; PRL, prolactin; TPO, thrombopoietin.

upregulated expression of inflammation mediators (*inos*, *il-1β*, *il-12p40*, *il-23p19*, *il-6*, *ccl2*, *cxcl10*, and *ifr5*) and an immune response dominated by Th1 and Th17 cells. Adoptive transfer of M2 macrophages into myeloid SOCS3-deficient mice delayed onset and reduced severity of disease [128].

JAC2 was activated by LPS and via Stat5 regulated LPS-induced IL-6 expression [82]. Resveratrol had anti-inflammatory effects in LPS-stimulated murine N13 microglial cells through up-regulating *socs-1* expression, as this effect was lost in the presence of *socs-1* siRNA [32]. Resveratrol treatment also prevented the pro-inflammatory effect of LPS or fibrillar Aβ on BV-2 and RAW 264.7 cells by inhibiting activation of STAT1 and STAT3, IκB phosphorylation, IL-1β, TNF-α and IL-6 secretion [17].

Protein tyrosine phosphatases such as Src homology 2 (SH2) domain-containing phosphatases (SHP)-1 and SHP-2 are other regulators of cytokine signaling. SHP-1 deficient mice (spontaneous mutations at the "motheaten" locus) had weaker microglial activation and were less susceptible to focal cerebral ischemia induced by MCAo [6]. LPS stimulated microglia from those mice produced more NO, IL-1β and TNF-α [178].

2. Transcriptional programs underlying microglia activation

The initiation of inflammation requires global activation of gene expression, postranscriptional regulation, epigenetic modifications and changes in chromatin structure. Analyses of many transcriptomics data derived from human and murine microglia show that microglia express a different set of genes than peripheral macrophages [15,38,55]. Microglial functions are regulated in a signal-specific manner, allowing these cells to develop specific functional programs [7,15,34]. The inflammatory activation of cultured microglia (stimulated with LPS) induced transcriptional responses that are similar to inflammatory responses of microglia under pathophysiological conditions *in vivo* []. Global gene expression profiling on the ischemic cortex/sham operated cortex and Gene Ontology functional analysis revealed overrepresentation of functions such as responses to wounding and external stimuli, inflammatory, immune system and defense response, response to stress, cell death [177]. The same categories of genes were overrepresented in LPS-stimulated microglia. The analysis of predominant signaling pathways in LPS-stimulated microglia revealed NF-κB and JAK/STAT pathways on the top of the ranking list. Transcriptomic analysis of microglia from the corpus callosum during demyelination/remyelination in the mouse cuprizone model, showed the presence of a microglia phenotype that supports early remyelination and continue throughout the remyelination process. The changes indicated microglia involvement in the phagocytosis of myelin debris and apoptotic cells during demyelination (*Lrp1*, *Calr*, *Cd14* and *Itgb2*, *Itgam*, and *Lgals3*). Microglial expression of cytokines and chemokines supporting recruitment of endogenous oligodendrocyte precursors to the lesions (e.g., *Cxcl10*, *Cxcl13*

and *Igf1*, *Tgfb1*, *Pdgfa*, and *Pdgfb*), and tissue remodeling (e.g., *Mmp12* and *Mmp14*) was detected [121].

Transcriptome analysis of microglial cultures stimulated to induce M1 (LPS stimulation) or M2-like activation (exposure to glioma-conditioned medium, GCM) uncovered two patterns of gene expression. Many inflammation- and immune system-related genes and signalling pathways characteristic for M1 inflammatory activation of microglia failed to be induced in glioma-exposed microglia. Glioma-induced activation was associated with induction of genes coding for transcription regulators: ID (inhibitor of DNA binding) 1/3, c-Myc, Smad7, markers of the M2 phenotype: Arg1, MT1-MMP, CXCL14, IGF-1, and numerous cytokines/chemokines implicated in immune cell trafficking [34]. Most of the genes upregulated by GCM were down-regulated in the inflammatory microglia. Gene expression patterns for the BV-2 microglial cell line stimulated with viral dsRNA Poly (I:C) or LPS (as evaluated by RNA-seq) were similar as gene patterns detected in LPS-stimulated rat microglia cultures. Transcription factor motif analysis (-950 to $+50$ bp of the 5′ upstream promoters) demonstrated that the DNA sequences for NF-κB, IRF1, and STAT1 were significantly enriched in TLR agonists-stimulated microglia [28].

