Innate Defense Regulator Peptide 1018 Protects against Perinatal Brain Injury

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Objective: There is currently no pharmacological treatment that provides protection against brain injury in neonates. It is known that activation of an innate immune response is a key, contributing factor in perinatal brain injury; therefore, the neuroprotective therapeutic potential of innate defense regulator peptides (IDRs) was investigated.
Methods: The anti-inflammatory effects of 3 IDRs was measured in lipopolysaccharide (LPS)-activated murine microglia. IDRs were then assessed for their ability to confer neuroprotection in vivo when given 3 hours after neonatal brain injury in a clinically relevant model that combines an inflammatory challenge (LPS) with hypoxia–ischemia (HI). To gain insight into peptide-mediated effects on LPS-induced inflammation and neuroprotective mechanisms, global cerebral gene expression patterns were analyzed in pups that were treated with IDR-1018 either 4 hours before LPS or 3 hours after LPS+HI.
Results: IDR-1018 reduced inflammatory mediators produced by LPS-stimulated microglia cells in vitro and modulated LPS-induced neuroinflammation in vivo. When administered 3 hours after LPS+HI, IDR-1018 exerted effects on regulatory molecules of apoptotic (for, eg, Fadd and Tnfsf9) and inflammatory (for, eg, interleukin 1, tumor necrosis factor α, chemokines, and cell adhesion molecules) pathways and showed marked protection of both white and gray brain matter.
Interpretation: IDR-1018 suppresses proinflammatory mediators and cell injurious mechanisms in the developing brain, and postinsult treatment is efficacious in reducing LPS-induced hypoxic–ischemic brain damage. IDR-1018 is effective in the brain when given systemically, confers neuroprotection of both gray and white matter, and lacks significant effects on the brain under normal conditions. Thus, this peptide provides the features of a promising neuroprotective agent in newborns with brain injury.

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Perinatal brain injury is a major clinical problem associated with high neonatal mortality and morbidity and an increased risk of lifelong chronic disabilities. Despite improved survival rates, the absolute number of neurological handicaps of perinatal origin has not decreased. Neonatal encephalopathy occurs in 1 to 6 infants per 1,000 term births, with the risk of long-term neurodevelopmental disabilities further markedly increased in preterm infants.[1] There is currently no pharmacological treatment that provides neuroprotection in neonates.

Susceptibility and progression of central nervous system injury in the fetus and newborn are closely associated with an exacerbated innate immune response.[2–5] Neuroinflammation can be elicited by infectious

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challenge as well as tissue damage.[6] Experimental studies that use animal models of neonatal brain injury suggest that the inhibition of microglia-associated proinflammatory processes is neuroprotective.[7–11] Results are, however, contradictory,[12] and activated microglia may also have protective properties.[13–15] Thus, neuroprotective treatments that aim to modulate, rather than eliminate, immune cells and processes are particularly attractive for the treatment of perinatal brain injury.

Novel innate defense regulator peptides (IDRs) are synthetic derivatives of endogenous cationic host defense peptides that work to selectively suppress inflammation by a wide range of infectious agents, while augmenting, rather than compromising, protective immunity to pathogens.[16–18] Likewise, the "enhanced" synthetic IDRs modulate inflammatory responses by augmenting the recruitment of immune cells to sites of infection and inflammation, and increasing their anti-infective and wound-healing properties. Simultaneously, IDRs suppress immune cell hyperactivation and the excessive production of proinflammatory cytokines.[17,19] Intriguingly, such peptides also promote the transition to adaptive immune responses[20] and were recently shown to increase survival while downregulating key inflammatory networks associated with fatality in a murine model of malaria.[21] To examine the impact of IDRs on cerebral inflammation and injury, we used a clinically relevant neonatal brain injury model in postnatal day 8 (PND8) mice that, in terms of brain development, are equivalent to (human) infants born close to term.[22] Herein, we provide evidence that IDR-1018 suppresses gene regulation of proinflammatory mediators and cell injurious mechanisms in the developing brain, and postinsult treatment is efficacious in reducing lipopolysaccharide (LPS)-induced hypoxic–ischemic brain damage.

Materials and Methods

Chemicals and Reagents

Ultrapure LPS from *Escherichia coli* 055:B5 was purchased from List Biological Laboratories (Campbell, CA). Synthetic peptides IDR-HH2 (VQLRIRVAVIRA-NH_2), IDR-1018 (VRLIVAVRIWRR-NH_2), P-1006 (VQLRIWVRR-NH_2), and IDR-1002 (VQRWLIVWRIRK-NH_2) were synthesized using F-moc chemistry at the Nucleic Acid/Protein Synthesis Unit (University of British Columbia, Vancouver, British Columbia, Canada). All peptides were selected from a library of >100 peptides based loosely on the weakly active bovine peptide Bac2A, with the same size and similar overall amino acid compositions. Peptides HH2, 1002, and 1018 were designated as immunomodulatory based on their superior ability to induce chemokines and suppress LPS-induced inflammatory responses in peripheral blood mononuclear cells (PBMC), whereas P-1006 was inactive in this assay (see Supplementary Table 1). All other reagents were purchased from Sigma-Aldrich (Stockholm, Sweden).

Animals

For brain injury models, C57/Bl6 male and female mice were purchased from B&K Universal (Stockholm, Sweden), and pups were bred at the Experimental Biomedicine animal facility (University of Gothenburg, Gothenburg, Sweden).[9] All experiments were approved by the local ethical committee at University of Gothenburg (Nos. 277–2007 and 374–2009) and performed according to the Guidelines for the Care and Use of Laboratory Animals.

In Vitro Study

Microglia were isolated from 1- to 2-day-old mice as previously described.[23] Microglia from 4 to 6 mice were pooled, seeded in 24-well plates (100,000 cells/well) for 24 hours (37°C, 5% CO_2, 95% relative humidity), then activated with 10ng/ml LPS ± IDRs (20μg/ml) for a subsequent 24 hours. Microglia-conditioned medium (MCM) was collected and stored at −80°C until analysis. The lactate dehydrogenase (LDH) assay was used according to the manufacturer's instructions (Roche, Stockholm, Sweden) to verify that IDRs did not compromise microglia viability. Inflammatory mediators in MCM were measured using the Cytokine Mouse 20-Plex Panel (Invitrogen, Carlsbad, CA) and a Luminex 100 analyzer (Luminex Corporation, Austin, TX), according to the manufacturer's instructions. The multiplex assay included basic fibroblast growth factor, granulocyte-macrophage colony–stimulating factor (GM-CSF), interferon (IFN)-γ, interleukin (IL)−1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, interferon-inducible protein-10 (IP-10), keratinocyte chemoattractant (KC), monocyte chemoattractant protein-1, monokine induced by gamma interferon (MIG), macrophage inflammatory protein-1α, tumor necrosis factor (TNF)-α, and vascular endothelial growth factor. Cytokine concentrations were calculated using Bio-Plex Manager 3.0 software (Bio-Rad Laboratories, Hercules, CA) with a 5-parameter curve-fitting algorithm applied for standard curve calculations. Analyses were performed in duplicates from 4 independent repeats, using 4 to 6 animals/repeat.

