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# Absence of Mitochondrial Uncoupling Protein 1 Affects Apoptosis in Thymocytes, Thymocyte/T-Cell profile and Peripheral T-Cell Number

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Abbreviations: BAT, brown adipose tissue; BSA, bovine serum albumin; DP, $CD4^+/CD8^+$ double positive ; DN, $CD4^-/CD8^-$ double negative ; FBS, fetal bovine serum; PBS, phosphate buffered saline; SP, single positive ; PI, propidium iodide ; PDH, pyruvate dehydrogenase; DEX, dexamethasone ;

UCP, uncoupling protein;

**Abstract**

Our laboratory has previously demonstrated the presence of constitutively expressed mitochondrial uncoupling protein 1 in mouse thymocytes. In our endeavours to understand the role of mitochondrial uncoupling protein 1 in thymocyte function, we compared cell profiles in thymus and spleen of wild-type with those of UCP 1 knock-out mice, which in turn led to comparative investigations of apoptotic potential in thymocytes from these mice. We demonstrate that spleen cell numbers were reduced ~3-fold in UCP 1 knock-out mice compared to wild-type mice. We record a halving of CD8 single positive cell numbers in thymus with a significant incremental increase in CD4/CD8 double positives cell numbers in the thymus of UCP 1 knock-out mice compared to wild-type mice. Theses data are mirrored by an approximate halving of CD8 single positive cell numbers and a doubling of CD4/CD8 double positive cell numbers in the spleen of UCP 1 knock-out mice compared to wild-type mice. These differences are most probably explained by our observations of decreased apoptotic potential and higher ATP levels in thymocytes of UCP 1 knock-out mice when compared to wild-type controls. We conclude that constitutively expressed UCP 1 is a factor in determining T-cell population selection in mice.

## Introduction

The thymus is a primary lymphoid tissue where progenitor thymocytes from bone marrow develop into mature thymocytes and via antigen selection processes develop to naïve T-cells which migrate to peripheral lymphoid tissue, such as spleen and lymph nodes [for reviews see 1-3]. Thus the thymus contains thymocytes that are at different stages of maturation. The most immature of these cells are easily identified due to the lack of the CD4/CD8 surface antigens (CD4/CD8 double negatives DN)) and account for up to ~5% of the cells present in the thymus. Progress to maturation can be followed firstly by the expression of both CD4 and CD8 antigens of the thymocyte surface (CD4/CD8 double positives (DP)), which account for up to 85% of cells in the thymus. The DP cells undergo positive selection for antigen and negative selection for self-antigen resulting in attrition of up to 95% of these cells through apoptosis. The remaining 5% of selected DP cells generate either naïve CD4 single positive (CD4 SP) or naïve CD8 single positive (CD8 SP) cells which enter the medulla of the thymus before they migrate to the peripheral immune tissues. In a peripheral immune tissue such as the spleen, CD4 SP T-cells account for approximately 20% of all cells, CD8 SP T-cells account for approximately 10% of all cells, whereas the amount of CD4/CD8 DP T-cells is negligible [4]. The remainder of the cells in the spleen are composed mainly of reticulocytes, monocytes and B-lymphocytes [5]. For many apoptotic events the Bcl-2 family of mitochondrial proteins play a central role [6]. For instance, Bim, a BH3-only Bcl-2 family member, is required for apoptosis of thymocytes in response to negative selection [7]. Furthermore, there is a hierarchy of ATP usage in thymocytes [8] and although ATP

is necessary for apoptosis [9], low intracellular ATP levels can trigger nitric oxide induced apoptosis [10].

Until relatively recently, mitochondrial uncoupling protein 1 (UCP 1) had only ever been associated with mitochondria of brown adipose tissue. Under conditions of cold-stress, brown adipose tissue UCP 1 activity results in uncoupling of mitochondrial ATP synthesis, from oxygen consumption, by dissipating the proton electrochemical gradient, generated by the electron transport, chain across the inner membrane. The resulting increased metabolic flux, increased heat production by brown adipose tissue and ultimately distribution of that heat throughout the whole body, is a process termed non-shivering thermogenesis [11-13]. UCP 1 has also, however, been shown to be present in thymocyte mitochondria, and although the original empirical evidence had been contested [14], the recent cell imaging evidence demonstrating UCP 1 present in thymocytes from wild-type mice but absent in thymocytes from UCP 1 knock-out mice, has proven very convincing [15-18]. Interestingly, despite the fact that T-cells in the spleen are derived from the thymus, no transcripts for UCP 1 have been detected in spleen [19]. Until this study we had no indication of a role for UCP 1 in thymus function, but we had established that UCP1 did not have a thermogenic role in thymus, as cold-acclimation did not result in increased UCP 1 expression there [20]. In this study we investigated whether the absence of UCP 1 in thymocytes resulted in a difference in thymus and spleen cell composition profile and function when compared to wild-type mice of the same background, sex, age and strain.

## 2. Material and Methods

### *2.1 Reagents*

All reagents and chemicals used were of analytical grade where possible and were obtained from Sigma Chemical Co. Ltd, Fancy Rd., Poole, Dorset, U.K. unless otherwise stated.

*2.2 Animal Source*

UCP 1 knock-out mice on a C57BL/6J background, originally provided by Dr Leslie Kozak (Pennington Biomedical Research Center, Baton Rouge, Louisiana, US) and wild-type C57BL/6J background mice were bred in-house. The background of the UCP 1 knock-out mice are essentially congenic with C57 [21]. UCP 1 knock-out mice were confirmed to be homozygous by PCR genotyping of tail DNA. All animals had access to food and water ad libitum. All animals were kept at room temperature. Only 3 week old female C57BL/6J wild-type and 3 week old female UCP 1 knock-out mice were used in this study.

*2.3 Thymocyte isolation*

Thymocytes were isolated from UCP-1 knock-out/wild type C57BL/6 mice essentially as described by Buttgereit and Brand [8]. The thymus was removed, trimmed clean of connective tissue and brown fat (if present) and transferred into Hanks' Balanced Salt Solution (Sigma) containing 10% (w/v) foetal bovine serum (FBS). A single cell suspension was prepared by passage through a 70μm nylon sieve (Falcon).

