Immune Cells & Hearing Disorders: Development of a Short *in vivo* Model for Evaluating the Anti-inflammatory Potential of Therapeutic Compounds

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Objectives

Following exposure to noise, ototoxic agents or aging, immune cells, particularly **macrophages**, are recruited to the cochlea to contribute to tissue repair. However, their excessive activation can lead to **chronic inflammation**, resulting in cochlear damage and **hearing loss**. A better characterization of macrophage recruitment kinetics, localization and signaling pathways is essential for optimizing the therapeutic potential of **anti-inflammatory compounds**.

This study aimed to establish a **short** and well characterized ***in vivo* model of cochlear inflammation to efficiently evaluate anti-inflammatory compounds**.

Methods

Different parameters were evaluated: Strains of mice, LPS concentrations, timepoints for monitoring hearing (ABR measures) and immune response, histological techniques for the characterization of immune cells.

Table 1: Parameters of Described LPS Studies

Studies	Study #1	Study #2
Mice (6 weeks old)	CBA/JRj (n=2/group)	CBA/JRj and C57Bl/6J (n=8/group)
LPS Concentration	5 and 10 µg/ear in both ears	10 µg/ear in both ears
Transtympanic (TT) Injections	5 µL/ear of LPS or NaCl at T_0 (both ears)	• 5 µL/ear of LPS or NaCl at T_0 (both ears) • 5 µL/ear of NaCl at $T_{+5HOURS}$ (both ears)
ABR Measures	Baseline, T_{+2DAYS}, T_{+4DAYS}, T_{+7DAYS}	Baseline, T_{+2DAYS}
Cochleae Sampling	T_{+2DAYS}, T_{+4DAYS}, T_{+7DAYS}	T_{+2DAYS}
Histological Analysis	Macrophages on flat surface preparations	• Macrophages on flat surface preparations • Macrophages, T and B lymphocytes on cross sections

ABRs (Auditory Brainstem Responses): ABRs were measured on both ears (5 animals/one ear at a time) at Baseline (BL), T_{+2DAYS}, T_{+4DAYS} and T_{+7DAYS} (Study #1) or BL and T_{+2DAYS} (Study #2) at 7 frequencies: 4, 8, 16, 25, 32, 40 and 45 kHz.

Histological analyses on flat surface preparations:

At T_{+2DAYS}, T_{+4DAYS} and T_{+7DAYS} (Study #1) or at T_{+2DAYS} (Study #2) cochleae were sampled, fixed, decalcified and microdissected. Hair cells (HCs) and macrophages were immunolabeled with anti-Myosin-VIIa and anti-Iba1 antibodies, respectively. Images were acquired with a confocal microscope. Macrophage analysis was performed either qualitatively at the apex, middle and base of cochleae (n=4/group, Study #1) or quantitatively by measuring the corresponding fluorescence intensity at 8, 16, 25, 32, 40 and 45 kHz, using ImageJ software (n=8/group, Study #2) .

Histological analyses on cross sections:

At T_{+2DAYS} (Study #2) cochleae were sampled (n=4/group), fixed and decalcified. After paraffin embedding, cochleae were cross-sectioned and immunolabeled with anti-Iba1 (macrophages), anti-Pax 5 (B lymphocytes) or anti-CD3 (T lymphocytes) antibodies. Images were acquired with a NanoZoomer slide scanner and immune cell counts were performed at the apex, middle and base of each cochleae, for the region of spiral ganglion neurons (SGN), organ of Corti (OC), stria vascularis and spiral ligament (NDP view software).