In Vivo Model

Neonatal LPS-sensitized hypoxic–ischemic (LPS+HI) damage was induced in C57/Bl6 male and female mice at PND9 as a clinically relevant animal model as previously described.[11] In this model, LPS (0.3mg/kg) is administered 14 hours prior to unilateral HI induced by carotid ligation followed by 20 minutes of hypoxia in 10% oxygen. IDR-1018 (8μg/g, intraperitoneally) or vehicle (NaCl, 0.9%) were administered 3 hours after LPS+HI. On PND16, both regional brain damage and total tissue loss were examined. Regional brain injury (striatum, hippocampus, thalamus, and cerebral cortex) was assessed blindly by 2 independent researchers in acid fuchsin/thionin–stained sections using a well-established semiquantitative neuropathological scoring system.[8] In brief, damage to the hippocampus, striatum, and thalamus was assessed by grading both the

degree of atrophy (scale from 0 to 3) and the observable cell injury/infarction (0–3), yielding a neuropathological score (0–6) for each of these 3 regions. Injury to the cerebral cortex was graded on a scale from 0 to 4. Scores from all 4 regions of the brain (the cerebral cortex, hippocampus, striatum, and thalamus) were combined for a total injury score in the range from 0 to 22. For quantitative, unbiased stereological assessment of gray and white matter damage, adjacent sections were stained with microtubule-associated protein-2 (MAP2; 1:2,000, mouse-anti-MAP2; Sigma-Aldrich), a marker of neurons and dendrites, and myelin basic protein (MBP; 1:10,000, SMI-94R; BioSite, San Diego, CA), respectively.[7] The area of MAP2 or MBP (subcortical staining) positive staining was measured in each hemisphere in each section. Total tissue loss was calculated by subtracting the MAP2/MBP–positive area of the ipsilateral hemisphere from the contralateral hemisphere. Every 50th brain section throughout the forebrain was analyzed, and tissue volume loss was calculated as previously described.[7]

Microarray

PND8 mice were pretreated with IDR-1018 at 4 hours before LPS or treated with IDR-1018 3 hours after LPS+HI, as described (in vivo model). Six hours after LPS alone or 6 hours after mice were subjected to LPS+HI, brains were removed, rapidly frozen on dry ice, and total RNA extracted (RNeasy Lipid Tissue Mini Kit, including DNase treatment; QIAGEN, Manchester, UK). Total brain RNA (n = 5/group) from animals treated with LPS $\pm$ IDR-1018 as well as RNA from both injured (left) and uninjured (right) hemispheres from animals treated with LPS+HI $\pm$ IDR-1018 were analyzed separately on Affymetrix GeneChip Mouse Gene 1.0ST Array (Swegene, Lund, Sweden). Gene expression data were submitted to the National Center for Biotechnology Information's Gene Expression Omnibus database (accession number GSE36215).

Network Analysis

Gene expression data were analyzed with Bioconductor software in the R statistical language, as implemented in our MetaGEX analysis software (http://marray.cmdr.ubc.ca/metagex/). Differential expression (DE) of genes was assessed using the affy and limma Bioconductor packages. Normalization was done with the RMA method (affy package). Statistical analysis used linear models fit to compute t statistics and log-odds of differential expression of probes using empirical Bayes moderation of standard errors (limma package), with probability values adjusted for multiple testing using the false discovery rate method. Probes were mapped to Entrez Gene IDs using Bioconductor annotation packages. Gene expression patterns were first compared to vehicle control (LPS vs vehicle, IDR-1018 vs vehicle), then contrasted to one another (IDR-1018 vs LPS). Network analysis using counts of node connections and minimum-spanning tree analysis (BioNet package from Bioconductor) identified IDR-1018–responsive genes ($p < 0.05$) that encoded protein products, termed *hubs*, that had the greatest number of interactions with other differentially expressed genes/proteins; thus, these hub genes represent key proteins involved in the trafficking of signals and gene expression responses. Enrichment (also known as over-representation analysis [ORA]) for DE genes in pathways used Kyoto Encyclopedia of Genes and Genomes database annotations from Bioconductor for pathway information and a threshold for DE genes of $p = 0.05$ (unadjusted), using the hypergeometric distribution to calculate probability values (as the chance of seeing this level of enrichment by chance). Network analysis of DE genes used the interactions downloaded from InnateDB.ca (the interactome), and significantly affected subnetworks were identified using the Bioconductor BioNet package. Results from the network and ORA analysis were visualized using InnateDB employing the Cerebral plugin of Cytoscape.[24]

Tracking

^{3}H-Radiolabeled IDR-1018, tritiated at alanine-6 during synthesis, was synthesized at the Centre for Drug Research and Development (specific activity 0.329μCi/μg of peptide; peptide concentration 0.44μg/μl; purity by high-performance liquid chromatography > 95%). The peptide was formulated in physiological saline solution containing 0.01% (vol/vol) Tween 80. Naive and LPS+HI-treated mice at PND9 (3–4 per time point) were injected into the intraperitoneal cavity, at 3 hours after LPS+HI, with IDR-1018 at 10μl/g per animal to achieve a target dose of 2mg/kg IDR-1018 (40μCi/kg ^{3}H). Blood, liver, spleen, and brain (left hemisphere and right hemisphere were collected separately) were collected 5, 15, 60, and 240 minutes after peptide injection. Blood and tissue samples were analyzed for peptide content by scintillation counting after digestion with Solvable tissue solubilizer and decolorization with hydrogen peroxide; tissues were homogenized prior to tissue solubilization. Tissue peptide concentrations were corrected for residual blood volume space. Pharmacokinetic parameters were calculated using PK Solutions (Eugene, OR) software.

Statistics

Differences between groups were analyzed by t test or 1-way analysis of variance (ANOVA) followed by Dunnet correction, and differences in frequency distribution between groups were analyzed by the chi-Square test. A probability value <0.05 was considered statistically significant. Differential expression of microarray values was assessed with the limma package[25] from the Bioconductor project, and reported probability values were adjusted for multiple comparison with the Benjamini–Hochberg method.

Results

IDR-1018 Suppresses LPS-Induced Inflammatory Mediators in Primary Murine Microglia

Three biologically active IDRs (IDR-HH2, IDR-1002, and IDR-1018) and 1 inactive, control peptide (P-1006) were selected for this study. The active IDRs have been shown to possess antiendotoxin effects on human PBMC in the absence of direct antimicrobial activity or direct activation of immune cells,[16,17] but their effects on microglia were not known. To investigate whether IDRs could modulate innate immune cells of the brain, thus showing neuroprotective potential, the effect of IDRs on

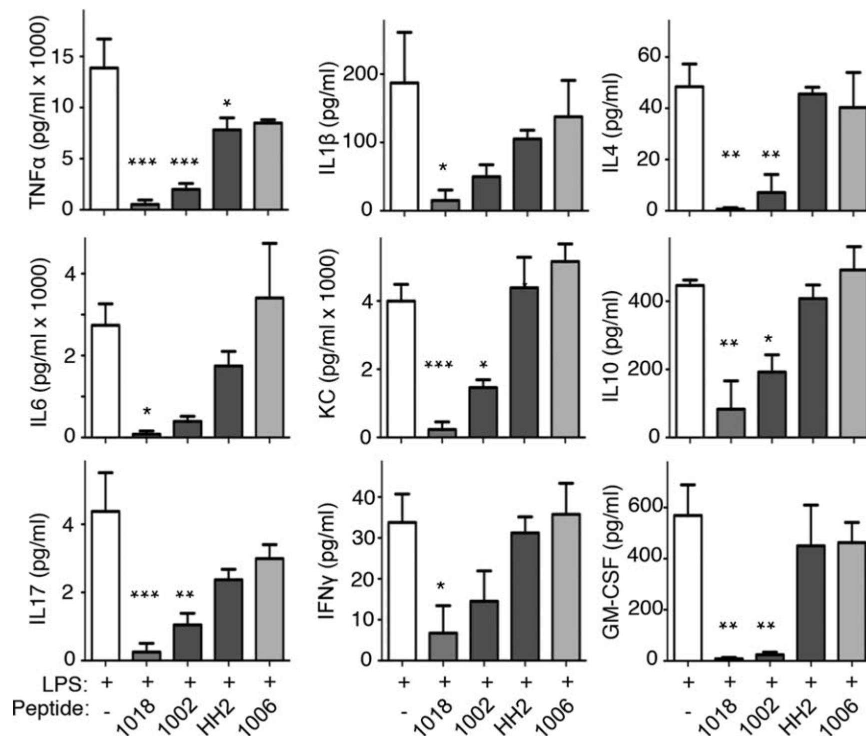


FIGURE 1: Anti-inflammatory potency of innate defense regulator peptides (IDRs). Prototypic inflammatory mediators produced by lipopolysaccharide (LPS)-activated microglial cells in the absence (−) and presence of IDRs 1018, 1002, and HH2 and a negative control peptide, 1006 (x-axis) were measured (pg/ml, y-axis) by 20-plex immunoassay. Data represent the mean concentration of proteins ± standard error of the mean from 4 independent experiments. Asterisks indicate a statistically significant reduction in LPS-induced inflammatory mediators by individual IDRs (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$, 1-way analysis of variance followed by Dunnet correction). GM-CSF = granulocyte-macrophage colony–stimulating factor; IL = interleukin; IFN = interferon; TNF = tumor necrosis factor, KC = keratinocyte chemoattractant.

