Systemic Inflammation Induces Axon Injury During Brain Inflammation

Beatriz Moreno, John-Paul Jukes, Nuria Vergara-Irigaray, Oihana Errea, Pablo Villoslada, V. Hugh Perry, and Tracey A. Newman
Ann Neurol 2011;70:932-942

Patrick Altmann
January 2013
Overview

- Background
- Methods
- Results
- Discussion
- Conclusion
Model for inflammatory demyelinating disease

**MS:**

- Relapsing-remitting disease
- Progressive in one half of patients
  - axonal damage
  - secondary progressive MS
- Approx. 1/3 of relapses are preceded by a systemic infection
Infection

- PAMPs such as LPS elicit activation of the innate IS
- Systemic challenge can be achieved with
  - LPS, TNFα, IL-12
- Axonal damage in MS plaques is associated with macrophage/microglia activation
Patient criteria in clinical trials included the ability to complete two trials of the 25-foot walk in 8-45 seconds.²,³
FIGURE 1: Experimental timeline. Animals were inoculated at day 0, peak disease occurred between day 15 and day 20. All animals entered clinical remission by day 25. Animals were challenged with LPS or saline (control groups), at 1, 3, and 6 weeks into remission. Animals were assigned for LPS or control challenge to ensure matched clinical scores and weight losses between groups. Tissue was harvested from the lumbar spinal cord and mid-brain for histological analysis. Animals with clinical scores ≥2 in the first phase of the disease were eliminated from the study to avoid unacceptably severe clinical disease; 216 animals comprising 8 experimental matched groups were used in the study. LPS = lipopolysaccharide.
Challenge started in the remission phase (after day 26)

FIGURE 1: Experimental timeline. Animals were inoculated at day 0, peak disease occurred between day 15 and day 20. All animals entered clinical remission by day 25. Animals were challenged with LPS or saline (control groups), at 1, 3, and 6 weeks into remission. Animals were assigned for LPS or control challenge to ensure matched clinical scores and weight losses between groups. Tissue was harvested from the lumbar spinal cord and mid-brain for histological analysis. Animals with clinical scores ≥2 in the first phase of the disease were eliminated from the study to avoid unacceptably severe clinical disease; 216 animals comprising 8 experimental matched groups were used in the study. LPS = lipopolysaccharide.
EAE Induction

- Inoculation with guinea pig spinal cord homogenate in CFA containing M. tuberculosis
- Animals weighed daily
- Neurological assessment:
  - 0... normal
  - 0,5... partial limp tail
  - 1... fully limp tail
  - 1,5... + loss of righting reflex
  - 2... mild hind limp paraparesis
  - 3... hind limp paraplegia
  - 4... quadruplegia
  - 5... moribund
Immuno-histochemistry

- brain; spinal cord; spleen ... all in paraffin wax
- 10µm mid-brain and lumbar spinal cord sections

stained for:
- MHC II; IL-1β; iNOS; APP; CD68/ED1; CD3+ T-cells;
- nitrotytosine; rat IgG

Immunohistochemical findings (animals, n ≥ 4 per group/time point) were analyzed using Leica QWin (Leica, Wetzlar, Germany), to calculate the signal per unit area of lesion after image capture (×40 magnification) of individual lesions. Nitrotyrosine signals were quantified using ≥4 animals per experimental condition.
RT qPCR

- Total RNA from spleen; brain; spinal cord
- Extracted from 20µm sections
- To detect mRNA for cytokines in lesions
FIGURE 2: Change in weight (left) and clinical scores (right) over the course of the experiments. All animals exhibited clinical symptoms and weight loss associated with development of monophasic EAE. Any animal that had a clinical score ≥2 during the initial phase was eliminated from the study. A total of 216 animals were included in the 8 subsequent experiments. Animals were assigned to groups to receive either LPS or control (saline) challenges; these groups were matched for weight loss and first disease peak symptoms. The experimenter handling the animals was blinded to the type of challenge received. The re-emergence of symptoms and associated weight loss, when present, persisted beyond what would be expected for an equivalent LPS challenge in a naive animal. Some animals challenged at (A) 1 week, (B) 3 weeks, and (C) 6 weeks into remission still exhibited symptoms up to 1 week after the challenge. (Solid circles = controls; empty circles = LPS challenged). EAE = experimental allergic encephalomyelitis; LPS = lipopolysaccharide.
Results I

- EAE symptoms on days 6-25
- Peak disease (n=12) between days 15-20
- Onset of remission by day 26
- LPS injection resulted in re-emergence of mild EAE in 60%
- Controls remained in remission
- Histology at peak of disease revealing perivascular inflammatory infiltrates (macrophages/microglia and T cells)
Results I
Results II

T cell (CD3-positive cells) numbers (see Fig 4C, D) were steady 24 hours after the LPS challenge but rose significantly by 96 hours ($p < 0.001$) (see Fig 4E). This identified a delay in the increase in T cells after LPS, indicating secondary activation of the adaptive immune system after CNS damage.
LPS/control challenge:

After 24 hours: **ED1** same in LPS and control
After 96 hours: **ED1** ↓ in control

= **ED1** ↑ in LPS

After 24 hours: **T cells** same in LPS and control
After 96 hours: **T cells** ↓ in control

= **T cells** ↑ in LPS
After 96 hours: ED1↑ in LPS

After 96 hours: T cells↑ in LPS

This delay indicates:

– Secondary activation of the adaptive immune system after CNS damage

... did the recruitment of inflammatory cells occur due to BBB leakage?
...No, it did not!

