

Uncoupling Protein 2 Has Protective Function during Experimental Autoimmune Encephalomyelitis

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Uncoupling protein 2 (UCP2) is a member of the mitochondrial transporter superfamily that is expressed in many tissues, including immune cells. UCP2 prevents oxidative stress by reducing reactive oxygen species. Using UCP2-deficient mice, it was shown that UCP2 is involved in the regulation of insulin secretion, in the resistance to infection, and in atherosclerosis. Here, we investigated the role of UCP2 in experimental autoimmune encephalomyelitis, a murine model of multiple sclerosis. Immunized C57BL/6J UCP2-deficient mice showed a slightly delayed onset during experimental autoimmune encephalomyelitis (13.0 ± 0.6 versus 11.5 ± 0.8 in wild-type controls) and developed significantly higher disease scores than littermate controls (maximum disease score of 2.9 ± 0.2 versus 1.7 ± 0.2 , $P = 0.001$). Higher levels of infiltrating T cells into the spinal cord meninges and parenchyma were observed. The T-cell proliferative response to the specific antigen was increased in UCP2-deficient mice compared with littermate controls, and CD4 cells of UCP2 knockout mice produced significantly higher levels of pro-inflammatory cytokines, eg, tumor necrosis factor- α and interleukin-2, resulting from a Th1 response. Mice lacking UCP2 also developed a higher B-cell response. Concomitantly, CD4 and CD8 cells of the UCP2-deficient mice showed increased production of reactive oxygen species. These results suggest a protective function of UCP2 in chronic inflammatory diseases such as multiple sclerosis. (*Am J Pathol* 2006, 168:1570–1575; DOI: 10.2353/ajpath.2006.051069)

environmental factors that affects more than 2 million people worldwide. Susceptibility to MS is controlled by multiple genetic factors, as indicated by numerous studies showing higher rates of disease concordance in monozygotic than dizygotic twins and higher incidence in offspring of MS patients.¹ The primary genetic contribution to MS susceptibility is thought to be linked to the HLA locus. Identification of the non-MHC genetic loci regulating MS has been complicated by genetic heterogeneity, incomplete penetrance, and environmental factors.^{2,3} Experimental models of MS in mice, such as experimental autoimmune encephalomyelitis (EAE), have been extensively used in dissecting the genetic basis of disease. In recent years, genetic linkage analysis and gene expression profiling approaches identified many quantitative trait loci and potential susceptible genes for EAE and MS, respectively. One such putative susceptible gene could be uncoupling protein 2 (UCP2). This common gene is polymorphic and differentially expressed in EAE.⁴ UCP2 is located on mouse chromosome 7 at cM 50 within the EAE susceptible locus EAE 4.² UCP2 protein is expressed in a wide variety of tissues, eg, spleen and lung^{5,6} and immune cells, like macrophages and T cells.⁷ It is also expressed in the nervous system: in the spinal cord and lymph nodes of EAE mice and in human MS brain lesions.⁴ UCP2 protein shares 60% identity with the well-known UCP1, which uncouples the ATP production from the mitochondrial respiration chain in brown adipocyte mitochondria. Although the uncoupling activity of UCP2 has been evidenced after overexpression in yeast⁸ or in proteoliposome,⁹ UCP2 has no detectable *in vivo*

Supported by grants from The Federal Ministry of Education and Research (FKZ 01ZZ0108 to S.M.I.), Centre National de la Recherche Scientifique (INSERM), Institut de Recherche Servier (to D.R.), and the European Union Sixth Framework Program (contract LSHM-CT-2003-503041 to D.R.).

Accepted for publication February 1, 2006.

