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# CD8$^+$ T cell help is required for efficient induction of EAE in Lewis rats

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ABSTRACT

The role of CD8$^+$ T cells in multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE) is still unclear. We describe here significantly reduced disease activity of EAE both in Lewis rats depleted of CD8$^+$ T cells by monoclonal antibodies and CD8 knockout rats, which was accompanied by reduced leukocyte infiltration into the spinal cord. We detected myelin basic protein (MBP)-specific CD4$^+$ T cells in peripheral lymphoid organs of CD8-depleted animals which, however, failed to differentiate into interferon-γ-producing effector cells. Our results indicate that CD8$^+$ T cells interact with myelin-specific CD4$^+$ T cells early in EAE enabling them to differentiate into pathogenic effector cells.



## 1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is an animal model of the immunopathology of human multiple sclerosis (MS) (Wekerle, 2008). The hallmarks of the early stages of MS and of EAE are inflammation and demyelination of the central nervous system (CNS) (Merkler et al., 2006; Storch et al., 2006). Although EAE does not mimic all features of MS, studies in this animal model have given important new insights into the pathogenesis of this neurological disorder and have also provided the basis for efficacious therapeutic concepts (Gold et al., 2006; Hohlfeld, 2009).

CD4$^+$ T cells with specificity for myelin antigens are primarily responsible for the induction of EAE (Ben-Nun et al., 1981). It was demonstrated that myelin-specific CD4$^+$ T cells are activated in the periphery and differentiate into interferon-γ (IFN-γ)-secreting TH1 cells before they infiltrate the CNS (Merrill et al., 1992). More recently, two additional CD4$^+$ T cell subsets have been shown to contribute to the induction of EAE: (i) TH17 cells which differentiate from naïve T cells in the presence of interleukin-6 (IL-6) and TGF-β and secrete the effector cytokines IL-17A and F and GM-CSF (Langrish et al., 2005; Murphy et al., 2010), and (ii) TH9 cells which differentiate from naïve CD4$^+$ T cells upon the cooperative stimulation with IL-4 and TGF-β leading to the production of large amounts of IL-9 and IL-10 (Jager et al., 2009).

In contrast to abundant data on the role of CD4$^+$ T cells, only recently more extensive studies have been initiated investigating the functional role of CD8$^+$ T cells in the pathogenesis of MS and EAE (Zozulya and Wiendl, 2008). Auto-aggressive cytotoxic CD8$^+$ T cells have been shown to be of relevance in the effector phase of MS and EAE. In MS patients, CNS-infiltrating CD8$^+$ T cell clones could be identified in CNS lesions and were also found in the cerebrospinal fluid (Babbe et al., 2000). Moreover, recent studies revealed that CD8$^+$ T cells are in close contact with demyelinated axons and can induce axonal loss via "collateral bystander damage" (Sobottka et al., 2009). The role of myelin-specific CD8$^+$ T cells outside of the CNS, e.g. in peripheral lymphoid organs, still remains to be elucidated. While several studies indicate that CD8$^+$ T cells might play a role as immune regulators dampening inflammation (Jiang et al., 1992; Koh et al., 1992; Hu et al., 2004; Tennakoon et al., 2006; Correale and Villa, 2008), it remains to be shown whether myelin-specific CD4$^+$ T cells may actually need help from CD8$^+$ T cells in the periphery to fully differentiate into effector cells capable of infiltrating the CNS.

To formally address this question, we utilized the two variants of EAE. In Lewis rats, EAE is a monophasic disease that can be induced either by immunization with an encephalitogen (e.g. guinea pig MBP) in complete Freund's adjuvant (CFA) or by adoptive transfer of encephalitogenic CD4$^+$ T cells into recipient naïve animals (AT (adoptive transfer) EAE) (Ben-Nun et al., 1981; Swanborg, 2001). Here, the disease is initiated by activated autoreactive CD4$^+$ T cells secreting proinflammatory cytokines and crossing the blood

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brain barrier (Wekerle et al., 1987; Rigolio et al., 2008) together with macrophages and granulocytes. The subsequent inflammatory response in the CNS dose-dependently leads to neurological deficits of the animals, which is followed by recovery (Gold et al., 2006).

