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Continued clearance of apoptotic cells critically depends on the phagocyte Ucp2 protein

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Abstract

Rapid and efficient removal of apoptotic cells by phagocytes plays a key role during development, tissue homeostasis, and in controlling immune responses^{1–5}. An important feature of efficient clearance is the capacity of a single phagocyte to ingest multiple apoptotic cells successively, and to process the increased load of corpse-derived cellular material^{6–9}. However, factors that influence sustained phagocytic capacity or how they in turn influence continued clearance by phagocytes are not known. Here we identify that the ability of a phagocyte to control its mitochondrial membrane potential is a critical factor in the capacity of a phagocyte to engulf apoptotic cells. Changing the phagocyte mitochondrial membrane potential (genetically or pharmacologically) significantly affected phagocytosis, with lower potential enhancing engulfment and higher membrane potential inhibiting uptake. We then identified that Ucp2, a mitochondrial membrane protein that acts to lower the mitochondrial membrane potential^{10–12}, is upregulated in phagocytes engulfing apoptotic cells (but not synthetic targets, bacteria, or yeast). Loss of Ucp2 limited the capacity of phagocytes to continually ingest apoptotic cells, while overexpression of Ucp2 increased the capacity for engulfment and the ability to engulf multiple apoptotic cells. Mutational and pharmacological inhibition of Ucp2 uncoupling activity reversed the positive effect of Ucp2 on engulfment capacity, suggesting a direct role for Ucp2-mediated mitochondrial function in phagocytosis. Macrophages from Ucp2-deficient mice^{13, 14} were impaired in their capacity to engulf apoptotic cells *in vitro*, and Ucp2-deficient mice displayed profound *in vivo* defects in clearing dying cells in the thymus and the testes. Collectively, these data suggest that phagocytes alter the mitochondrial membrane potential during engulfment to regulate uptake of sequential apoptotic cells, and that Ucp2 is a key molecular determinant of this step *in vivo*. Since Ucp2 function has also been linked to metabolic diseases and atherosclerosis^{14–16}, these data identifying a new role for Ucp2 in regulating apoptotic cell clearance may provide additional insights toward understanding the complex etiology and pathogenesis of these diseases.

When one cell engulfs another, the phagocyte essentially doubles its cellular contents. Remarkably, phagocytes often seem to be capable of ingesting and processing multiple

targets sequentially. However, the cellular features that regulate the ‘capacity’ of a given phagocyte to eat apoptotic cells, or regulate sequential phagocytosis of multiple dying cells are not understood. Since metabolites derived from the ingested apoptotic cells are likely to influence mitochondrial function in the phagocyte, we first asked whether there are any alterations to the mitochondrial membrane potential within phagocytes that have ingested apoptotic cells. Phagocytes labeled with the dye JC-1 (whose ratio of red to green fluorescence is dependent on mitochondrial membrane potential¹⁷ and reversible, Supplementary Fig. 1a), or another dye TMRE were incubated with apoptotic thymocytes in a time course. To focus specifically on phagocytes with internalized targets, we labeled the apoptotic thymocytes with a pH-dependent fluorescent dye, cypHer5E. The cypHer5E fluorescence of the labeled cells is significantly enhanced during phagocytosis, after the apoptotic corpse enters the acidic environment of the phagolysosome¹⁸. The relative mitochondrial membrane potential within phagocytes that contained engulfed apoptotic cells was significantly increased (1.99-fold $\pm$ 0.11, $p < 0.001$, $n=6$) compared to those phagocytes without ingested targets within the same population (Fig. 1a and Supplementary Fig. 1b). It is noteworthy that the contribution to the enhanced mitochondrial membrane potential signal seen within phagocytes from the mitochondria of ingested targets (i.e. apoptotic thymocytes) was minimal (Supplementary Fig. 1c). Interestingly, there was no increase in the mitochondrial membrane potential within phagocytes that had ingested synthetic targets (2 μ m carboxylate-modified beads) (Fig. 1a and Supplementary Fig. 1b). Since these synthetic targets have previously been shown to use components of the apoptotic cell engulfment machinery¹⁹, yet do not bring in a metabolic load for the phagocytes, these data suggest that the increase in relative mitochondrial membrane potential is specific to the uptake of cellular material from apoptotic cells, and not a general effect of engulfment.

