Christian Doppler Laboratory for Cardiac and Thoracic Diagnosis & Regeneration

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Systemic Inflammation Induces Axon Injury During Brain Inflammation

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Overview

- Background
- Methods
- Results
- Discussion
- Conclusion

EAE

- Model for inflammatory demyelinating disease

MS:

- Relapsing-remitting disease
- Progressive in one half of patients

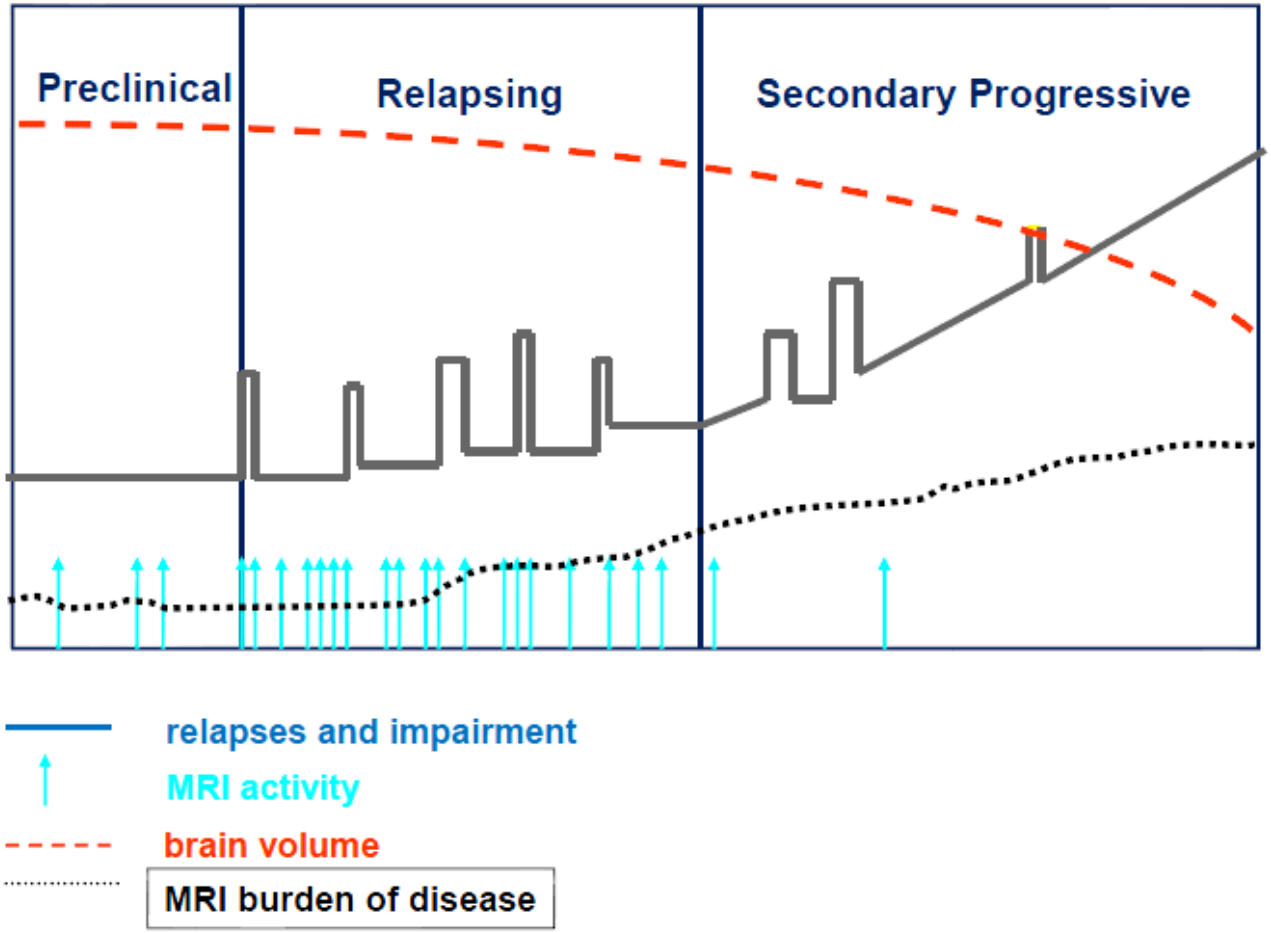
  → axonal damage

  → secondary progressive MS
- Approx. 1/3 of relapses are preceded by a systemic infection