Conclusion

In cochleae treated with 10 µg of LPS, a huge recruitment of **inflammatory cells**, mainly **macrophages**, was reported from T_{+2DAYS} to T_{+4DAYS}, thanks to histological tools and **correlated with transient hearing loss**. These results validate the local use of LPS as a rapid and efficient method to **induce cochlear inflammation**, providing a **relevant *in vivo* model to evaluate anti-inflammatory compounds *in vivo* and their effects on hearing**. Immune response characterization is now being applied to our noise- and cisplatin-induced hearing loss models to **better define the therapeutic window and treatment efficacy.**

Results: Effects of LPS on Hearing & Macrophage Recruitment

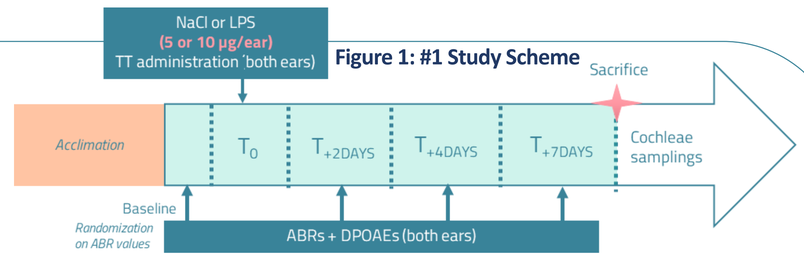


Figure 1: #1 Study Scheme

ABRs

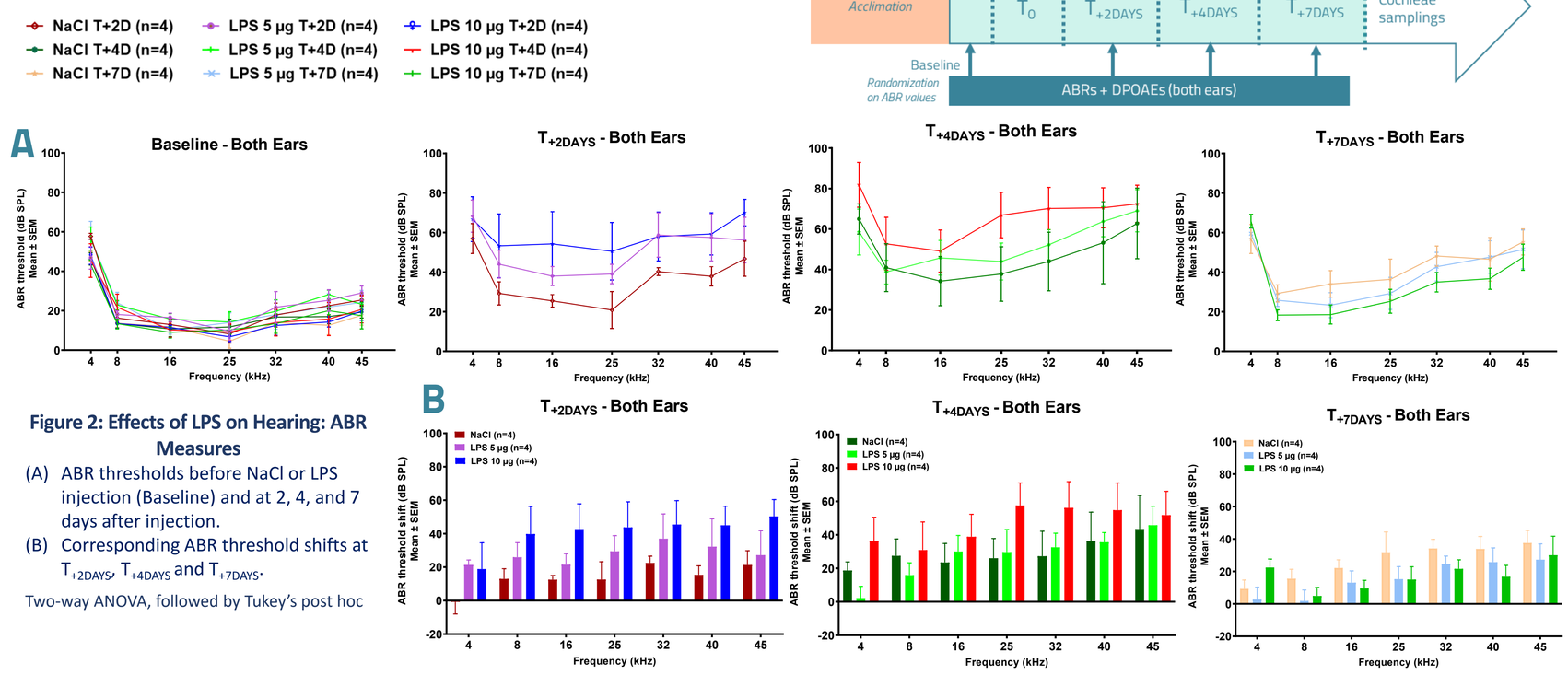


Figure 2: Effects of LPS on Hearing: ABR Measures

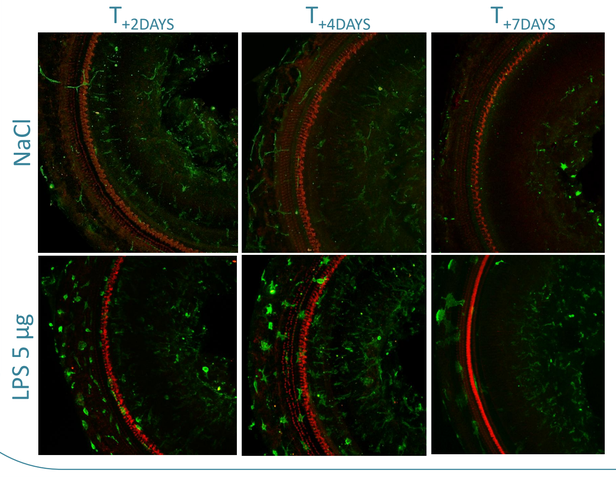
(A) ABR thresholds before NaCl or LPS injection (Baseline) and at 2, 4, and 7 days after injection.

(B) Corresponding ABR threshold shifts at T_{+2DAYS}, T_{+4DAYS} and T_{+7DAYS}.

Two-way ANOVA, followed by Tukey's post hoc

