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**Mitochondria contribute to LPS-induced MAPK activation via uncoupling protein UCP2 in macrophages**

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**Short Title:** UCP2 modulates LPS-induced MAPK signaling

**Keywords :** LPS, MAPK, mitochondria, ROS, signaling, UCP2

## Synopsis

The mitochondrion is a major organelle contributing to energy metabolism but also a main site of reactive oxygen species (ROS) production. Lipopolysaccharide (LPS)-induced ROS signaling is a critical event in macrophage activation. We report here that part of LPS-mediated ROS signaling comes from mitochondria inside a signal amplification loop that enhances mitogen-activated protein kinase (MAPK) activation. More precisely, we identified the inner mitochondrial membrane uncoupling protein 2 (UCP2) as a physiological brake on ROS signaling. Stimulation of murine bone marrow-derived macrophages by LPS quickly downregulated UCP2 through the JNK and p38 pathways. UCP2 downregulation was shown to be necessary to increase mitochondrial ROS production in order to potentiate MAPK activation. Consistent with this, UCP2-deficient macrophages exhibit an enhanced inflammatory state characterized by increased nitric oxide production and elevated migration ability. Additionally, we found that absence of UCP2 renders macrophages more resistant to NO-induced apoptosis.

**Abbreviations** : The abbreviations used are: ATF2, activating transcription factor-2; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; ERK, extracellular signal-regulated kinase; HUVEC, human umbilical vein endothelial cells; I- $\kappa$ B, inhibitor of  $\kappa$ B; iNOS, inducible nitric oxide synthase; IL, interleukin; IFN, interferon; JNK, Jun N-terminal kinase; LPS, lipopolysaccharides; MAPK, mitogen-activated protein kinase; MCP1, monocyte chemoattractant protein-1; Mnk-1, MAPK-interacting kinase; mTOR, mammalian target of rapamycin; NF- $\kappa$ B, nuclear factor- $\kappa$ B; NO, nitric oxide; ROS, reactive oxygen species; SNAP, S-nitroso-N-acetyl-penicillamine; TNF, tumor necrosis factor; TLR, Toll-like receptor; UCP2, uncoupling protein 2.

## Introduction

Innate immunity, the host's first line of defense against pathogens, relies on many cells such as neutrophils and macrophages. Activation of macrophages finally induces production of nitric oxide (NO), cytokines and reactive oxygen species (ROS) as prime tools of antimicrobial function [1]. Innate activation of macrophages occurs with the recognition of microbial products [2,3] such as lipopolysaccharides (LPS) [1-4]. LPS stimulation triggers signaling pathways leading to activation of the three members of the mitogen-activated protein kinase (MAPK) family [3,5], which are extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK) and p38 [6-9], and also to activation of the nuclear transcription factor NF- $\kappa$ B [2-3]. Furthermore, LPS induces an early ROS production, which constitutes a primordial signaling pathway potentiating MAPK activation [10-12]. Both NADPH oxidase [13-15] and mitochondria [12,16,17] are associated with this ROS signaling.

Uncoupling protein 2 (UCP2) is a carrier protein located in the inner mitochondrial membrane [18] which negatively regulates ROS production [19-21]. Several lines of evidence underline its role in immunity. First, UCP2 is expressed in immune cells such as phagocytes and lymphocytes [22]. Second, *Ucp2*(-/-) mice are more resistant to a *Toxoplasma gondii* or *Listeria monocytogenes* infection than *Ucp2*(+/+) mice [20,22]. Third, the development of unstable atherosclerotic plaques is greater in a *Ucp2*(-/-) mouse model of atherosclerosis [23]. Finally, transgenic mice overexpressing UCP2 show a reduced inflammatory response after LPS treatment [24].

The aim of our present work was 1/ to provide mechanistic evidence to explain the immune phenotypes of *Ucp2*(-/-) and *Ucp2*(+/+) macrophages, 2/ to demonstrate the involvement of mitochondria in LPS-induced ROS signaling, and 3/ to identify the mitochondrial protein UCP2 as a physiological brake on this phenomenon. Therefore, we propose a signal amplification loop model in which UCP2 is downregulated in response to LPS through the JNK and p38 pathways. This downregulation increases mitochondrial ROS production, which in turn enhances MAPK activity in order to enable the macrophage response.

## Experimental procedures

### *Reagents*

LPS from *E. coli* (serotype 0111:B4), S-nitroso-N-acetylpenicillamine (SNAP), 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), SP600125, SB202190, PD98059, rapamycin, rotenone, antimycin A. Recombinant murine interferon- $\gamma$  (IFN $\gamma$ ) was from PBL. Monocyte chemoattractant protein-1 (MCP-1) and interleukin-10 (IL-10) were from RnD Systems. Synthetic double-stranded RNA, p(I:C), was from Amersham Pharmacia.

### *Animals and macrophage culture*

Studies on mice were performed in agreement with the institutional CNRS guidelines defined by the European Community guiding principles and by the French decree No. 87/848 of October 19, 1987. Authorization to perform animal experiments was given by the French Ministry of Agriculture, Fisheries and Food (A92580 issued February 2 1994 and 92-148 issued May 14, 2002). Ucp2(-/-) and Ucp2(+/+) littermate mice raised in a C57BL/6J genetic background were maintained under a 12-h light, 12-h dark schedule, with food and water ad libitum. Bone marrow-derived macrophages were obtained as previously described [25]. Briefly, mice were euthanized, femurs were dissected and bone marrow tissue was flushed with RPMI medium. Cell aggregates were dispersed by passing through a 25-gauge needle. Cells were cultured in RPMI 1640 with glutamax containing 10% FCS and 15% L929 cell conditioned medium as M-CSF source. After 7 days of culture, macrophages were obtained as an adherent cell population and were synchronized by an overnight M-CSF deprivation. Macrophages were trypsinized and plated at  $4 \times 10^6$  cells/mL, except when stated otherwise. When Ucp2(-/-) and Ucp2(+/+) macrophage responses were compared, macrophages were cultured in fresh medium two hours prior to any experiment.