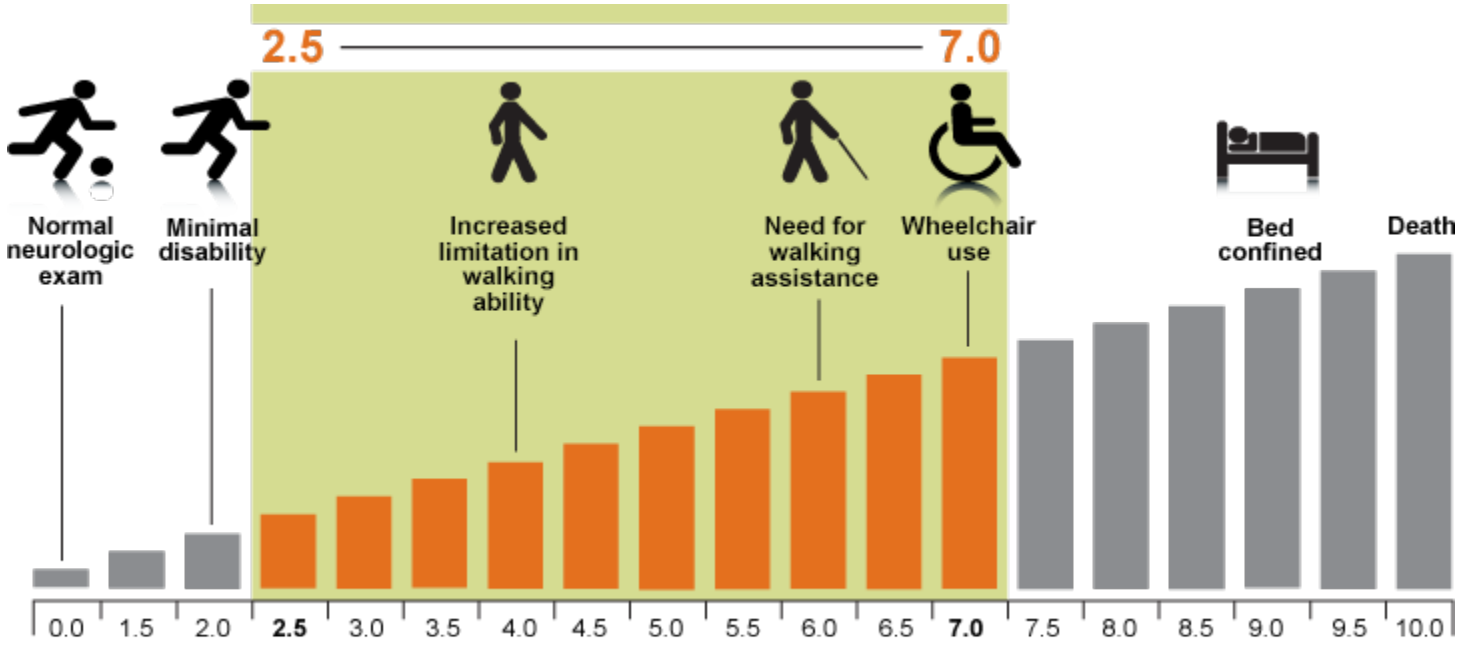
Infection

- 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

MS Progression



EDSS



Patient criteria in clinical trials included the ability to complete two trials of the 25-foot walk in 8-45 seconds.[2,3]

http://ampyra-hcp.com/local/images/hcp/about/patient-demongraphics_lg.png (27/01/12(

Study Design

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 $\geq$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)*

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

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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

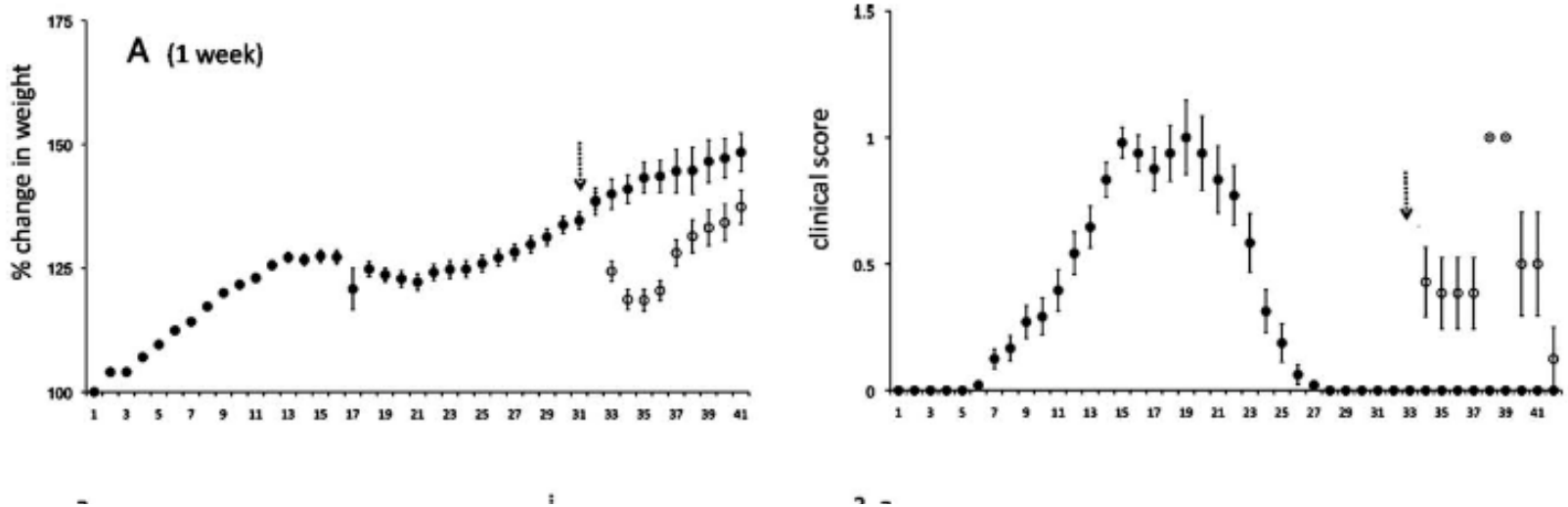
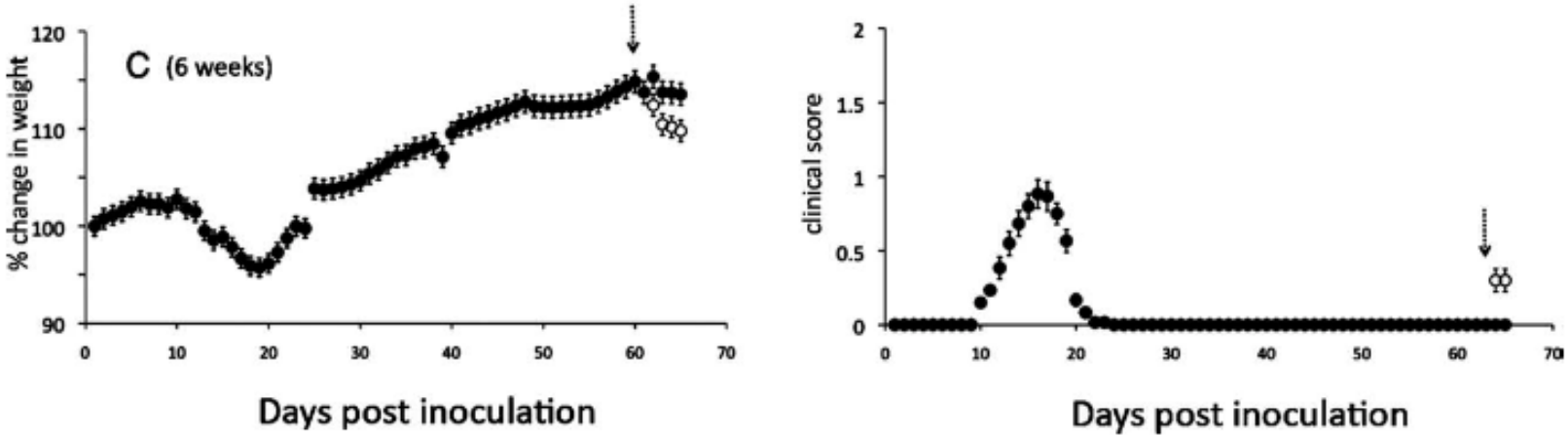


