Video Article

# An *Ex vivo* Model of an Oligodendrocyte-directed T-Cell Attack in Acute Brain Slices

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## Abstract

Death of oligodendrocytes accompanied by destruction of neurons and axons are typical histopathological findings in cortical and subcortical grey matter lesions in inflammatory demyelinating disorders like multiple sclerosis (MS). In these disorders, mainly $CD8^+$ T-cells of putative specificity for myelin- and oligodendrocyte-related antigens are found, so that neuronal apoptosis in grey matter lesions may be a collateral effect of these cells. Different types of animal models are established to study the underlying mechanisms of the mentioned pathophysiological processes. However, although they mimic some aspects of MS, it is impossible to dissect the exact mechanism and time course of ‘‘collateral’’ neuronal cell death. To address this course, here we show a protocol to study the mechanisms and time response of neuronal damage following an oligodendrocyte-directed $CD8^+$ T cell attack. To target only the myelin sheath and the oligodendrocytes, *in vitro* activated oligodendrocyte-specific $CD8^+$ T-cells are transferred into acutely isolated brain slices. After a defined incubation period, myelin and neuronal damage can be analysed in different regions of interest. Potential applications and limitations of this model will be discussed.

## Video Link

The video component of this article can be found at https://www.jove.com/video/52205/

## Introduction

Death of oligodendrocytes and destruction of the myelin sheath accompanied by loss of neurons and axons are typical pathological findings in grey matter lesions in individuals suffering from multiple sclerosis (MS)[1,2]. Cortical lesions can be divided so far in three different subtypes[2]: subpial, intracortical and leukocortical lesions. In comparison to white matter plaques, infiltrates are characterized by a predominance of $CD8^+$ T-cells, suggesting their possible decisive role in grey matter inflammation[3]. Furthermore, oligoclonal expansions in blood, cerebrospinal fluid (CSF) and within inflammatory lesions can be found for $CD8^+$ T-cells themselves[4-6].

In line with this, it is assumed that $CD8^+$ T-cells may be specific for different myelin proteins[7,8]. Indeed, $CD8^+$ T-cells are found near oligodendrocytes and myelin sheaths[9,10] that show MHC I expression[11] and might therefore be responsible for the loss of the myelin sheath. This process is often seen together with extensive ‘‘collateral’’ neuronal and axonal damage within the central nervous system (CNS) grey matter[1,2]. In fact, direct and indirect death of oligodendrocytes and neurons is induced by $CD8^+$ T-cells via two different mechanism: (i) cell membrane swelling and rupture due to the formation of cytotoxic granules following the release of perforins and granzymes and (ii) ligation to the Fas receptors or exposition of FasL on their surface[8,12,13].

Different types of animal models are established to study the underlying mechanism of the mentioned processes. In this respect, primed $CD8^+$ T-cells specific for autoantigens with induced expression in CNS glial cells, like oligodendrocytes or astrocytes, can be adoptively transferred to analyse ‘‘collateral’’ neuronal and axonal death in grey matter subsequently[14,15]. To perform such *in vivo* experiments is a big help to mimic some pathophysiological aspects of MS, however, this approach is not suited to resolve the underlying mechanism and kinetics of axonal damage and neuronal apoptosis.

To overcome these restrictions, an *ex vivo* approach was established to study the mechanisms and time course of neuronal cell death following a oligondendrocytes-directed $CD8^+$ T-cell attack. Since only oligodendrocytes and therefore myelin sheath production should be targeted by immune cells, MHC class-I-restricted, ovalbumin (OVA)-reactive OT-I Tcells are used[16]. These cells are subsequently transferred into brain slices obtained from mice selectively expressing OVA in oligodendrocytes (ODC-OVA mice)[17].

 

## Protocol

All experiments using mice should be performed in accordance with the guidelines of the respective institutional animal care and use committee.

## 1. General Comments for Mouse Experiments

1. Keep the mice under pathogen-free conditions and enable them access to food and water ad libitum.
   NOTE: It is important to use age- and sex-matched mice in experimental groups because immunological patterns can vary with age and gender.