### *Endothelial cell culture and macrophage transmigration*

Human umbilical vein endothelial cells (HUVEC) were cultured using the endothelial cell growth media system from Clonetics (Cambrex Bio Science, Verviers, Belgium) containing 2% FCS according to the manufacturer's protocol. HUVEC were used in the fourth passage for experiments. Transmigration of macrophages through a confluent monolayer of HUVEC was analyzed upon colorimetric labeling of the transmigrated macrophages using the QCM<sup>TM</sup> 24-Well Collagen-Based Cell Invasion Assay (CHEMICON<sup>®</sup> International, Inc.). Briefly,  $5 \times 10^4$  HUVEC were seeded into invasion chamber inserts of the assay and were grown to

confluence. Afterwards,  $5 \times 10^5$  macrophages were added to the confluent endothelial monolayer and cells were incubated overnight with or without MCP-1 (10 ng/mL) and/or IFN $\gamma$  (100 U/mL). The number of transmigrated macrophages was analyzed photometrically ( $\lambda=550$  nm) after cell staining according to the manufacturer's protocol.

#### *Western blot analysis*

Whole-cell extracts were obtained by cell lysis with Roti-load (Carl Roth). Mitochondrial protein extracts were loaded onto polyacrylamide gels, separated by electrophoresis and transferred onto nitrocellulose membrane as previously described [26]. Western blots were hybridized with appropriate antibodies under the following conditions: UCP2, hUCP2-605 antibody (at 1/2000 dilution) and rabbit anti-IgG (at 1/3000 dilution); porin, anti-porin antibody (Molecular Probes, at 1/4000 dilution) and mouse anti-IgG (Sigma Aldrich, at 1/3000 dilution). The UCP2 amount was normalized by using antibodies against porin. Inducible Nitric oxide synthase (iNOS), inhibitor of  $\kappa$ B (I- $\kappa$ B) and  $\beta$ -actin were detected with a rabbit polyclonal antibody respectively from Sigma, Cell Signaling Technology and Santa Cruz Biotechnology. Direct recording of the chemiluminescence (ECL kit, Amersham Pharmacia Biotech) was performed using the CCD camera of GeneGnome analyzer and quantification was achieved using Genetool software (Ozyme).

#### *Determination of MAPK and ATF-2 phosphorylation*

p38, ERK and activating transcription factor-2 (ATF-2) phosphorylation was determined with Pathscan Sandwich ELISA kits specific to phosphorylated forms of p38, ERK1/2 and ATF-2 (Cell Signaling Technology) according to the manufacturer's protocol.

#### *Determination of NF- $\kappa$ B activity*

NF- $\kappa$ B DNA binding activity in nuclei of Ucp2(-/-) and Ucp2(+/+) macrophages was determined with TransAM NF- $\kappa$ B kit (Active Motif) according to the manufacturer's protocol.

#### *Cytokine measurement and nitric oxide production*

Macrophages were treated with LPS for 24 hours. Culture media was collected and centrifuged at 1000g to remove any detached cells. Cytokine (IL-6, TNF- $\alpha$  and MCP-1) production was assayed by ELISA using the R&D systems kit according to the manufacturer's

instructions. NO production was estimated by measuring nitrite in macrophage media. Samples were incubated with an equal volume of Griess reagent and absorbance at 550 nm was measured.  $0.15 \times 10^6$  macrophages were cultured in RPMI 1640 without phenol red to avoid interference with Griess absorbance. Nitrite concentration was obtained by comparison to a standard curve.

#### *Mitochondrial ROS production*

Mitochondrial ROS production was estimated by analysis of aconitase and fumarase activities. Macrophages stimulated or not with LPS were resuspended in isolation buffer (320 mM sucrose, 1 mM EGTA, 10 mM Tris-Base, 0.2% BSA pH 7.4) with protease inhibitors (1  $\mu\text{g}/\text{mL}$  pepstatin, 4  $\mu\text{g}/\text{mL}$  aprotinin, 2  $\mu\text{g}/\text{mL}$  leupeptin, 5  $\mu\text{g}/\text{mL}$  bestatin) and homogenized in a Dounce. Unbroken cells and nuclei were removed by centrifugation at 750g. The supernatant was centrifuged at 12000g and mitochondria were resuspended in isolation buffer then lysed in 0.2% Triton X-100. Enzymatic activities were measured as previously described [27] by following optical density increase at 240 nm for 20 min in appropriate media (30 mM sodium isocitrate, 50 mM Tris-HCl, 0.6 mM  $\text{MnCl}_2$  at pH 7.4 for aconitase, and 50 mM sodium L-malate, 50 mM sodium phosphate at pH 7.4 for fumarase). The linear rate allowed evaluation of enzymatic activities. The fumarase/aconitase ratio was expressed as the ratio of respective rates of optical density increase.

#### *Measurement of apoptosis*

Apoptosis was quantified by an ELISA technique (Cell Death Detection ELISA Plus, Roche) directed against cytoplasmic histone-associated DNA fragments. The loss of plasma membrane asymmetry was assayed by annexin-V and propidium iodide staining with the Annexin V-FITC Apoptosis Detection Kit I (Pharmingen).

#### *Statistical analysis*

Values were expressed as mean  $\pm$  s.e.m and “n” represents the number of independent experiments. Comparisons among the different groups were assessed by one-way ANOVA. The level of significance (*P*-value) was \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ), \*\*\* ( $p < 0.005$ ).

## Results

### *Ucp2(-/-) macrophages exhibit increased inflammatory response*

To determine the involvement of UCP2 in immunity and more precisely in macrophages, we used primary bone marrow-derived macrophage cultures. The suitability of our cellular model was first checked by analyzing known phenotypes. To this end, we examined the inhibitory role of UCP2 in NO and cytokine production in response to LPS [21,28]. When cells were stimulated with LPS for 24 hours, we observed a 2.1-fold higher NO production (Fig. 1A) and a 2.5-fold higher iNOS protein expression (Fig. 1B) in Ucp2(-/-) macrophages compared to Ucp2(+/+) macrophages. At the same time, TNF- $\alpha$  (Fig. 1C) and IL-6 (Fig. 1D) releases were significantly higher in Ucp2(-/-) macrophages. Our data are strongly in line with recent reports [21,28], proving the accuracy of our cellular model for further investigations.