We then determined the duration of this increased mitochondrial membrane potential within a phagocyte after engulfment of apoptotic cells. After the initial incubation of apoptotic thymocytes with the phagocytes for 3 hours (to allow time for engulfment), the unbound or excess targets were washed off, and we monitored the mitochondrial membrane potential in a time course. Following the engulfment phase, mitochondrial membrane potential decreased over time, returning to the baseline level of non-engulfing phagocytes by 2 hours during this chase time (Fig. 1b). Thus, there is a transient increase in the mitochondrial membrane potential within the phagocytes with ingested apoptotic cells.

In general, cellular ATP levels are maintained within a fairly narrow range and we found this to be true in engulfing phagocytes despite their increased metabolic load. Engulfing phagocytes had a modest 10 % increase in cellular ATP levels only at the 2 hour time point after engulfment (Fig. 1c). The lack of ATP generation concomitant with a decrease in mitochondrial membrane potential subsequent to engulfing apoptotic cells suggested that phagocytes possess a rapid mechanism for uncoupling nutrient oxidation from ATP generation¹¹.

A well-known endogenous mechanism for uncoupling the proton gradient and ATP generation is mediated by uncoupling proteins (UCPs)^{11, 20}. The UCPs regulate the mitochondrial membrane potential via dissipation of the proton gradient across the inner mitochondrial membrane, without generation of ATP. Although the function of Ucp1 in mitochondrial uncoupling and heat generation is well described²¹, Ucp1 expression is restricted to brown adipose tissue. Since Ucp2 is more ubiquitously expressed¹¹, including in multiple key primary phagocytic cell populations (Supplementary Fig. 2a), we examined its possible involvement in phagocytes during the engulfment of apoptotic cells. Interestingly, while the mRNA level of Ucp2 was not detectably altered, the mitochondrial Ucp2 protein levels in phagocytes were increased during engulfment, in a time course after incubation with apoptotic cells (Fig. 1d **left** and Supplementary Fig. 2b–c). It is important to

note that the increased Ucp2 levels were not derived from the targets being ingested as the levels of Ucp2 in thymocytes were below detection and feeding Ucp2 deficient apoptotic thymocytes to the phagocytes also resulted in enhanced Ucp2 levels in phagocytes (Fig. 1d and Supplementary Fig. 2d). Under the same conditions, Ucp2 protein levels did not change in phagocytes engulfing synthetic targets, which is consistent with a lack of effect of synthetic targets on mitochondrial membrane potential (Fig. 1d **right**).

To directly address whether Ucp2 may regulate engulfment of apoptotic cells, we knocked down endogenous Ucp2 expression in phagocytes by siRNA (Fig. 1e). We first noted that the decreased levels of Ucp2 within NIH/3T3 cells led to increased mitochondrial membrane potential both at the basal state and during engulfment (Fig. 1f and Supplementary Fig. 2e). We also observed two key phenotypes in engulfment by Ucp2-depleted cells. First, the Ucp2 knockdown phagocytes were less efficient engulfers, as measured by the percentage of phagocytes ingesting apoptotic cells over a 6-hour time course (Fig. 1g **left**). Second, the Ucp2 knockdown cells showed reduced phagocytic ‘capacity’, i.e. lower amount of apoptotic material ingested per phagocyte, as measured by the mean fluorescence intensity (MFI) derived from the labeled apoptotic cells (Fig. 1g **right**). It is noteworthy that the phagocytes with Ucp2 knockdown showed no defect in engulfing synthetic targets and the number of particles taken up per phagocyte was also unaltered (Fig. 1h). Collectively, these data suggest that phagocytes engulfing apoptotic cells upregulate the Ucp2 protein and that Ucp2 expression level contributes to the capacity of a phagocyte to ingest apoptotic cells.

