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Full-length Article

Live imaging of the innate immune response in neonates reveals differential TLR2 dependent activation patterns in sterile inflammation and infection

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ABSTRACT

Activation of microglial cells in response to brain injury and/or immune stimuli is associated with a marked induction of Toll-like receptors (TLRs). While in adult brain, the contribution of individual TLRs, including TLR2, in pathophysiological cascades has been well established, their role and spatial and temporal induction patterns in immature brain are far less understood. To examine whether infectious stimuli and sterile inflammatory stimuli trigger distinct TLR2-mediated innate immune responses, we used three models in postnatal day 9 (P9) mice, a model of infection induced by systemic endotoxin injection and two models of sterile inflammation, intra-cortical IL-1β injection and transient middle cerebral artery occlusion (tMCAO). We took advantage of a transgenic mouse model bearing the dual reporter system luciferase/GFP under transcriptional control of a murine TLR2 promoter (TLR2-luc-GFP) to visualize the TLR2 response in the living neonatal brain and then determined neuroinflammation, microglial activation and leukocyte infiltration. We show that in physiological postnatal brain development the *in vivo* TLR2-luc signal undergoes a marked ~30-fold decline and temporal-spatial changes during the second and third postnatal weeks. We then show that while endotoxin robustly induces the *in vivo* TLR2-luc signal in the living brain and increases levels of several inflammatory cytokines and chemokines, the *in vivo* TLR2-luc signal is reduced after both IL-1β and tMCAO and the inflammatory response is muted. Immunofluorescence revealed that microglial cells are the predominant source of TLR2 production during postnatal brain development and in all three neonatal models studied. Flow cytometry revealed developmental changes in $CD11b^+/CD45^+$ and $CD11b^+/Ly6C^+$ cell populations, involvement of cells of the monocyte lineage, but lack of $Ly6G^+$ neutrophils or $CD3^+$ cells in acutely injured neonatal brains. Cumulatively, our results suggest distinct TLR2 induction patterns following PAMP and DAMP – mediated inflammation in immature brain.

1. Introduction

Neuroinflammation caused by infection, hypoxia-ischemia and stroke during the perinatal period contributes to increased risk for neurological and neuropsychiatric deficits and long term disabilities in children (Hagberg et al., 2015). Injury-induced inflammatory response is characterized by marked activation of the resident immune cells, microglial cells, and peripheral leukocytes, and production of inflammatory cytokines, events that may contribute to brain damage (Dirnagl et al., 1999; Iadecola and Anrather, 2011; Kriz and Lalancette-Hebert, 2009; Lo et al., 2003). Microglial cells are the principal immune cells of the brain. The current view is that once activated, microglial cells may acquire a variety of different stimulus- and context dependent immune profiles ranging from pro-inflammatory/cytotoxic to more alternative and neuroprotective phenotypes (Fernandez-Lopez et al., 2014; Pierre et al., 2017). The role of microglial cells is even more complex in immature brain, as growing evidence suggests that microglial cells play active role during brain development and early CNS homeostasis, in part by enabling neuronal synapse remodelling and phagocytosis of neurons that undergo still

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on-going programmed cell death in the developing brain (Paolicelli et al., 2011). Thus, inflammation and microglial activation can actively contribute to both normal physiological development and to injury in neonatal brain.

Upregulation of the pattern-recognition receptors, such as Toll-like receptors (TLRs), by activated microglial cells has been shown in injured adult brain (Akira and Takeda, 2004; Arumugam et al., 2009; Gordon, 2002; Janeway and Medzhitov, 2002; Lalancette-Hebert et al., 2009; Stridh et al., 2011; Ziegler et al., 2007) and in immature brain after H-I (Stridh et al., 2011). TLRs act via recognition of two types of ligands, the pathogen-associated molecular pattern (PAMP) ligands in response to infectious particles (Medzhitov et al., 1997) and the endogenous danger-associated molecular pattern (DAMP) ligands in response to stress or injury-derived molecules (Arumugam et al., 2009; Asea et al., 2002; Hanisch et al., 2008; Kariko et al., 2004; Matzinger, 2002). While some data suggest that immune cascades following activation of TLR receptors, like TLR2 in microglia, may have harmful effect on neonates (Du et al., 2011; Mallard et al., 2009; Mottahedin et al., 2017; Pierre et al., 2017), the role of the innate immune response in neonatal brain development and/or response to injuries remains poorly understood.