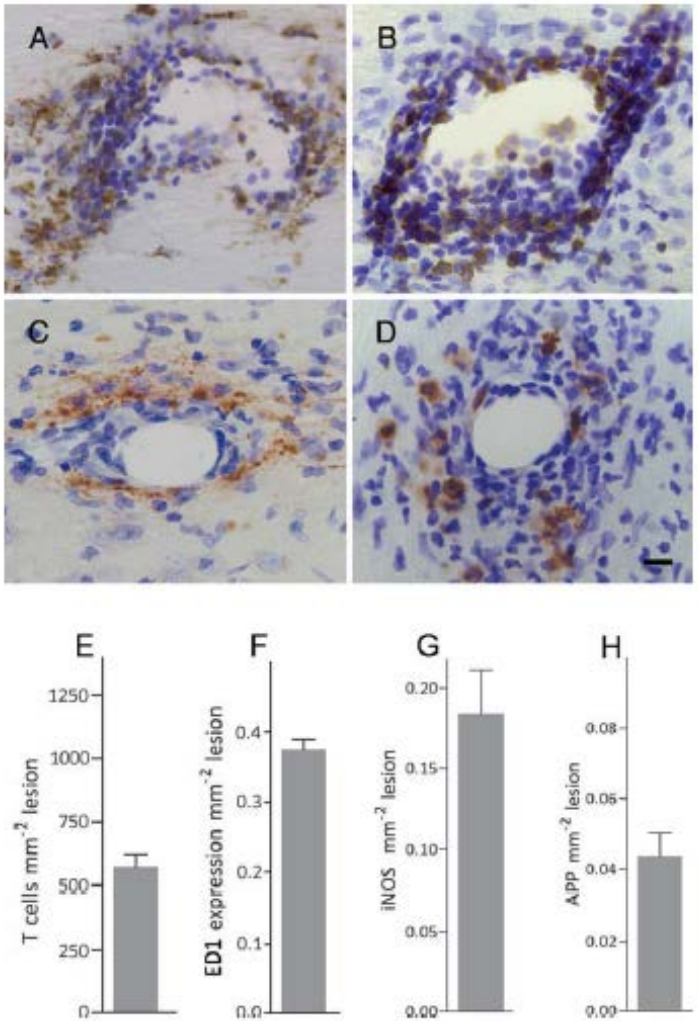
FIGURE 2: Change in weight (left) and clinical scores (right) over the course of the experiments. All animals exhibited clinical symptoms and weight loss associated with development of monophasic EAE. Any animal that had a clinical score ≥2 during the initial phase was eliminated from the study. A total of 216 animals were induded 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

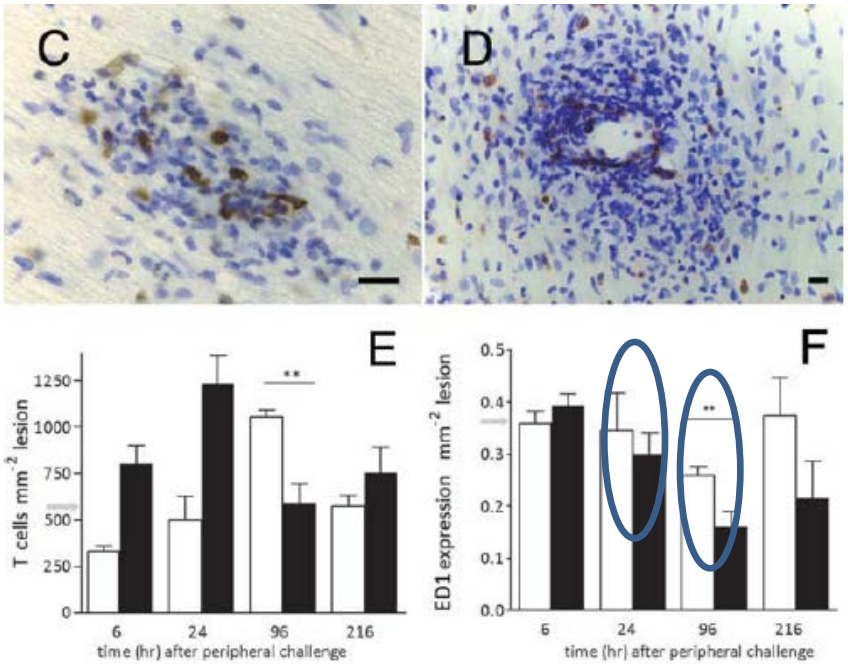
- EAE symptoms on days 6-25
- Peak disease (n=12) between days 15-20
- Onset of remission by day 26
- LPS injection resulted in re-emergence of mild EAE in 60%
- Controls remained in remission
- Histology at peak of disease revealing perivascular inflammatory infiltrates (macrophages/microglia and T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

Results I


A
B
C
D
E
T cells mm-2 lesion
1250
1000
750
500
250
0
F
ED1 expression mm-2 lesion
0.4
0.3
0.2
0.1
0.0
G
iNOS mm-2 lesion
0.20
0.15
0.10
0.05
0.00
H
APP mm-2 lesion
0.08
0.06
0.04
0.02
0.00

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.