The above data prompted us to ask whether Ucp2 overexpression in phagocytes may provide a ‘gain of function’ for engulfing apoptotic cells. We transiently expressed Flag-tagged Ucp2 in LR73 phagocytes. Ucp2^{Flag} was detected in the mitochondrial fraction (and not in the phagosomes), and the overexpressed Ucp2^{Flag} co-localized with another mitochondrial inner membrane protein, AIF, indicating proper targeting of ectopically expressed Ucp2 (Fig. 2a and Supplementary Fig. 3). Ucp2 overexpressing phagocytes showed three striking phenotypes with respect to engulfment. First, a greater percentage of the Ucp2-transfected LR73 cells ingested apoptotic cells compared to the control transfected cells; this enhanced uptake was specific for apoptotic cells and not seen with synthetic targets, and depended on the known engulfment machinery components (Fig. 2b and Supplementary Fig. 4a–b). Second, in a time course engulfment assay, the Ucp2 overexpressing cells continued to ingest apoptotic targets compared to control transfected cells (Fig. 2c **left**). Moreover, while the control-transfected LR73 cells displayed a plateau in the accumulation of apoptotic cell-derived fluorescence beyond 2 hours, the Ucp2 transfected LR73 cells continued to accumulate more apoptotic cells up to 6 hours (as determined by the mean fluorescence intensity) (Fig. 2c **right**). This was also confirmed by microscopy, where Ucp2 overexpressing cells consistently displayed more ingested apoptotic cells per phagocyte compared to control transfected cells (Fig. 2d). Quantitation of the number of targets per phagocyte in this assay revealed that a higher fraction of Ucp2 expressing cells showed more than two apoptotic cells per phagocyte compared to GFP expressing cells (Fig. 2e). Third, there was no difference in the rate of uptake of synthetic targets over a time course between Ucp2 and control-transfected cells (Fig. 2f), further supporting the notion that Ucp2 plays a specific role in sensing the metabolic load derived from apoptotic cells. The increased uptake of apoptotic cells was also observed with overexpression of other homologues of Ucp2 (Supplementary Fig. 4c). Taken together, these data revealed that overexpression of Ucp2 provides a ‘gain of function’ for phagocytes and that Ucp2 levels can confer a capacity to ‘continue to eat’ apoptotic cells on phagocytes, which is dependant on the canonical engulfment machinery.

We then asked whether the increased phagocytic capacity of Ucp2 overexpressing cells correlates with reduced mitochondrial membrane potential. As expected, Ucp2 cells showed

decreased mitochondrial membrane potential compared to controls (assessed by JC-1 fluorescence) (Fig. 2g). In a second approach, we directly visualized the mitochondrial uptake of MitoTracker, a dye that accumulates as the mitochondrial membrane potential increases²². The MitoTracker signal was distinctly weaker in Ucp2^{Flag} expressing cells compared to neighboring non-transfected cells (Fig. 2h). These data indicate that Ucp2 overexpression decreases the mitochondrial membrane potential. In previous studies, the drug genipin has been shown to inhibit the proton leak or mitochondrial uncoupling mediated by Ucp2²³. Genipin strongly inhibited the enhanced engulfment seen in Ucp2-overexpressing cells (both the percentage and the capacity for engulfment) (Fig. 2i). As another approach, we generated a D28N mutant of Ucp2 that has previously been shown to decrease the uncoupling activity of Ucp2^{24, 25}. Although Ucp2^{D28N} was expressed comparably to wild-type Ucp2 and localized to the mitochondria, Ucp2^{D28N} failed to enhance the percentage of phagocytes engulfing apoptotic cells (Fig. 2j). These data suggest that a decrease in mitochondrial membrane potential by Ucp2 correlates with the 'gain of function' phenotype, manifested as enhanced and continued uptake of apoptotic cells by Ucp2 expressing phagocytes.

Since UCP2 is also reported to have other functions²⁶, we next asked whether the effect of Ucp2 could be mimicked by synthetic uncouplers that lower the mitochondrial membrane potential^{27, 28}. We initially tested two different synthetic uncouplers, 2,4-DNP and FCCP, over a range of concentrations where the total cellular ATP levels were unaltered and the phagocytes appeared morphologically similar to vehicle control treated cells (Supplementary Fig. 5a–b). Both 2,4-DNP and FCCP significantly enhanced the uptake of apoptotic thymocytes by LR73 phagocytes in a dose-dependent manner (Fig. 3a); this increased uptake correlated with the decreased mitochondrial membrane potential induced by these drugs (Supplementary Fig. 5c–d). To determine if mitochondrial membrane potential itself, i.e. independent of increased proton leak and increased electron transport chain flux, was the signal for enhanced engulfment capacity we tested sodium azide, an inhibitor of complex IV of the electron transport chain that slows electron transport flux and lowers mitochondrial membrane potential. Increasing concentrations of sodium azide that did not affect cellular ATP levels enhanced engulfment by phagocytes along with a progressive decrease in the mitochondrial membrane potential within the phagocytes (Fig. 3b and Supplementary Fig. 5e–f). Higher concentrations of sodium azide, which decrease the cellular ATP levels, did inhibit apoptotic cell engulfment (since engulfment is an ATP-dependent process) (Fig. 3b and Supplementary Fig. 5f). Importantly, synthetic uncoupling also enhanced the ability of phagocytes to continue to ingest apoptotic cells in a time course, similar to Ucp2 overexpression (Fig. 3c). This was also confirmed by microscopy and flow cytometry, which revealed that phagocytes treated with the synthetic uncoupler FCCP have an increased capacity to ingest multiple apoptotic cells (Fig. 3d–e).