Interpretation of microglial transcriptome-based studies have to be considered with a caution. Comparison of the transcriptomes of FACS-sorted CX3CR1-GFP labeled microglia isolated from the brainstem of adult mice to laser capture microdissection (LCM) demonstrated majority of shared transcripts but also transcripts unique to FACS-isolated and LCM-captured microglia. A half of LCM-isolated microglial transcripts represented genes characteristic for neurons and glia, and these transcripts were not translated into protein. Levels of oligodendrocyte and neuronal transcripts were increased in microglia following the induction of EAE, while only the myelin basic protein transcript level increased in microglia after traumatic brain injury [143]. A very useful resource in studying microglial transcriptome is a recently published Glia Open Access Database (GOAD) (www.goad.education) which contains a collection of published and unpublished transcriptomic data, including data sets from isolated microglia, astrocytes and oligodendrocytes under physiological and pathological conditions [59]

Convergence of neuroinflammation-related signaling pathways on specific transcription factors discussed above and transcription factor motif analysis of co-regulated genes in various microglial data sets established transcription factors NF-κB/Rel, AP-1, IRFs, and STATs as master regulators of inflammatory gene expression in microglia under neuroinflammatory conditions.

2.1. The NF-κB transcription factor

The NF-kappaB (NF-κB) protein family consists of distinct members, including NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB, and c-Rel, that form homo- and heterodimers. In resting cells, NF-κB dimers are sequestered in the cytoplasm through interaction with IκB proteins (IκBα, IκBβ and IκBε) or the precursor proteins p100 and p105. Induction of NF-κB depends on the phosphorylation of IκBs on serine residues resulting in IκBs ubiquitination by the E3 ubiquitin ligase and degradation by the proteasome [53]. Activation of NF-κB in microglia, through the increase of IKKβ expression, induced a classical M1 pro-inflammatory phenotype [37] The early phase of inflammatory response is predominantly associated with the nuclear accumulation of p50/RelA dimer, known to activate transcription of proinflammatory genes coding for cytokines TNFα, IL-1β and IL-6, iNOS, and proteolytic enzymes in microglia. NF-κB precursors are processed by the proteasome to produce the mature transcription factors p50 and p52 [101]. Both TLR-dependent and -independent pathways engage the IKK complex and related kinases TBK-1 and IKKε. Activation of the NF-κB and IRF transcription factor pathways are essential early steps of immune activation [77].

There is the extensive literature on the involvement of NF-κB in regulating cytokine cascades during neurodegeneration and different contribution of distinct NF-κB subunits to inflammatory responses in glial cells, neuronal cell death or survival [111,124,133]. Agents that inhibit NF-κB usually suppress LPS-induced Cox-2 expression, but the role of microglial NF-κB in neuronal injury is complicated by the fact that activated microglia produce neurotrophic factors such as NGF, bFGF, and modulation of NF-κB have been shown to prevent neuronal death in various experimental models of neurodegenerative disorders [111].

There are a few studies directly assessing a role of NF-κB in microglial functions. LPS, IL1β and TNF-α activated NF-κB and stimulated RANTES (regulated upon activation of normal T cell expressed and secreted) production in a NF-κB dependent manner in human fetal brain microglia [61]. The expression of Jmdj3, a transcription factor and histone demethylase that controls the inflammatory responses, is regulated by NF-κB in LPS-stimulated primary rat microglial cultures similarly as in peripheral macrophages [127].

2.2. The AP-1 transcription complex

The AP-1 (activator protein 1) complex, composed of Fos (c-Fos, Fos, Fra1/2) and Jun (c-Jun, JunB, JunD) proteins, binds to regulatory DNA sequences (AP-1 and ATF/CRE-like consensus motifs) and activates the basal transcription machinery. While c-Jun is mostly activated by phosphorylation, other components of the AP-1 complex are transcriptionally activated. ETS-domain ternary complex factors (TCFs), including Elk-1 and SAP-1 are phosphorylated and activated by ERK, JNK and p38, or ERK and p38, respectively, which leads to enhanced transcription of the c-*fos* gene. Up-regulation of c-*jun* and c-*fos* followed by formation of the AP-1 complex is required to target promoter elements of many genes. Phosphorylation of c-Jun and ATF-2 proteins by JNK and p38 MAPK leads to increased transcriptional activity of the AP-1 transcription factor [54,70]. The AP-1 target genes include genes coding for many cytokines, chemokines and their receptors, metalloproteinases and their inhibitors, and are upregulated during neuroinflammation [54] but their regulation by AP-1 complex in microglial cells has not been evaluated. Alternative splicing of *Fosb* mRNA generates *Fosb* and *ΔFosb*, encoding full-length FosB and ΔFosB proteins, respectively. Microglia express equivalent levels of *Fosb* and *ΔFosb* mRNAs to hippocampal neurons and six microglial genes were identified as dependent on FosB, including *c5ar1 and c5ar2*, that encode receptors for complement C5a. Microglia isolated from Fosb-KO had lower chemotactic responsiveness toward C5a. Microglial activation after kainate-induced excitotoxicity was significantly diminished in Fosb-KO hippocampus, levels of *il6* and *tnf* mRNAs were reduced. The results suggest that FosB contributes to a neuroinflammatory response in the hippocampus through regulation of microglial C5ar1 and C5ar2 expression [119].