Results II

- 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

Results II

- **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)

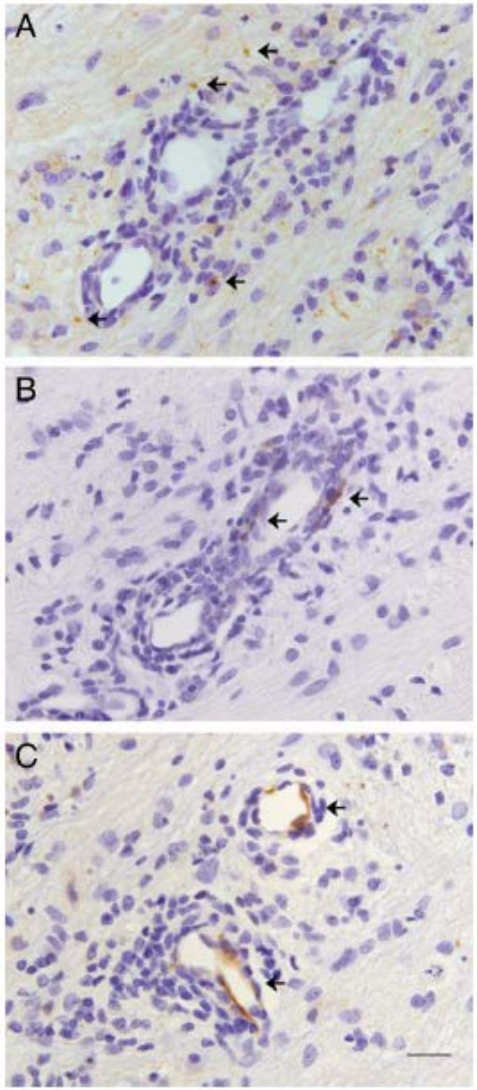


FIGURE 5: Blood-brain-barrier integrity in APP+iNOS+ lesions 24 hours after an LPS challenge at 1 week. (A) APP. (B) iNOS. (C) IgG. Bar = 20μm. APP = amyloid precursor protein; IgG = immunoglobulin G; iNOS = inducible nitric oxide synthase; LPS = lipopolysaccharide.

Results III

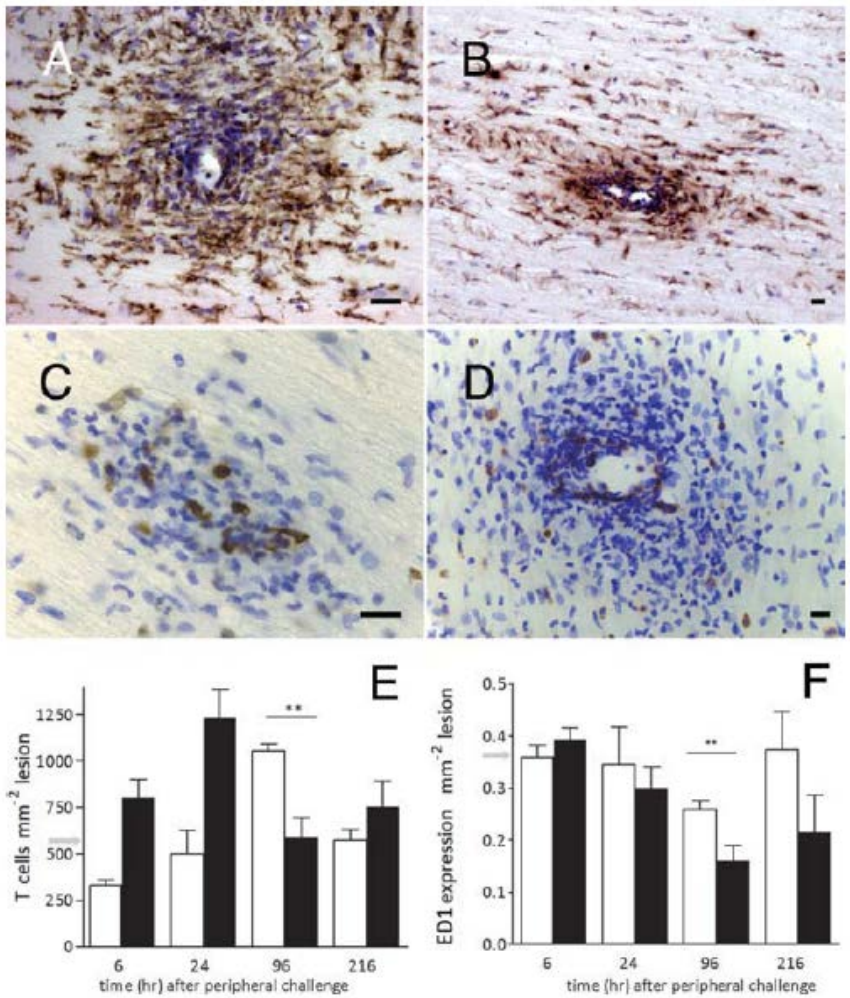
- ***What about the effects of LPS on the peripheral and CNS immune response?***