We next asked whether artificially increasing the mitochondrial membrane potential of phagocytes would inhibit engulfment. Treatment of cells with oligomycin, an inhibitor of ATP synthase, increased mitochondrial membrane potential and potentially inhibited engulfment (Fig. 3f). Notably, the concentration of oligomycin chosen for these experiments affected the mitochondrial membrane potential but not the total cellular ATP levels (Supplementary Fig. 5gh). In addition to LR73 cells, we also assessed the effect of the synthetic uncouplers DNP and FCCP, as well as sodium azide and oligomycin, on phagocytosis by NIH/3T3 cells; again, decreasing the mitochondrial membrane potential strongly promoted the uptake of apoptotic cells (data not shown).

We also asked whether modulating the metabolic state of the phagocytes would affect the engulfment of apoptotic cells. Intriguingly, culturing the phagocytes in glucose free medium, which decreased the mitochondrial membrane potential, resulted in enhanced

engulfment of apoptotic cells (Fig. 3g and Supplementary Fig. 6a). Conversely, phagocytes cultured in the presence of excess of glucose (which showed higher mitochondrial membrane potential) blocked the enhanced uptake of apoptotic cells either by Ucp2 overexpression or by FCCP (Fig. 3h–i and Supplementary Fig. 6b–c). Taken together, these data suggest that the mitochondrial membrane potential within phagocytes is a critical determinant in regulating the phagocytic capacity and continued uptake by phagocytes.

Increased metabolic load in phagocytes during engulfment may affect many aspects of metabolism and mitochondrial signaling such as β -oxidation and reactive oxygen species (ROS) production. Since Ucp2 is known to increase β -oxidation and decrease ROS production¹¹ one hypothesis could be that excess cellular lipids or ROS feedback could slow down the engulfment pathway. First, we tested whether fatty acid oxidation may be altered during engulfment and how this relates to the phenotypes seen with Ucp2 over/under expression and changes to mitochondrial membrane potential. Incubation of phagocytes with apoptotic thymocytes increased the rate of fatty acid oxidation and this was not observed when phagocytes were fed synthetic targets (Fig. 3j–k). However, we did not find any correlation between β -oxidation and Ucp2 levels; cells overexpressing Ucp2 as well as *Ucp2* deficient phagocytes displayed higher fatty acid oxidation rate (Supplementary Fig. 7a–b), even though they have opposite phenotypes with respect to their ability to engulf apoptotic cells. This suggested that lipid oxidation alone is not a major regulator of apoptotic cell clearance. Ucp2 has also been shown to negatively regulate ROS levels and decreasing ROS could also be a potential mechanism by which Ucp2 promotes continued engulfment. However, we could not establish a link between mitochondrial ROS levels in phagocytes and engulfment of apoptotic cells. Increasing phagocyte mitochondrial ROS levels via addition of the drugs rotenone or antimycin A (which block complexes I or III within the electron transport chain, respectively) did not decrease apoptotic cell engulfment (Supplementary Fig. 8a–b). In fact, these drugs modestly increased the engulfment of apoptotic cells, likely due to decreased mitochondrial membrane potential. Furthermore, neither ameliorating ROS with FCCP, nor scavenging ROS with well-known scavengers Tiron or MitoTEMPO enhanced the ability of phagocytes to engulf apoptotic cells (Supplementary Fig. 8a–f). Furthermore, overexpression of the mitochondrial antioxidant enzyme superoxide dismutase 2 (SOD2) did not affect engulfment of apoptotic cells (Supplementary Fig. 8g). We also noted that the master regulatory transcription factor for mitochondrial biogenesis, PGC1 α , did not change during engulfment and overexpression of PGC1 α in phagocytes did not promote engulfment of apoptotic cells (**Data not shown** and Supplementary Fig. 8h). Moreover, neither the AMPK nor the mTOR signaling pathways were activated during engulfment, which was determined by phosphorylation of AMPK and P70S6K, respectively (Supplementary Fig. 8i–j). Collectively, while these data cannot completely rule out some contribution of β -oxidation and ROS levels in regulating apoptotic cell clearance, none of these pathways in isolation could account for the engulfment phenotype regulated by Ucp2. Rather, the Ucp2 mediated regulation of the phagocyte mitochondrial membrane potential correlated best with the ability of phagocytes to continue to engulf apoptotic cells.