We looked for other immune phenotypes of Ucp2(-/-) macrophages. A pivotal event in the inflammatory response is transmigration of immune cells through the endothelial barrier. Therefore, we studied the ability of macrophages to migrate upon stimulation by the chemokine MCP-1. Interestingly, transmigration of Ucp2(-/-) macrophages through a confluent monolayer of endothelial cells (HUVEC) was 2-fold higher than that of the Ucp2(+/+) macrophages (Fig. 1E). In other respects, MCP-1 release of Ucp2(-/-) macrophages was higher than that of Ucp2(+/+) macrophages after 24 hours of LPS stimulation (Fig. 1F), indicating better ability of Ucp2(-/-) macrophages to recruit immune cells and better ability to migrate.

### *LPS-induced mitochondrial ROS production is controlled by UCP2*

Since UCP2 is described as a regulator of ROS production [20,21], the mitochondrial ROS generation of macrophages after LPS stimulation was investigated. We used the assay of fumarase/aconitase ratio since it is a specific measurement of the imbalance between mitochondrial ROS production and antioxidant defense rather than an assay of overall cellular ROS production. Mitochondrial aconitase, an enzyme of the Krebs cycle, is a target of mitochondrial ROS because of its Fe-S center [29,30] whereas fumarase, another Krebs cycle enzyme, is ROS-insensitive [31]. The ratio of fumarase/aconitase activities provides physiologically relevant information since it tests the activity of a known mitochondrial ROS target [31,32]. Under basal conditions, mitochondrial ROS production in Ucp2(-/-) macrophages was higher than in Ucp2(+/+) macrophages (Fig. 2). Macrophage stimulation



with LPS for one hour increased the mitochondrial ROS production of Ucp2(+/+) macrophages (Fig. 2). In contrast, ROS production by Ucp2(-/-) mitochondria was not modified upon LPS stimulation. As a result, once the macrophage is activated, the ROS level of LPS-treated Ucp2(+/+) macrophage mitochondria reached that of Ucp2(-/-) macrophage mitochondria (Fig. 2). This result demonstrates that LPS stimulation suppresses the difference in mitochondrial ROS production between Ucp2(-/-) and Ucp2(+/+) macrophages and suggests an early role for UCP2 in macrophage activation.

#### *Macrophage activation by LPS leads to early downregulation of UCP2*

We next investigated the reason why the difference in mitochondrial ROS production was not maintained after one hour of LPS treatment. UCP2 protein level began to decrease 30 minutes after LPS treatment (Fig. 3), reached its nadir (50% decrease) after one hour of LPS treatment and remained low until 48 hours (data not shown). Furthermore, in our conditions, UCP2 mRNA level was not affected by short LPS stimulation (data not shown), suggesting a decrease in the translation of UCP2 or an increase of the rate of UCP2 degradation. Anyway, the former hypothesis is in agreement with a half-life of UCP2 of 30 minutes (Rousset et al. submitted). Then, one hour after LPS, Ucp2(+/+) macrophages exhibit negligible UCP2 level and are comparable to Ucp2(-/-) macrophages. Finally, these results highlight a role of UCP2 in early phase of macrophage activation.

#### *Ucp2(-/-) macrophages show stronger activation of p38 and ERK in response to LPS*

The quick downregulation of UCP2 protein level led us to investigate any changes in signaling pathways. LPS-induced cell activation depends on MAPK and NF- $\kappa$ B activation [2-3,5]. We investigated the kinetics of the activation of these signaling pathways in response to LPS. Kinetics of I- $\kappa$ B degradation or in NF- $\kappa$ B DNA binding activity were similar in Ucp2(-/-) and Ucp2(+/+) macrophages (figure 4A-B), therefore excluding the influence of NF- $\kappa$ B signaling pathway.

We next analysed MAPK activation by studying phosphorylation of p38, ERK and JNK. In Ucp2(+/+) macrophages, p38 and ERK phosphorylation was maximal 60 minutes after LPS stimulation whereas phosphorylation was maximal only 30 minutes after LPS stimulation in Ucp2(-/-) macrophages (Fig. 5A-B). As LPS-induced mitochondrial ROS production, the differences were not maintained after 60 minutes of treatment. No difference was detected in JNK activation (data not shown).

To confirm that the enhanced activation of the p38 pathway had physiologic consequences, we analyzed a downstream target of p38 named activating transcription factor-2 (ATF-2). ATF-2 is a transcription factor implicated in iNOS induction in response to LPS [33]. Phosphorylation level of ATF-2 was significantly enhanced in Ucp2(-/-) macrophages after 60 minutes of LPS treatment (Fig. 5C). The weak increase in p38 phosphorylation after 30 minutes of LPS treatment resulted in an even stronger ATF-2 activation after 60 minutes of LPS treatment.

Our results show that LPS-induced activation of ERK and p38 signaling pathways is earlier in Ucp2(-/-) macrophages leading to a stronger activation of downstream targets of these kinases.

#### *UCP2 downregulation is mediated by p38 and JNK signaling pathways*

Since UCP2 is rapidly downregulated in response to LPS (Fig. 3), the signaling pathways that may be implicated in this phenomenon were investigated. Macrophages were treated for one hour with MAPK inhibitors prior to one hour of LPS stimulation. The use of a JNK inhibitor (SP600125) and a p38 inhibitor (SB202190) prevented the LPS-mediated downregulation of UCP2 protein (Fig. 6). In contrast, there was no significant effect of an ERK inhibitor (PD98059) on UCP2 protein level. Macrophage preincubation with IL-10, an anti-inflammatory cytokine which inhibits MAPK activation [34], confirmed the dependency of UCP2 protein expression on MAPK pathways.

We previously showed that UCP2 mRNA level did not change after LPS treatment [20, data not shown]. We tested the involvement of two MAPK-induced protein kinases involved in translational control in response to LPS [35,36]. Neither inhibition of the mammalian target of rapamycin (mTOR) by rapamycin nor inhibition of MAPK-interacting kinase (Mnk-1) by its specific inhibitor CGP057380 affected UCP2 downregulation (Fig. 6).