Here we show that Lewis rats depleted of $CD8^+$ T cells by monoclonal antibodies (mAb) and CD8 knockout (CD8KO) Lewis rats both displayed less severe clinical signs with diminished infiltration of immune cells into the CNS in actively induced EAE. Importantly, MBP-specific $CD4^+$ T cells could be detected in the draining lymph nodes of CD8-depleted animals but these did not produce the pro-inflammatory cytokine IFN-γ. Our results indicate that $CD8^+$ T cells are required for effector cell differentiation in the periphery and CNS infiltration of MBP-specific $CD4^+$ T cells.

## 2. Material and methods

### 2.1. Animals

Lewis rats were purchased from Charles River (Sulzfeld, Germany) and used at 6–8 weeks of age and a body weight between 125 g and 160 g. A chemically-induced null mutation of rat CD8α (Zan et al., 2003; Taurog et al., 2009) was produced at the University of Texas Southwestern Medical Center, Dallas, USA, backcrossed >10 generations to the Lewis background, and transferred to the animal facilities of the Institute for Virology and Immunobiology at the University of Würzburg. The knockout genotype was checked before each experiment. Animals were kept under SPF conditions with 12 h of lighting per day and fed with commercial food pellets and water ad libitum. All animal experiments were conducted according to German law and approved by the Regierung von Unterfranken as the responsible authority.

### 2.2. T cell culture

The encephalitogenic MBP-specific T cell line MBP-V1 was established from Lewis rats as described earlier (Gold et al., 1995; Jung et al., 1995). Specificity of all T cells used for transfer (cell line/isolated cells) was tested in vitro in 96-well microtiter plates with $1.5 \times 10^4$ responder T cells, $7.5 \times 10^5$ irradiated (3000 rad) thymocytes and graded doses of guinea pig (gp)MBP (Eylar et al., 1974) using RPMI 1640 medium supplemented with 1% normal rat serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine. For the adoptive transfer (AT) experiments, T cells were always used at the same stage of activation.

### 2.3. Induction of EAE and experimental design

For active EAE induction, Lewis rats were inoculated s.c. in both footpads with a total volume of 100 μl of an emulsion containing equal volumes of saline with 25–75 μg of gpMBP (Weishaupt et al., 2000) and CFA with *Mycobacterium tuberculosis* (0.1–1.0 mg/ml; Difco, Detroit, USA). I.v. treatment with mAbs OX8 (0.45 mg/rat in PBS; BioXcell, West Lebanon, USA), 341 (0.45 mg/rat in PBS; InVivo BioTech Services GmbH, Hennigsdorf, Germany) or PPV06 (0.45 mg/rat IgG1 isotype control in PBS, EXBIO, Praha, Czech Republic) and MOPC-21 (0.45 mg/rat IgG1 isotype control in PBS, InVivo BioTech Services GmbH, Hennigsdorf, Germany) respectively was performed two days before immunization. OX8 was used in excess for inducing complete depletion of $CD8^+$ $CD4^-$ αβ T cells (≥99.9%) in spleens and lymph nodes which lasted for at least 16 days. Even after clinical remission of EAE more than 75% of $CD8^+$ T cells were still depleted. 341 was even more efficient than OX8 at depleting $CD8^+$ $CD4^-$ αβ T cells but, of course, spared $CD8\alpha\alpha^+$ $CD4^-$ αβ T cells (about 99% depletion of $CD8^+$ $CD4^-$ αβ T cells).