Recent studies using mice lacking *JunB* in myeloid cells showed that JunB is a modulator of both classical and alternative macrophage activation. JunB was required for expression of *il1b* and several M1 inflammation-related genes in macrophages treated with LPS and other immunostimulatory molecules. Furthermore, JunB modulated expression of canonical markers of M2 activation in macrophages treated with IL-4 [36]. Myeloid-restricted deletion of *Junb* reduced type 1 immune activation (an experimental cerebral malaria model), which was associated with reduced cerebral pathology and improved survival during infection. Myeloid JunB deficiency also compromised M2 activation (a hookworm infection model) leading to diminished cytokine production, eosinophil recruitment and increased parasite load. These results demonstrate that JunB in myeloid cells shapes host responses and outcomes during type 1 and type 2 infections [36].

2.3. Signal transducers and activators of transcription

STATs (Signal Transducers and Activators of Transcription) are a family of transcription factors (STAT 1, 2, 3, 4, 5a, 5b, 6) that are

activated in response to over 40 different cytokines or growth factors. Each STAT protein has a DNA binding domain, a src-homology 2 (SH2) domain necessary for homo- or heterodimerization and a conserved tyrosine residue 705 phosphorylated by tyrosine kinases. Phosphorylated STAT dimers translocate to the nucleus and bind to a consensus DNA element upstream of regulated genes. Stat5a and Stat5b proteins alone are weak activators of transcription, and they act predominantly by cooperating and interacting with other proteins. The activation of individual STATs is temporary and usually lasts from a few minutes to several hours under normal physiological conditions [99,123].

Hundreds of genes have been identified by knockout and biochemical studies as potential Stat3 targets, but only a small fraction has been proved to be direct Stat3 targets in various cells. A meta-analysis of 29 ChIP-seq data sets of STATs DNA binding in macrophages, B and T lymphocytes, embryonic fibroblasts, embryonic cells and liver cells revealed that most of the classical STAT-binding sites were located near genes with cell-specific expression, and STAT binding pattern is unique in each cell type [74]. Studies of Stat1 and Stat3 binding sites in LPS-activated primary microglial cultures, using chromatin immunoprecipitation and promoter array hybridization (ChIP-chip), revealed novel Stat-target genes including *jmjd3*, *ccl5*, *ezr*, *ifih1*, *irf7*, *uba7*, and *pim1* []. Functional manipulations of Stat signaling (silencing or overexpression of Stats) were performed on BV2 cells. While knockdown of individual Stat had little effect on the expression of tested genes in BV2 microglial cells, knockdown of both Stat1 and Stat3 inhibited the expression of *jmjd3* and other inflammatory genes. In BV2 cells overexpression of constitutively active Stat1 and Stat3 was capable of recapitulating global gene expression profiles and cytokine production (except IL-10). Transcriptional regulation of Jmjd3, a transcription factor and histone demethylase [30], by Stat1 and Stat3 is a novel mechanism of transcriptional control of inflammatory gene expression in microglia [127].

2.4. Interferon responsive factors

Interferon regulatory factors (IRFs) comprise a family of transcription factors that recognize DNA similar in sequence to the IFN-stimulated response element (ISRE) and modulate different sets of genes, depending on the cell type and/or the nature of cellular stimuli. Four IRFs: IRF1, IRF3, IRF5, and IRF7 have been implicated as positive regulators of type I IFN elicited gene transcription [66,184]. Gene-targeting study demonstrated that in haematopoietic cells from Irf5-deficient mice, the induction of pro-inflammatory cytokines *il-6*, *il-12* and *tnf-α* by various TLR ligands was impaired, whereas induction by interferon-α unimpaired [151].