inflammatory mediators produced by LPS-activated murine microglia was measured. None of the IDRs affected microglia viability, as determined by an LDH activity assay (data not shown), and none of the IDRs stimulated cytokine release by microglia, with the exception of IDR-1002, which induced a modest IP-10 release (1-way ANOVA, $p < 0.05$). As expected, the control peptide P-1006 did not affect the production of LPS-induced inflammatory mediators (Fig 1). IDR-HH2 reduced LPS-induced TNF-α production but not the production of other inflammatory mediators. In contrast, IDR-1002 and IDR-1018 substantially reduced the release of GM-CSF, IL-4, IL-10, TNF-α, IL-17, and KC (1-way ANOVA). In addition, IDR-1018 reduced IFN-γ, IL-1β, and IL-6 release (1-way ANOVA). Thus, IDR-1018, and to a somewhat lesser extent IDR-1002, exerted broad anti-inflammatory effects, as measured by suppression of LPS-induced inflammatory mediators that are produced in vitro by primary murine microglia.

IDR-1018 Confers Neuroprotection In Vivo

In an initial experiment to determine whether IDR-1018 and/or IDR-1002 could influence the injury process in vivo, peptides were given systemically by intraperitoneal injection 4 hours before a clinically relevant model of neonatal brain injury that combines inflammatory challenge (LPS) with HI.[11,26] There was no mortality associated with either the in vivo model or IDR administration (data not shown). A single prophylactic dose of IDR-1018, but not IDR-1002, caused a significant reduction in the number of animals that suffered severe insult to the hippocampus (see Supplementary Fig S1). Consistent with these results, global gene analysis revealed that injury-associated pathways were altered in LPS+HI-injured brain hemispheres that were pretreated with IDR-1018. Most notably, the presence of IDR-1018 prior to LPS+HI was associated with the downregulation of Ca^{2+} signaling ($p = 0.0027$) and p53 signaling ($p = 0.039$; see Supplementary Fig S2).

IDR-1018 Enters the Brain after Peritoneal Injection

To determine whether IDR-1018 is present in the brain after injection, IDR-1018 was isotope-labeled, and its distribution was tracked in the blood and brain following intraperitoneal injection. Under control conditions, the concentration of the labeled peptide rapidly increased in the blood to ~1μg/g of tissue within 2 minutes of delivery, peaking at ~2.5μg/g of tissue after 1 hour (Fig 2A).

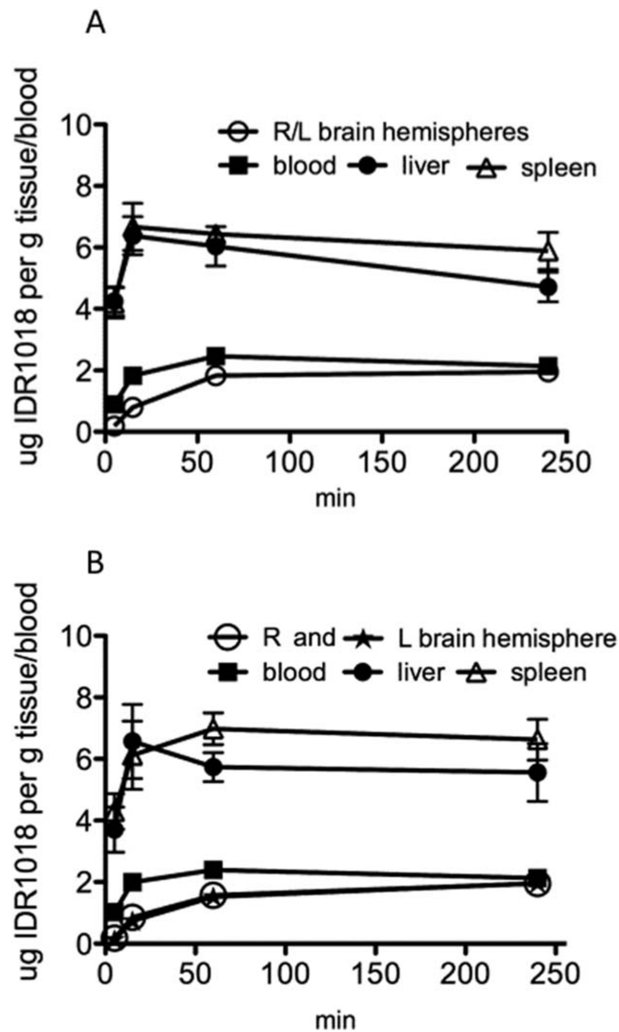


FIGURE 2: Tracking of IDR-1018 in vivo. The distribution of ^{3}H-1018 (y-axis; micrograms of 1018) in blood *(closed squares)*, liver *(closed circles)*, spleen *(open triangles)*, and brain hemispheres was monitored over time (x-axis, minutes) in nontreated naive pups (A) and pups subjected to lipopolysaccharide + hypoxia–ischemia (LPS+HI; B). Peptide was distributed by intraperitoneal injection 3 hours after LPS+HI. For controls, ^{3}H-1018 was measured in the combined left (L) and right (R) hemispheres of the brain (*open circles* in A). In the LPS+HI model (B), ^{3}H-1018 was measured separately in the right, uninjured *(open circles)* and left, injured *(stars)* brain hemispheres. Data represent the mean quantity of 1018 ± standard error of the mean (n = 3–4 per time point).

Labeled IDR-1018 also appeared in the brain soon after delivery and remained at a peak level up to 4 hours after injection. A similar pattern was observed in blood and brain when the labeled peptide was injected after LPS+HI (see Fig 2B). The peptide was distributed both in the ipsilateral hemisphere (injured) and contralateral hemisphere (noninjured). A similar pattern of distribution was also seen when labeled IDR-1018 was given to adult mice (see Supplementary Fig S3). These data demonstrate that in vivo IDR-1018 delivered to the periphery can transit to the brain and reside there for an extended period. It also suggests that this does not require breakdown of the blood–brain barrier and can occur in both the developing and mature (adult) brain.

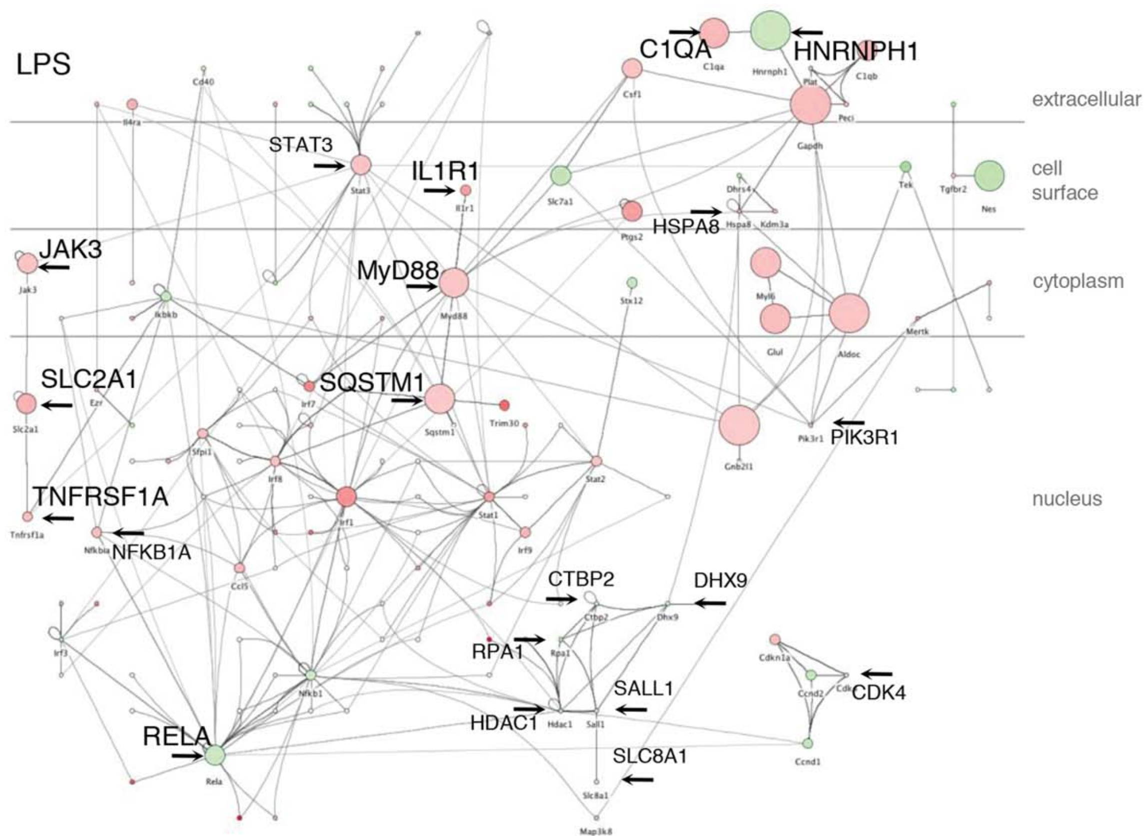
IDR-1018 Administration Is Associated with Suppressed Inflammatory Signaling