Adoptive transfer- (AT-) EAE was induced by injecting $8 \times 10^6$ freshly activated MBP-specific $CD4^+$ T cells (Weishaupt et al., 2000) into the tail vein of the rats. 0.45 mg of OX8 or PPV06 as a control was administered i.v. on day −2 before adoptive transfer of the encephalitogenic T cells. In AT-EAE experiments using $CD4^+$ T cells from draining lymph nodes of CD8KO rats MBP-specific $CD4^+$ T cells were generated freshly. For this purpose $CD4^+$ T cells were isolated from draining lymph nodes of CD8KO and WT littermates at day 10 after immunization and propagated for five rounds with restimulation by 10 μg/ml gpMBP (Weishaupt et al., 2004) before transfer of $8 \times 10^6$ cells.

Animals were weighed daily and inspected for signs of EAE. The severity of EAE was assessed by a masked observer employing an established scale ranging from 0 to 10: 0 = healthy; 1 = reduced tone of tail; 2 = limp tail, impaired righting; 3 = absent righting; 4 = gait ataxia; 5 = mild paresis of hindlimbs; 6 = moderate paraparesis; 7 = severe paraparesis or paraplegia; 8 = tetraparesis; 9 = moribund; 10 = death (Linker et al., 2008). The correlations between the severity of EAE in control groups and the protection from disease in the CD8-deficient groups was assessed by using the following calculation: {Σ(score of control group d11 to 18) − Σ(score of CD8-depleted group d11-18)} / Σ(score of control group d11 to 18) × 100 = % of protection of the CD8-depleted group. Data/results from every group were normalized to n = 5 animals.

### 2.4. Proliferation assay

For the proliferation assay $1 \times 10^5$ $CD4^+$ T cells negatively purified using MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) from draining lymph nodes of gpMBP-immunized Lewis rats were cultured in 200 μl/well in triplicate together with $1 \times 10^6$ irradiated thymocytes from a naive rat in a 96 well flat bottom plate. 10 μg/ml gpMBP was added to the wells as stimulating antigen. Cell cultures without antigen and cell cultures of $CD4^+$ T cells from superficial lymph nodes of a naive rat with or without addition of antigen were used as controls. Proliferation was assessed on the basis of [$^3$H] thymidin incorporation, added during the last 18 h of a 72 h culturing period. The DNA of the [$^3$H] thymidine-pulsed cells was harvested onto fiberglass filters and the amount of incorporated radioactivity was quantified using a β-plate reader.

### 2.5. Cytokine assay

For IFN-γ measurements 50 μl supernatant was taken from the cultures described above right before addition of [$^3$H] thymidin and analyzed by Cytometric Bead Array (BD, Heidelberg, Germany) or ELISA (Life Technologies, Carlsbad, USA) according to the manufacturer's instructions.

### 2.6. Flow cytometry

For external surface staining antibodies R73 (αβTCR, BD Heidelberg, Germany), OX38 (CD4, BioLegend, Fell, Germany), OX39 (CD25, BD, Heidelberg, Germany), OX8 (CD8, BioLegend, Fell, Germany) and mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, USA) were used. Intracellular staining was performed with mAb against Ki-67, IFN-γ (both antibodies from BD, Heidelberg, Germany) and Foxp3 (eBioscience, Frankfurt, Germany). Stainings were carried out as described (Schmidt et al., 2003a; Beyersdorf et al., 2005, 2009).

For measuring the kinetics of T cell activation after immunization with gpMBP in CFA in vivo, cells were isolated from draining lymph nodes on days 1, 3, 7 and 10 and $1 \times 10^6$ lymph node cells were restimulated with 5 ng/ml PMA and 500 ng/ml Ionomycin in a 48 well plate for 4 h in the presence of Golgi Plug (1:1000, BD, Heidelberg, Germany) for the last 2 h followed by staining of surface molecules and detection of intracellular IFN-γ (anti-IFN-γ FITC) expression was also performed after PMA/ionomycin restimulation. Proliferation