At Baseline, mice exhibited ABR thresholds corresponding to normal hearing. After **LPS injection at 5 and 10 µg/ear**, a **dose effect** was observed on the **increase of ABR thresholds and ABR threshold shifts, at all frequencies, from T_{+2DAYS} to T_{+4DAYS}** compared to mice injected with NaCl. **At T_{+7DAYS}, a recovery of ABR thresholds and ABR threshold shifts was observed** in mice administered with LPS, regardless of the dose, compared to mice injected with NaCl.

Macrophage recruitment



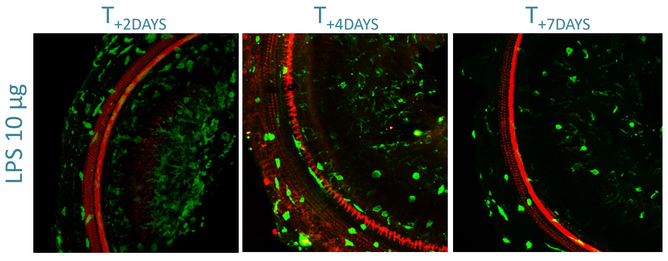


Figure 3: Effects of LPS on Inflammation: Histological Evaluation

Representative pictures of cochleae sections from the middle for mice injected with either NaCl, LPS 5 µg or LPS 10 µg and sampled at T_{+2DAYS}, T_{+4DAYS} or T_{+7DAYS}. Cochleae were immunostained for Myosin-VIIa (HCs, red) and Iba1 (macrophages, green).

Qualitative analyses of macrophages showed:

- **In NaCl-mice: Few macrophages** observed from T_{+2DAYS} to T_{+7DAYS}. Macrophages showed a **stellate morphology** with long processes, characteristics of non-inflammatory conditions.
- In **LPS-mice: Increase of macrophage recruitment** at **T_{+2DAYS} and T_{+4DAYS}, with a greater number of macrophages with the highest dose of LPS**. Macrophages **presented round cell bodies** with processes shortened. **At T_{+7DAYS}, decrease of the number of recruited macrophages** compared to T_{+2DAYS} and T_{+4DAYS}.