Functions of IRFs in microglial cells are still poorly known. In cultured microglial cells, signaling through the prostaglandin-E2 (PGE2) EP4 receptor attenuated levels of Aβ-induced inflammatory factors and potentiated Aβ phagocytosis. Microarray analysis demonstrated that EP4 stimulation had an opposite effect on Aβ-driven gene expression changes in microglia. The genes modulated by EP4 stimulation were enriched in targets of IRF1, IRF7, and NF-κB transcription factors [166]. The expression of IRF7 was increased in LPS treated microglia *in vitro* and *in vivo*. Knockdown of IRF7 using siRNA suppressed the expression of LPS-induced genes and reduced phosphorylation of STAT1 [153]. Also IRF8 is strongly implicated in initiation of a reactive phenotype of microglia [114]. Loss of IRF8 did not affect development and colonization of CNS by microglia but IRF8-deficient microglia had less elaborated processes with reduced expression of microglial marker IBA1, reduced phagocytic capacity and were less proliferative in mixed glial cultures than wild-type microglia. Exogenous macrophage colony stimulating factor (CSF1) restored defective proliferation of IRF8-deficient microglia in mixed glial cultures and granulocyte-macrophage colony stimulating factor (CSF2) intensified microglial proliferation. Cultured IRF8-deficient microglia had reduced *Il12B* mRNA levels in response to IFNγ and LPS. Defective scavenging activity of IRF8-deficient microglia was confirmed in the cuprizone-induced demyelination model in mice [60]. IRF8 was upregulated in the spinal cord microglia following a peripheral nerve injury. Its overexpression in cultured microglia promoted the inflammatory gene expression (*il1b*,*tlr2*, *p2rx4*, *p2rx12*, *cx3cr1*, *ctss*) and deficiency prevented these gene expression in the spinal cord. Furthermore, IRF8-deficient mice were resistant to neuropathic pain, and transfer IRF8-overexpressing microglia to a spine of normal mice produced pain [109]. IRF8 has been implicated in regulating microglial migration. Chemotaxis induced by ATP and a complement component C5a was suppressed in microglia lacking IRF8 (Irf8-KO). Consistently, phosphorylation of Akt (a kinase participating in ATP-induced chemotaxis) and the expression of motility-related genes such *p2ry12p*,*p2rx4* and *adora3* (coding for purinergic and adenosine receptors, respectively) was abolished in Irf8-KO microglia. Moreover, Irf8-KO microglia showed reduced expression of *antpd1* coding for an ectonucleotidase ENTPdase1, which hydrolyzes ATP to ADP and adenosine monophosphate and is predominantly expressed in microglia [110].

3. Epigenetic control of microglia activation

3.1. Epigenetic mechanisms control transcriptional responses

Epigenetic mechanisms are mediated by non-coding RNA, post-translational modifications (mainly methylation, acetylation, and phosphorylation) of histones and other chromatin proteins that bind DNA, and by methylation (5-methylcytosine [5mC]) and hydroxymethylation (5-hydroxymethylcytosine [5hmC]) of DNA [11]. Global changes of DNA methylation were detected in Alzheimer disease. Global levels of 5mC and 5hmC positively correlated with amyloid β, tau, and ubiquitin loads. Immunoreactivity for 5mC and 5hmC were significantly increased in affected structures of AD patients, mostly in neurons [25].

3.2. A role of miRNAs in microglia activation

MicroRNAs (miRNAs), a family of non-coding RNAs ~22 nucleotides in length, are post-transcriptional regulators of gene expression. miRNAs silence target genes by binding to their 3′-untranslated region (3′-UTR) of messenger RNA (mRNA) [49]. Over the past decade, a growing body of evidence has revealed the important role of miRNAs in inflammation and immunity [8,102], including their capability of regulating the activation of macrophages [102]. However, only in the past couple of years microglia have been a matter of study, and thus the relevance of miRNAs on specifically microglial activation is still not fully understood. Table 2 summarizes the current state-of-the-art on this subject.

Cardoso et al. were the first to report the pro-inflammatory effect of miR-155 *in vitro* [18]. LPS stimulation of N9 murine microglial cell line and primary microglia cultures promoted the expression of miR-155. The overexpression of miR-155 led to a decrease of SOCS-1, a negative regulator of inflammation. miR-155 was shown to be responsible for the production of pro-inflammatory mediators, as transfection of N9 cells with anti-miR-155 oligonucleotides lead to a decrease of IFNβ, TNFα, IL-6, and INOS after incubation with LPS. Furthermore, the inhibition of miR-155 decreased the LPS-stimulated microglia-mediated toxicity on primary cortical neurons, suggesting that miR-155 can be a target for neuroprotection [18]. This notion was reinforced by observation of the miRNA expression profile of primary murine microglia cultures after M1-polarization with LPS and M2a-polarization with IL-4. Microglial activation towards the M1-phenotype was accompanied by up-regulation of miR-155 and down-regulation of miR-689 and miR-124.,miR-155 seemed to be the main miRNA driving this polarization. On the other hand, M2a-polarization of microglia resulted in a higher expression of miR-145 and miR-214, and lower expression of miR-711 [38].