*2.4. Indirect Immunofluorescence*

Isolated thymocytes were prepared for indirect immunofluorescence essentially as described in Adams et al. [17]. Thymocytes were suspended in Hanks medium (and

10% FBS). 0.5% (v/v) paraformaldehyde was added to 1ml of thymocytes and incubated on ice for 5 minutes. 20µl of cells were added to the slides (poly-*l*-lysine coated) and allowed to dry (phosphate crystals form). Slides were then dipped in (-20$^{o}$C) methanol at -20$^{o}$C for 5 minutes. Slides were then dipped in (-20$^{o}$C) acetone at -20$^{o}$C for 5 minutes. Slides were then allowed to dry at room temperature.

The cells on the cover-slips were then incubated with blocking buffer (PBS containing 5% (w/v) BSA and methylamine, 0.1M) for 2 hours at room temperature in petri dishes, to inactivate any remaining formaldehyde and block non specific binding. The cells on the cover-slips were incubated with primary antibody diluted in PBS containing 5% (w/v) BSA overnight at room temperature. Following washing with PBS the cells on the cover-slips were incubated with the secondary antibody conjugated to a fluor diluted with PBS for 3 h at room temperature. The cells on the cover-slips were then washed with PBS (and sodium azide) and finally mounted onto glass slides using 5ul of anti-quench solution.

*2.5 Preparation of anti-quench*

The anti-oxidant n-propyl gallate (4%, v/v) was dissolved in PBS buffer containing sodium azide (15 mM) and glycerol (50%, w/v). This solution was always made fresh on the day and stored at 4 $^{o}$C in the dark until required.

*2.6 Confocal microscopy*

Confocal microscopy was performed essentially as described by Adams et al [16]. The 12 well plates with anti-quench solution were covered with cover-slips and were affixed to the glass slide by applying a thin film of nail varnish to the edges of the cover-slip. This procedure also prevented the samples from drying out. The slides

were examined by phase contrast and confocal microscopy with an Olympus Fluoview FV1000 Imaging system. The excitation light for imaging was provided by the 457-514 nm lines of a multi-line Argon laser, the 543nm line of a Green Helium - Neon laser and the 633 nm line of a red helium-Neon laser. Images were collected and processed with the Olympus Fluoview software (version 1.3c). Thymocytes were probed with FITC-conjugated anti-CD4, R-PE-conjugated anti-CD8 (both from BD Biosciences) and UCP 1 antibody (Calbiochem) using Alexa 647 labelled secondary antibody (Invitrogen-magenta)

*2.7 Flow Cytometry- Cell detection*

Thymocytes/splenocytes were washed and adjusted to 5 x $10^6$/ml in PBSA (1%BSA-PBS) and 50 µl was used for each sample. Cells were incubated on ice for 15 mins in the dark with the surface staining antibodies (CD4-FITC and CD8-R-PE). Fixation medium (Caltag A; Invitrogen) was added to each tube, followed by incubation on ice for 15 mins. Cells were washed twice in PBSA, followed by resuspension in permeabilization medium (Caltag B; Invitrogen) and anti-UCP 1 for 15 mins on ice in the dark. Cells were washed twice in PBSA and resuspended in Caltag B and Alexa 647 labelled secondary antibody and incubated on ice for 15 mins. Cells were washed twice and 30 000 events collected on a DakoCyan ADP flow cytometer. Data was analysed using Flowjo software.

*2.7 Thymocyte incubations with dexamethasone*

Thymocytes were isolated as previously described [8]. Thymocytes were seeded in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2μM glutamine, penicillin (100U/ml) and streptomycin (100μg/ml) in the presence or absence (just vehicle, ethanol) of 0.1μM dexamethasone for up to six hours in a 37$^{o}$ C humidified incubator under an atmosphere of 5% $CO_2$ .

*2.8 Isolation of Bone Marrow and Spleen Cell Mitochondria*

All bone marrow and spleen cell homogenate, red cells, monocytes and lymphocytes fractions were centrifuged at 300 x *g* for 5 minutes at 4$^{o}$C. The supernatants were discarded and all cellular fractions were resuspended in ice-cold (0-4$^{0}$C) STE buffer (250 mM Sucrose, 5 mM Tris-HCl, 2 mM EGTA, pH 7.4). Mitochondria were isolated according to the protocol of Chappell & Hansford [22].

*2.9 Mitochondrial Protein Determination using the Markwell Assay*

Mitochondrial protein concentration was determined by the reduction of Folins-Ciocalteau phosphomolybdic-phosphotungstic reagent according to the method of Markwell et al. [23].

*2.10 SDS-PAGE and Western Blot analysis:*

One-dimensional SDS-PAGE under reducing conditions was used to separate proteins prior to immunoblot analysis, as described by Cunningham et al. [24] Following SDS-PAGE, resolved proteins were transferred onto polyvinylidene difluoride membranes (Immobilin-P$^{SQ}$; Millipore) as described by Cunningham et al. [24]. Commercial anti-UCP 1 (amino acids 145-159) was purchased from Calbiochem. The pyruvate dehydrogenase (PDH) $E_1$□ monoclonal antibody was from MitoSciences. The

primary antibodies were all used at 1:1000 dilution. Following blocking and overnight primary antibody incubation, the blots were incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000) in Tris-buffered saline (TBS), 0.5% (v/v) Tween 20, 5% (w/v) bovine serum albumin for 1h at room temperature. Blots were developed using an ECL detection system (Amersham Biosciences), and immunoreactions were visualized by exposure to Kodak X-Omat LS film.

*2.11 Densitometry*

Following Western blot analysis, the relative abundance to protein was determined using densitometry. The band intensities of the exposed film were analysed using Scion Imaging Software.

*2.12 Flow Cytometry FITC-Annexin V /Propidium Iodide (PI) Staining*

The apoptosis of isolated thymocytes treated with dexamethasone, was examined. To determine early membrane and DNA changes, whole thymocytes were stained with FITC-conjugated Annexin V and propidium iodide (PI) according to manufacturer's instructions (Molecular Probes). Cells ($0.75 \times 10^6$) were suspended in 100µl of annexin-binding buffer, and 5µl of FITC-annexin V and 1µl of PI (100µg/ml) was added to each 100µl cell suspension. Cells were incubated for 15 min at room temperature. Stained cells were suspended in 400µl of binding buffer and immediately processed by flow cytometry.