Results: Effect of LPS on Hearing Depending on the Mouse Strain

ABRs

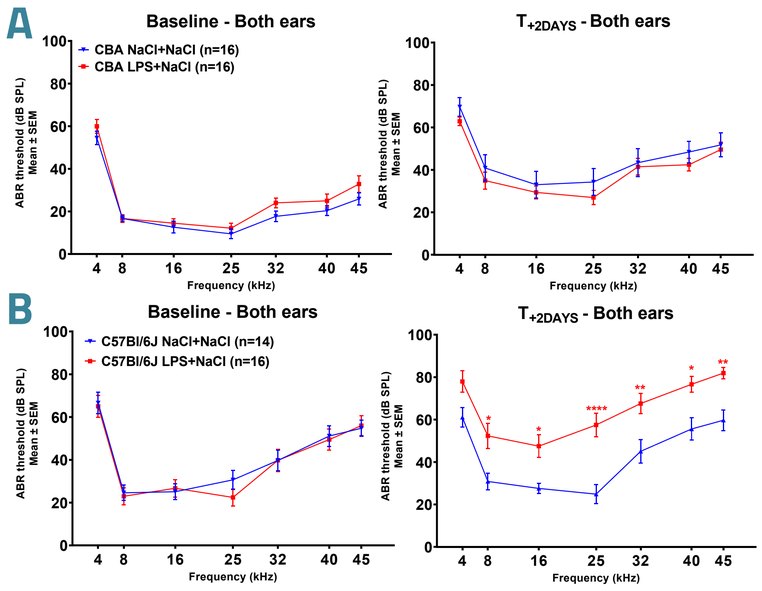


Figure 4: #2 Study Scheme

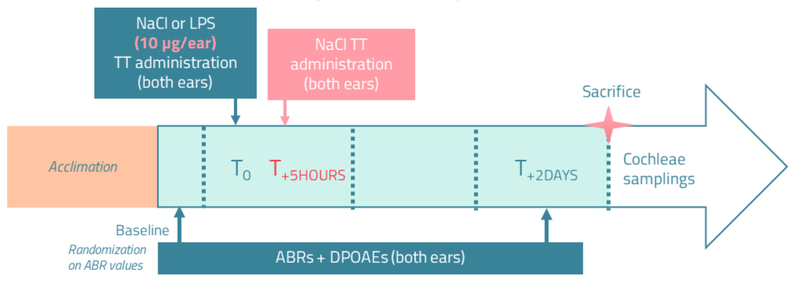


Figure 4: Effects of LPS on Hearing of CBA and C57Bl/6J Mice : ABR Measures

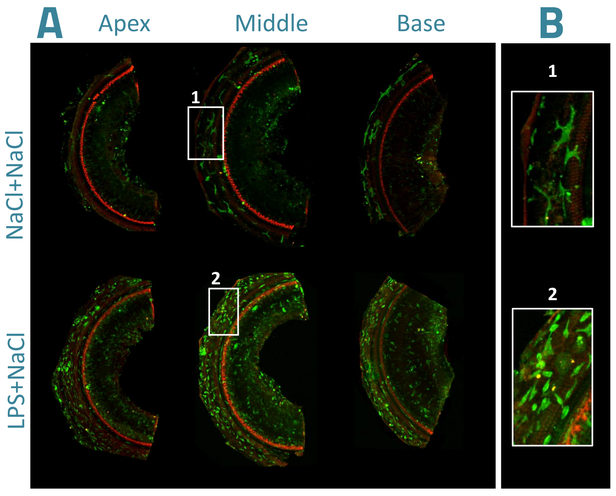
ABR thresholds before NaCl or LPS injection (Baseline) and 2 days after injection in CBA **(A)** and C57Bl/6J **(B)** mice.