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Multiple sclerosis (MS) is a complex multifactorial polygenic disease influenced by age, gender, hormonal, and

uncoupling activity¹⁰ and consequently does not play a primary role in the regulation of the energy metabolism like UCP1. Instead, UCP2 has been shown to participate in the regulation of the production of reactive oxygen species (ROS) during infection¹¹ and the development of atherosclerosis.¹² UCP2 might also be involved in nitric oxide production of lipopolysaccharide-stimulated macrophages⁷ and in preventing neuronal death.¹³ Reactive oxygen species are thought to induce cellular damage and to play a pathological role in several human diseases. For its ability to decrease reactive oxygen species, UCP2 is a likely candidate to be protective with respect to development of neurodegenerative diseases. Therefore, we evaluated in this study the role of the UCP2-mediated reactive oxygen species modulation in the inflammatory pathogenesis of EAE using the UCP2-deficient mice. We investigated the effect of the mutation on the clinical course of disease, on the immune response to myelin oligodendrocyte glycoprotein (MOG₃₅₋₅₅), and on histopathological features of the spinal cord.

Materials and Methods

Animals and Induction of EAE

The production of UCP2^{-/-} mice was previously published.¹¹ They were kept on the susceptible C57Bl/6 background. The knockout mice and their littermate controls were kept at the animal facility of the University of Rostock. The Rostock's Animal Care Committee approved all experimental procedures. EAE was induced according to established protocols.⁴ Seven-week-old mice were immunized subcutaneously with 150 μ g of myelin oligodendrocyte glycoprotein (MOG₃₅₋₅₅) (American Peptide Company, Sunnyvale, CA) dissolved in water and mixed with an equal volume (50 μ l) of CFA (IFA with 4 mg/ml *Mycobacterium tuberculosis*; Difco Laboratories, Detroit, MI) and 400 ng of PTX (Sigma-Aldrich, Taufkirchen, Germany) two times (days 0 and 2). Disease score was recorded as follow: 0, normal; 1, flaccid tail; 2, waddle; 3, moderate paraparesis; 4, severe paraparesis; 5, tetraparesis; and 6, moribund.

Biochemical Methods

UCP2 protein detection was performed as described by Apues-Guerra et al⁴⁴ using the mitochondrial porin (20B12; Molecular Probes, Eugene, OR) as control. Total RNA was extracted from spinal cord and lymph nodes using RNeasy Mini kit (Qiagen, Valencia, CA). Quantitative real-time polymerase chain reaction was performed using ABI PRISM TM 7700 sequence detection system (Applied Biosystems, Foster City, CA). UCP2 primers are as follows: 5'-GGG CCT CTG GAA AGG GAC T-3', 5'-ACC AGC TCA GCA CAG TTG ACA-3', and probe 5'-TCC CAA TGT TGC CCG TAA TGC CA-3'. For ROS measurement, blood was treated with erythrocyte lysis buffer (Qiagen) and resuspended in RPMI without supplements. White cells were preheated to 37°C, treated

with dihydrorhodamin 123 (5 nmol/L) and incubated for 5 minutes. After addition of phorbol myristate acetate (PMA) (0.5 nmol/L), cells were incubated for another 20 minutes, stained with anti-CD4-phycoerythrin (PE), anti-CD8-PE, and anti-CD11b-PE, respectively, and analyzed by FACS Calibur (Becton-Dickinson).

Immunological Methods

For cell proliferation assay, spleen cells (day 10) were stimulated with 5 μ g of concanavalin A (Difco) and 30 μ g of MOG₃₅₋₅₅, pulsed for 12 hours with [³H]methyl-thymidine (10 μ l, 100 μ Ci/ml; Amersham) and harvested onto glass fiber strips (Lierbyen, Norway). The radioactivity was counted in triplicate on a Wallace 1214 counter (Rackbeta, Sweden). At day 30, antibody titer against MOG₃₅₋₅₅ was determined by enzyme-linked immunosorbent assay. CD4 and CD8 cells were determined by FACS analysis of lymph nodes cells as described previously.¹⁵ Cytokine measurements were performed on lymph nodes cells treated for 2 hours with PMA (1 ng/ml) and ionomycin (500 ng/ml) followed by brefeldin A (10 μ g/ml) and stained with anti-tumor necrosis factor (TNF)- α -PE, anti-interferon- γ -fluorescein isothiocyanate and anti-IL-10-fluorescein isothiocyanate, anti-IL-2-PE.