To determine whether the recognition and uptake of apoptotic cells via specific engulfment receptors may be sensed or integrated into Ucp2/mitochondrial signaling, we generated LR73 cells overexpressing the phosphatidylserine receptor Tim-4. Consistent with previous reports^{8, 29, 30}, overexpression of the engulfment receptor Tim-4 led to increased uptake of apoptotic cells. However, we found three pieces of data that suggested a link between Tim-4 mediated apoptotic cell recognition and Ucp2. First, while there was no difference in the basal Ucp2 level between control and LR73 cells overexpressing Tim-4, when incubated with apoptotic cells, the Tim-4 overexpressing cells upregulated Ucp2 to a much higher level (Fig. 3l **top**). Importantly, incubation with synthetic targets did not increase Ucp2

expression in phagocytes, even though the uptake of these targets is also promoted by Tim-4 overexpression (Fig. 3l **bottom**). Second, the Tim-4 overexpressing cells also continued to take up multiple apoptotic cells (indicated by MFI). This continued uptake, without reaching a plateau that is seen in the control cells, is consistent with the increased upregulation of Ucp2 in Tim-4 overexpressing cells and very similar to LR73 cells transfected with Ucp2 (Fig. 3m). Third, an Ucp2 inhibitor drug genipin blocked the increased uptake of apoptotic cells seen due to Tim-4 (Fig. 3n). This prompted us to ask whether the enhanced engulfment seen with synthetic uncoupling or Ucp2 overexpression could be due to enhanced levels of Tim-4 surface expression. However, the surface expression of endogenous Tim-4 or HA-Tim-4 was not affected by Ucp2 overexpression, Ucp2 deficiency, synthetic uncoupling or by scavenging ROS (Supplementary Fig. 9a–e). Collectively, these data suggest a link between apoptotic cell recognition at the phagocyte membrane and the mitochondrial membrane potential with Ucp2 serving as a key molecular intermediate.

We next addressed the relative importance of Ucp2 in regulating the mitochondrial membrane potential and apoptotic cell clearance *in vivo* using *Ucp2* deficient mice^{13, 16}. Bone marrow-derived macrophages (BMDMs) from *Ucp2*^{−/−} mice had a higher relative mitochondrial membrane potential compared to BMDMs from *Ucp2*^{+/+} littermates (Fig. 4a). We then assessed the *in vitro* phagocytic capacity of BMDMs from *Ucp2*^{+/+} or *Ucp2*^{−/−} mice to engulf different targets and found that in a time course of engulfment, *Ucp2*^{−/−} macrophages were consistently less efficient and showed lower phagocytic capacity (Fig. 4b). By contrast, the BMDMs from *Ucp2*^{−/−} mice showed no defect in the uptake of synthetic targets, either live or dead bacteria or zymosan A particles (Fig. 4c and Supplementary Fig. 10a–f). This suggested that during apoptotic cell clearance Ucp2 requires both the metabolic load (absent in synthetic targets) and sensing of the nature of entry of targets (e.g. bacteria and yeast, which do carry a metabolic load but are taken up via other types of receptors). It is notable that there was still some level of basal engulfment that was unaffected in the *Ucp2* null phagocytes, which may in part be explained by the upregulation of *Ucp3* that was observed in 6-day cultures of both splenocytes and BMDMs from *Ucp2*^{−/−} mice (Supplementary Fig. 11a–c).