Table 2
A summary of miRNAs effects in microglia.

miRNA	Effect	Potential mechanism	Reference
miR-155	Pro-inflammatory	Decrease of SOCS-1	[18]
miR-204	Pro-inflammatory	Inhibition of SIRT1	[100]
let-7c-5p	Anti-inflammatory Anti-apoptotic	Inhibition of pro-inflammatory mediators Reduction of caspase 3	[117]
miR-203	Anti-inflammatory	Decrease of MyD88	[173]
miR-21	Anti-apoptotic	Decrease of FasL	[180]
miR-181-c	Anti-inflammatory	Suppression of TLR4	[182]
miR-424	Anti-inflammatory Inhibition of microglial activation	Reduction of pro-inflammatory mediators Reduction of CDC25A, CDK6 and cyclin D1	[183]

Abbreviations: CDC25A, cell division cycle 25A; CDK6, cyclin-dependent kinase 6; FasL, Fas ligand; miRNAs, microRNAs; MyD88, myeloid differentiation factor 88; SIRT1, sirtuin 1; SOCS-1, suppressor of cytokine signaling 1; TLR4, Toll-like receptor 4.

Microglial activation by OGD (oxygen-glucose deprivation) was associated with the release of Fas ligand (FasL), which has a pro-apoptotic effect in neurons, and the decrease of miR-21 mRNA expression [180]. Transfection of microglia with miR-21 mimics, and consequent over-expression of miR-21, decreased both mRNA expression and FasL protein level [180]. Neurons treated with conditioned media (CM) from miR-21-transfected microglia subjected to OGD presented reduced apoptosis, when compared to neurons treated with CM from non-transfected cells [180]. In primary microglial cultures subjected to OGD, miR-203 reduced MyD88-mediated inflammation. Additionally, miR-203 mimics were protective in experimental stroke in mice, decreasing protein levels of the inflammatory cytokines IL-8 and TNFα, whilst being able to reduce the expression of MyD88 and NF-κB in the ischemic brain [173].

Earlier this year, Li et al. reported miR-204 as an inhibitor of SIRT1 expression. miR-204 upregulates pro-inflammatory markers, such as iNOS and IL-1β, in N9 cells. Furthermore, the inhibition of miR-204 was able to restore the LPS-induced reduction of SIRT1, suggesting that the pro-inflammatory effect of miR-204 can be partly due to a inhibition of SIRT1, and that resveratrol can restore SIRT1 levels in microglia and, thus, decrease microglia-driven inflammation [100]. *In vitro*, LPS-stimulated and oxygen and glucose-deprived BV2 cells and primary microglia cultures showed a decrease in let-7c-5p expression. Treatment of microglia with CM from neurons subjected to OGD produced similar results. Over-expressing let-7c-5p in BV2 cells led to a decrease of both LPS and OGD-induced inflammation. In addition, let-7c-5p reduced the expression of caspase 3, by directly targeting its 3′-UTR [117]. Considering that miRNAs are important components of inflammation, NF-κB pathway appears as an ideal target of miRNA regulation in microglia. Consistent with this premise, the 3′-UTR of *MyD88* mRNA contains binding sites for miR-203 [173], and miR-181c targets the 3′-UTR of *TNF-α* [181] and *TLR4* [182] mRNAs.

The expression of miRNAs is controlled by promoters that in some cases are regulated by NF-κB. OGD-activation increased TLR4 and NF-κB/p65 protein expression in primary microglia cultures, which was blocked by over-expressing miR-181c [182]. miR-181c-induced decrease in inflammatory markers was mediated by TLR4 suppression [182].

Although there is a clear need for further research on this subject, an application of miRNAs appears to be a promising strategy for the treatment of diseases characterized by microglia-mediated toxicity. Recent *in vivo* studies have addressed this growing potential. Ponomarev and colleagues have recently shown that miR-124 regulates microglial activation, both *in vitro* and *in vivo*. miR-124 expression was elevated in activated microglia. In macrophages, miR-124 was responsible for inhibiting C/EBP-alpha directly and, subsequently, transcription factor PU.1. In addition, over-expression of miR-124 induced phenotypic changes in macrophages, displaying features like those of ramified resting microglia, and shifted their polarization from the classical M1 activation to the alternative M2 [126]. *In vivo*, treatment with miR-124 reduced inflammation and ameliorated clinical symptoms in diverse mouse models of EAE. Concomitantly, miR-124-treated mice presented mostly $CD11b^{+}CD45^{low}$resting microglia, as opposed to activated microglia in the sham group [126]. Zhao et al. identified miR-424 as a regulator of microglia-mediated inflammation in ischemic stroke. The level of miR-424 decreased in plasma of patients after ischemic stroke, and similar findings were reported in mice following MCAo Pre- and post-treatment of mice with miR-424 was protective and decreased microglial activation. Over-expression of miRNA-424 attenuated the increase in the level of Iba-1 and TNFα 8 h after MCAo, as well as decreasing cell-cycle activators CCND1, CDC25A and CDK6, which could be the mechanism responsible for the decrease of microglial activation [183]. These results were reproduced *in vitro* in BV2 microglial cells, where miR-424 mimics reduced the mRNA expression of TNFα and IL-1β after 1 h of OGD, and decreased cell-cycle activators expression [183].