- Investigation of cytokine profiles within 24 hours

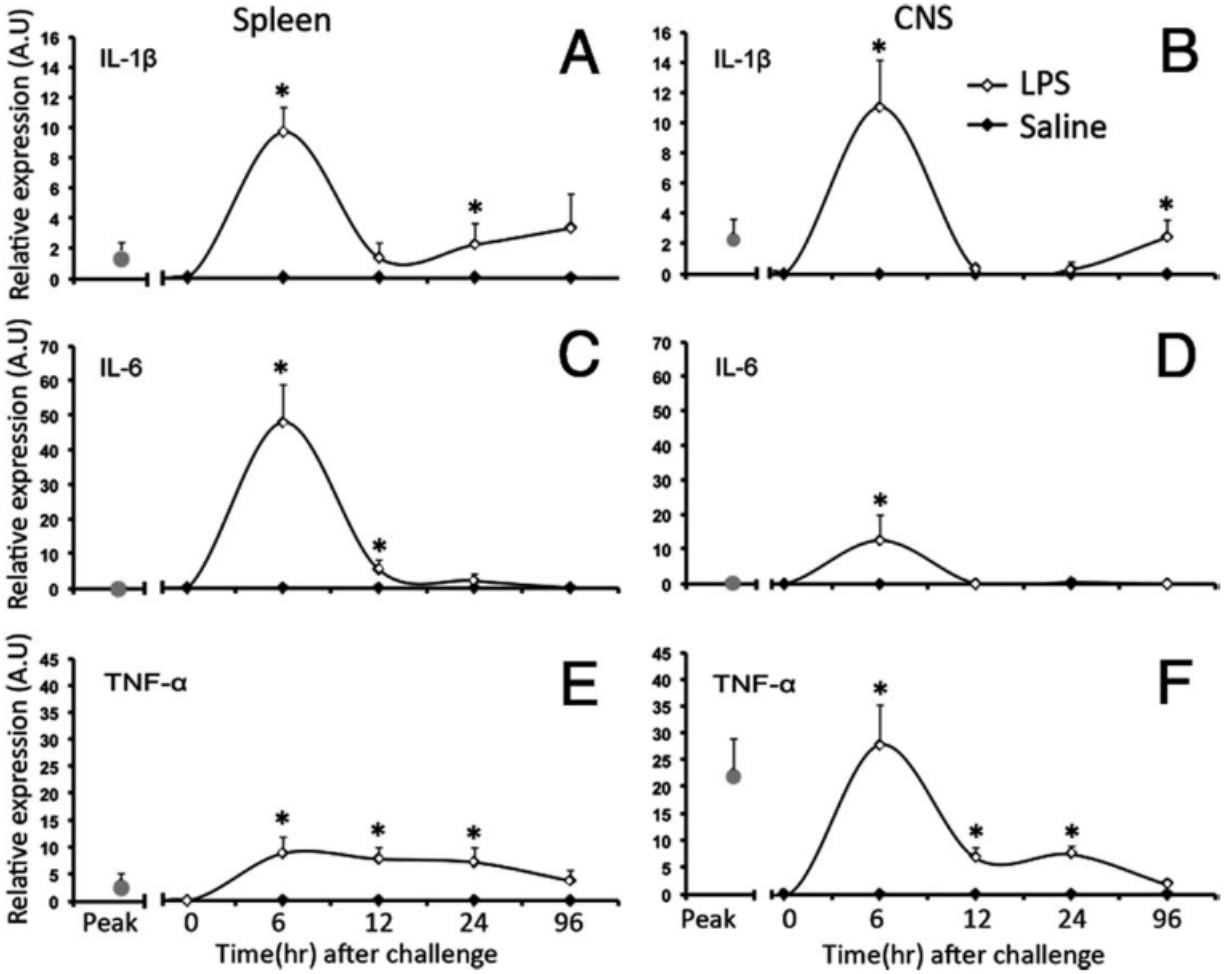
  … in the spleen and the cord

- Remember: there were **no alterations in cell recruitment** within 24 hours!

Results III


A
B
C
D
E
T cells mm-2 lesion
1250
1000
750
500
250
0
**
6
24
96
216
time (hr) after peripheral challenge
F
ED1 expression mm-2 lesion
0.5
0.4
0.3
0.2
0.1
0.0
**
6
24
96
216
time (hr) after peripheral challenge

Results III


Spleen
CNS
A
B
C
D
E
F
IL-1β
IL-6
TNF-α
LPS
Saline
Relative expression (A.U)
Peak
0
6
12
24
96
Time(hr) after challenge

Results III

- 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!

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Effects of LPS on Oxidative Stress and Axon Injury