Then, p38 and JNK pathways are the signaling pathways involved in UCP2 downregulation observed after LPS treatment. Meanwhile the absence of UCP2 is associated to increased activation of MAPK pathways, suggesting the existence of a self-amplification phenomenon.

#### *Increased ROS production of Ucp2(-/-) macrophages mediates the greater resistance to NO-induced apoptosis*

A late event that follows LPS-dependent activation of macrophages is NO-mediated apoptosis [25], which resolves the inflammatory reaction. Since NO production by Ucp2(-/-)

macrophages is 2-fold higher than that of Ucp2(+/+) macrophages (Fig. 1A), we studied LPS-induced apoptosis. Macrophages were stimulated for 24 and 48 hours with LPS and DNA fragmentation was assayed by measuring the presence of histone-associated DNA fragments. Surprisingly, despite the higher NO production of Ucp2(-/-) cells (Fig. 1A), LPS-induced apoptosis of Ucp2(-/-) macrophages was similar to apoptosis of Ucp2(+/+) macrophages (Fig. 7A). These results suggest a better resistance of Ucp2(-/-) macrophages to NO-induced apoptosis.

To test this hypothesis, both types of macrophages were treated with SNAP, a NO donor. Apoptosis was assessed by quantification of DNA fragmentation (Fig. 7B), as previously described [25]. There was no difference in apoptosis of Ucp2(-/-) and Ucp2(+/+) macrophages after 24 hours of SNAP treatment. Interestingly, after 48 hours of treatment, there were significantly more Ucp2(+/+) macrophages undergoing apoptosis than Ucp2(-/-) macrophages (Fig. 7B), confirming our hypothesis. The detection of the loss of plasma membrane asymmetry by annexin-V/propidium iodide staining was in line with a better resistance of Ucp2(-/-) macrophages to NO (Fig. 7C).

It was previously reported that increased superoxide production by macrophages is in part responsible of their enhanced resistance to NO-induced cell death [37]. Moreover appropriate concentrations of NO and superoxide was able to reduce NO-induced apoptosis [38]. So we investigated whether this phenomenon could explain the better resistance of Ucp2(-/-) macrophages to NO. Ucp2(+/+) macrophages were treated as described above with SNAP in the presence of DMNQ, a ROS donor (Fig. 7D). After SNAP treatment in the presence of 0.5  $\mu$ M DMNQ, DNA fragmentation was significantly reduced compared to macrophages treated with SNAP alone. After 48 hours, a higher concentration of DMNQ led to increased apoptosis, considering the cytotoxic/pro-apoptotic effects of ROS at high concentrations [39] (Fig. 7D). We suggest that Ucp2(-/-) macrophages may be better protected against NO-mediated cell death than Ucp2(+/+) macrophages because of their higher basal ROS production.

## Discussion

Beyond antimicrobial defense during the oxidative burst, ROS are key players in immune cell signaling [40]. In macrophages, the origin of LPS-induced ROS production is not limited to NADPH oxidase [13-15] and the involvement of mitochondria has been proposed [12,16,17]. In this study, we aimed 1/ to demonstrate the involvement of mitochondria in LPS-induced ROS signaling 2/ to propose the mitochondrial protein UCP2 as a key regulator of this process, and 3/ to provide mechanistic evidence to explain the immune phenotypes of Ucp2(-/-) and Ucp2(+/+) macrophages.

Our primary macrophage culture model was adequate since we reproduced previously published phenotypes [21,28]. Indeed, we confirmed the negative influence of UCP2 on NO and cytokine production in response to LPS. Moreover, UCP2 has been described as a negative regulator of cellular ROS production in macrophages [20,21] as well as in spleen mitochondria [28]. Here, we show for the first time that mitochondria of Ucp2(-/-) macrophages produce more ROS than mitochondria of Ucp2(+/+) macrophages.

We previously reported that UCP2 mRNA starts to decrease in peritoneal macrophages about 4 hours after LPS treatment [20] and Kizaki et al. described a downregulation of UCP2 in RAW264 cells, a macrophage cell line, stimulated with LPS for 6 hours [21]. In fact, no intermediate times were tested. Here, we showed that UCP2 protein downregulation is an early event required for macrophage activation and the protein level is downregulated before mRNA level (respectively 30 minutes and 4 hours). Our results indicates that macrophage activation requires an early need to get rid of UCP2 protein. We propose a signal amplification loop, in which UCP2 downregulation is required to enable LPS-induced mitochondrial ROS signaling (Fig 8A).

Stimulation of macrophages with LPS for 1 hour, once UCP2 protein level reached its minimum level, eliminated the differences in fumarase/aconitase activities observed under basal conditions between Ucp2(+/+) and Ucp2(-/-) macrophages. This interesting result indicates that 2-fold UCP2 downregulation is sufficient to enable mitochondrial ROS production since UCP2 protein expression is not completely removed in LPS-treated macrophages. Therefore, similar MAPK phosphorylation after 1 hour of LPS stimulation in Ucp2(-/-) and Ucp2(+/+) macrophages is a direct consequence of similar ROS production.

The enhanced ROS signaling promoted p38 and ERK activity in Ucp2(-/-) macrophages. These two MAPK are known to play a key role in LPS-induced signal transduction pathways in macrophages [3,5,41]. Accordingly, ATF-2 phosphorylation, a

transcription factor involved in iNOS induction [33] and a downstream target of p38, was clearly increased in LPS-treated Ucp2(-/-) macrophages. As a consequence, the inflammatory response of Ucp2(-/-) macrophages is upregulated (Fig. 1; 8B). They produce two times more NO, a major mediator in the antimicrobial response [42]. Their 2-fold higher transmigration ability is also a very important result since extravasation is an early key event in inflammation [43]. Finally, they are more resistant to NO-mediated cell death than Ucp2(+/+) macrophages. Thus, taken together, it becomes clear that Ucp2(-/-) macrophages are more powerful immune cells than Ucp2(+/+) macrophages. However, despite a higher ROS production under basal conditions, Ucp2(-/-) macrophages have neither elevated MAPK activity nor NO and cytokine production or iNOS expression. Therefore, the difference in the redox state of Ucp2(-/-) and Ucp2(+/+) macrophages is not sufficient to induce macrophage activation in the absence of an appropriate stimulus. This may explain why Ucp2(-/-) mice do not develop inflammatory events in basal conditions. UCP2 is only a brake that has to be switched off in order to allow mitochondrial ROS to amplify MAPK signaling and the set-up of the inflammatory response (Fig. 8A).