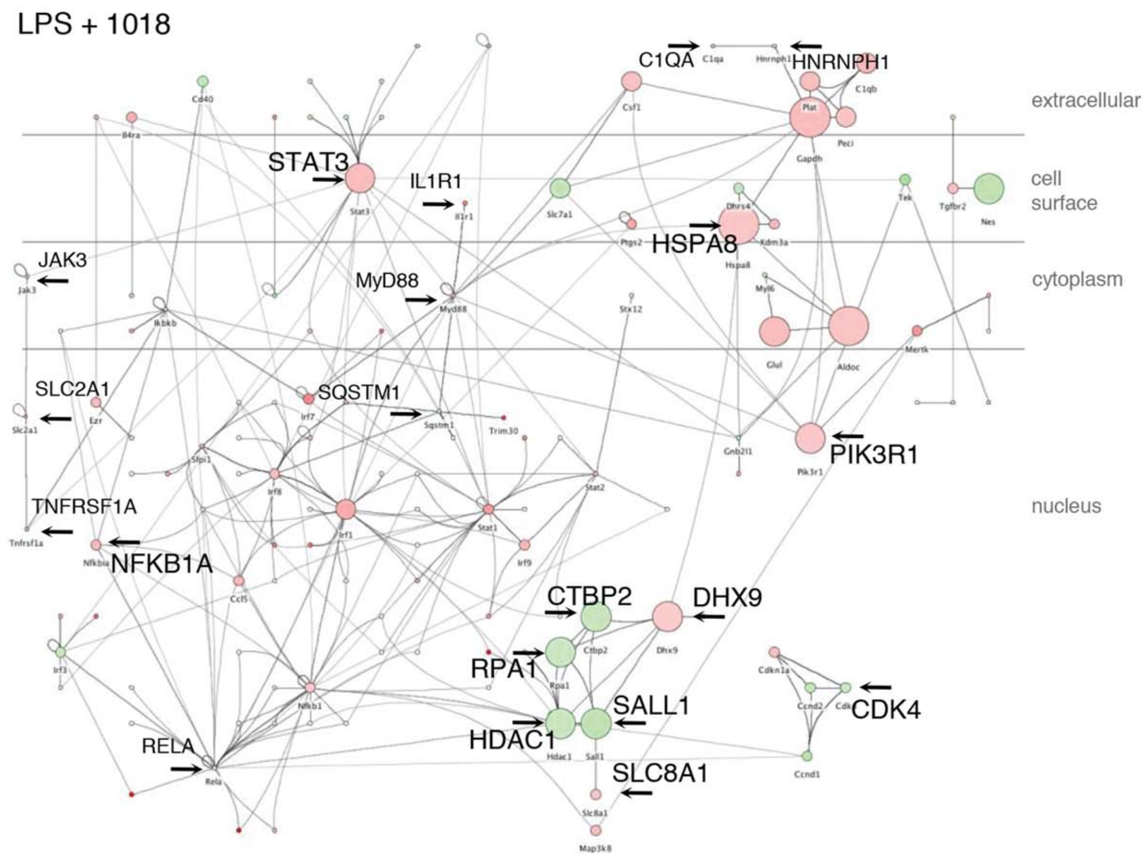
To gain insight into the mechanisms of IDR-1018 actions in the brain, global cerebral gene expression patterns were analyzed in neonatal pups that were pretreated with IDR-1018 4 hours before inflammatory challenge (LPS only), and compared to pups treated with vehicle control. The effect of IDR-1018 on gene expression associated with neuroinflammation was evaluated 6 hours after LPS injection. Administration of IDR-1018 alone (without LPS) led to the differential expression (fold change ≥ 1.2 or ≤ -1.2 with $p < 0.05$) of approximately 50 genes compared to vehicle control. This is consistent with other observations that in the absence of an inflammatory or infectious stimulus, the peptides alone have very mild effects on host gene expression.[16,17]

In contrast, there were hundreds of changes in gene expression in response to LPS, and this response involved 44 key hub genes (hubs are key proteins involved in trafficking of information that are identified by a bioinformatic network analysis; Fig 3). Pretreatment with IDR-1018 increased the importance of 1 LPS-specific hub (STAT3) and diminished the relative role of 24 other hub proteins including the TLR-signaling adapter MyD88, transcription factor nuclear factor κ B (NFκB) subunit p65/RelA, scaffold protein sequestosome-1 (SQSTM1), TNF-related signaling intermediate genes TRAF5 and TNFRSF1A, and the IL1 receptor IL1R1. Intriguingly, IDR-1018 treatment in association with LPS evoked the participation of 26 novel hubs, including negative regulators of NFκB (NFκBIA, PIK3R1, HDAC1, and SALL1), heat shock protein HSPA8, cell cycle regulator CDK4, Ca^{2+}-ion exchanger SLC8A1, and the transcription factor IRF3. These data provide evidence that systemic administration of IDR-1018 can disengage genes/proteins that drive LPS-mediated inflammatory responses in the brain.

IDR-1018 Provides Therapeutic Neuroprotection

Encouraged by these results, the neuroprotective properties of IDR-1018 were tested when given after LPS+HI, as this would mimic the clinical situation where treatment could only be initiated after the primary insult had occurred. Experimental studies indicate a therapeutic window following perinatal insults of up to 6 hours,[27] which has been confirmed in clinical studies of postinjury hypothermia.[28] Thus, IDR-1018 was administered by intraperitoneal injection 3 hours after LPS+HI (Fig 4). Under





Hub degree: >500 100-500 50-100 10-50 <10 Gene expression: -5 0 +5

FIGURE 3: Network analysis of effects of IDR-1018 on lipopolysaccharide (LPS)-associated gene expression in the neonatal brain in vivo. (A) Selected genes (hubs) that are central to LPS-responsiveness in brain tissue in the absence (upper image, LPS) and the presence (lower image, LPS+1018) of IDR-1018. IDR-1018 altered the importance (hub degree; represented as relative size) of various genes, with larger hubs being most important. Node color indicates downregulation (green) or upregulation (red) of genes (relative to vehicle control).

these conditions, IDR-1018 reduced the overall semiquantitative injury score, confirming that IDR-1018 was able to confer therapeutic neuroprotection after neonatal brain injury. As the pattern of perinatal brain injury can vary with insult and brain maturity, the effect of IDR-1018 on the volume of cerebral white matter and gray matter was separately determined by unbiased stereological investigation of MBP and MAP2, respectively. IDR-1018 reduced the white matter tissue loss as well as injury in the gray matter. Neuroprotection was evident in all brain regions examined, including cerebral cortex, hippocampus, thalamus, and striatum, and was most dramatic in the thalamus and striatum, the areas most commonly affected in infants with severe sequels following birth asphyxia.[6]

Postinjury Treatment with IDR-1018 Reduced the Expression of Genes Associated with Immune Cell Trafficking, Endothelial Function, and Apoptosis