*2.13 Caspase 3/7 Activity*

The *Caspase-Glo 3/7* Assay (Promega) is a luminescent assay that measures caspase 3 and caspase 7 activities in suspension cells. This assay provides a proluminescent caspase μ3/7 substrate, which contains the tetrapeptide sequence DEVD. The substrate is cleaved to release aminoluciferin, a substrate of luciferase used in the production of light. The addition of the single *Caspase-Glo 3/7* reagent results in cell lysis, followed by caspase cleavage of the substrate and generation of a luminescent signal. Thymocytes from UCP 1 -/- mice and C57BL/6 mice were isolated and induced into apoptosis with either (a) 0.1μM dexamethasone, (b) vehicle alone (ethanol) or (c) no addition (untreated control) at 37$^{o}$C in a 5% $CO_2$ environment . After 5 hours, cells were resuspended at 2x10$^{6}$/ml in RPMI-1640. 100μl of *Caspase-Glo 3/7* reagent was added to each well of a white walled 96-well plate containing 100μl of control or drug treated thymocytes in RPMI-1640. Contents of the wells were gently mixed on a plate shaker for 30 seconds, and incubated at room temperature for 1 hour. The luminescence of each well was measured on a plate reading luminometer.

*2.14 DNA Fragmentation*

DNA electrophoresis was performed using $10^{7}$ thymocytes incubated at 37$^{o}$ C in medium containing ethanol (vehicle) or 0.1μM dexamethasone-supplemented medium. Cells were harvested and resuspended in 1ml cell lysis buffer (20mM EDTA, 100mM Tris pH 8.0 0.8% (w/v) sodium lauryl sarcosinate) and incubated at 37$^{o}$ C for 1 hour. 0.5mg/ml RNAse was then added to each sample and left for a further 2 hours at 37$^{o}$ C. finally the samples were treated with 6 mg/ml proteinase K and were left overnight at 37$^{o}$ C. An aliquot of each sample (45μl) was mixed with

5µl DNA loading dye and resolved on a 1% agarose gel in TAE, pre-stained with ethidium bromide. DNA was electrophoresed at a constant voltage of 55V for 2 hours 30 min.

*2.15 Measurement of ATP levels*

The ApoSENSOR ADP/ATP ratio assay kit (BioVision) utilizes the enzyme luciferase to catalyze the formation of light from ATP and luciferin, and the light can be measured using a luminometer. Thymocytes from UCP 1 -/- mice and C57BL/6 mice were isolated and resuspended at $1x10^6$ in PBS. Triplicate cell samples (10 µl per well) were placed in a white walled 96 well luminometer plate. Nucleotide releasing buffer was then added to each well (100µl), to lyse the cells, and left at room temperature for 5 minutes. To measure the ATP levels in the thymocytes from UCP 1 -/- mice and C57BL/6 mice, 1µl of the ATP monitoring enzyme was added to the resulting lysates. The resulting luminescence was measured one minute later.

*2.18 Measurement of oxygen consumption rates of cells*

All measurements of respiration were made using an Oxygraph-2k respirometer (Oroboros Instruments, Innsbruck, Austria), and oxygen flux was resolved using DATLAB software. The oxygraph-2k is a two chamber titration-injection respirometer with a limit of oxygen flux detection of 1pmol/$ml^{-1}$. Standardized instrumental and chemical calibrations were performed to correct for back diffusion of oxygen into the chamber from the various components ; leak from the exterior, oxygen consumption by the chemical medium, and sensor oxygen

consumption. The cell suspension was stirred using a PDVF magnetic stirrer and thermostatically maintained at 28$^{o}$C. Before each experiment medium was equilibrated for 30-40 min with air in the oxygraph chambers until a stable signal was achieved to calibrate for oxygen saturation. Quiescent thymocyte steady state oxygen consumption rates were then measured in RPMI medium ($5x10^{6}$ cells/ml).

**RESULTS**

Three week old mice were selected for this study based on the observation that UCP 1 expression in mitochondria from thymi of wild-type mice was persistently evident in mice of less than 5 weeks old (Figure 1A and 1B). We also confirm previously reported RT-PCR evidence that UCP 1 protein is only evident in mitochondria from thymus and not in mitochondria from bone marrow or spleen cells (Figure 1C and 1D).

Figure 2A give a typical profile of the thymocyte cells present in the thymus of mouse, indicating, as expected, that the majority of cells are CD4/CD8 double positive. Figure 2B demonstrates that mitochondrial UCP 1 is associated with all thymocyte cell types: CD4/CD8 double positive (DP) thymocytes, CD4/CD8 double negative (DN) thymocytes, CD4 single positive (CD4 SP) thymocytes and CD8 single positive (CD8 SP) thymocytes. The association of UCP 1 with CD4/CD8 double positive (DP) thymocytes is visually demonstrated in the confocal images in Figure 2C.

In light of the observation that mitochondrial uncoupling protein 1 (UCP 1) is present in thymocytes (but not spleen) of mouse, and because the T-cell profile of the spleen is contributed to by the thymus, we compared a variety of tissue and cell parameters from both thymus and spleen from C57BL/6 wild-type and UCP 1 knock-out mice, to ascertain whether there were differences in cell profile and function. Table 1 holds data for thymus and spleen tissue masses from C57BL/6 wild-type and UCP 1 knock-out mice. No significant difference was observed in a comparison of tissue mass from C57BL/6 wild-type and UCP 1 knock-out mice, however there is a trend for the spleen mass of knock-out mice to be lower than that from wild-type mice.

Despite the fact that we saw no significant difference in thymus or spleen tissue mass in a comparison of C57BL/6 wild-type and UCP 1 knock-out mice (Table 1), we nevertheless performed a cell count from these tissues. We observed no difference in cell numbers in a comparison of the thymus from C57BL/6 wild-type and UCP 1 knock-out mice (Figure 3A), but we did observe a significant ~3-fold decrease in cell number in a comparison of the spleen cell number from C57BL/6 wild-type and UCP 1 knock-out mice (Figure 3B).