## 2. Preparation and Activation of OVA-specific $CD8^+$ T-cells (OT-I)

1. Perform stimulation of OT-I T cells as described below 5 days before preparing brain slices.
   NOTE: $5 \times 10^5$ activated effector $CD8^+$ OT-I T-cells ($CD8^+$ T-cells of transgenic mice recognizing only the $OVA_{257-264}$ peptide in the context of MHC-I molecules) are needed per slice The concentration $5 \times 10^5$ was chosen experimentally as optimal one to favor a good cell-cell interaction once that the cells start migrating into the slice and, at the same time, the amount of cells is not too high to induce an overreaction allowing single cell counting[8,18].
2. Prepare the medium for culturing OT-I T-cells. Supplement 500 ml DMEM with 5% fetal calf serum (FCS), 10 mM HEPES, 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 1% nonessential amino acids and 25 µg/ml gentamicin. Store the medium at 4 °C until use.
3. Remove the spleen of OT-I transgenic mice as per reference[16]. Transfer them into the prepared medium.
4. Generate single cell suspension by mashing spleens through a 70 µm strainer and centrifuge suspension at 300 x g for 5 min, 4 °C.
5. Incubate cell suspension with 5 ml ammonium-chloride-potassium (ACK) buffer (150 mM $NH_4Cl$, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3) for 5 min to lyse red blood cells.
6. Stop incubation with 10 ml medium and centrifuge it again as described above.
7. Dilute cell suspension in medium and calculate numbers. Plate cells at a density of $3 \times 10^7$ cells/well in a 12-well plate and prime them by incubation with $OVA_{257-264}$ (SIINFEKL; 1 nM) and interleukin 2 (IL-2) (500 IU/ml) for 5 days.
8. After 4 days, add IL-2 at a concentration of 500 IU/ml again.
9. Subsequent to stimulation, purify OT-I T-cells from cell suspension by using a negative selection based mouse $CD8^+$ T-cell isolation kit following manufacturer's instructions. Purification is based on the depletion of all non-$CD8^+$ cells by using a cocktail of antibodies against CD4, CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, MHC Class II, Ter-119, and TCRγ/δ which are then discarded by using magnetic columns for the elution of the purified $CD8^+$ T-cells.
   NOTE: Critical steps in this procedure are indicated by the manufactures: it is important that the reaction involves only single cells because the presence of clumps could block the column during the elution step; counting of the cell before adding the Byotin cocktail is important for the degree of purity of the sample and the procedure should be performed on ice to minimize unspecific antibody bindings.

## 3. Preparation of Acute Brain Slices and Co-culture with OT-I T-cells

1. Prior to the preparation of acute brain slices[19], prepare placedine ice-cold physiological saline solution using 200 mM sucrose, 20 mM PIPES, 2.5 mM KCL, 1.25 mM $NaH_2PO_4$, 10 mM $MgSO_4$, 0.5 $CaCl_2$ and 10 mM dextrose and artificial cerebrospinal fluid (ACSF) by using 125 mM NaCl, 2.5 KCl, 1.25 $NaH_2PO_4$, 24 mM $NaHCO_3$, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM dextrose. Adjust pH of each solution to 7.35 by using NaOH. Before adjusting pH of ACSF, solution must be bubbled with a mixture of 95% $O_2$ and 5% $CO_2$.
2. Pre-cool the solutions.
3. Anesthetize 8 - 10 week old mice (*e.g.,* C57Bl/6 as control or transgenic ODC-OVA mice[17] with inhalation using 5.0% isoflurane, 5.0% halothane or inject 100 mg/kg ketamine and 10 mg/kg body weight xylazine via i.p. Wait for anesthesia and use a toe pinch to assess the level of anesthesia and decapitate them at once. Euthanize the mice as per institutional animal care and use committee criteria for euthanasia.
4. Put mouse in ventral position. Disinfect and cut scalp sagittally. Open the cranial bone sagitally, remove the brain quickly with the help of a scoop and fix it on the plate of a vibratome by using glue.
5. Fill the plate with the prepared placedine ice-cold physiological saline solution.
6. Cut 300 µm coronal slices with the vibratome.
   NOTE: This procedure is known to yield viable intact tissue specimens appropriate for functional cellular studies within a time interval of at least 8 hr[19-21]. Indeed, after this time period, already starting after 6 hr, the preparation shows reduced quality, namely changes in basic and functional properties like action potential generation and propagation.
7. After sectioning, transfer one slice immediately into each well of a 12-well plate filled with ACSF.
8. Add carefully $5 \times 10^5$ OT-I T cells per slice.
9. Incubate slices for up to 8 hr in an incubator (37 °C, 5% $CO_2$).
10. After the incubation period, harvest slices and embed them by using OCT compound tissue-tek and freeze them in liquid nitrogen. Store them at -20 °C for further histological studies to *e.g.,* evaluate neuronal structure (*e.g.,* using anti-MAPII or anti-synaptophysin antibodies) or apoptosis (*e.g.,* using anti-Caspase-3 antibodies and anti-NeuN antibodies) according to standard procedures.