To study gene regulation associated with neuroprotective postinjury treatment, we performed microarray analysis on brain tissue harvested 6 hours after LPS+HI with and without IDR-1018 administration 3 hours after the insult. LPS+HI significantly induced regulation of 37 pathways in the injured hemisphere compared to the contralateral (noninjured) hemisphere (Fig 5A, Table 1). Some of the identified pathways are associated with normal cell functions, such as RNA regulation, RNA transport, and protein synthesis and metabolism. Other pathways that were engaged are associated with cellular/neuronal regulation and communication (for, eg, gap junction, long-term potentiation, axon guidance, Ca^{2+} signaling), inflammation (for, eg, African and American trypanosomiasis, osteoclast differentiation, B-cell receptor signaling, leukocyte transendothelial migration, focal adhesion), and cell cycle regulation (for, eg, apoptosis, p53 signaling, cancers). Postinjury treatment with IDR-1018 induced 2 novel pathways (glycerophospholipid metabolism and dilated cardiomyopathy), and reduced the total number of injury-associated pathways from 37 to 16, completely eliminating the significance of 21 pathways (see Fig 5B, Table 1). Of importance, several of the abrogated pathways are associated with cell death (for, eg, apoptosis and p53 signaling) and inflammation. More specifically, we also investigated the effect of the peptide in the injured brain hemisphere by direct comparison of gene transcription in the injured hemispheres of vehicle-treated and IDR-1018–treated pups. We found that 8 pathways (cytokine–cytokine receptor interaction, prion disease, malaria, rheumatoid arthritis, apoptosis, chemokine signaling pathway, Chagas disease, and hematopoietic cell lineage) were significantly downregulated by the peptide, and no pathways were significantly upregulated. As depicted in Figure 6 and Table 2, the regulated pathways were characterized by genes encoding cytokines, chemokines, and proteins involved in immune cell trafficking/cell–cell interactions, angiogenesis/endothelial function, apoptosis, and brain development. In support, an ORA of the regulated pathways demonstrated that one of the most significantly affected hubs was NFκB and its associated signaling pathway (see Fig 6).

Discussion

This study provides evidence for a new approach to treat neonatal brain injury using a selective immunomodulatory peptide, IDR-1018. Herein we demonstrate that IDR-1018 provides significant neuroprotection in neonatal LPS-induced HI brain damage. Our findings demonstrate that IDR-1018 has potential clinical applicability, because a single, peripheral dose of IDR-1018 given after the initial insult was sufficient to confer neuroprotection. Importantly, neuroprotection was evident in all brain regions examined including cerebral cortex, hippocampus, thalamus, and striatum, and was most dramatic in the thalamus and striatum, the areas most commonly affected in infants with severe sequels following birth asphyxia. IDR-1018 efficiently suppressed LPS-induced release of proinflammatory mediators from microglia in vitro, and in vivo, IDR-1018 had beneficial effects on the developing brain by disengaging genes that drive LPS-mediated inflammatory responses while promoting neuroprotective mediators. IDR-1018 significantly altered the expression of several genes encoding inflammatory and cell-death signaling molecules that are known drivers of neonatal brain injury.

The action of IDR-1018 on key inflammatory pathways evoked by both LPS and LPS+HI was investigated by gene expression analysis, and was consistent with the conclusion that IDR-1018 can protect against neuroinflammation and subsequent injury elicited by both of these insults. With respect to a brain insult provoked by LPS, IDR-1018 diminished the importance of several key proinflammatory, and potentially injurious, mediators including IL-1R1 and MyD88. MyD88 is essential in LPS-induced HI brain damage,[11] and exposure to IL-1 has detrimental effects on neonatal brain development.[4] Intriguingly, we also identified IDR-1018–mediated downregulation of SQSTM1 (also known as p62) and transcription factor NFκB p65/RelA as central hubs in the inflamed brain. SQSTM1 is a complex scaffold protein that together with atypical protein kinase C signaling plays a critical role in NFκB activation.[29] Early NFκB activation significantly contributes to brain injury after neonatal HI.[30] SQSTM1 has also recently been shown to be essential for MyD88-dependent signal transduction.[31] Concurrent with the

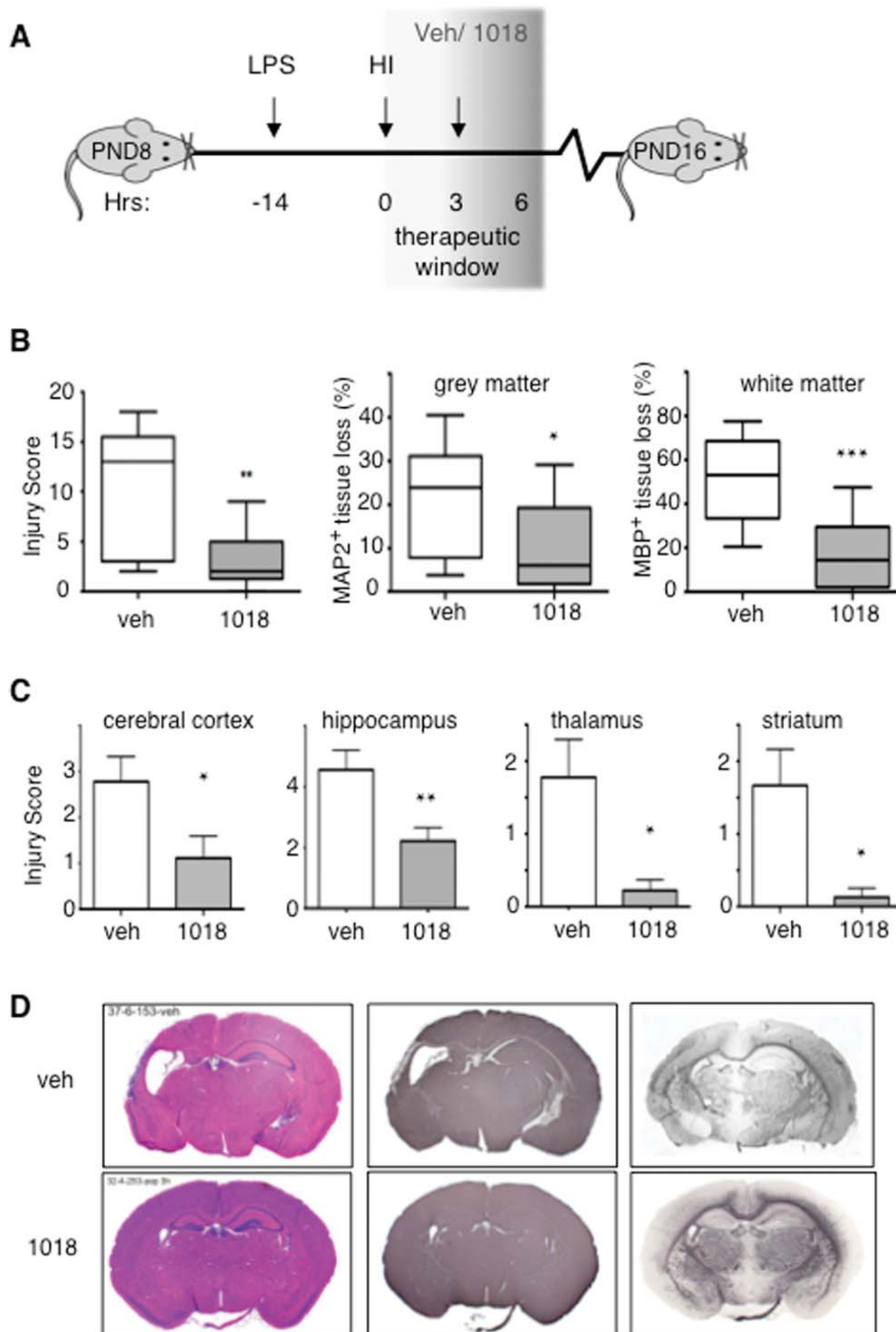


FIGURE 4: IDR-1018 protected both white and gray matter in vivo. (A) In the postinjury treatment schema, vehicle (veh) and IDR-1018 were given to postnatal day 9 (PND9) pups within a clinically relevant therapeutic window, 3 hours after lipopolysaccharide (LPS) + hypoxia–ischemia (HI). (B) Total histological injury score ± standard error of the mean (SEM; y-axis) in the injured brain hemisphere (left), and tissue volume loss in gray matter (middle; measured as microtubule-associated protein-2 [MAP2]-positive tissue loss) and in white matter (right, measured as myelin basic protein [MBP]-positive tissue loss) from animals treated with vehicle (veh, n = 9) and IDR-1018 (1018, n = 8–11). Horizontal lines show the mean injury score or mean percentage loss for the groups. (C) Mean injury score ± SEM (y-axis) in different regions of brain tissue in the absence (veh, n = 9) and presence of IDR-1018 (n = 11). (D) Microscopic images representative of untreated (veh) and treated (1018) brains stained for acid fuchsin/thionin (left), MAP2 (middle), and MBP (right). Asterisks indicate a statistically significant reduction in injury in IDR-1018–treated pups (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$, Student t test).