→ IgG only in the vessel lumen (at 24 hours)

FIGURE 5: Blood-brain-barrier integrity in APP+iNOS+ lesions 24 hours after an LPS challenge at 1 week. (A) APP. (B) iNOS. (C) IgG. Bar = 20μm. APP = amyloid precursor protein; IgG = immunoglobulin G; iNOS = inducible nitric oxide synthase; LPS = lipopolysaccharide.
What about the effects of LPS on the peripheral and CNS immune response?

Investigation of cytokine profiles within 24 hours in the spleen and the cord.

Remember: no alterations in cell recruitment within 24 hours!
Results III

**Background** – **Methods** – **Results** – **Discussion** – **Conclusion
LPS challenge resulted in an *elevated production of IL-1β, IL-6, and TNF-α at both sites within 6 hours*

... despite inflammatory cell recruitment not being altered until 96 hours after the LPS challenge!
Effects of LPS on Oxidative Stress and Axon Injury

- We know that $\text{IL-1}\beta$ and $\text{TNF}\alpha$ induce $\text{iNOS}$.
- It was shown that $\text{IL-1}\beta$ and $\text{TNF}\alpha \uparrow$ within 6 hours.

... does an iNOS induction happen here too?

→ Yes, it does!

- $\text{iNOS} \uparrow$ within 6 hours.
- NO release is implicated in axon injury in MS.
- Axon injury can be measured by APP accumulation.
Results IV

(A) (1 week)

(B) (3 weeks)

(C) (6 weeks)
Results IVb
Results IV

![Graph showing nitrotyrosine signal over time after peripheral challenge]

**Graph Details:**
- **X-axis:** Time (hr) after peripheral challenge
- **Y-axis:** Nitrotyrosine signal (integrated intensity)
- **Legend:** EAE-LPS, EAE-SAL

**Annotations:**
- Marked with an asterisk (*)

**Images:**
- Image I: [Image 1]
- Image J: [Image 2]
- Image K: [Image 3]
Microglia Switching

Does the microglia/macrophage switching contribute to axonal injury?

- Remember: There is iNOS↑, APP↑ + cytokines↑ within 6 hours after LPS challenge but inflammatory cells are recruited only 96 hours after the challenge!
- M/M become morphologically activated in response to inflammatory events...
- ... IL-1β, iNOS and MHC II are markers associated with microglial activation and axonal damage
Results VI

A

% of positive cells

INOS+ vs INOS-

0 6 12 24 96

B

% of positive cells

MHC II+ vs MHC II-

0 6 12 24 96

C

% of positive cells

IL-1β+ vs IL-1β-

0 6 12 24 96

Time (hr) after LPS challenge
What are the mechanisms underlying axonal damage as a consequence of M/M activation?

Lesions were assayed for mRNA of proinflammatory mediators iNOS, IL-1β and TNF-α and anti-inflammatory cytokines TGF-β and IL-10
The differential expression of the proinflammatory and anti-inflammatory mediators in the lesions infers a link between cytokine expression and ongoing axonal injury. The differences, between physically adjacent lesions, after LPS challenge indicates that it is the local microenvironment that critically determines the induction of the tissue damaging (axon injury) profile during an inflammatory episode.
Systemic inflammation induced by LPS activates the CNS innate immune response
(IL-1β, IL-6 and TNF-α↑ in spleen and cord)
This switches the phenotype of microglia/macrophages of animals with EAE to an aggressive proinflammatory phenotype
(iNOS, MHC II, IL-1β↑ in APP+ lesions)
This switch happens within 6 hours of the LPS challenge and prior to any significant recruitment of T-cells to the lesions
In our model, about 60% of the animals challenged with LPS had a clinically detectable relapse; however, LPS leads to the generation of tissue damage independently of detectable clinical signs. Furthermore, tissue damage did not occur in all lesions in the LPS-challenged animals and was not associated with BBB breakdown. This dissociation indicates that the microglia/macrophage phenotype after systemic challenge with LPS might be sensitive to regulation by the local CNS microenvironment or to a stochastic process, leading to heterogeneity of CNS lesions, as has been observed in the brain of patients with MS.48
This is of interest because Buljevac and colleagues\textsuperscript{5} have shown that infection-associated relapses do not lead to an increase in gadolinium-enhancing lesions or evidence of BBB breakdown, but may lead to long-term deficits. It is important to note that despite the presence of tissue-damaging lesions not all the animals showed an increase in clinical signs and in those where these signs appeared they were mild. This infers that systemic events which modify the activity of the immune system may drive an increase in tissue damage in the CNS without producing overt clinical relapses, which may in turn help explain the clinical-pathological/MRI paradox.\textsuperscript{49}
During remission, the main contributor to axonal damage is chronic microglia activation.

Microglia/macrophages, associated with lesions, respond to circulating cytokines produced by inflammation outside the CNS.

These activated M/M release immune mediators that lead to tissue damage.

Preventing and stringently managing systemic infectious diseases may slow down disease progression.
Thank you for your attention!