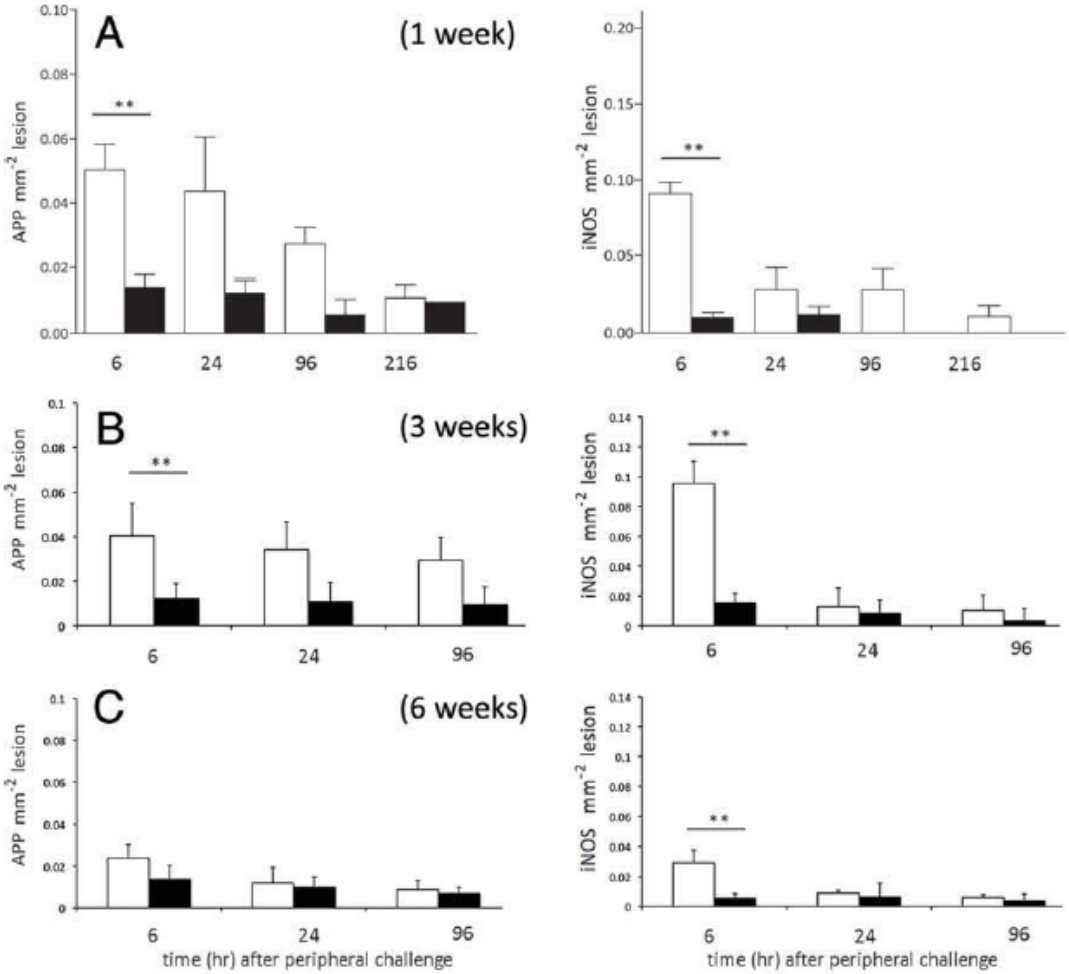
- We know that **IL-1β** and **TNFα** induce **iNOS**
- It was shown that **IL-1β** and **TNFα↑** within 6 hours

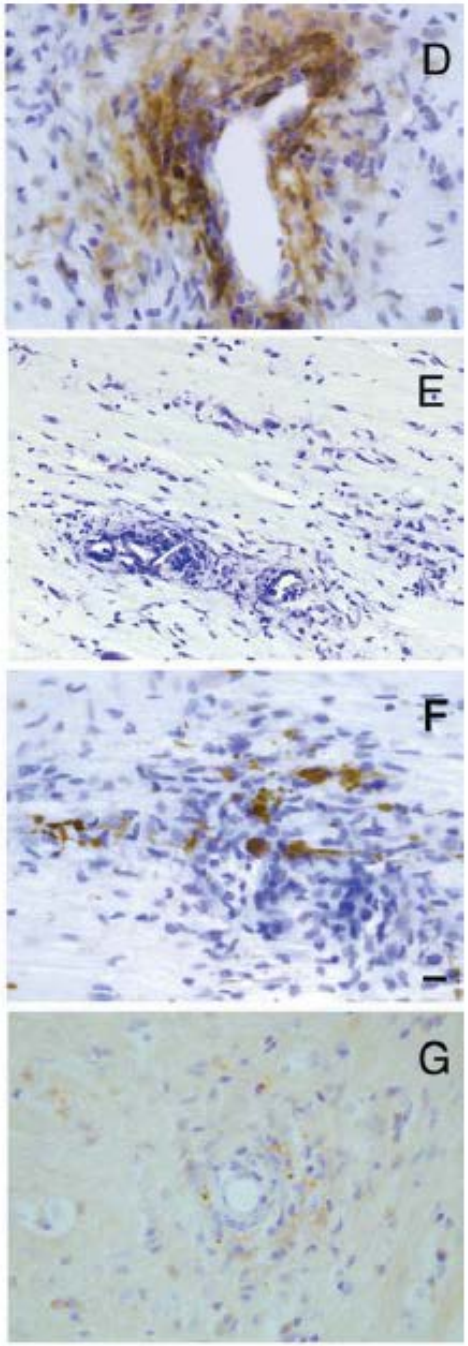
- ***… does an iNOS induction happen here too?***

→ Yes, it does!