Histological Analysis and Immunohistochemistry

Spinal cords tissues were arranged in a tissue microarray (TMA) for parallel processing.¹⁴ Slides were conventionally stained with Luxol-Nissl stain, and immunohistochemical analysis was performed using a Nexus machine (Ventana, Germany) with antibodies against amyloid precursor protein (Boehringer, Mannheim, Germany), myelin basic protein (DAKO, Carpinteria, CA), neurofilament (NF200; Sigma, St. Louis, MO), IBA1 (Wako, Switzerland), CD3 (LabVision, Fremont, CA), and B220 (CD45R; BD Biosciences, Heidelberg, Germany). Slides were developed using the Ventana DAB MAP kit or the DAKO iView kit. The staining intensities were evaluated as follows: 0, no infiltration; 1, moderate infiltration; and 2, high infiltrations.

Statistical Analysis

The Mann-Whitney *U*-test was used except for histological analysis where scoring data were adjusted by the Student's *t*-test. A value of *P* < 0.05 was considered significant.

Results and Discussion

In multiple sclerosis disease, myelin destruction and neurodegeneration occur after a long period of chronic inflammation. Therefore, we investigated UCP2 levels in spleen, lymph node, and spinal cord of mice during EAE (Figure 1A). Twelve days after immunization, UCP2 significantly decreased in spleen mitochondria. At day 20 after immunization, UCP2 increased again in spleen but