Unlike Bai et al., who noted increased NF- $\kappa$ B activity in spleen of Ucp2(-/-) mice [28], we observed differences neither in NF- $\kappa$ B activity nor in I- $\kappa$ B degradation under basal conditions or after LPS stimulation in our *in vitro* macrophage culture system. The observation of Bai et al. is probably a consequence of interactions between the diversity of immune cells present in spleen.

IFN $\gamma$  pretreatment of macrophages eliminated the differences in NO production and transmigration between Ucp2(-/-) and Ucp2(+/+) macrophages (data not shown). IFN $\gamma$  is responsible for macrophage priming through the Jak/Stat signaling pathway [44,45]. In this way it increases the responsiveness of macrophages to LPS (21) without modification of UCP2 protein level, eliminating the benefits of Ucp2(-/-) over Ucp2(+/+) macrophages. Therefore, IFN $\gamma$ -activation of macrophages is independent of UCP2 (Fig. 8A).

NO and ROS are two well-known pro-apoptotic agents. We found that Ucp2(-/-) macrophages are more resistant to NO-induced apoptosis than Ucp2(+/+) macrophages and we suggest that it may be a consequence of their intrinsically higher ROS production. Indeed previous studies demonstrated that adequate amount of ROS production protects against NO-mediated apoptosis [37,38]. Thus, UCP2 downregulation leads to participation of a modified intracellular redox state in the inflammatory response as well as in the protection against pro-apoptotic effects of NO.

Our present results clarify previous studies by giving a mechanistic explanation of their findings. We have shown that Ucp2(-/-) mice resist *Toxoplasma gondii* infection due to higher elimination of *T. gondii* by Ucp2(-/-) macrophages [20]. In order to escape the immune response, *T. gondii* inhibit MAPK phosphorylation by promoting phosphatase activity in macrophages [46]. Since phosphatases are ROS-sensitive [12, 14], enhanced ROS signaling in Ucp2(-/-) macrophages can counteract the action of *T. gondii*. In addition, a major weapon against *T. gondii* is NO. Therefore the difference in mouse survival is not simply a matter of microbicidal ROS production but rather is due to cell activation, leading to higher NO production, as shown here. In another model, i.e. brain ischemia, inhibition of TLR signaling contributes to ischemic tolerance [47]. Cerebral ischemia triggers acute inflammation which exacerbates primary brain damage [47,48]. Therefore, unlike in Ucp2(-/-) mice, the LPS-induced ROS and MAPK signaling pathways are probably decreased in mice overexpressing UCP2. This can explain the reduced inflammatory response of mice overexpressing UCP2 [24] and their greater resistance to ischemic brain damage [49]. Finally, the relevance of our *in vitro* data is corroborated by the increased NO and TNF- $\alpha$  production in Ucp2(-/-) mice in response to LPS [28].

In summary, our study provides evidence of a crucial role of UCP2 as regulator of mitochondrial ROS production and its signaling in early events leading to macrophage activation. Our results should be extended to any immune cell activation since TLR signaling and ROS signaling [40,50] are both important in the function of lymphocytes and phagocytes, which are UCP2-expressing cells [22].

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## Figure legends

Fig. 1. Ucp2(-/-) macrophages exhibit increased inflammatory response. *A-D*, Macrophages were stimulated with LPS (1  $\mu\text{g}/\text{mL}$ ) or not for 24 hours. NO production (*A*) was determined (n=4) and iNOS protein (*B*) was quantified by Western blot. The amount of  $\beta$ -actin served as loading control. Western blots represent the results of one experiment representative of 4 independent experiments. TNF- $\alpha$  (*C*) and IL-6 (*D*) secretions of macrophages were measured by ELISA (n=3). *E*, Transmigration of macrophages ( $\Delta$  OD 550 nm) through an endothelial cell monolayer in the presence or absence of MCP-1 was determined as described in experimental procedures (n=3). *F*, MCP-1 release of macrophages stimulated with LPS (1  $\mu\text{g}/\text{mL}$ ) or not for 24 hours was measured by ELISA (n=3). \* (p<0.05), \*\* (p<0.01).

Fig. 2. LPS-induced mitochondrial ROS production is controlled by UCP2. Macrophages were stimulated with LPS (1  $\mu\text{g}/\text{mL}$ ) or not for 60 minutes. Mitochondrial ROS production was measured as the ratio of mitochondrial fumarase/aconitase activities as described in experimental procedures (n=4). \* (p<0.05), \*\* (p<0.01).

Fig. 3. Macrophage activation by LPS leads to early downregulation of UCP2. Mitochondrial fractions were prepared prior to immunoblotting. Immunoblot analysis of UCP2 protein after LPS treatment (1  $\mu\text{g}/\text{mL}$ ). The amount of porin served as loading control. Western blots represent the results of one experiment representative of 4 independent experiments. \* (p<0.05), \*\* (p<0.01).

Fig. 4. NF- $\kappa$ B signaling pathway is not modified in Ucp2(-/-) macrophages. Macrophages were stimulated with LPS (1  $\mu\text{g}/\text{mL}$ ) for the indicated time periods. *A*, I- $\kappa$ B degradation was analysed by western blot. The amount of  $\beta$ -actin served as loading control. Western blot represents the results of one experiment representative of 3 independent experiments. *B*, NF- $\kappa$ B DNA binding activity in nuclei of macrophages treated or not with LPS (1  $\mu\text{g}/\text{mL}$ ) was analysed as described in experimental procedures.(n=2).