miRNA let-7c-5p was decreased in patients with ischemic stroke and in mice after MCAo [117] Over-expression of let-7c-5p was able to protect mice against ischemic injury. Moreover, it also reduced post-ischemic inflammation, by significantly reducing the expression of *il-6*, *cox-2*, *tnf-α and inos* mRNAs in the cortical penumbra, and decreasing Iba1 staining in the ipsilateral cortex [117].

Taken together, these results suggest that targeting miRNAs can be a neuroprotective therapeutic approach worth considering in neurological diseases characterized by microglial activation.

3.3. Histone modifications

The balance of positive and negative histone marks at gene promoters and distal regulatory elements regulates transcription rates. Combinations of histone marks define chromatin states, which determine basal transcription rates, magnitude and kinetics of gene expression [69,156]. Epigenetic marks are typically more stable than the rapidly modulated post-translational modifications of signaling proteins and may persist after the original stimulus has resolved. Epigenetics provides a mechanism for converting transient short-lived signals into more persistent cellular responses lasting several hours or days (or longer). Histones are subject to post-translational modifications that greatly influence gene expression, as they allow or deny access of the transcriptional apparatus to DNA. These modifications include methylation, acetylation, phosphorylation, ubiquitination, amongst others [79,80].

Histone acetylation and methylation are the most studied modifications of histone. The state of histone acetylation depends on histone acetyltransferases (HATs) and histone deacetylases (HDACs), enzymes responsible for acetylation and deacetylation, respectively. HATs, that transfer an acetyl group from acetylCoA on lysine histone tails, have shown distinctive functions in active genes and include pCAF, CBP, p300, Tip60, MOF and GCN5 [165]. HDACs act through the removal of the acetyl group from lysine residues on histones, which promotes chromatin condensation and thus makes DNA less accessible for transcription factors, decreasing gene expression. Human HDACs are divided into five main subtypes [78]. Class I (HDAC1, 2, 3 and 8) and class II HDACs are Zn2 + −dependent deacetylases and share similar structure. The latter presents tissue-specific expression [107] and can be subdivided into class IIa (HDAC4, 5, 7 and 9) and IIb (HDAC6 and 10). Class IV (HDAC11) shares characteristics of both class I and class II HDACs [40]. Lastly, class III HDACs (Sirtuin1-7), unlike other HDACs, have a NAD+-dependent activity, displaying distinctive structure and function [120]. Several HDAC inhibitors (HDACi) have been developed over the last years, with different HDAC inhibition selectivity [186].

The acetylation state is tuned by the opposing activities of HATs and HDACs [125] working in equilibrium. Neurodegeneration, a state characterized by death of neurons and neuroinflammation, is associated with a loss of acetylation equilibrium [137], mainly due to a decrease of HAT activity [135] With the aim to investigate the effect of increasing acetylation, a number of studies have shown the beneficial activity of HDAC inhibition in animal models of AD [85,129,148], HD [58,157], and multiple sclerosis ([16,41] Particularly, a significant body of data comes from models of acute CNS injury such as stroke [35,88,131,171, 175] and trauma [27,140,162]. Data are summarized in the Table 3.

These studies support the notion of HDACi as a valid treatment for neurodegenerative disorders. Furthermore, some HDACi are already approved for human use in diseases such as epilepsy [42] and cancer [31, 87]. Recent published data showed improvements in adding valproate to the standard post-insult stroke treatment in humans [96]. However, due to the low number of patients included in the study, further clinical trials with larger number of patients are needed to demonstrate the efficacy of valproate in stroke.

3.4. Microglia as a target of HDACi treatment in ischemic stroke

The acetylation level of histone H3 decreases in animal models of ischemic stroke [88,131]). HDAC expression was shown to be altered in models of stroke both *in vivo* and *in vitro* [5]. Moreover, microglia can be cellular targets of HDAC inhibition, and two studies demonstrated an HDACi-induced protection against ischemic damage that depends on anti-inflammatory effects on microglia [88,171]. However, little is known about the effect of HDAC inhibition in stroke-induced microglia inflammation.