Furthermore, we profiled the cells within the thymus (Figure 4A-F) and compared those from C57BL/6 wild-type and UCP 1 knock-out mice. Figure 4A and 4B are typical examples of the primary FACS analysis data for mouse thymus. From our collated data we were able to demonstrate a significant ~2-fold decrease in the CD8 single positive cells (Figure 4C) and a significant ~10% increase in CD8/CD4 double positive cells (Figure 4D) from UCP 1 knock-out mice when compared to thymocytes from C57BL/6 wild-type mice. No significant difference was observed in the proportion of CD8/CD4 double negative or CD4 single positive thymocytes isolated from C57BL/6 wild-type mice compared to UCP 1 knock-out mice (Figure 4E and Figure 4F)

As there is a significant contribution to T-cells in the spleen from the thymus, we identified and determined the relative abundance of CD8 single positive, CD4 single positive and CD8/CD4 double positive cells within the spleen (Figure 5A-F) and compared those from C57BL/6 wild-type and UCP 1 knock-out mice. An example of the FACS analysis primary data for mouse spleen is given in Figure 5A and 5B. We

observed a significant ~2-fold decrease in the CD8 single positive cells (Figure 5C) and a significant ~2-fold increase in CD8/CD4 double positive T-cells (Figure 5D) in the spleen of UCP 1 knock-out mice compared to cells from C57BL/6 wild-type mice. No significant difference was observed in the proportion of other cells (which includes CD8/CD4 double negative T-cells)(Figure 5C) or CD4 single positive T-cells (Figure 5D) isolated from C57BL/6 wild-type compared to UCP 1 knock-out mice.

The decrease in the proportion of CD8 single positive thymocytes in the thymus (Figure 4C) and CD8 single positive T-cells in the spleen (Figure 5C), together with the increase in CD8/CD4 double positive thymocytes in the thymus (Figure 4D) and the doubling of CD8/CD4 double positive circulating T-cells (Figure 5D) led us to question whether there was a differential effect in apoptotic potential in a comparison of thymocytes from C57BL/6 wild-type and UCP 1 knock-out mice. Figure 6 contains primary data and collated data for annexin V plasma membrane phosphatidylserine binding and propidium iodide DNA staining, as measures of the extent of apoptosis, after 5hours (Figure 6 A, B and C) and 18hours (Figure 6 D, E and F) for dexamethasone and vehicle treated thymocytes from C57BL/6 wild-type and UCP 1 knock-out mice. Our data show no significant difference in apoptotic potential in a comparison of dexamethasone treated thymocytes (uncorrected for vehicle) from C57BL/6 wild-type and UCP 1 knock-out mice after 5hours (Figure 6C) and no significant difference in apoptotic potential in a comparison of dexamethasone treated thymocytes (uncorrected for vehicle) from C57BL/6 wild-type and UCP 1 knock-out mice after 18hours (Figure 6F). However there is a significant decrease in apoptotic potential in vehicle treated thymocytes from UCP 1 knock-out mice after 5 hours compared to cells from wild-type mice (Figure 6C) and a significant decrease in

apoptotic potential in vehicle treated thymocytes from UCP 1 knock-out mice after 18 hours when compared to thymocytes from C57BL/6 wild-type mice (Figure 6F).

An additional index of apoptosis, namely caspase 3 and 7 activity in dexamethasone-treated, vehicle-treated and untreated control thymocytes from C57BL/6 wild-type and UCP 1 knock-out mice, indicated less caspase activity in thymocytes from UCP 1 knock-out mice for dexamethasone-treated, vehicle-treated and untreated control thymocytes (Figure 7). These data, depicting caspase activity in Figure 7, are consistent with the independent indices of apoptosis, namely annexin V binding and propinium iodide staining laid out in Figure 6.

The former data in Figures 6 and 7 are further endorsed by our observation of increased DNA laddering in dexamethasone treated and control thymocytes from C57BL/6 wild-type mice compared to UCP 1 knock-out mice (Figure 8).

The decreased apoptotic potential of thymocytes from UCP 1 knock-out mice when compared to thymocytes from C57BL/6 wild-type mice (Figures 6 to 8) are consistent with our further observation of an increased abundance of ATP in thymocytes from UCP 1 knock-out mice when compared to thymocytes from C57BL/6 wild-type mice (Figure 9A). However, there appears to be no impact of the absence of UCP 1 on the total oxygen consumption rate by thymocytes (Figure 9B).

## DISCUSSION

We have previously demonstrated empirically and using confocal microscopy that thymocytes from mice have uncoupling protein 1 (UCP 1) associated with their mitochondria [15-18]. In light of this observation we set out in this study to determine

whether there was any difference in thymus function by comparing profiles and function of thymocytes from C57BL/6 wild-type and UCP 1 knock-out mice. We firstly demonstrate that in our hands profiling of thymus from C57BL/6 wild-type have UCP 1 prominently present for up to 5weeks (Figure 1 A and 1 B) and give a typical profile to those in the literature [1] with CD4/CD8 double positive cells being the predominant cell type in the thymus (Figure 2A). We also clearly demonstrate that mitochondrial UCP 1 is apparent in all thymocytes (Figure 2B) with a clear microscopic demonstration of UCP 1 in CD4/CD8 double positive cells (Figure 2C), but is not present in bone marrow (the site of thymocyte progenitor cells) nor in spleen cells (a location for naive T-cells) (Figure 1B). Initial anatomical investigations gave no indication of any cellular phenotypic difference as there was no detectable difference in mass of thymus and spleen from C57BL/6 wild-type and UCP 1 knock-out mice (Table 1). However, when cell numbers from thymus and spleen are compared, there is a significant ~3-fold reduction in cell numbers in spleen, with no significant difference in cell number in thymus in a comparison of C57BL/6 wild-type and UCP 1 knock-out mice (Figure 3). Preliminary data from our laboratory indicate an increase in cell size (results not shown) but further work is required to cement this observation. Interpreting the data so far would suggest that the absence of UCP 1 in thymus may affect cell number in spleen.

Analysis of the cell populations in a comparison of thymocytes (Figure 4) and spleens (Figure 5) clearly demonstrates a significant incremental increase in CD4/CD8 double positives cells in the thymus (Figure 4) and a significant doubling of CD4/CD8 double positives cells in this peripheral tissue of UCP 1 knock-out mice compared to wild-type mice (Figure 5). We conclude from these data that there is either an

alteration in the positive or negative selection process in the thymus so that CD4/CD8 double positive cells are in excess in the thymus and spill over into the spleen.