## Representative Results

After incubation of brain slices with oligodendrocyte-directed $CD8^+$ T-cells, oligodendrocytes as well as neurons undergo apoptosis (**Figures 2A and 1C**, respectively). Histological signs of apoptosis (*e.g.,* Caspase-3, Tunel) can be earliest detected after 3 hr of incubation. Incubation period should not be longer than 8 hr in order to guarantee a good quality of the preparation and reproducible results. Apoptotic cells can be found all

over the slice with preponderance in myelinated areas. Exemplary histological staining of the slice for structural integrity and apoptotic neurons is depicted in **Figure 1A-C**.

There are different time points during incubation which are of interest for assessing outcome parameters. A typical time course performed in our lab includes parameters analyses, namely, cell-cell interactions at time 0, then 3, 4 and 6 hr (the maximal interaction observed) after interaction started. Some typical assays beyond histological analyses which can often be applied are described very briefly:

T-cell recovery and analysis

After an incubation period up to 8 hr, T-cells can be recovered from slices and can be evaluated to analyse parameters relative to the intracellular effector molecules such as cytokine production or proliferation. In order to retrieve T-cells, slices were dissociated mechanically and pooled together for every experimental condition. Cells were isolated from the interface a 30% to 50% Percoll (Amersham, Freiburg, Germany) after density gradient centrifugation for 30 min at 2,500 rpm. In this way, it is possible to obtain mononuclear cells which were washed and stained immediately using flow cytometry techniques (FACS) using a different number and type of antibodies which vary depending on the study we want to address, in this protocol, for example $CD8^{+}$ [18].

Neuronal collateral damage and functionality

Information about neuronal health and functionality can be assessed by performing electrophysiological recording *e.g.,* to evaluate basic properties of the neurons such as action potentials (APs) generation, as shown in **Figure 1D**.