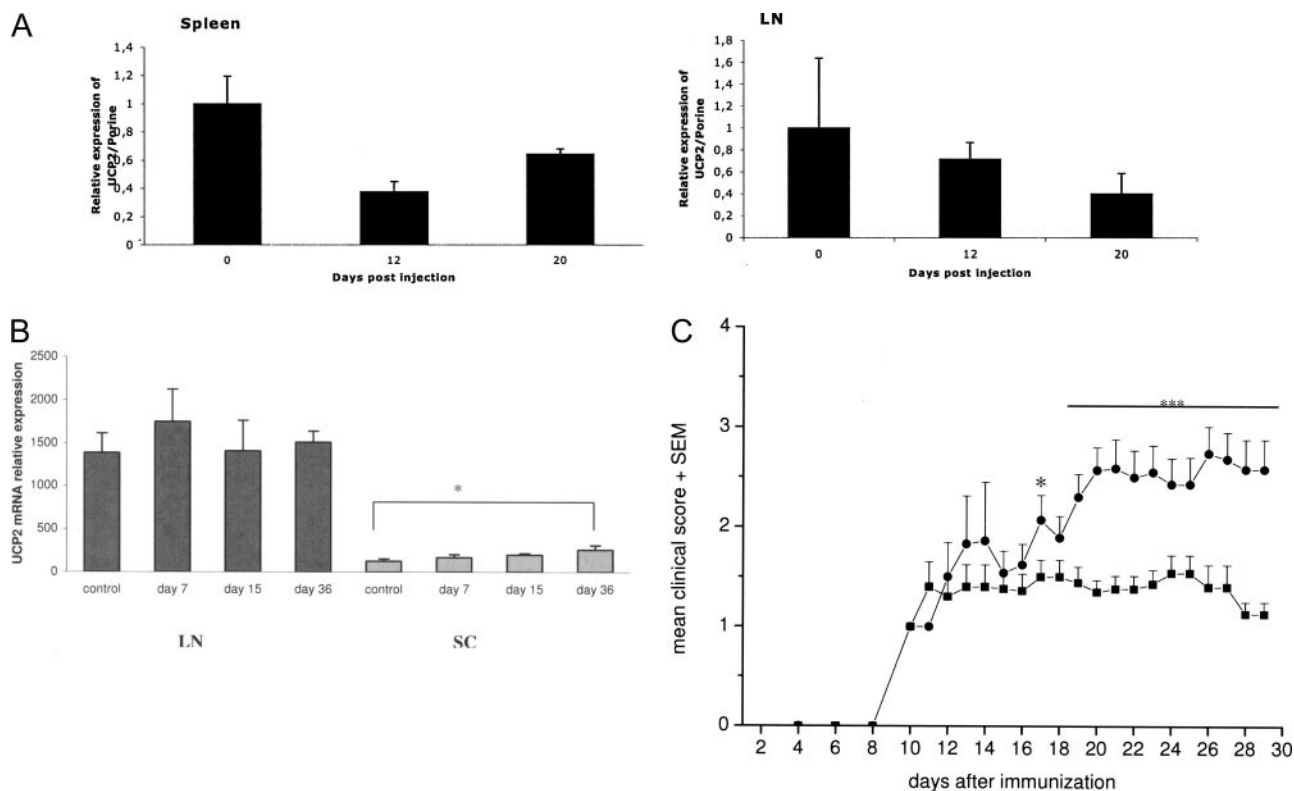


Figure 1. Role of UCP2 in EAE. Quantification of UCP2 protein expression in spleen lymph nodes (LN) (A) and of UCP2 mRNA in LN and spinal cord (SC) (B) at different time points after the immunization of C57BL/6J. For UCP2 protein quantification, the relative UCP2 expression was normalized with the mitochondrial porin. Data are expressed as mean \pm SEM of five mice per time point. UCP2 mRNA expression levels in LN and SP were measured by quantitative real-time polymerase chain reaction. The relative UCP2 expression was normalized with the GAPDH expression. Data are expressed as mean \pm SEM of three mice per time point, measured in triplicate. C: Clinical scoring data from UCP2-deficient mice and the control group. C57BL/6J-UCP2^{-/-} (circle) and C57BL/6J (square) mice were scored for EAE lesions as described in Materials and Methods. The data presented are a combination of two independent experiments ($n = 40$ and 25 for UCP2^{-/-} and controls, respectively). * $P < 0.05$, ** $P < 0.001$.

did not return to its basal level ($P < 0.05$). In lymph node, UCP2 protein expression tends to decrease, whereas UCP2 mRNA remained unchanged, which illustrates further the posttranscriptional regulation of UCP2 previously described.⁶ In spinal cord, UCP2 protein was undetectable, but UCP2 mRNA significantly increased ($P = 0.01$) during the time course of the disease (Figure 1A), suggesting either that immune cells infiltrate the spinal cord or that the neurons themselves express UCP2 to counterbalance the oxidative stress induced by the inflammation. To confirm the importance of UCP2 in EAE and MS, we induced EAE in 7-week-old C57BL/6J UCP2^{-/-} mice and in C57BL/6J background littermate controls. Sixty-five mice with clinical signs of EAE were examined daily from days 0 to 30 after immunization. More severe disease was developed in the C57BL/6J UCP2-deficient mice than in the littermate controls (Figure 1C). Consistent with the protective role of UCP2, the mean maximum severity score was 2.9 ± 0.2 for the C57BL/6J UCP2 knockout mice and significantly higher ($P = 0.001$) than the maximum score value of 1.7 ± 0.2 in the C57BL/6J controls. Significant differences between the UCP2 mutant mice and controls were also observed when analyzing disease score over time, ie, area under the curve. Knockout mice had higher area under the curve values, $P = 0.02$. The onset of the disease was delayed until day 13.0 ± 0.6 after immunization in UCP2 knockout mice