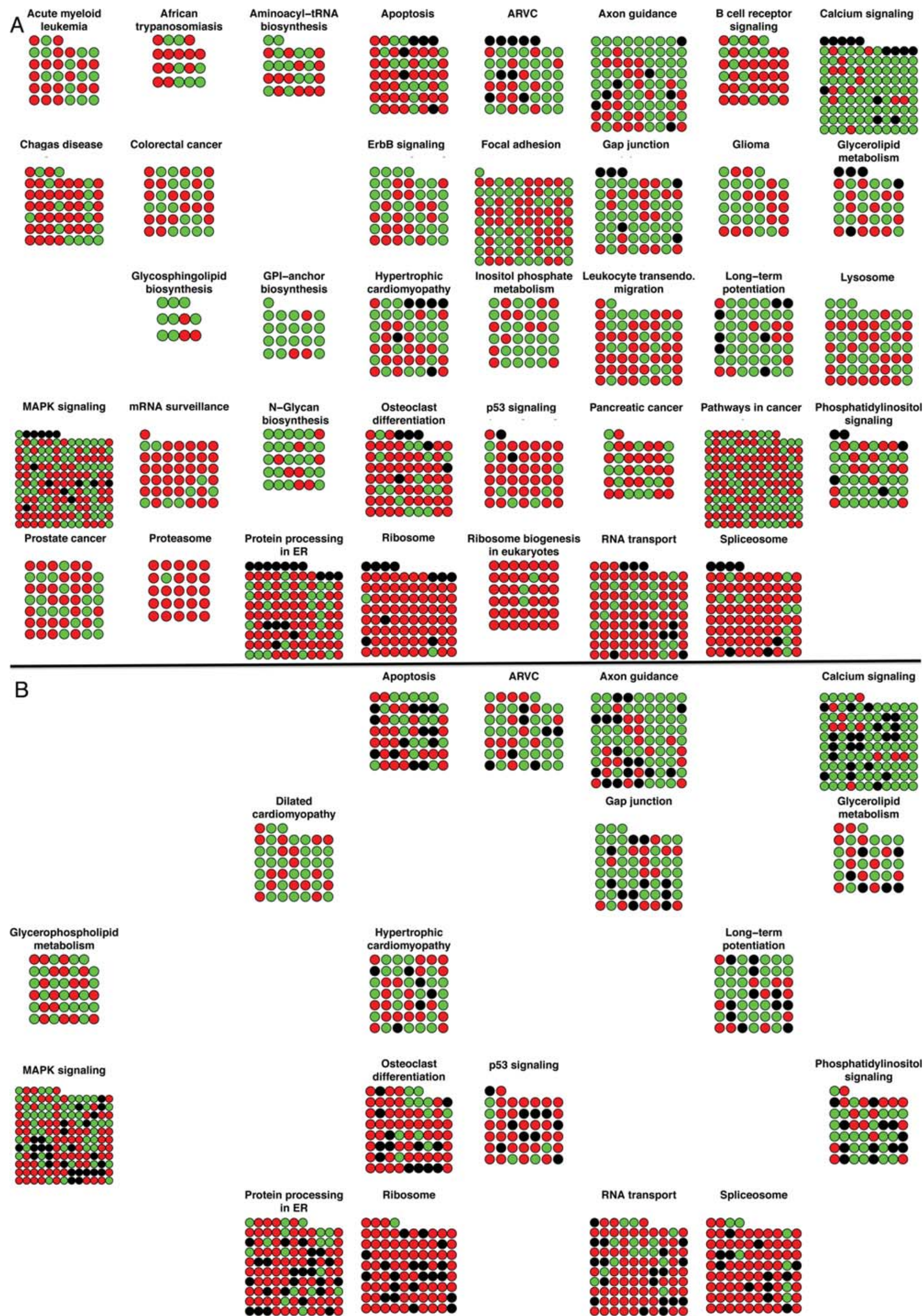


FIGURE 5: IDR-1018–mediated effects on Kyoto Encyclopedia of Genes and Genomes pathways associated with neonatal brain injury in vivo. Microarray analysis on brain tissue harvested 6 hours after lipopolysaccharide + hypoxia–ischemia (LPS+HI) with and without IDR-1018 administration 3 hours after the insult was performed. (A) LPS+HI significantly induced regulation of 37 pathways in the injured hemisphere compared to the contralateral (noninjured) hemisphere. (B) Postinjury treatment with IDR-1018 induced 2 novel pathways (glycerophospholipid metabolism and dilated cardiomyopathy), reduced the total number of injury-associated pathways from 37 to 16, and completely eliminated the significance of 21 pathways. Node color indicates downregulation (green), upregulation (red), and no significant change ($p > 0.05$; black) in gene regulation in the injury response compared to control. ARVC = arrhythmogenic right ventricular cardiomyopathy; ER = endoplasmic reticulum; GPI = glycosyl-phosphatidylinositol; MAPK = mitogen-activated protein kinase; transendo. = transendothelial.

reduced significance of proinflammatory mediators, IDR-1018 elevated the importance of STAT3 and PI3K-mediated signaling hubs. Both these pathways are known to be neuroprotective.[32] Furthermore, STAT3 is the key mediator of the anti-inflammatory effects of IL-10,[33] suggesting that IDR-1018 may mediate resolution of LPS-induced inflammation. Thus, it appears that in vivo in a clinical inflammatory context, IDR-1018 modulates

TABLE 1. Pathway Activation without and with Postinjury IDR-1018 Treatment

KEGG ID	% Expression[a] –	% Expression[a] 1018	Pathway Name	Function
3010	84.1	72.7	Ribosome	Protein synthesis
4020	51.1	46.1	Ca^{2+} signaling	Membrane depolarization, IC signaling
4010	51.7	46.4	MAPK signaling	IC signaling pathway
4380	54.8	48.7	Osteoclast differentiation	Monocyte–macrophage lineage
4070	57.7	48.7	PI signaling system	Cell signaling, membrane trafficking
4210	57.6	49.4	Apoptosis	Cell death
4360	56.9	50.0	Axon guidance	Brain development, neuronal growth
3013	55.8	50.0	RNA transport	Transport from nucleus to cytoplasm
5414	N/A	50.6	Dilated cardiomyopathy	ECM interactions
4141	56.2	50.6	Protein processing in the ER	Protein folding
564	N/A	51.3	Glycero-PL metabolism	Lipid metabolism
4115	63.6	51.5	p53 signaling	Induced by stress signals
5410	51.8	51.8	Hypertrophic cardiomyopathy	ECM interactions
561	54.9	52.9	Glycerolipid metabolism	Cell membrane lipid
5412	51.4	54.1	ARV-cardiomyopathy	ECM interactions, gap junctions
4720	60.9	55.1	Long-term potentiation	Neuronal signaling
4540	60.9	55.2	Gap junction	Intercellular communication
3040	62.1	58.1	Spliceosome	RNA regulation, splicing
604[b]	73.3	NR	Glycosphingolipid biosynthesis	Cell membrane glycolipid
563[b]	68.0	NR	GPI-anchor biosynthesis	Post-translational modification
562[b]	63.2	NR	IP metabolism	Calcium metabolism
970[b]	61.9	NR	Aminoacyl-tRNA biosynthesis	tRNA, protein synthesis
5143[b]	61.3	NR	African trypanosomiasis	TLR9, Fas, apoptosis
510[b]	60.0	NR	N-Glycan biosynthesis	Protein folding
5221[b]	57.9	NR	Acute myeloid leukemia	STAT3, PI3K-Akt, MAPKs
5210[b]	57.1	NR	Colorectal cancer	Apoptosis
3050[b]	56.8	NR	Proteasome	Protein degradation
3008[b]	56.8	NR	Ribosome biogenesis	Protein synthesis
5215[b]	53.9	NR	Prostate cancer	Apoptosis, p53, GSK3
4662[b]	53.3	NR	B-Cell receptor signaling	Adaptive immune functions
4012[b]	52.9	NR	ErbB signaling	Growth factor receptors
5212[b]	52.9	NR	Pancreatic cancer	Apoptosis, p53, Erb receptors
5214[b]	52.3	NR	Glioma	Apoptosis, p53, Erb receptors
5142[b]	52.0	NR	Chagas disease	Complement, TLRs, Ca^{2+} signaling
3015[b]	51.2	NR	mRNA surveillance pathway	Quality control of mRNA
4670[b]	49.6	NR	Leukocyte TEM	WBC exit blood to tissue
4142[b]	48.4	NR	Lysosome	Breakdown and disposal of cellular debris