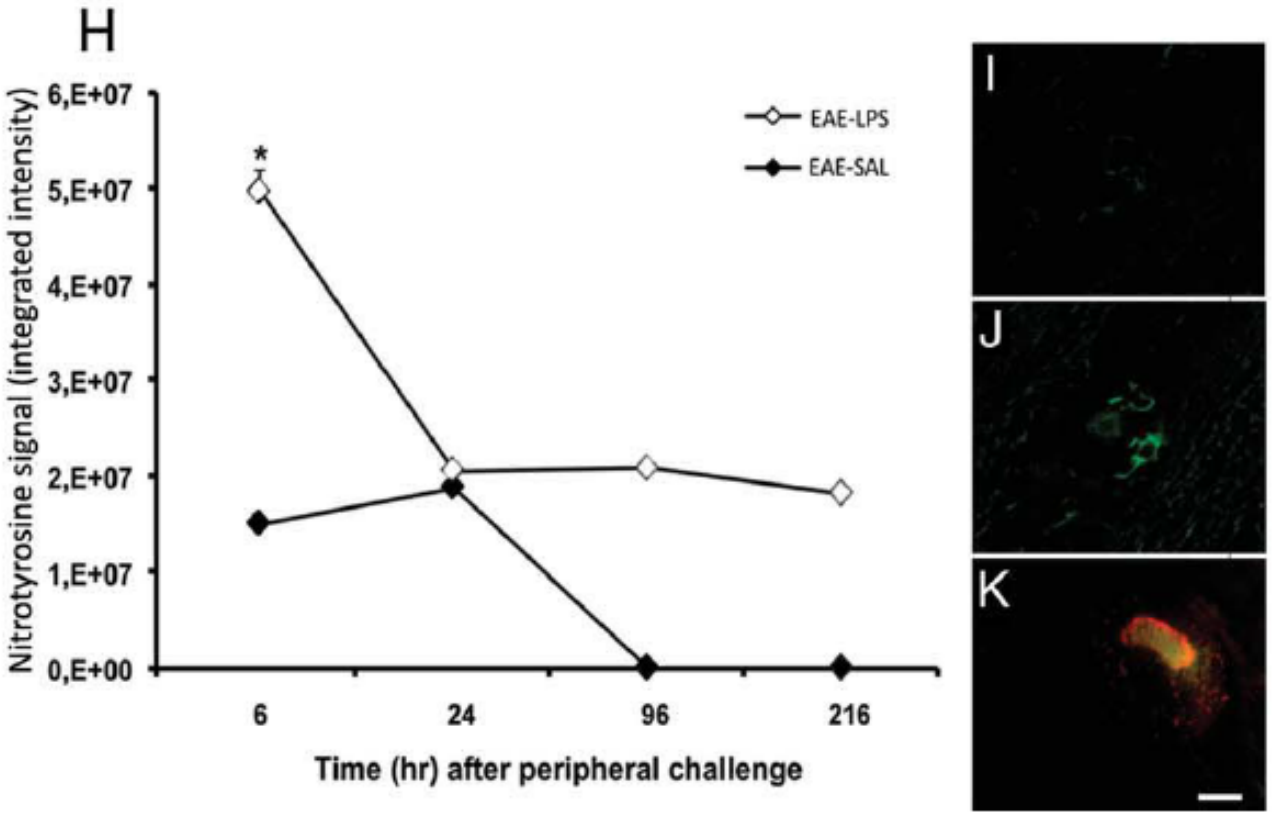
- **iNOS↑** within 6 hours
- **NO release** is implicated in **axon injury** in MS
- Axon injury can be measured by **APP accumulation**

Results IV


A
(1 week)
APP mm-2 lesion
iNOS mm-2 lesion
**
6
24
96
216
B
(3 weeks)
C
(6 weeks)
time (hr) after peripheral challenge


D
E
F
G

Results IV

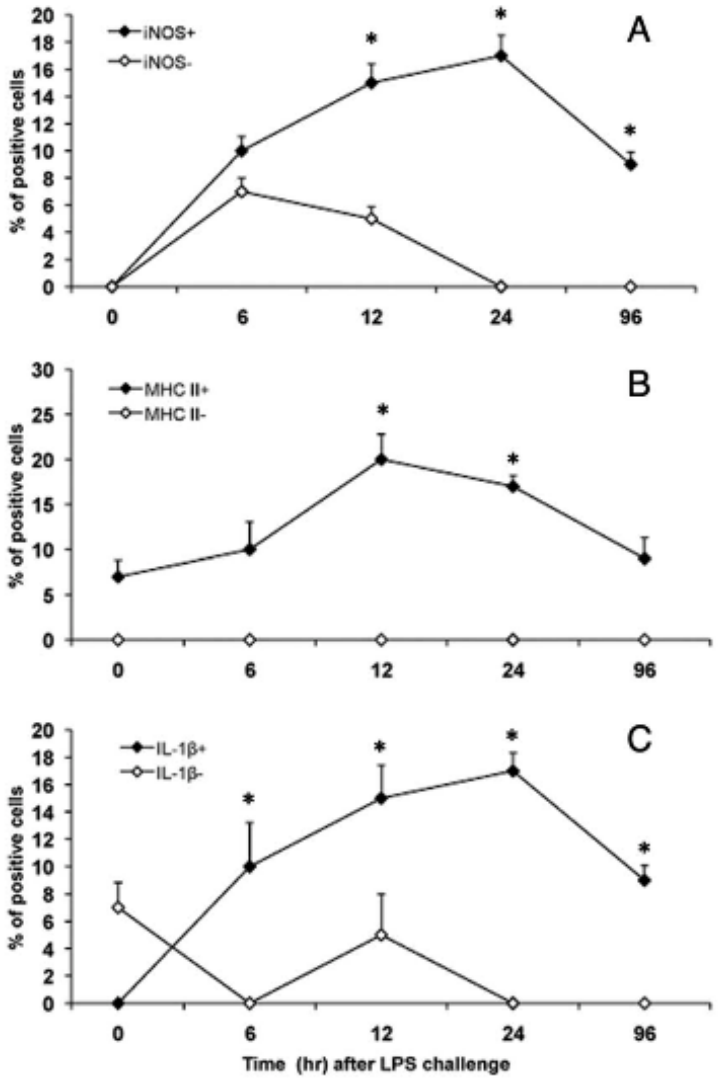

H
Nitrotyrosine signal (integrated intensity)
6,E+07
5,E+07
4,E+07
3,E+07
2,E+07
1,E+07
0,E+00
*
EAE-LPS
EAE-SAL
6
24
96
216
Time (hr) after peripheral challenge
I
J
K