Two-way ANOVA, followed by Bonferroni's post hoc, *vs. NaCl+NaCl group, *p<0.05,** p<0.01, **** p<0.0001

- At Baseline, mice exhibited ABR thresholds corresponding to normal hearing.
- **At T_{+2DAYS}, in CBA mice: no significant difference of ABR thresholds** in LPS-treated mice, from 4 to 45 kHz, compared to NaCl-injected mice.
- **At T_{+2DAYS}, in C57Bl/6J mice: significant increase of ABR thresholds in LPS-treated mice**, from 8 to 45 kHz, compared to NaCl-injected mice.

Results: Characterization of LPS-induced Cochlear Inflammation in C57Bl/6J Mice

Results on flat surface preparations



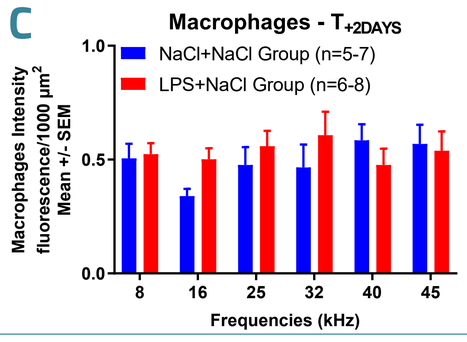


Figure 5: Macrophage Recruitment in LPS-Injected Cochleae

(A) Representative pictures of flat surface preparations of cochleae from C57Bl/6J mice injected with NaCl or LPS at 10 µg/ear.

(B) Magnified images of macrophages. Cochleae were immunostained for Myosin-VIIa (HCs, red) and Iba1 (macrophages, green).

(C) Quantification of fluorescence intensity corresponding to macrophages.

Results on cross sections

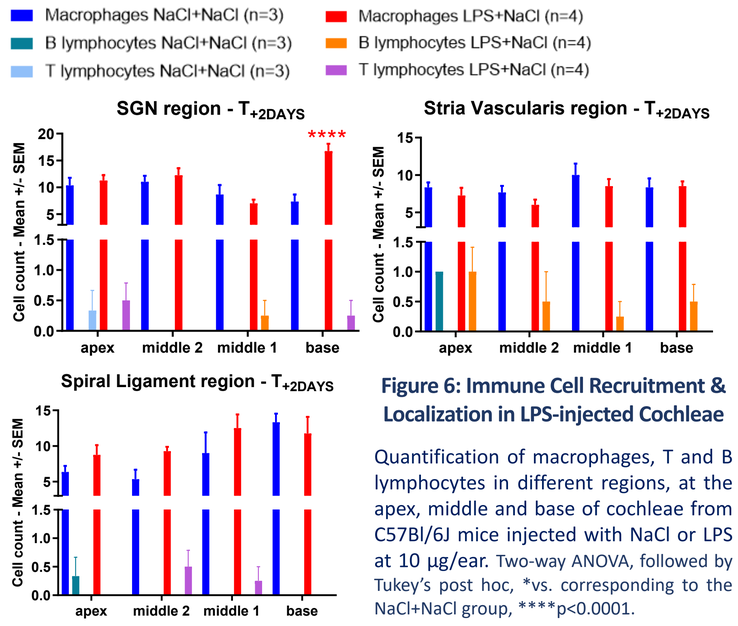


Figure 6: Immune Cell Recruitment & Localization in LPS-injected Cochleae

Quantification of macrophages, T and B lymphocytes in different regions, at the apex, middle and base of cochleae from C57Bl/6J mice injected with NaCl or LPS at 10 µg/ear. Two-way ANOVA, followed by Tukey's post hoc, *vs. corresponding to the NaCl+NaCl group, ****p<0.0001.

Few macrophages were observed in cochleae of **NaCl-mice**. Macrophages presented a **stellate morphology** with long processes. After the LPS injection, an **increase of macrophage recruitment, presenting round cell bodies,** was observed in cochleae.

Macrophage quantification indicated an **increase of the number of macrophages at 16, 25 and 32 kHz in LPS-treated mice**, compared to NaCl-injected mice.

After the LPS injection, **recruited immune cells were mainly macrophages but no T or B lymphocytes**. Macrophages were primarily located in the SGN, stria vascularis and spiral ligament regions, with almost no immune cells detected in the OC region (data not shown).