TABLE 1: Continued

KEGG ID	% Expression[a] −	% Expression[a] 1018	Pathway Name	Function
5200[b]	47.7	NR	Pathways in cancer	Apoptosis
4510[b]	45.7	NR	Focal adhesion	Cell motility, cell matrix interactions

[a]Represents the percentage of observed genes/total genes in each pathway in the injured brain (lipopolysaccharide + hypoxia–ischemia) without (−) and with (1018) IDR-1018.
[b]Postinjury treatment with IDR-1018 reduced the total number of injury-associated pathways from 37 to 16 by completely eliminating the significance of these 21 pathways.
ARV = arrhythmogenic right ventricular; ECM = extracellular matrix; ER = endoplasmic reticulum; GPI = glycosylphosphatidylinositol; IC = intracellular; IP = inositol phosphate; KEGG = Kyoto Encyclopedia of Genes and Genomes; MAPK = mitogen-activated protein kinase; N/A = not available; NR = not regulated; PI = phosphatidylinositol; PL = phospholipid; TEM = transendothelial migration; TLR = Toll-like receptor; WBC = white blood cells.

the LPS-induced inflammatory response so that proinflammatory responses are limited, whereas wound healing is enhanced.

With respect to brain injury caused by LPS+HI, post-treatment with IDR-1018 reduced expression of many of the inflammatory genes that have previously been associated with immune cell interactions and brain injury. Previous studies have demonstrated upregulation of cytokines and chemokines in the brain after neonatal HI,[34–36] and genetic deletion of these entities can be neuroprotective.[8] In support, we found in association with peptide-mediated neuroprotection downregulation of a large number of chemokines in the injured hemisphere. Many of these genes are associated with inflammatory cell recruitment/activation, suggesting that the peptide may affect the entry of peripheral immune cells into the brain following LPS+HI. Furthermore, several adhesion molecules and regulators of endothelial function were differentially regulated, also indicating that the peptide may have effects on the interaction between peripheral inflammation and the brain. IDR-1018 also affected the transcription of several proinflammatory cytokines

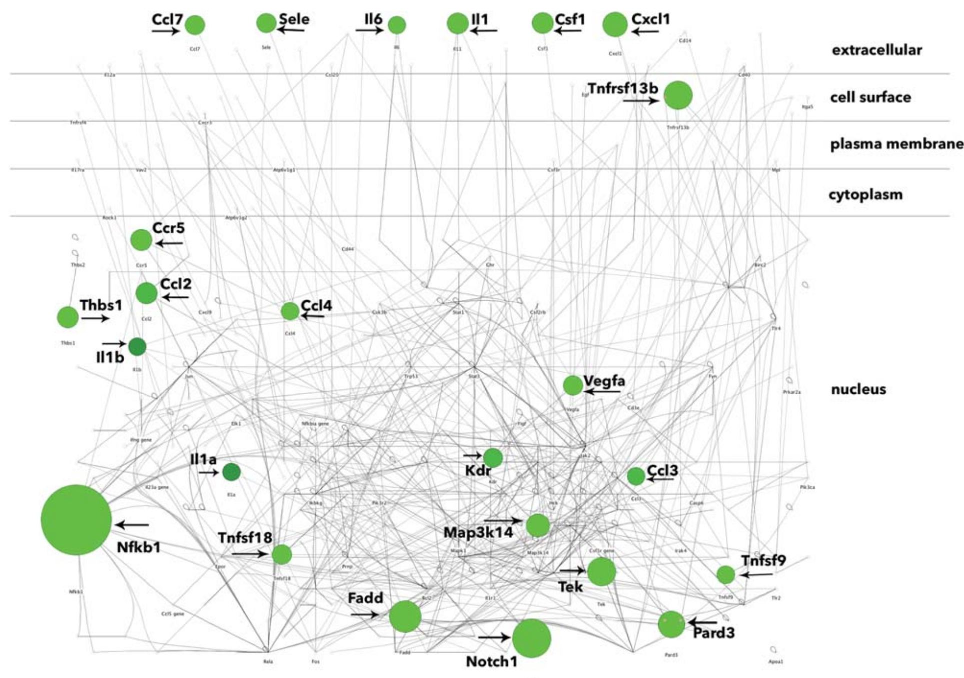


FIGURE 6: IDR-1018–mediated effects on gene expression in vivo in the injured neonatal brain. Differentially expressed genes in brain tissue harvested 6 hours after lipopolysaccharide + hypoxia–ischemia with and without IDR-1018 administration 3 hours after the insult was performed. Comparisons are between the injured, ipsilateral hemispheres without and with IDR-1018 treatment. Genes in affected pathways that are significantly downregulated by IDR-1018 are colored ($p < 0.05$) and adjusted for size based on the hub degree of the gene or protein, with larger hubs being most important for IDR-1018–mediated neuroprotection.

TABLE 2. Genes in Kyoto Encyclopedia of Genes and Genomes Pathways Affected by IDR-1018

Gene Symbol	Gene Name	Function
Chemokines		
Ccl2	Monocyte chemotactic protein 1	Chemotactic for monocytes and basophils; regulated after neonatal HI[45]
Ccl3	Macrophage inflammatory protein 1α	Attracts macrophages to sites of injury; regulated after neonatal HI[34,36,46]
Ccl4	Macrophage inflammatory protein-1β	Chemotactic for macrophages; regulated after neonatal HI[34]
Ccl6		Leukocyte recruitment, expressed in microglia[47]
Ccl7	Monocyte chemoattractant protein 3	Macrophage attractant, expressed in brain of MS patients[48]
Ccl12	Monocyte chemotactic protein 5	Stimulates microglia[49] HIF-1 target (astrocytes) + hypoxia[50]
Ccl19	Macrophage inflammatory protein 3β	T-Cell trafficking, expressed in leukocytes, astrocytes, microglia in EAE[51]
Cxcl1	Neutrophil-activating protein Gro-α/KC	Neutrophil attractant; increased in stroke[52]; found in neonatal brain after LPS/HI[41]
Cxcl11	Small-inducible cytokine B11	Induced by IFN-γ; increased in CSF of MS patients[53]
Ccr5	C-C motif chemokine receptor 5	Receptor for Ccl3, Ccl4, and Ccl5; expressed after neonatal HI[36]
Cytokines & associated regulators		
IL1a	Interleukin 1α	Increased after neonatal HI[54]
IL1b	Interleukin 1β	Increased after neonatal HI[54]; neonatal IL1β administration results in brain damage[4]
IL6	Interleukin 6	Increased in infants with asphyxia[55]; bimodal effects in cerebral ischemia[56]; beneficial in neonatal HI[57]
IL11	Interleukin 11	Anti-inflammatory cytokine, by regulation of IkappaB-beta[58]
Tnfsf10	TNF-related apoptosis-inducing ligand	Proapoptotic to oligodendrocytes,[59] induced on $CD68^+$ microglia/macrophages after perinatal HI[60]
Tnfrsf13b	TNF receptor 13B	Binds to TACI and APRIL, associated with auto-immunity in MS lesion[61]
Tnfsf18	TNF receptor 18, GITR	Induces microglia activation[38] $GITR^+CD4^+T$ cells harmful to poststroke neurogenesis[40]
Tnfsf9	TNF receptor 9, CD137	NK-mediated inflammation[62]; activates microglia, induces oligodendrocyte apoptosis[39]
Map3k14	Nuclear factor-kappa-B–inducing kinase	Activation of NFκB by proteolytic processing of NFκB2/P100
NFkb1	Nuclear factor-kappa-B p105 subunit	Major regulator of inflammation
Adhesion molecules & endothelial cell factors		
Sele	E-Selectin	Cell adhesion
Icam1	Intercellular adhesion molecule 1	Cell adhesion