In addition we observed a significant halving of CD8 single positive cells in the thymus (Figure 4) concomitant with a significant halving of CD8 single positive cells in the spleen (Figure 5) but no significant changes in CD4 SP cells in the thymus when comparing cell profile from UCP 1 knock-out mice with those from wild-type mice. We conclude from these data that the process of determining SP cell type after negative selection is affected by the absence of UCP 1.

The high attrition rate of cells in the thymus, through positive and negative selection by apoptosis, led us the investigate whether our observations of decrease spleen cell numbers (Figure 3) and the decreased CD8 single positive and CD4/CD8 double positive cells in the thymus and spleen of UCP I knock-out mice (Figure 4 and 5), might be due to differences in the apoptotic potential of thymocytes from C57BL/6 wild-type and UCP 1 knock-out mice. Our data clearly demonstrate that apoptotic potential is reduced in thymocytes from UCP 1 knock-out mice which compared to C57BL/6 wild-type mice (Figures 6, 7 and 8). Our interpretation from these data, in the context of the Figures 6, 7 and 8, is that the reduced apoptotic potential of thymocytes from UCP 1 knock-out mice probably explains (a) the halving of cell numbers in spleen (b) the disrupted selection processes of CD4/CD8 double positive cells and (c) the maturation of SP cell type following negative selection.

In light of the fact that we have observed a significant decrease in spontaneous, non-dexamethasone-treated, apoptosis in thymocytes from C57BL/6 wild-type mice compared to thymocytes from UCP 1 knock-out mice, and in light of the fact that

steady-state UCP 1 activity may reduce intracellular ATP status in thymocytes, we measured the ATP levels in quiescent thymocytes from C57BL/6 wild-type and UCP 1 knock-out mice. Our observation that quiescent thymocytes from UCP knock-out mice have higher ATP levels (Figure 9A) is consistent with an increased steady-state mitochondrial efficiency in thymocytes from UCP 1 knock-out mice compared to wild-type controls. Furthermore, in light of the fact that nitric oxide mediated apoptosis in thymocytes is dependent on efficient mitochondria [25] and reduced intracellular ATP levels trigger nitric oxide induced apoptosis [10], our observations of higher intracellular ATP levels in cells of a lower apoptotic potential is interesting and may hint at mechanism. If we thus presume that the higher ATP concentrations in thymocytes of UCP 1 knock-out mice are instrumental in reducing apoptosis, we would hypothesis that the absence of UCP 1 increases the efficiency of the in situ mitochondria resulting in the higher steady-state ATP concentration in knock-out mice. Consequent to that, one might expect the proton leak in these quiescent thymocytes of UCP 1 knock-out mice to be reduced compared to thymocytes from wild-type mice which in turn might manifest itself as a decrease in oxygen consumption at the whole cell (thymocyte) oxygen consumption level. This was not apparent though, as there was no significant difference in the whole cell oxygen consumption rates in a comparison of thymocytes from UCP 1 knock-out mice and from wild-type mice (Figure 9B). However, an equivalent observation was made by Krauss et al. [26] who observed no significant difference in the rate of thymocyte oxygen consumption rates in a comparison of UCP2 knock-out and wild-type mice, yet they did manage to detect a difference in in situ proton leak. Future work by us will explore whether in situ proton leak is affected in thymocytes by the absence of UCP 1.

One could argue that the effects we observe on thymus and spleen cell profiles, numbers and apoptotic potential may not in fact due to absence of UCP 1 directly, but due to secondary effects on BAT function in vivo. There is no doubt that brown fat has been reported to play a role in the development of immune potential [27] and that the ablation of UCP 1 can effect mitochondrial proton leak in skeletal muscle, a tissue that does not contain UCP1 [28] . Such a metabolic influence, if it exists, my well explain differences in profiles and numbers of the immediately accessed thymus and spleen cells. However, it is probably less likely that brown adipose tissue is affecting our observed apoptotic potential differences in thymocytes, as in this instance we are working with isolated cells, no longer under the influence of whatever regulation might be imparted by brown adipose tissue.

Finally, the potential consequence of increased circulating DP cells and reduced CD8SP T-cells in the UCP 1 knock-out mice (compared to wild-type controls) are, on the face of it, quite similar, in that CD8SP T-cells are anti-cancer and anti-viral whilst increased circulating DP T-cells have been associated with increased cancers and viral infection [4,29]. Thus extrapolating from our cell data to the whole animal, one might expect UCP 1 knock-out mice to be more susceptible to cancers and viral infection compared to wild-type controls, however, I am not aware of any whole animal studies, as yet, indicating such vulnerability, but in light our observations, future investigations may highlight them.

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**Figure 1. UCP 1 protein expression in mitochondria from thymi of mice.**

Mitochondria (10μg) from thymus were isolated from 1, 2, 3, 4, 5, 6, 8 and 16 week old C57bl/6 mice, subjected to SDS-PAGE and Western blotting using **(A)** a 1:1000 dilution of an anti-UCP 1 peptide antibody (Sigma) and **(B)** PDH peptide antibody (Cambridge Biosciences). Mitochondria from brown fat, bone marrow, thymus, spleen and liver were isolated and subjected to SDS-PAGE and Western blotting using **(C)** a 1:1000 dilution of an anti-UCP 1 peptide antibody (Sigma) and **(D)** PDH peptide antibody (Cambridge Biosciences).