Fig. 5. LPS-induced p38, ERK and ATF-2 phosphorylation is higher in Ucp2(-/-) macrophages. Macrophages were stimulated with LPS (1  $\mu\text{g}/\text{mL}$ ) for the indicated time periods. Phosphorylation of p38 (p-p38, *A*), ERK (p-ERK, *B*) and ATF-2 (p-ATF-2, *C*) was

determined by an ELISA-based method as described in experimental procedures (n=3). \* (p<0.05).

Fig. 6. UCP2 downregulation is mediated by the p38 and JNK signaling pathways. Immunoblot analysis of UCP2 protein after 1 hour of LPS (1  $\mu\text{g}/\text{mL}$ ) stimulation of macrophages preincubated with vehicle or IL-10 (100 ng/mL) or inhibitors of ERK (PD98059, 50  $\mu\text{M}$ ), JNK (SP600125, 25  $\mu\text{M}$ ), p38 (SB202190, 10  $\mu\text{M}$ ), mTOR (rapamycin 1  $\mu\text{M}$ ) or Mnk-1 (CGP057380, 10  $\mu\text{M}$ ), (n=3).

Fig. 7. Ucp2(-/-) macrophages are more resistant to NO-induced apoptosis. *A*, DNA fragmentation in macrophages stimulated with LPS (1  $\mu\text{g}/\text{mL}$ ) was analyzed by ELISA as described in experimental procedures (n=3). *B-C*, Macrophages were treated with SNAP (50  $\mu\text{M}$ ), an NO donor, for the indicated times. *B*, DNA fragmentation was analyzed by ELISA (n=5). *C*, Annexin-V and propidium iodide staining was analyzed by flow cytometry 48 hours after SNAP treatment (n=5). *D*, Apoptosis was induced by treating Ucp2(+/+) macrophages with SNAP (50  $\mu\text{M}$ ) in the presence or absence of different concentrations of DMNQ, a ROS donor. DNA fragmentation was analyzed by ELISA (n $\geq$ 3). \* (p<0.05), \*\*\* (p<0.005).

Fig. 8. Model for the role of UCP2 in LPS-induced mitochondrial ROS signaling. *A*, Recognition of LPS by TLR-4 activates JNK, p38 and ERK signaling pathways. Meanwhile, a ROS signaling pathway is set up. UCP2 is quickly downregulated in response to LPS via the JNK and p38 pathways to increase mitochondrial ROS production. The mitochondrial part of this signal is regulated by UCP2, a brake physiological that has to be switched off in order to enhance mitochondrial ROS production. This mitochondrial ROS generation, as a feedback signal, stimulates the ERK and p38 pathways. In this way, mitochondria contribute to the macrophage inflammatory response. *B*, Diagram showing the amplified signaling cascade responsible for the enhanced inflammatory state of Ucp2(-/-) macrophages.