We then asked how *Ucp2*^{−/−} phagocytes would clear apoptotic cells *in vivo* under conditions where a significant population of cells within a tissue is undergoing apoptosis. Injection of dexamethasone (Dex) in mice induces rapid and synchronous death of thymocytes, and the subsequent clearance of apoptotic thymocytes by resident phagocytes can provide a reproducible and quantitative *in vivo* model of apoptotic cell clearance³¹. Using this model, we tested the efficiency of apoptotic cell clearance in control and *Ucp2*^{−/−} mice. Compared to control mice, which show a decrease in overall thymic size at 6 hours after Dex injection, the *Ucp2*^{−/−} mice showed only a limited reduction in thymic size after Dex injection (Fig. 4d). Assessing the absolute numbers of cells in the thymi confirmed that the Dex-injected *Ucp2*^{−/−} mice had much higher total thymic cell number than that of Dex-injected *Ucp2*^{+/+} mice (Fig. 4e). Importantly, we found that thymocytes from *Ucp2*^{−/−} mice underwent apoptosis to the same extent as the control littermates *ex vivo* (Supplementary Fig. 12a–b); moreover, the migration of monocytes or macrophages toward find-me signals from apoptotic cells was not affected by Ucp2 levels or mitochondrial uncoupling (Supplementary Fig. 13a–e). These data suggests that increased thymic cellularity most likely resulted from defective clearance in the *Ucp2*^{−/−} mice. We then determined the status of uncleared apoptotic cells in the thymus of *Ucp2*^{+/+} and *Ucp2*^{−/−} mice by TUNEL, and found a consistently higher level of unengulfed apoptotic cells within the thymi of Dex treated *Ucp2*^{−/−} mice without altered density of F4/80 positive cells (Fig. 4f and Supplementary Fig. 13f–h). Quantitation of the TUNEL fluorescence over multiple thymic sections of several mice revealed a three-fold increase in the amount of TUNEL staining in the thymi of Dex-treated *Ucp2*^{−/−} mice compared to *Ucp2*^{+/+} controls (Fig. 4g). We also tested the ability of

Ucp2^{-/-} mice to clear apoptotic cells in other tissues *in vivo*; acute apoptosis of germ cells in the testes was induced by testicular torsion and the clearance of these dying germ cells by the Sertoli cells of the testes was assessed. The testes of *Ucp2*^{-/-} mice contained the increased number of uncleared apoptotic cells per seminiferous tubule compared to *Ucp2*^{+/+} (Fig. 4h–i). These data demonstrate an essential role for *Ucp2* in apoptotic cell clearance *in vivo*.

While significant progress has been made in recent years regarding how phagocytes recognize and engulf apoptotic cells^{32–34}, the data presented in this report provide several new insights toward our understanding of the dynamic nature of apoptotic cell clearance. First, our studies using multiple approaches suggest that the mitochondrial membrane potential critically controls how well a phagocyte can engulf, with an inverse correlation between mitochondrial membrane potential and the capacity for engulfment. Second, these data identify the mitochondrial protein *Ucp2* as a critical molecular determinant of the mitochondrial membrane potential within phagocytes during apoptotic cell engulfment. At the organism level, genetic ablation of *Ucp2* in mice led to severe defects in the ability to clear apoptotic cells in the thymus and the testes. Thus, these data assign a new role to the mitochondria within phagocytes in controlling the engulfment capacity and identify *Ucp2* as a key molecular rheostat of phagocyte engulfment capacity. Third, our data identify a previously unappreciated crosstalk between the mitochondria and the engulfment machinery within phagocytes. Our work establishes that ‘sensing’ of the total mitochondrial membrane potential critically influences the capacity of phagocytes to engulf apoptotic cells, and identify *Ucp2* as a key determinant in phagocytes for continued uptake of apoptotic cells. This has broad implications for apoptotic cell clearance *in vivo* since failed clearance of apoptotic cells has been linked to inflammation and autoimmune diseases.

Since uncoupling proteins regulate the energetics within the mitochondria^{15, 35–37}, understanding the relationship between metabolic diseases and uncoupling proteins is an area of intense study^{38–42}. Loss of *Ucp2* expression has been linked to impaired pancreatic β cell function and glucose-induced insulin secretion. The data presented here provide a new and unexpected link between mitochondrial function and the process of apoptotic cell clearance. Thus, our new observations that *Ucp2* protein levels can be modulated downstream of engulfment receptors may be relevant toward understanding complex etiology of some of these metabolic diseases. Loss of *Ucp2* has been linked to atherosclerosis, with mice deficient *Ucp2* displaying a greater severity of disease^{14–16, 43}; similarly, failed clearance of apoptotic cells within atherosclerotic plaques and the resultant inflammatory milieu have been associated with the severity of atherosclerosis. Thus, our studies providing a link between *Ucp2* and apoptotic cell clearance may help identify a possible mode of treatment for atherosclerosis and other diseases by enhancing the phagocytic potential via regulation of *Ucp2* activity.