**Figure 2 Characterization of thymocytes isolated from wild-type C57BL/6 mice**

**A.** Representative two colour flow cytometry analysis of thymocyte subsets from wild-type C57BL/6 mice**.** Numbers in the dot plot represent percentage of cells in each quadrant using 10,000 cells. Thymocytes were probed with FITC-conjugated anti-CD4, R-PE-conjugated anti-CD8 (both from BD Biosciences) **B.** All thymocytes were simultaneously labelled for mitochondrial UCP1, and a profile of the proportion of each thymocyte subset containing UCP 1 was expressed as a percentage. Each thymocyte subset was defined by the number of cells in each quandrant. Intensity of fluorescence is proportional to the amount of UCP 1 present. The graph shows the FACS Profiles for UCP 1 in CD4/CD8 double positive (DP) thymocytes (blue), CD4/CD8 double negative (DN) thymocytes (green), CD4 single positive (CD4 SP) thymocytes (red) and CD8 single positive (CD8 SP) thymocytes (yellow). UCP 1 was detected with anti-UCP 1 antibody (Calbiochem) and secondary Alexa-647 antibody (Invitrogen-magenta). The intensity of fluorescence due to non-specific binding by the secondary antibody (negative control) is depicted by the grey lined profile. **C.** Isolated thymocytes were fixed and permeabilized as described in the methods section. Thymocytes were probed with FITC-conjugated anti-CD4 (green), R-PE-

conjugated anti-CD8 (red) [both from BD Biosciences] and UCP 1 antibody (Calbiochem) using Alexa 647 labelled secondary antibody (Invitrogen-magenta)(4200 zoom; Bar 5µm)

**Figure 3 Number of cells per thymus and spleen from 3 week old wild-type C57BL/6 and UCP 1 -/- mice**

The bar chart depicts the collated data for cell number for (A) thymus and (B) spleen of wild-type (■) and UCP 1 knock-out (□) mice. Data are from three separate experiments each performed in at least triplicate (mean ± sem (3)). *p* values ≤ 0.05 on an unpaired Students 't'-test were deemed significant.

**Figure 4 Cell subsets in thymus of 3 week old wild-type C57BL/6 and UCP 1 -/- mice.**

Thymocytes from 3 week old wild-type C57BL/6 (■) and UCP 1 knock-out (□) mice were isolated and the cells were stained for with FITC-conjugated anti-CD4, R-PE-conjugated anti-CD8 (both from BD Biosciences) and analyzed on the FACS Cyan. Representative dot plots for thymocytes from (A) wild-type C57BL/6 and (B) UCP 1 knock-out mice are shown. Numbers represent percentage of total cells in each quadrant using 10,000 cells. The percentage of cells that are (C) CD8 single positive (CD8 SP), (D) CD8/CD4 double positive (DP), (E) CD8 and CD4 double negative (DN) and (F) CD4 single positive (CD4 SP) are indicated. Data are mean ± sem of six

experiments performed in at least triplicate. *p* values ≤ 0.05 on an unpaired Students 't'-test were deemed significant.

**Figure 5 Cell subsets in spleen of 3 week old wild-type C57BL/6 and UCP 1 -/- mice.** Spleen cells from 3 week old wild-type C57BL/6 (■) and UCP 1 knock-out (□) mice were isolated and the cells were stained for with FITC-conjugated anti-CD4, R-PE-conjugated anti-CD8 (both from BD Biosciences) and analyzed on the FACS Cyan. Representative primary data for spleen cell from (A) wild-type C57BL/6 and (B) UCP 1 knock-out mice are shown. Numbers represent percentage of total cells in each quadrant using 10,000 cells. The percentage of T-cells that are (C) CD8 single positive (CD8 SP), (D) CD8/CD4 double positive (DP), (E) Other cells including CD8 and CD4 double negative (other cells) and (F) CD4 single positive (CD4 SP) are indicated. Data are mean ± sem of six experiments performed in triplicate. *p* values ≤ 0.05 on an unpaired Students 't'-test were deemed significant.

**Figure 6. Percentage of apoptosis in vehicle-treated and dexamethasone-treated thymocytes from C57BL/6 wild-type and UCP 1 -/- mice, as determined by annexin V and propidium iodide (PI) staining.**

Thymocytes from wild-type C57BL/6 (■) and UCP 1 knock-out (□)mice were isolated and treated with either the vehicle (VEH) or dexamethasome (DEX)(0.1µM ) for 5 (A-C) and 18 (D-F) hours. Cells were collected and stained with Annexin V (conjugated to Alexa 488- Millipore) and Propidium iodide (PI). 10000 events per measurement were then collected on a FACS Cyan. Representative dot plots of dexamethasone treated thymocytes after (5 h)(A and B) and 18 hours (D and E) are

indicated. Collated data for dexamethasone and vehicle treated thymocytes after (5 h)(C) and 18 hours (F) are indicated. Data are mean ± sem of four experiments performed in at least triplicate. *p* values ≤ 0.05 on an unpaired Students 't'-test were deemed significant.

**Figure 7 Caspase activation in dexamethasone-treated, vehicle-treated and untreated (control) thymocytes from 3 week old C57BL/6 wild-type and UCP 1 -/- mice, as determined by the *Caspase Glo 3/7 assay*.**

Thymocytes from wild-type C57BL/6 (■) and UCP 1 knock-out (□) mice were isolated and treated with either dexamethasome (0.1µM), vehicle or were untreated. After 5 hours , $10^4$ cells were placed in each well and lysed. A substrate peptide for caspase 3 and 7 was added. Data are mean ± sem of three experiments performed in at least triplicate. *p* values ≤ 0.05 on an unpaired Students 't'-test were deemed significant.

**Figure 8. DNA Fragmentation in untreated (control), vehicle-treated and dexamethosone treated thymocytes from C57BL/6 wild-type and UCP 1 -/- mice.**

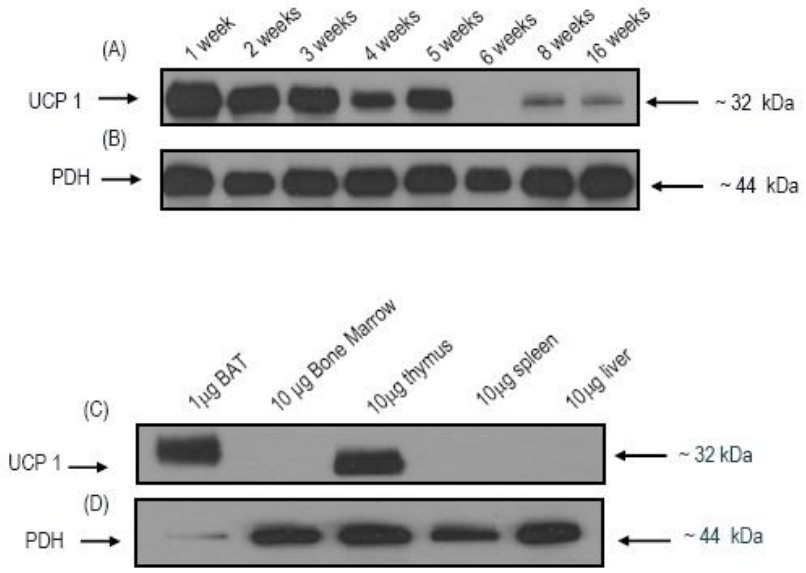
The lanes on the agarose gel show DNA markers and DNA isolated from untreated (0h), vehicle-treated (4h) and dexamethasone treated (4h) thymocytes from C57BL/6 (WT) and UCP 1 -/- (KO) mice.