We asked if DAMP and PAMP mediated TLR2 responses lead to distinct neuroinflammatory injury patters in neonatal mice. To visualize TLR2 response in the living neonatal brain we took advantage of a transgenic mouse model bearing the dual reporter system luciferase/green fluorescent protein under transcriptional control of a murine TLR2 promoter (TLR2-luc-GFP), a reporter mouse model that we used to identify induction of TLR2 and associated microglial activation by biophotonic/bioluminescence imaging in living adult mice subjected to stroke (Lalancette-Hebert et al., 2009, 2012). We show that TLR2-luc signal undergoes marked ~30-fold decline and regional redistribution during the second and third postnatal weeks and that there are striking differences in the TLR2 response to endotoxin (LPS) challenge compared to sterile inflammation induced by intra-cerebral injection of IL-1β or transient middle cerebral artery occlusion (tMCAO) in postnatal day 9 (P9) mice—a robust upregulation of TLR2-luc by LPS but, surprisingly, a significant down-regulation of the TLR2-luc signal following IL-1β or tMCAO. Together with distinct LPS compared to IL-1β or tMCAO patterns of induction of inflammatory and anti-inflammatory cytokines our results strongly suggest distinct TLR2 induction patterns following PAMP and DAMP- mediated inflammation in immature brain.

2. Material and methods

2.1. Mouse model

TLR2-luc-GFP transgenic reporter mice were used to visualize TLR2 induction/microglial activation, as we described before (Lalancette-Hebert et al., 2009). Transgenic animals were identified by polymerase chain reaction (PCR) detection of the luciferase transgene with the following primers: 5′-CAG-CAG-GAT-GCT-CTC-CAG-TTC-3′ AND 5′-GGC-GCA-GTA-GGC-AAG-GTG-GT-3′. Genotyping was performed as previously described (Lalancette-Hebert et al., 2009). All experimental procedures were approved by the Laval University animal care ethics committee and are in accordance with The Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care. All experimental procedures conducted at the University of California San Francisco were approved by the University of California San Francisco Institutional Animal Care and Use Committee and followed in accordance with the Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services). Animals were given ad libitum access to food and water; housed with nesting material and shelters, and kept in rooms with temperature control and light/dark cycles.

2.2. In vivo bioluminescence/biophotonic imaging

As previously described (Cordeau et al., 2008; Lalancette-Hebert et al., 2009), bioluminescence/biophotonic images were captured using IVIS® 200 Imaging System (PerkinElmer, MA, USA). Twenty-five minutes prior to imaging session, mice received intraperitoneal (i.p.) injection of luciferase substrate D-luciferine (150 mg/kg; 20 mg/ml of D-luciferine dissolved in 0.9% saline was injected) (CaliperLS-Xenogen). 3D reconstruction of bioluminescent signal in the brain was accomplished by using diffuse luminescent imaging tomography (DLIT) algorithms (Living Image 3D Analysis Software, CaliperLS-Xenogen) (Cordeau and Kriz, 2012).

2.3. Surgical procedures

2.3.1. Stereotaxic IL-1β brain injection

P9 TLR2-luc-GFP pups of both sexes were anesthetized with 2% isoflurane in 100% oxygen at a flow rate of 1.5 L/min and placed in a stereotaxic apparatus (David Kopf Instruments). Mice received stereotaxic intracerebral injection of recombinant IL-1β (1 ng) (R&D systems, MN, USA) or sterile saline solution (0.9%) into the right parietal region of cerebral cortex. The coordinates for stereotaxic injection were the following: 2.0 mm posterior, 2.0 mm lateral (right) and −1.25 mm dorsoventral to the bregma. The injections were performed using a 33-gauge stainless steel cannula (Plastics One) connected to a 25-ml Hamilton syringe. A volume of 2 μl was infused over 2 min using a microinjection pump (model A-99; Razel Scientific Instruments). The animals were then longitudinally imaged by *in vivo* bioluminescence for a week.

2.3.2. Transient middle cerebral artery occlusion (tMCAO)

P9-P10 mice of both sexes with confirmed presence of TLR2-luc-GFP transgene were subjected to 3 h tMCAO as originally described for P7 rats (Derugin et al., 1998, 2005) and modified for P9-P10 mice (Woo et al., 2012). Mice were administered D-luciferine (150 mg/kg; i.p.) and were imaged 30 min later before tMCAO to obtain baseline and were imaged at 24 and 72 h after reperfusion. Mice were either perfusion-fixed for histology or tissue collected from injured regions and from matching contralateral regions for biochemical analysis.

2.4. LPS injection

P9 TLR2-luc-GFP pups of both sexes were injected intraperitoneally (i.p.) 1 mg/kg of LPS dissolved in 0.9% sterile saline or saline. Mice were longitudinally imaged *in vivo* before and 1 and 3 days after LPS administration.

2.5. Tissue collection

For immunofluorescence analysis, anaesthetized pups were transcardially perfused with 15 ml of 0.1 M PBS, followed by 4% paraformaldehyde (PFA, pH 7.4, phosphate buffered saline, PBS). Brains were postfixed overnight in 4% PFA and cryoprotected in 30% sucrose/ PBS for 48 h, embedded into Tissue-Tek (O.C.T. compound, Sakura, USA) and frozen at −20C, cut into coronal section with a Cryostat (15-μm thick) and stored at −20C.

Pups that underwent tMCAO were deeply anesthetised with Euthasol (100 mg/kg; Virbac) and perfused transcardially with 4% PFA in 0.1 M PBS (pH 7.4). Brains were post-fixed in 4% PFA over-