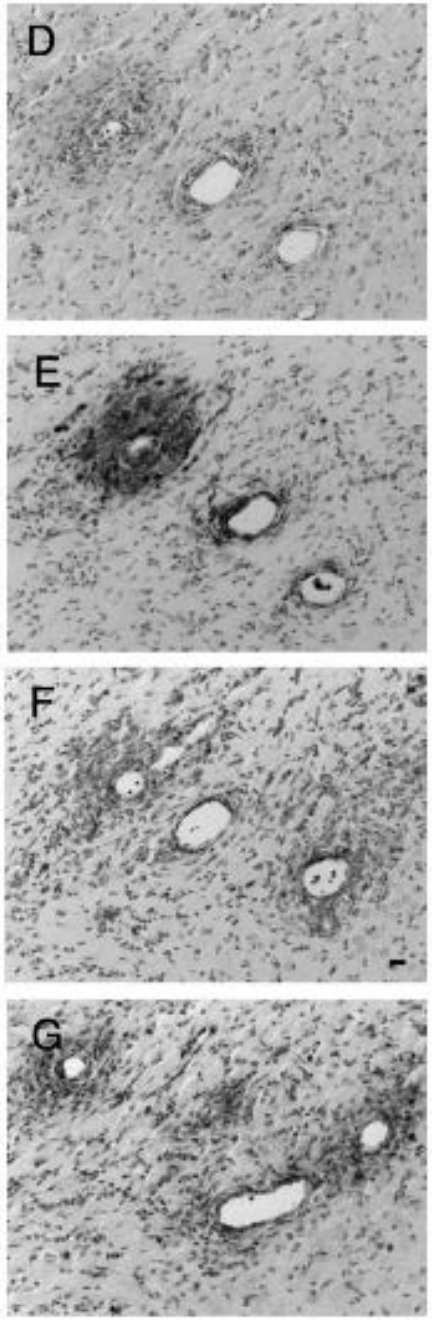
Microglia Switching

- ***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

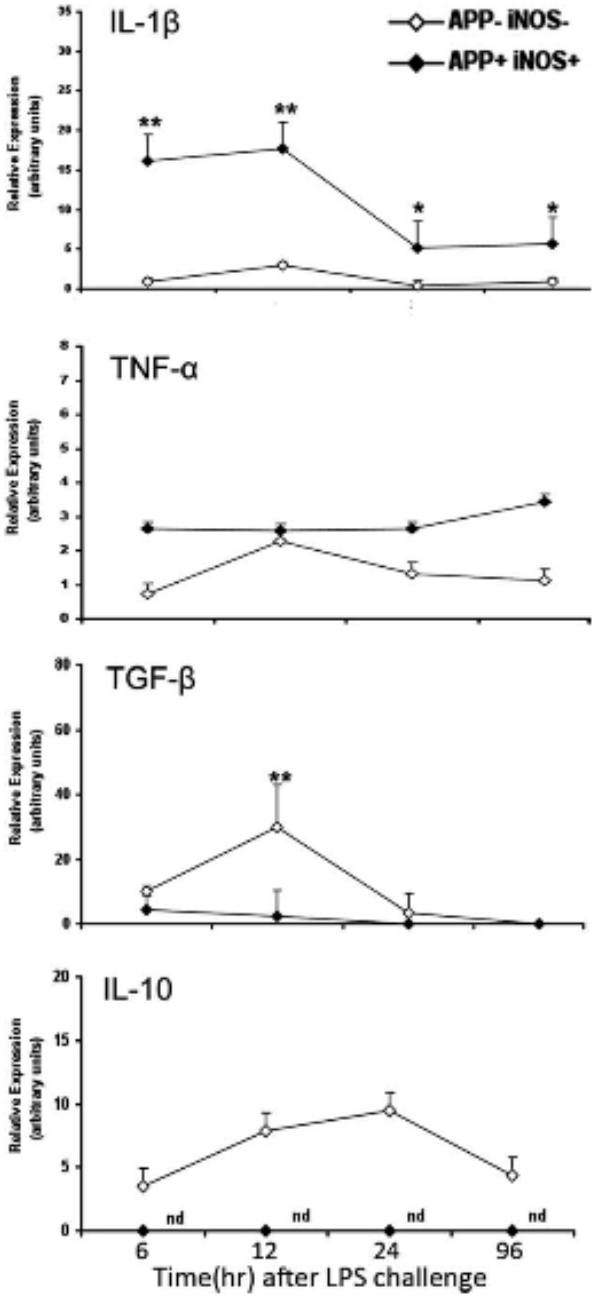

A
iNOS+
iNOS-
% of positive cells
B
MHC II+
MHC II-
% of positive cells
C
IL-1β+
IL-1β-
% of positive cells
Time (hr) after LPS challenge


D
E
F
G

Consequences of Microglial Activation

- ***What are the mechanisms underlying axonal damage as a consequence of M/M activation?***

→ Lesions were assayed for mRNA of **pro**inflammatory mediators **iNOS**, **IL-1β and TNF-α** and **anti**-inflammatory cytokines **TGF-β and IL-10**


IL-1β
APP- iNOS-
APP+ iNOS+
Relative Expression (arbitrary units)
**
**
*
*
TNF-α
Relative Expression (arbitrary units)
TGF-β
Relative Expression (arbitrary units)
**
IL-10
Relative Expression (arbitrary units)
nd
nd
nd
nd
6
12
24
96
Time(hr) after LPS challenge

Local Microenvironment

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.

Discussion

- 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

Discussion

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]

Discussion

This is of interest because Buljevac and colleagues[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.[49]

Conclusion

- 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**

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Thank you for your attention!