Methods

Cell culture and transfections

LR73 cells were cultured in Alpha-MEM, while NIH/3T3 cells and J774 macrophage cells were maintained in DMEM, and Jurkat cells were culture with RPMI, along with 10 % FBS and 1 % penicillin-streptomycin-glutamine. SCI cells were cultured in alpha-MEM and 1 mM of Na-pyruvate. LR73 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. NIH/3T3 cells were nucleofected using amaxa nucleofector (kit R, program U-030) or transfected with Lipofectamine 2000 according to the manufacturer’s manual.

Primary cell culture

Bone marrow cells from 6-week old mice were culture in RPMI medium with 10 % FBS, 1 % penicillin-streptomycin-glutamine, and 10 % L929 cell conditioned medium and then plated. Two or six days later, more than 85 % of the adherent cells were F4/80 positive and 95 % of the cells were CD11b positive. Resident peritoneal cells were collected from 6-week-old mice and plated with RPMI supplemented with 10 % FBS and 1 % penicillin-streptomycin-glutamine. Four hours later, floating cells were washed with warm PBS twice and adherent cells were regarded as resident peritoneal macrophages and used in further assays.

Plasmids and mutagenesis

All constructs generated were sequenced to confirm fidelity and the presence of the appropriate mutations. The pEBB-Ucp2 and pEBB-Ucp2-Flag constructs were generated from the *Ucp2* cDNA templates by a PCR-based strategy in the pEBB-Flag vector. pEBB-Ucp2^{D28N} was generated by site-directed mutagenesis. ELMO, Dock180, Rac, BAI1, and Tim-4 constructs used in this study have been described previously¹⁹.

Immunoblotting and antibodies

The antibodies used were anti-Ucp2 (Santa Cruz Biotechnology Inc., C-20), anti-Flag (Sigma, M-2), anti-Porin (Abcam, ab15895), anti-Erk2 (Santa Cruz, C-14), and anti-AIF (Cell Signaling, D39D2). The phospho-AMPK α (Thr172), AMPK α Antibody, phospho-p70 S6 Kinase (Thr389), p70 S6 Kinase, were purchased from Cell Signaling Technology. To detect endogenous Ucp2, it is necessary to isolate mitochondria. For the isolation of mitochondria, the cells were resuspended in 500 μ l of TS buffer (10 mM Tris, pH 7.5, 250 mM sucrose, 2 μ g/ml DNase and protease inhibitor cocktail), and then subjected to three cycles of 5-minute freezing in liquid nitrogen followed by 10-minute thawing at 37 °C. Unbroken cells and nuclei were removed by centrifugation at 800g for 10 min and mitochondria were collected from the supernatant by centrifugation at 10,000g for 20 min. The isolated mitochondria were lysed in RIPA buffer and subjected to immunoblotting. To measure induction of Ucp2 during apoptotic cell engulfment, 2.0×10^6 LR73 cells were plated on the 100 mm culture dish two days before 1.0×10^8 apoptotic thymocytes in 10 ml of alpha-MEM were added. After incubation for 3 hours, the mitochondria were isolated and the Ucp2 levels were analyzed. For BMDM, 5.0×10^6 BMDM were plated on the 100 mm Petri-dish 1 day before the assay. Next day, 1.0×10^8 apoptotic thymocytes in 10 ml of RPMI were added to BMDM and incubated for 1 hour. The cells were extensively washed with cold PBS and trypsinized and analyzed for Ucp2 levels. To detect overexpressed Ucp2, LR73 cells were transiently transfected with Flag tagged Ucp2 using Lipofectamine 2000 (Invitrogen). One day after transfection, the cells were lysed and subjected to immunoblotting against the respective tags or endogenous proteins.

Immunofluorescence microscopy

NIH/3T3 fibroblasts were plated on glass chamber slides and transfected with FLAG-Ucp2 either alone or in combination with YFP-Rab5 or YFP-Rab7. To stain lysosomes or mitochondria, cells were incubated with LysoTracker Red (1:10,000 dilution) or Mitotracker Deep Red (100 nM) in DMEM/10%FBS for 20 minutes. Cells were then fixed with 3% paraformaldehyde (Sigma) in PBS for 30 min, permeabilized with 0.1% Triton X-100 (Sigma) and blocked with 5% milk that had been clarified by high-speed centrifugation. Antibody staining was then performed using antibodies to FLAG (clone M5, Sigma) and/or AIF (D39D2, Cell Signaling) detected with either Alexa 488 or Alexa-555 goat anti-mouse and/or Alexa-555 goat anti-rabbit antibodies (highly cross-absorbed, Invitrogen),

respectively. YFP was detected using an Alexa-488 anti-GFP antibody (Invitrogen). The stained cells were analyzed by Axio imager 2 with Apotome (Zeiss).