TABLE 2: Continued

Gene Symbol	Gene Name	Function
Vcam1	Vascular cell adhesion molecule 1	Cell adhesion
Selp	P-Selectin	Cell adhesion
Thbs1	Thrombospondins 1/2	Mediates cell–cell and cell–ECM interactions; KO has impairments after stroke[63]
VEGFA Kdr	Vascular endothelial growth factor A, VEGF receptor 2	Endothelial growth factor, neuroprotective following neonatal stroke[64]; VEGF receptor
Itga1	Integrin alpha 1	Cell adhesion, upregulated in astrocytes with thrombospondin in the injured brain[65]
Apoptosis		
Aifm1	Apoptosis-inducing factor 1	Proapoptotic, involved in neonatal HI[66]
Cflar	CASP8 and FADD-like apoptosis regulator	Abrogate Fas/TNFR-induced apoptosis[67]; involved in neonatal HI[68]
Fadd	Fas-associating protein with death	Recruits caspase 8 via Fas and Trail receptors[37]
Other		
Notch1	Notch homolog 1	Induced after neonatal HI[69]; attenuation may be beneficial for neurogenesis in the long term[70]
Cd1d1	Antigen-presenting glycoprotein CD1	Interacts with NK T-cell receptors[71]
Cd9	CD9 molecule, tetraspanin	T-Cell activation[72]
Csf1	Macrophage colony-stimulating factor 1	Increases proliferation and phagocytosis in adult human microglia[73]
Tek	Tyrosine kinase, endothelial	Cell growth, differentiation, development; suppression protects neurodegeneration[74]
Pard3	Par-3 (partitioning defective 3)	Tight junctions[75]; neuronal polarity[76]; synapse development[77]
Prkx	Serine/threonine-protein kinase	Cell proliferation, apoptosis, differentiation, and tumorigenesis
Hspa1a	Heat shock protein 70	Overexpression neuroprotective[78]
Ifnab	Interferon alpha B	Type I IFN, neuroprotective?[79]
C6	Complement 6	Membrane attack complex, cell lysis

APRIL = a proliferation-inducing ligand; CSF = cerebrospinal fluid; EAE = experimental autoimmune encephalomyelitis; ECM = extracellular matrix; HI = hypoxia–ischemia; HIF-1 = hypoxia-inducible factor 1; IFN = interferon; IL1β = interleukin 1β; KO = knockout; LPS = lipopolysaccharide; MS = multiple sclerosis; NFκB = nuclear factor κ B; NK = natural killer; TACI = Transmembrane Activator and CAML Interactor; TNFR = tumor necrosis factor receptor; VEGF = vascular endothelial growth factor.

such as IL-1 and members of the TNF family and the general immune modulatory transcription factor NFκB and its inducer MAP3K14. The peptide had the strongest effect on the NFκB hub, and the affected gene products tended to be extracellular or in the nucleus (see Fig 6). As discussed above, both IL-1 and NFκB activation are known to contribute to neonatal brain damage.[4,30]

Cell death in the immature brain has been closely linked to apoptotic mechanisms. We found that IDR-1018 induced downregulation of several genes connected with apoptosis, oligodendrocyte death, and negative effects on neurogenesis (for, eg, FADD, Tnfsf9, Tnfrsf13b, and Tnfsf18). FADD is a proapoptotic adaptor molecule that recruits caspase-8 activated Fas (CD95) or TNF-receptor 1 (TNFR-1)[37]; TNF receptors (Tnfsf9, Tnfrsf13b, and Tnfsf18) have been shown to be able to induce microglia activation[38] and oligodendrocyte death[39] and have harmful effects on neurogenesis.[40] Overall, regulation of TNF family members appears to be important in neonatal brain injury, and in support it was shown that TNF gene cluster deletion ameliorates LPS-induced HI insult in neonatal mice.[41] Furthermore,

our data indicated that IDR-1018 reduced the number of regulated genes in the p53 cell-death signaling pathway. Transcription factor p53 is involved in the expression of proapoptotic proteins and may also induce mitochondrial dysfunction and promote apoptosis in a transcription-independent manner after neonatal HI. p53 has been targeted as a key mediator of perinatal brain damage, and inhibition of p53 is protective in animal models for perinatal brain injury.[42] Together, these data demonstrate that systemic administration of IDR-1018 can modulate key molecules and pathways associated with both neuroinflammation and injurious responses in the neonatal brain.

The cellular target through which IDR-1018 mediates neuroprotection in vivo has yet to be determined conclusively. We show that IDR-1018 was effective at blocking microglial cell responsiveness to LPS in vitro, and peripherally administered isotope-labeled IDR-1018 was found in the brain soon after injection (in naive control animals and after LPS+HI) and remained there for at least 4 hours after injection. Thus, IDR-1018 has the potential to exert neuroprotective effects directly on brain immune cells in vivo. It is also possible, however, that the peptide acts via inhibition of peripheral responses to LPS and/or HI. Our gene array data indicated that IDR-1018 altered the expression of cell adhesion molecules and regulators of endothelial cell function, events that could also occur in the periphery.

Finally, our data indicate that IDR-1018 in the absence of LPS or LPS+HI caused only minor changes in gene expression in cerebral tissue; thus, unlike most other anti-inflammatory approaches, IDR-1018 is not expected to nonspecifically suppress inflammatory responses or compromise immunity. These results also indicate that IDR-1018 may have limited effects on healthy neonatal brains, which is clinically significant, as the identification of "risk infants" in need of treatment inadvertently will include some infants who would otherwise develop normally.

Despite improved survival rates, the absolute number of children with neurological impairments following perinatal brain injury has not decreased. Therapeutic hypothermia is a significant advance in treating term infants suffering from neonatal encephalopathy; however, hypothermia offers only an 11% reduction in the risk of death or disability,[43,44] and there is no pharmacological neuroprotective treatment available for infants suffering brain injury. Thus, there is an urgent need for other treatment options. We show that a novel immunomodulatory peptide, IDR-1018, provides the features of a promising neuroprotective agent in newborns with brain injury. It confers neuroprotection of both gray and white matter when given after the insult, it is effective in the brain when given systemically, and it lacks significant effects on the brain under normal conditions.

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Authorship

H.B., K.S., A.T., N.M., E.D., H.H., R.E.W.H., K.L.B., and C.M. designed the experiments. R.E.W.H. provided peptides for the study. H.H. and R.E.W.H. provided financial support. H.B., K.S., W.W., A.T., C.J.E., and N.M. performed the experiments. K.S. and C.M. conducted the statistical analysis. C.D.F. and K.L.B. carried out the gene expression analysis. K.S., R.E.W.H., K.L.B., and C.M. interpreted data and wrote the manuscript. All authors revised and approved the final manuscript. H.B. and K.S. contributed equally to this work. C.M. and K.L.B. contributed equally to this work.

Potential Conflicts of Interest

R.E.W.H.: speaking fees, list available on request. The peptides described here have been filed for patent protection and assigned to R.E.W.H.'s employer, the University of British Columbia (UBC). IDR-1018 and IDR-HH2 have been out-licensed to Elanco Animal Health (Greenfield, IN) for use in treating infections of animals. Peptides IDR-1002 and IDR-HH2 have been licensed to the Pan-Provincial Vaccine Enterprise (Saskatoon, Saskatchewan, Canada) for development as a component of a single-dose vaccine adjuvant for animal viral diseases. Patents have been issued for IDR-1018 in New Zealand and the USA.

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