Figure 1

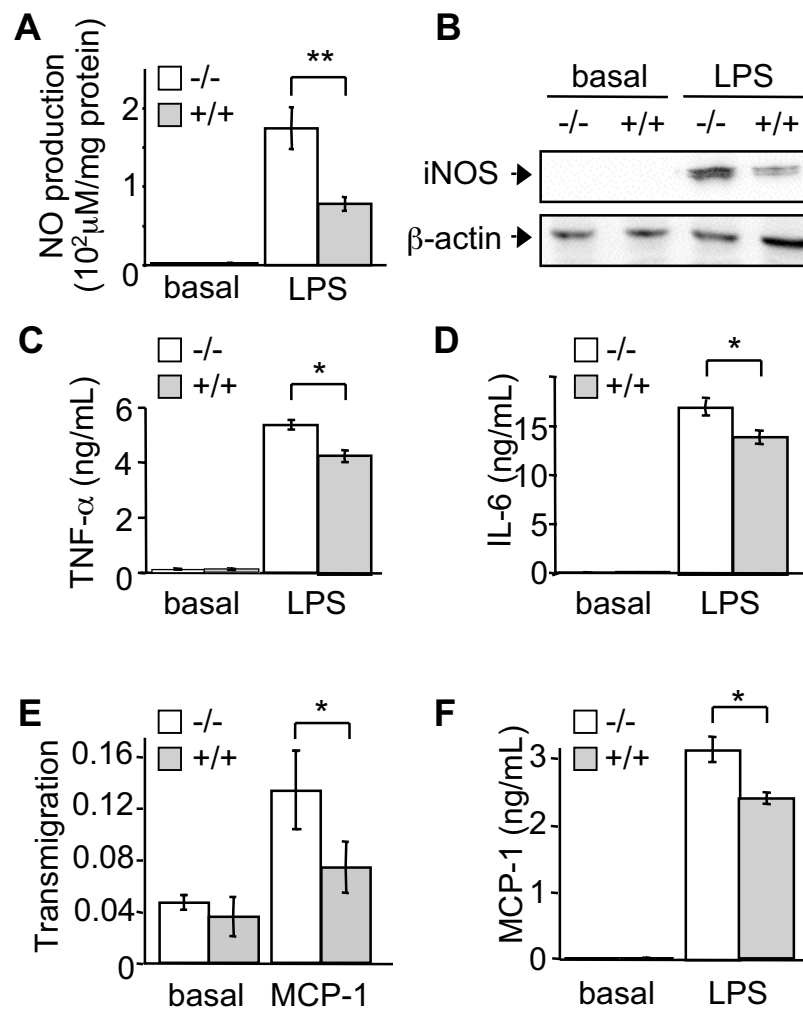


Figure 2

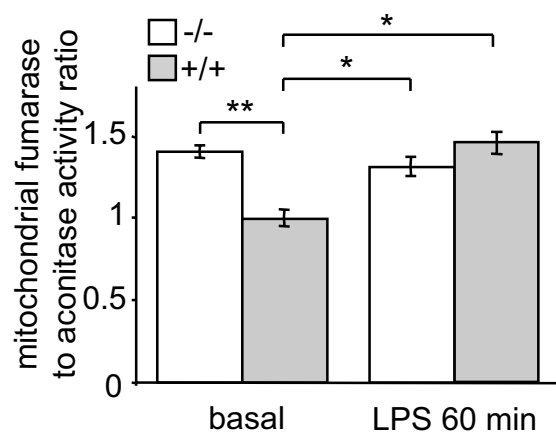


Figure 3

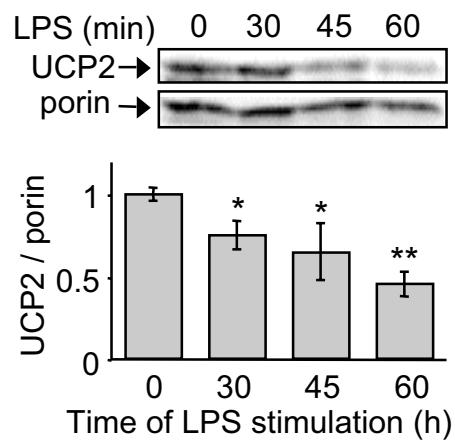




Figure 4