Three different HDACi, valproic acid (VPA), sodium butyrate (SB) and trichostatin A (TSA), reduced ischemic injury when administered after pMCAO [88]. VPA and SB reduced the number of OX42-positive cells in the ischemic corpus callosum both 24 and 48 h after the ischemic insult. These drugs decreased the activity of iNOS. The authors also reported that VPA increased the number of caspase-3-positive microglia, suggesting microglial apoptosis, which could explain, at least partially, an anti-inflammatory effect of VPA. HDACi were able to block the decrease in the acetylation of histone H3 seen after MCAo, which supports the notion that protection was mediated by an inhibition of HDAC activity. However, there is no data yet on the role of HDACi on histone modifications at the promoters of apoptotic genes in microglia. VPA, SB and TSA induced apoptosis in primary rat microglial cultures. This effect occurred at HDACi concentrations that increased histone H3 acetylation, that suggests HDAC inhibition as a mode of action [20].

3.5. Effects of HDAC inhibition on microglia-induced inflammation

Treatment of microglia with HDACi was shown to amplify LPS-induced inflammation in various cellular models [141]. Experiments performed by our research group are in line with this finding (unpublished data). In contrast, a treatment with HDACi, such as scriptaid, SAHA, VPA [164], and pretreatment with TSA [147], led to a decrease in inflammatory markers. The complex nature of HDAC inhibition in microglia *in vitro* must be highlighted. Opposing effects of the treatment of microglia with HDACi were reported by the same research groups [20,150]. Chen *et al.* [20] described both a decrease and increase in the LPS-induced inflammatory response of microglia, depending on whether cells were pretreated with SB or TSA, or if they were added concomitantly with LPS. Suuronen *et al.*, [150] reported similar findings, where TSA and SAHA enhanced the inflammatory effects of LPS when added at the same time. TSA induced the expression of *il-6 and inos* mRNA and the secretion of IL-6, TNFα and NO. This potentiating effect was blocked by NF-kB inhibitors [149,150]. However, when microglia were pretreated with TSA before LPS stimulation, TSA had an anti-inflammatory effect [150]

While Huuskonen *et al.* [63] reported that SB, either in pretreatment or added at the same time to LPS, could potentiate LPS-induced inflammation in N9 microglial cells, they also reported different effects in transformed vs. non-transformed microglial cells, as pretreatment with SB reduced LPS-induced inflammation in rat primary microglial cultures. Simultaneous treatment of SB with LPS did not reduce inflammation to the same extent, as only IL-6, but not TNFα or NO, decreased. To understand the mechanisms underlying the dual effect of HDACi on microglia activation when applied in diverse "temporal" windows deserves further investigation. Most important will be to understand if *in vitro* findings can be reproduced *in vivo*, and whether the HDACi-induced protection in stroke animal models, is because or in spite of their effect on microglia.

The role of HDAC and effects of HDACi on the M1-M2 microglial polarization are still for the most part unknown, and have been a matter of study only in the last couple of years. Singh *et al.* [141] reported that VPA and SB treatment results in shifting pro-M1 and anti-M2 activation in LPS-activated rat microglia, as both drugs increased *tnfα* and *il-1β* mRNA levels and decreased *il-10* gene expression. On the other hand, TSA pretreatment could diminish not only LPS-induced inflammation but also IL-4-induced expression of *arginase-1* and *Fizz1* mRNA, coding for M2 phenotype markers [72], suggesting that pretreatment with HDACi could induce deactivation of microglia and turn them into less reactive cells, thus inducing microglial immunosuppression, and contributing to the overall anti-inflammatory effects HDACi *in vivo*. Recently, Wang and colleagues reported [164] that Scriptaid decreased the expression of M1-polarization markers (iNOS, CD16) and increased those of M2 (CD206, IL-10) in a model of traumatic brain injury. With such opposing and scarce data, it is premature to comment on the role of HDACs in microglia activation, both *in vitro* and *in vivo*.

4. 5. NF-κB acetylation in the control of microglia activation

Acetylation of RelA on specific lysine residues (K122, 123, 218, 221, and 310) is a dynamic process that differently affects the RelA interaction with IκBα, the DNA-binding ability and specific transcriptional activity of the protein [21,83]. The acetylation state of RelA, likewise that of histones, is regulated by HATs and HDACs [50]. Members of class I HDACs, HDAC1, HDAC2 and HDAC3 inhibited by vorinostat and entinostat (MS-275), are responsible for the general deacetylation of NF-κB/RelA [21]. Sirtuin 1, an atypical class III HDAC that requires

Table 3
Dualistic effect of HDAC inhibition in LPS-stimulated microglia.