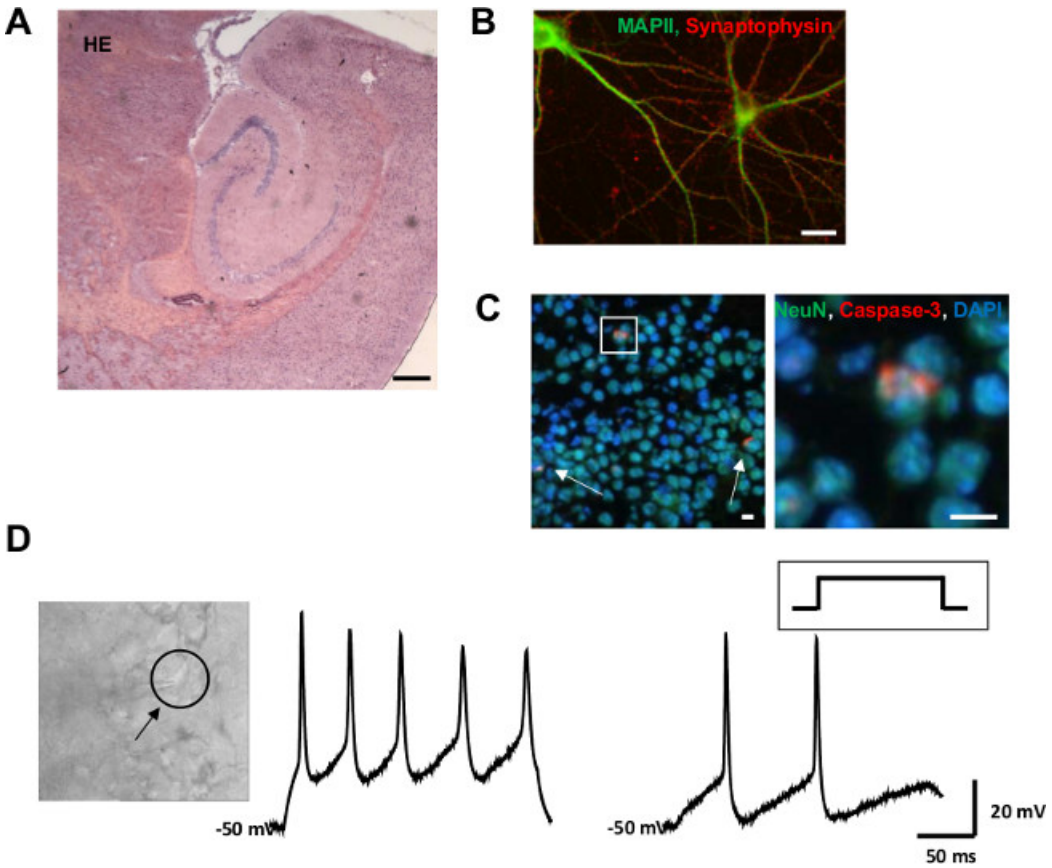


**Figure 1. Morphological structure of slice as well as representative results.** (**A**) Morphological structure of a slice 8 hr after incubation. Scale bar indicates 10 µm. (**B**) Neurons are synaptically interconnected as revealed by co-staining for MAPII (green) and synaptophysin (red) staining. Scale bar represents 10 µm. (**C**) Representative picture of apoptotic cells (Caspase-3, red) and neurons (NeuN, green) in the hippocampus after 6 hr incubation. Scale bar represents 10 µm. (**D**) Single cell patch-clamp recordings of neuronal action potential generation following depolarizing current steps in a murine cortical neuron. Please click here to view a larger version of this figure.