**Figure 9. Intracellular ATP levels and oxygen consumption rates of thymocytes from 3 week old C57BL/6 wild-type and UCP 1 -/- mice.**

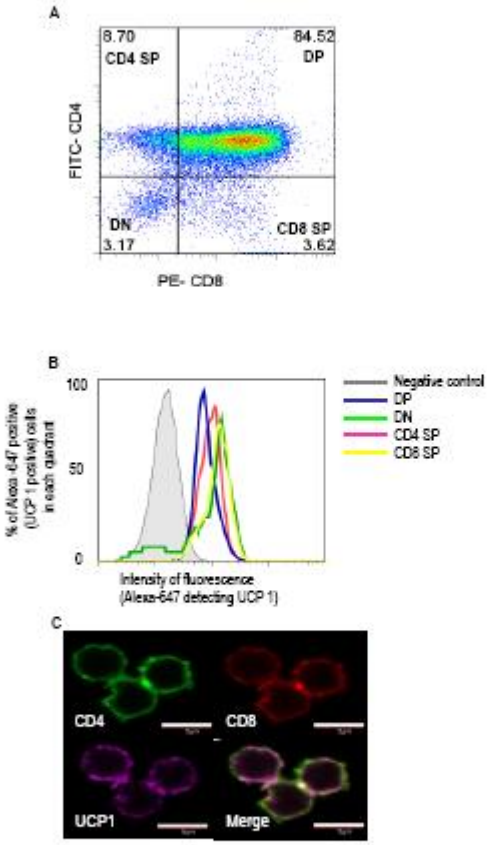
(A) ATP levels in quiescent thymocytes from wild-type C57BL/6 (■) and UCP 1 knock-out (□) mice were determined using an Aposensor ADP/ATP Ratio Assay Kit

from Biovision. 10,000 cells were used for each sample. (B) cellular oxygen consumption rates of quiescent thymocytes was measured in RPMI medium ($5x10^6$ cells/ml) using an Oxygraph Respirometer (Oroborus Instruments, Innsbrück, Austria). Data are mean ± sem of three experiments performed in at least triplicate. *p* values ≤ 0.05 on an unpaired Students 't'-test were deemed significant.

Adams et al. Figure 1



Adams et al. new Figure 2



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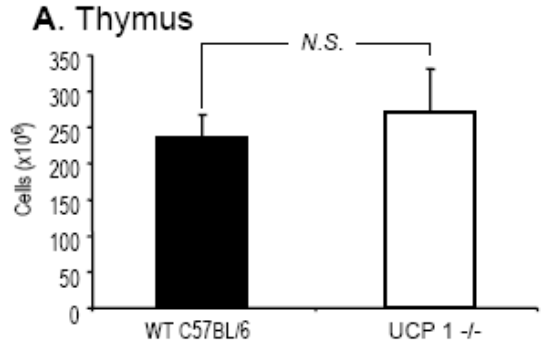


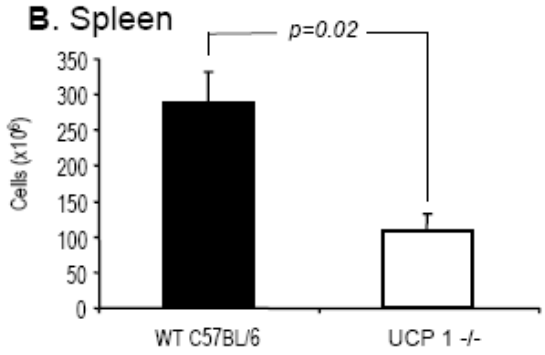

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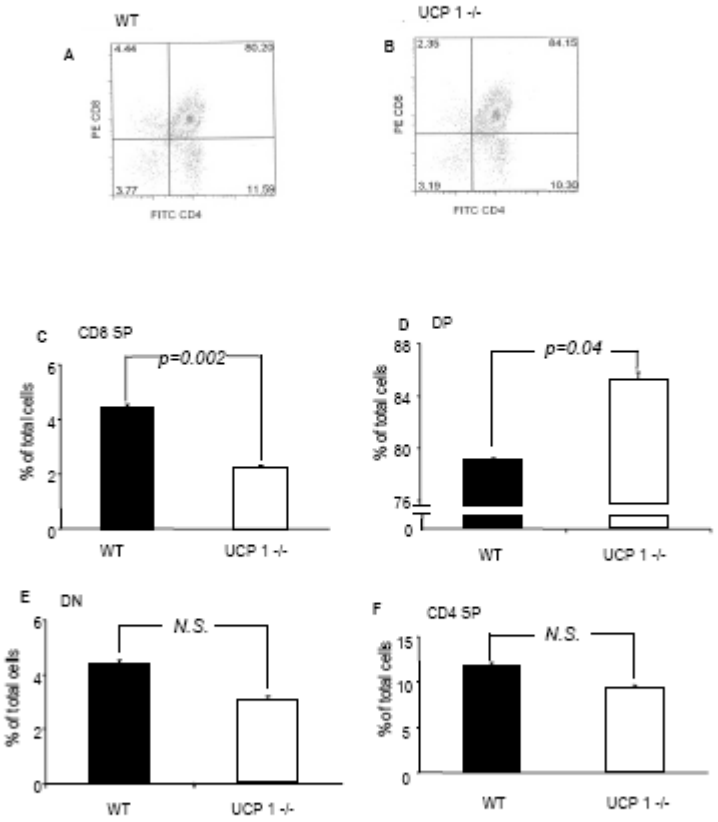