compared with day 11.5 ± 0.8 in the C57BL/6J control group, $P = 0.44$. The incidence of EAE induction was similar (75%) in both groups. The differences in the severity between the C57BL/6J UCP2 knockout mice and wild-type controls were also reflected in histopathological changes such as infiltration of inflammatory cells, eg, microglia. Multiple spinal cord cylinders of 40 C57BL/6J UCP2 knockout mice and 25 wild-type controls were analyzed in a single tissue microarray with 196 cores to allow controlled parallel processing of each sample. As presented in Figure 2 and Table 1, the C57BL/6J UCP2 knockout mice showed a higher amount of infiltrating inflammatory cells than wild-type control mice. A significantly higher amount of infiltrating T cells was observed in C57BL/6J UCP2 knockout mice in comparison with wild-type controls, whereas the B-cell infiltration was comparable in both groups (Table 1). The immunohistochemical stains for damaged axons (amyloid precursor protein), myelin protein, neuron filaments (NF200), and conventional stain for Luxol-Nissl revealed no differences between the groups in the degree of myelination and axonal damage (data not shown). These facts point toward a minor involvement of obvious demyelination and axonal damage for the disease progression in the EAE model.

Previous inflammatory challenges on UCP2^{-/-} mice suggested that overactivation of macrophages was suf-

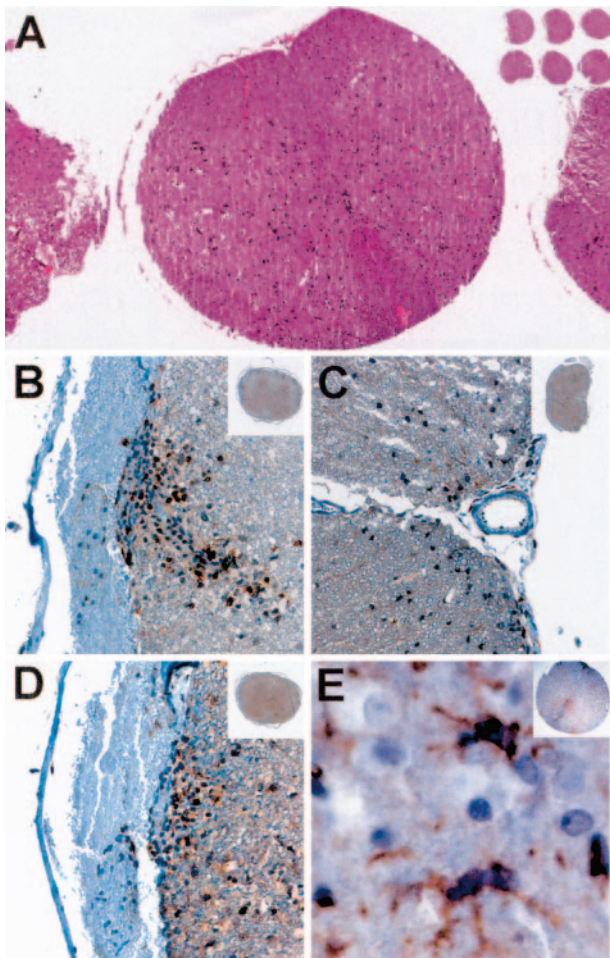


Figure 2. Morphological analysis of spinal cord sections in TMA. **A:** Overview of the spinal core cylinder arrangement in the TMA, single spinal core (hematoxylin and eosin, 4× objective, 1.3 mm diameter). **B:** CD3 (T-lymphocyte infiltration, ×20) high-infiltration example. **C:** CD3 (T-lymphocyte infiltration, ×20) low-infiltration example. **D:** B220 (B-lymphocyte infiltration, ×20) (consecutive slide of **B**). **E:** IBA1 stain for microglia infiltration (×60).