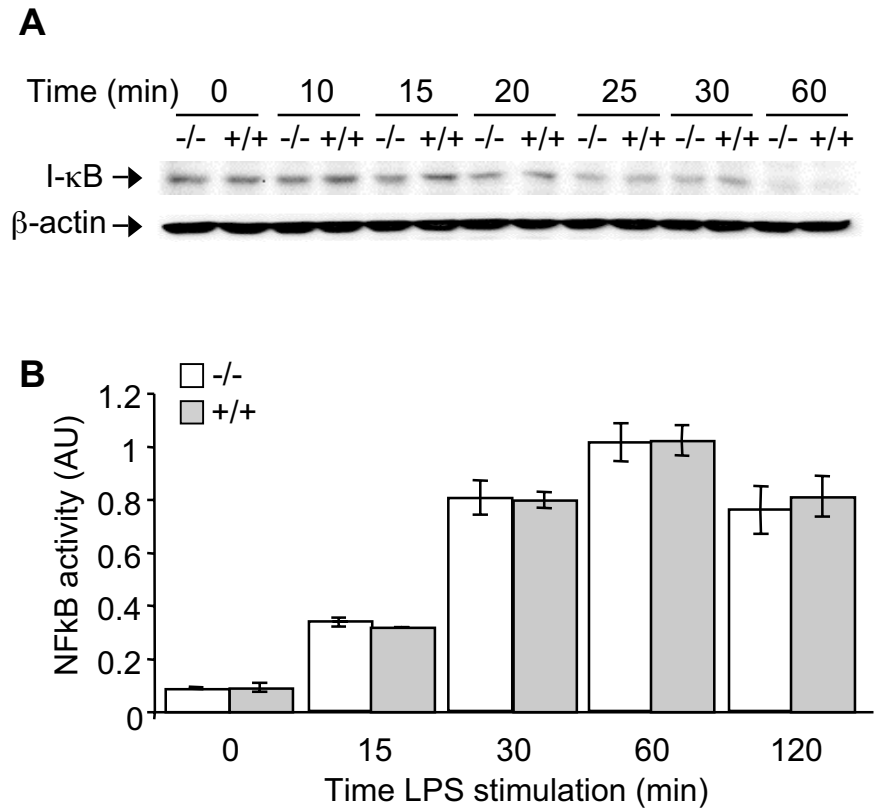


Figure 5

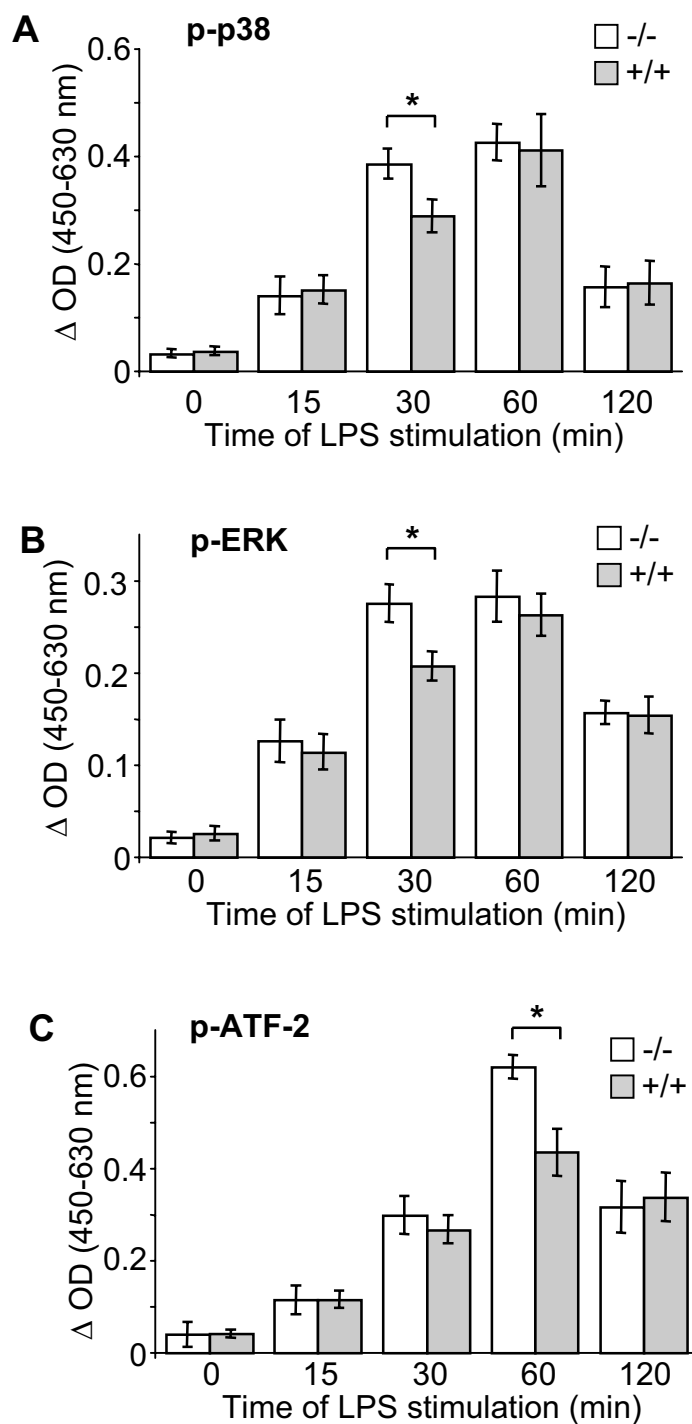


Figure 6

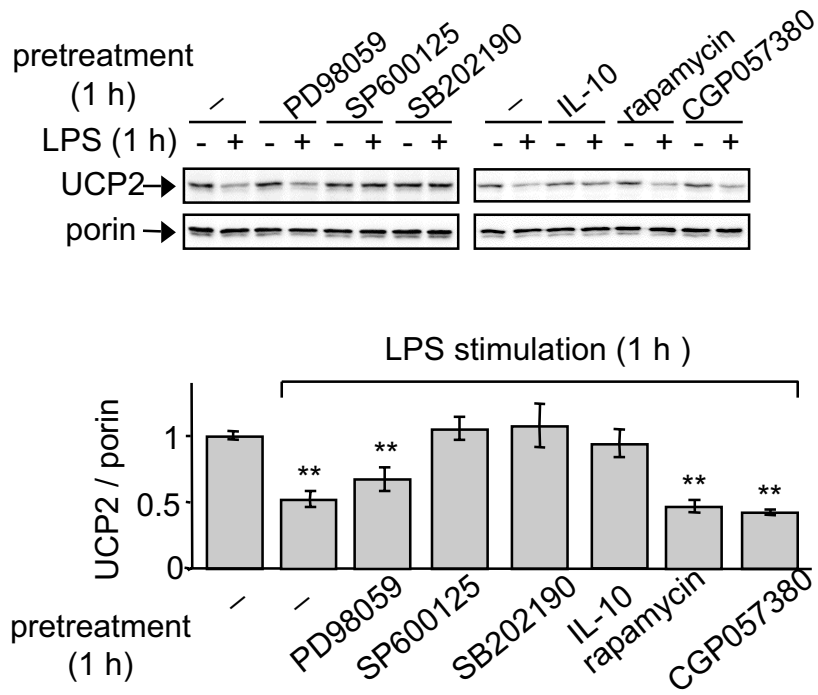


Figure 7

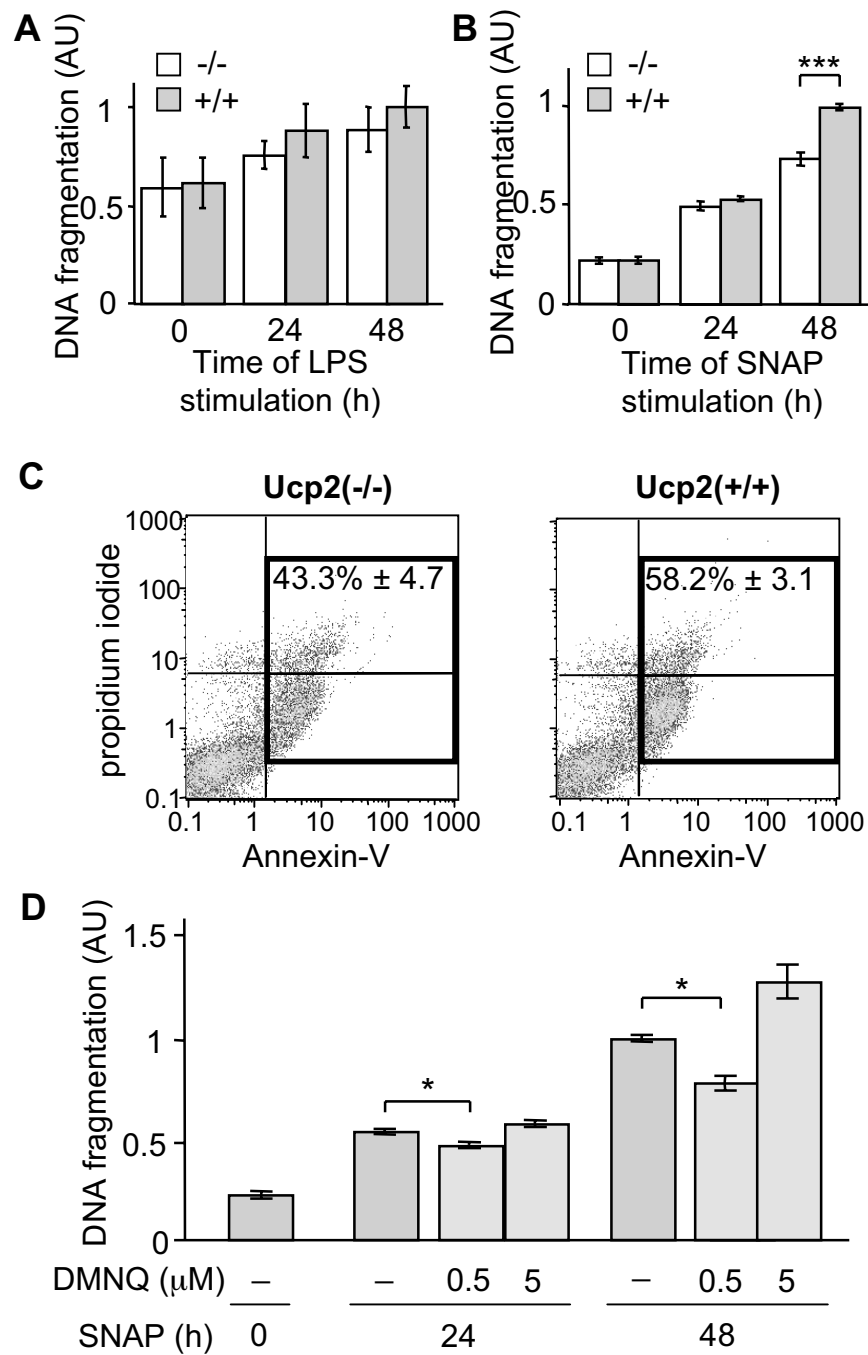


Figure 8