HDAC inhibitor	Cell type	Treatment schedule in relation to LPS stimulation	Effect	Reference
SB and TSA	Rat primary microglia	Pretreatment	Anti-inflammatory	[20]
SB	Murine N9 cell line	Pre and simultaneous treatment	Pro-inflammatory	[63]
SB	Rat primary microglia	Pretreatment	Anti-inflammatory	
TSA	Human primary microglia	Pretreatment	Anti-inflammatory	[147]
TSA	Murine N9 cell line Rat primary microglia	Simultaneous treatment	Pro-inflammatory	[150]
TSA	Murine N9 cell line	Simultaneous treatment	Pro-inflammatory	[149]
Scriptaid, SAHA, VPA	Mouse primary microglia	Posttreatment	Anti-inflammatory	[164]

Abbreviations: SAHA, suberoylanilide hydroxamic acid; SB, sodium butyrate; TSA, trichostatin A; VPA, valproic acid.

NAD+rather than zinc as a cofactor and is activated by resveratrol, selectively deacetylates RelA at lysine 310 (K310) [94,174]. Recent studies show that pro-apoptotic transcription induced by NF-κB p50/RelA in acute brain ischemia [187,188] relies on the specific derangement of normal RelA acetylation. The p50/RelA which translocates to the nucleus either in primary cortical neuronal cultures exposed to preconditioning OGD, as well as in cortices of mice subjected to preconditioning MCAo, showed a general deacetylation. Conversely, RelA activated after noxious ischemia displayed a general deacetylation associated with a site-specific acetylation on the K310 residue. The relevance of K310 acetylation to RelA-mediated effects during ischemia was demonstrated by mutagenesis analysis. The substitution of lysine with arginine at the RelA 310 residue impaired the acetylation at this site. In cells expressing the mutated RelA subunit, the OGD-mediated *dmt1* (divalent metal trasporter-1) transcription [67] as well as the cell damage were totally prevented [94]. It suggested that a mismatch between "general" and "site-specific" (K310) acetylation of RelA, could be responsible for deleterious effects of NF-*k*B in brain ischemia. By undergoing such aberrant acetylation, RelA detached from the anti-apoptotic *Bcl-xL* gene promoter to bind the pro-apoptotic *Bim* gene promoter. The RelA binding was specifically followed by promoter–associated histone acetylation [95] . In spite of changing the acetylation state of RelA, lethal ischemia produced a significant reduction of global H3 histone acetylation [95], in line with previous evidence [35]. We found that treatments with MS-275 (HDAC1-3 inhibitor) and resveratrol (AMPK/SIRT1 activator) in the post-ischemic period, corrected the aberrant RelA acetylation and displayed a significant neuroprotective activity [95]. MS-275 and resveratrol in combination showed a long-lasting efficacy and a wide therapeutic window. Their beneficial effects were still evident 72 h after the MCAO and when administered within 7 h from the ischemic onset. Besides correcting the aberrant RelA acetylation, the combination of MS275 and resveratrol displayed a concerted effect on H3 histones acetylation. The drugs produced a shift of RelA binding from the *Bim* to the *Bcl-xL* promoter followed by a concomitant acetylation of H3 histones associated with the *Bcl-xL* promoter.

While the RelA acetylation on microglia activation and polarization in brain ischemia has not been investigated yet, it has been proved to play a role in the microglia activation and microglia-dependent neurotoxicity in mixed neuron/glial cultures exposed to amyloid-β. In line with previous data [9], Aβ stimulated NF-κB in microglia and astrocytes [19]. The Aβ-induced neuronal toxicity was strictly dependent on NF-κB activation in microglia, through the acetylation at the Lys310 RelA. Either microglia-targeted overexpression of SIRT1, which specifically deacetylated RelA at Lys310 [174], or pretreatment with the SIRT1 activator resveratrol, effectively decreased the levels of Ac-RelA, without affecting total RelA content. Both treatments ultimately reduced microglia-dependent neurotoxicity [19].

Whether the acetylation of RelA at Lys310 demonstrated in ischemic brain has a relevance to the M1/M2 polarization of microglia remains to be established. If this hypothesis will be confirmed, the epigenetic drug combination, class I HDACi and resveratrol, might represent an innovative therapeutic approach to modulate microglia polarization and drive brain repair.

Acknowledgements

This work was supported by H607962-1 ITN NeuroInflammation, 2012/04/A/NZ3/00630 grant from the National Science Center, Poland (BK), Ricerca Finalizzata Ministero della Salute RF-2010-2315142; and Fondazione CARIPLO 2014-0769 (MP).

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