efficient to explain the resistance of UCP2^{-/-} mice to *Toxoplasma gondii* or the more rapid progression of atherosclerosis plaques in low density lipoprotein receptor-deficient mice transplanted with the bone marrow from UCP2^{-/-} mice.^{11,12} In the EAE model, we found that the lack of UCP2 modified the T- and B-cell responses. Thymidine incorporation in response to the autoantigen MOG₃₅₋₅₅ was significantly higher ($P = 0.012$) in splenocyte-derived T cells from immunized UCP2-deficient mice in comparison with the wild-type controls (Figure 3A). Maximal T-cell response induced with concanavalin A was similar in both groups of mice. B-cell response was assessed by measuring the anti-MOG₃₅₋₅₅ IgG. Figure

3B shows that specific anti-MOG₃₅₋₅₅ IgG had increased in UCP2-deficient mice ($P = 0.004$). The TNF- α production as well as the interleukin (IL)-2 production of the CD4 cells was also significantly increased in UCP2-deficient mice (TNF- α , $P = 0.001$; IL-2, $P = 0.04$) (Figure 3C). The interferon- γ and IL-10 production was below detection level in those cells (data not shown).

In principle, mice and humans contain more CD4 T cells than CD8 T cells in their thymuses and peripheral lymphoid tissues. However, the CD4-to-CD8 ratio can be affected by autoimmunity, infections, and protein expression, respectively.¹⁵ In our analysis no differences were found in UCP2-deficient mice regarding the CD4-to-CD8 ratio in comparison with the wild-type controls (data not shown). Wild type demonstrates that the change in the B- and T-cell response was linked to the lack of UCP2, we analyzed the ROS production in CD4, CD8 cells from UCP2-deficient mice and wild-type controls. The results show that CD4 and CD8 cells in UCP2-deficient mice have a significant increase in ROS production compared with the control group (Figure 4A), showing that the lack or the decreases of UCP2 can increase the ROS production in those immune cells, leading to a more intense response to the immunization and eventually to the disease. In contrast to the previously described *T. gondii* infectious model, ROS production from macrophages was not significantly increased in UCP2^{-/-} mice (Figure 4B). Because CD4 and CD8 cells from UCP2^{-/-} mice exhibit increased levels of ROS, it can be proposed that UCP2-mediated ROS participate in wild-type mice to the activation and to the resolution of the inflammation. The UCP2 protein levels in spleen and lymph node also support this hypothesis. Before immunization, UCP2 is highly expressed in those tissues, whereas 12 days after immunization, ie, when the immune system is activated, UCP2 is expressed at lower level. All together, the *in vivo* expression data are in agreement with the two-step model of UCP2 regulation in the LPS model¹⁶ in which UCP2 acts as a metabolic modulator of the immune response.

ROS contribution to brain pathology in EAE and MS patients is well documented. Gilgun-Sherki et al¹⁷ observed a decrease in EAE symptoms after oral administration of *N*-acetylcysteine amide (NAC), an antioxidant that reduced ROS production, thus substantiating the key function of ROS during EAE. As a matter of fact, simultaneous injection of LPS and NAC in mice prevented induction of UCP2 in spleen, intestine, and lung,¹⁶ showing that UCP2 acts as a sensor of oxidative stress. In addition to their involvement in the myelin phagocytosis by macrophages,¹⁸ they stimulate monocyte migration across the blood-brain barrier, a critical and early event in MS lesion formation.¹⁹ ROS can modulate cell functions as well.²⁰ For instance, controlled levels of ROS are necessary for signal-transduction pathways triggering T-cell activation, cytokine release, or apoptosis.^{21,22} Oxidative stress and ROS respectively affect the activity of NF- κ B and AP-1,^{21,23,24} which promote the transcription of the cytokine IL-2 and TNF- α , both cytokines that were more elevated in cells from UCP2^{-/-} mice during EAE. Together with a recent report of Collins laboratory showing increased NF- κ B activity in UCP2-deficient mice,²⁵ our

Table 1. Scoring Data of the Infiltration of Different Cell Types in the Spinal Cord