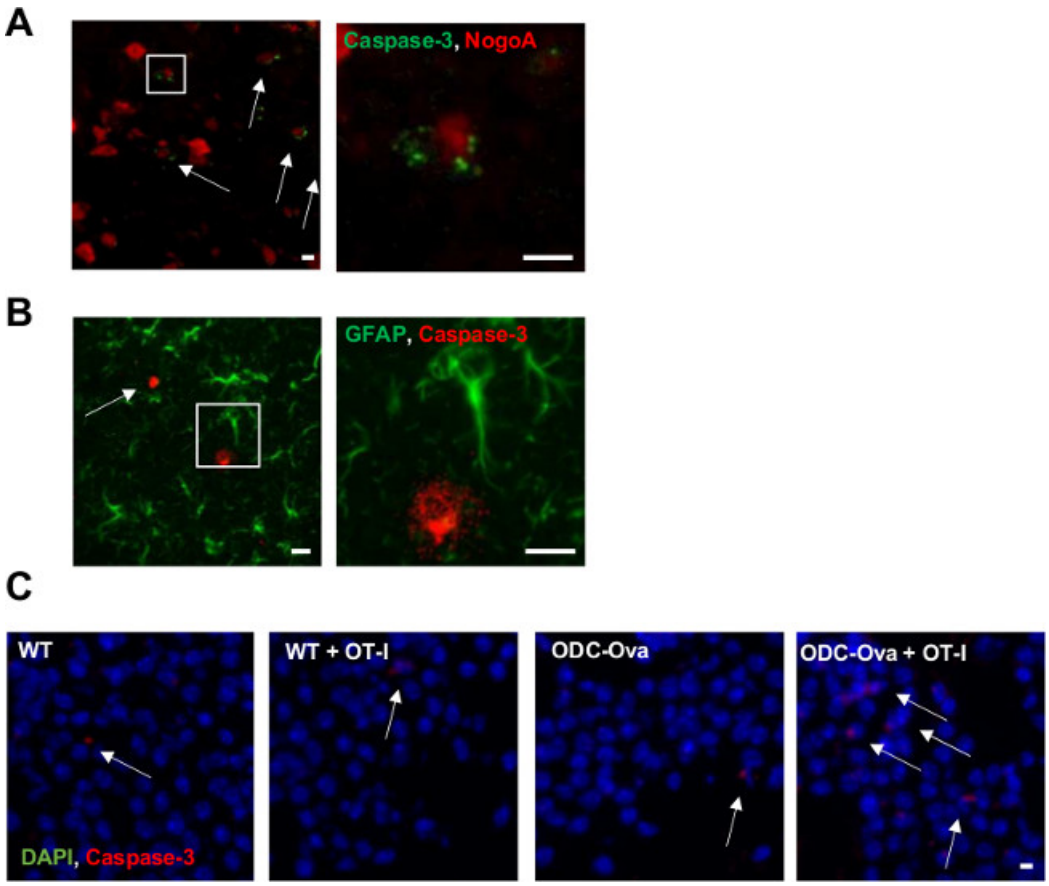


**Figure 2. Multiple stainings performed to assess specificity of the protocol.** (**A**) Co-staining of NogoA (red), a marker for mature oligodendrocytes (ODCs) and Caspase3 (apoptosis, green) shows that ODCs are attacked by $CD8^+$ T cells. (**B**) Interaction result is negative when Caspase3 (red) is co-stained with GFAP, a marker for glial cells (green). Scale bar represent 10 µm. (**C**) Specificity of the protocol is supported by the co-staining of neuronal cells (DAPI, blue) with Caspase3 (red), starting from the left: WT animals, WT animals exposed to OTI-I T cells, ODC-Ova and ODC-Ova exposed to OTI-I T cells. Please click here to view a larger version of this figure.

## Discussion

Different animal models have been described over the last decades to address the pathological features of inflammatory demyelinating disorders like MS. *In vivo* mouse and rat models are widely used to mimic pathophysiological features of the disease, namely, analysis of the consequences of demyelination and remyelination processes and of intermingled episodes of inflammation and neurodegeneration. Nevertheless, only an *ex vivo* approach allows to dissect the exact underlying mechanisms.

Preparation of acute brain slices is also a widely distributed model and can be considered as reliable and replicable. In combination with the isolation and incubation of oligodendrocyte-specific immune cells, it can be used to address specific experimental questions, especially in terms of studying mechanism and kinetics of an immune cell attack and cell – cell interactions. Other *ex vivo* approaches are known and widely used[22] but the novelty of our protocol in the specificity of the target that can be challenged by performing different "exclusion" co-stainings, namely verifying the Caspase3 immunoreactivity in WT exposed or not exposed to OT-I T cells (see **Figure 2C**).

Some critical points during the preparation should be taken into consideration, namely the experimental settings, to ensure that experiments are performed methodologically correct; for internal validity, isolation and stimulation of the used immune cells should be controlled on a regular basis via *e.g.,* flow cytometry analyses (FACS). Moreover, checking vitality of brain slices is highly recommended. Experimental groups should be age- and sex-matched. Experiments should always be performed in compliance with animal welfare regulations.

Some limitations of the protocol need to be kept in mind. Most importantly, the presented model is an *ex vivo* approach and is not suited to fully mimic the complex immunological situation of autoimmune central nervous system disorders. It should also be considered that the model uses only $CD8^+$ T-cell driven immunological response. $CD4^+$ T-cells and B cells play a less prominent role. Alternative protocols using other oligodendrocyte-specific immune cell subtypes should be considered when addressing these cell types. The expected histological finding is oligodendroglial (see **Figure 2A**) and neuronal apoptosis (see **Figure 1C**). Alternatively, apoptosis of other cell types can be achieved using *e.g.,* astroglial or neuronal antigen-specific immune cells and transgenic systems. In this specific protocol the immunoreactivity of Caspase3 in astroglial cells was negative due to the specificity of the system (**Figure 2B**).

The described protocol can be considered as a basic neuroimmunological experimental approach and may be modified for other applications. The experimental procedure can be easily applied to other protocols by varying brain slices (*e.g.,* different transgenic mice strains) in combination with different antigen-specific immune cell subtypes (*e.g.,* use myelin oligodendrocyte glycoprotein (MOG)- specific $CD4^+$ T-cells

and brain slices prepared from C57Bl/6 mice to address their effects on oligodendroglial and neuronal apoptosis). *In vitro* activation of immune cells can be varied for specific immunological questions (*e.g.,* polarization into $T_H1$ or $T_H17$ cells).

Sometimes, enhanced or reduced apoptosis in brain slices might be an experimental challenge. Some recommendations for troubleshooting are:

At first, quality of brain slices is highly dependent of preparation time as well as pH value, osmolarity and oxidation status of used solution. It should be ensured that conditions are comparable between the independent experiments. Furthermore, activation of immune cells should be controlled on a regular basis. Optimal antigen concentration may vary. Consider a titration of the used antigen when establishing the experiments.

As described above, the mentioned protocol can be used as starting point for analyzing the effect of further immune cell subtypes (*e.g.,* $CD4^+$ T-cells, B-cells) on central nervous system integrity. These approaches are especially suited for separating their effects on oligodendroglial and neuronal cells as histological analysis can be assessed thoroughly.

## Disclosures

The authors declare that they have no competing financial interests.

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