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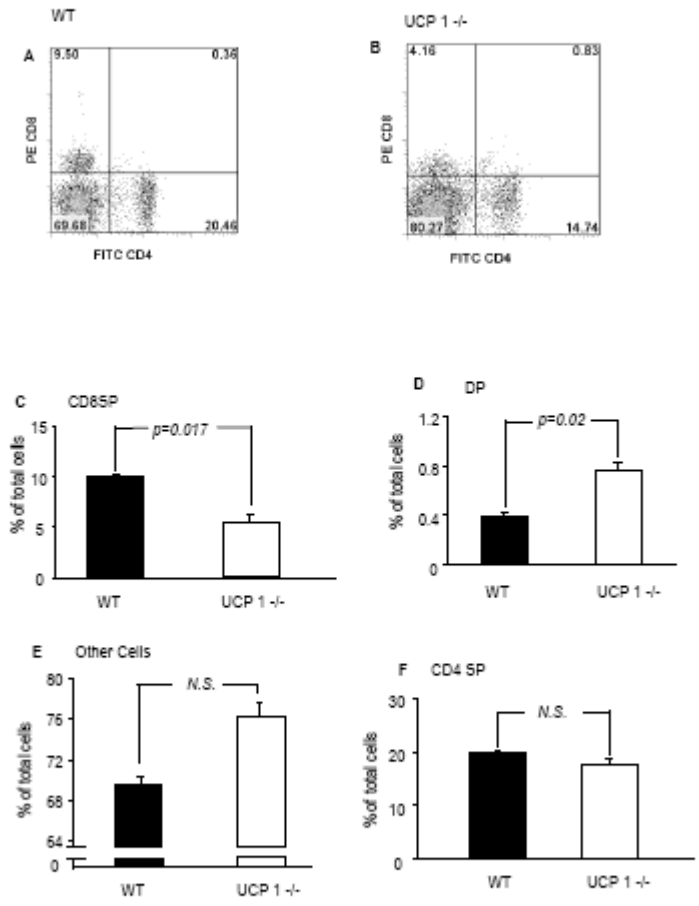

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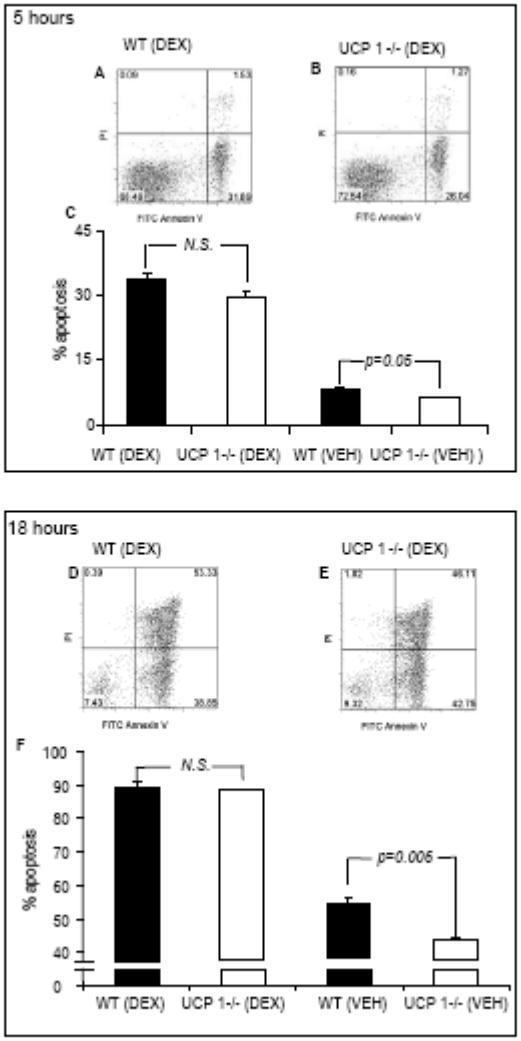

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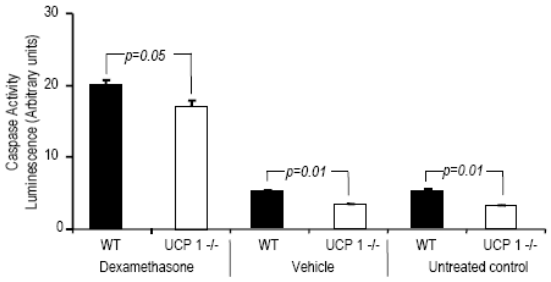


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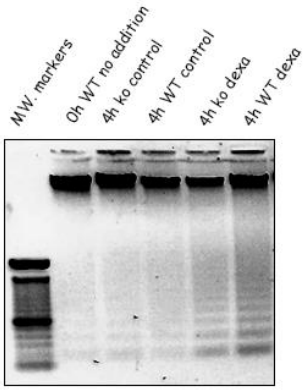

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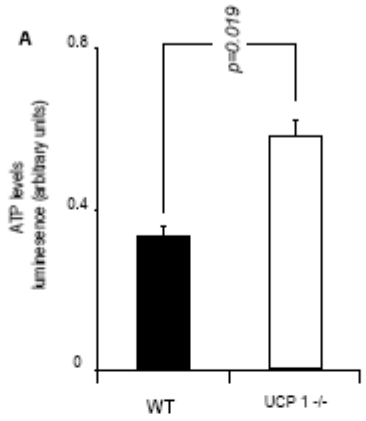


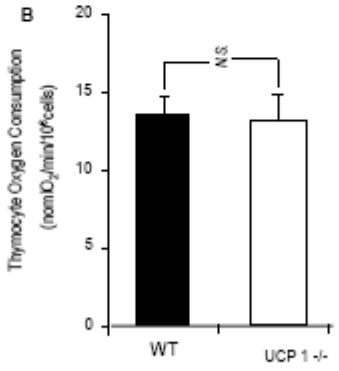

**Table 1. Thymus and spleen mass in C57BL/6 wild-type and UCP 1 knock-out mice**

| Animal source/Tissue | Thymus (grams) | Spleen (grams) |
|---|---|---|
| Wild-type mice | 0.073±0.006(9) | 0.073±0.009(9) |
| UCP 1 ko mice | 0.087±0.009(9) | 0.067±0.006(9) |

Data represents the mean ± sem of 9 separate measurements. $p$ values ≤ 0.05 on an unpaired Students 't'-test were deemed significant. No significant difference was observed in a comparison of tissue mass between C57BL/6 wild-type and UCP 1 knock-out mice