Cell type score	UCP2 ^{+/+}	UCP2 ^{-/-}	<i>P</i> value
T lymphocytes	0.5	0.8	0.047
B lymphocytes	0.7	0.7	—
Microglia	0.9	1.2	0.090

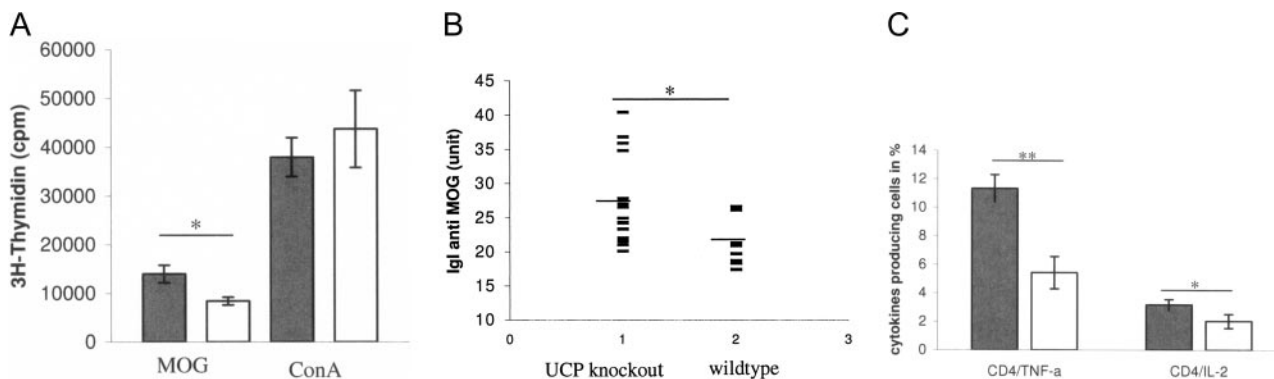


Figure 3. Analysis of the immune response in UCP2^{-/-} and UCP2^{+/+} mice. T-cell proliferation (**A**) and B-cell response (**B**) in UCP2 knockout mice (gray columns) and the control (white columns). Data are presented as mean ± SEM of *n* = 12 in the case of UCP2-deficient mice and *n* = 8 for the wild type. Statistical differences between the groups were analyzed using Mann-Whitney test. **C:** Cytokine productions in CD4 cells from immunized mice. The data presented are a combination of three independent experiments (*n* = 20 and 10 for UCP2^{-/-} and controls, respectively). **P* < 0.05, ***P* < 0.001.

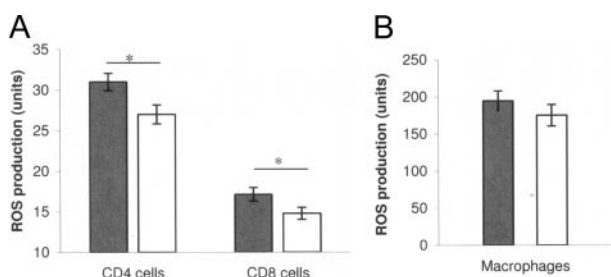


Figure 4. Comparison of the ROS production between the UCP2-deficient mice (gray columns) and the control (white columns) in T cells (**A**) and macrophages (**B**). Data are as mean ± SEM of 12 mice per group. **P* < 0.05.

data strongly support a signaling function of UCP2 in the immune system mediated by the mitochondrial ROS production.

Finally, the physiopathological data obtained in this study with the UCP2^{-/-} mice in the EAE model combined with the genetic linkage we observed between MS susceptibility and the human UCP2 promoter polymorphism -866 G/A²⁶ point out UCP2 as a target gene for the prevention and the treatment of multiple sclerosis.

Acknowledgments

We thank Caroline Aheng for technical help, Ilona Klamfuss and Eva Lorbeer for help with the animals, and Martina Storz for preparing the TMAs.

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