Quantitative RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and cDNA was generated from total RNA with Superscript III (Invitrogen) according to the manufacturers' protocol. *Ucp1*, *Ucp2*, and *Ucp3* mRNA were detected by PCR using cDNA as template. For siRNA transfected NIH/3T3 cells and *Ucp2* KO mice, relative *Ucp2* or *Ucp3* mRNA levels were determined by normalizing to *Hprt* using the StepOnePlus qPCR system (Applied Biosystems).

Phagocytosis assay

Phagocytosis assays were performed as described previously¹⁹. 1.0×10^5 LR73 cells were transiently transfected in triplicates with the indicated plasmids either with GFP or GFP fusion proteins in a 24-well plate. The cells were incubated with apoptotic thymocytes or 2 μ m carboxylate-modified red fluorescent beads (Invitrogen), which mimic the negative charge on apoptotic cells and can serve as simplified targets. For the induction of apoptosis, thymocytes were incubated with 50 μ M Dexamethasone (Calbiochem Inc.) at 37 °C for 4 hours. The thymocytes were resuspended to a final concentration of 1.0×10^7 cells/300 μ l with Alpha-MEM (or cell culture medium for specific cell types) supplemented with 2% FBS and 0.2% penicillin-streptomycin-glutamine for the phagocytosis assay of LR73. The transfected cells were incubated with 300 μ l of the apoptotic thymocyte resuspension in the 5% CO₂ incubator at 37 °C for desired times. After incubation of the phagocytes with targets, the phagocytes were extensively washed with cold PBS, trypsinized, resuspended in cold medium (with 1% NaN₃), and analyzed by two-color flow cytometry (FACSCalibur from BD). The transfected cells were recognized by their GFP fluorescence and targets were recognized by red fluorescence (carboxylate-modified red fluorescent beads or apoptotic thymocytes). Forward and side-scatter parameters were used to distinguish free unbound targets from phagocytes. The majority of 'double-positive' cells scored in the FACS assay represented targets engulfed by phagocytes, or targets in the process of being engulfed. The mean fluorescence intensity (MFI) in the red channel of the cells taking up targets provided an indication of the capacity of uptake (proportional to the number of particles taken up). For measuring phagocytosis by BMDM, 2.0×10^4 bone marrow derived macrophages were plated in the 24-well plate 1 day before the phagocytosis assay. Next day, the cells were incubated with 2.0×10^6 TAMRA-stained apoptotic thymocytes for desired times and analyzed for engulfment as above.

Internalization assay

Thymocytes were double stained with TAMRA (25 μ M) and cypHer5E (1 μ M, GE Healthcare) and phagocytosis assay was performed as described above. TAMRA and cypHer5E high double positive cells were considered as phagocytes internalizing the apoptotic cells, and TAMRA positive but cypHer5E low cells were regarded as bound but uninternalized targets on phagocytes.

Phagocytosis of dead *Escherichia coli*, *Staphylococcus aureus* and Zymosan

2.0×10^4 BMDMs from wild type or *Ucp2* deficient mice were plated on a well of a 24-well plate. Next day, BMDMs were incubated with alex-488 conjugated 5×10^6 *E. coli* and *S. aureus* or 5×10^5 zymosan particles (Invitrogen) for the desired times. Unbound targets were extensively washed with cold PBS five times and BMDMs were trypsinized and subjected to FACS analysis. Alex-488 positive BMDMs were considered as phagocytes engulfing targets.

Gentamicin protection assay for bacterial internalization

Quantitation of intracellular bacteria was done using the gentamicin protection assay. 2×10^5 BMDMs/well were seeded into a 24-well culture dish 18 hours prior to infection at a multiplicity of infection of 10 for 1 hour in antibiotic-free media in a 37 °C CO₂ incubator. Cells were then washed and incubated with gentamicin for 90 min to kill extracellular bacteria. Subsequently, cells were lysed in 1% Triton-X 100, lysates were serially diluted, and plated directly onto Luria-Bertani agar plates. Total colony-forming units (CFUs) were enumerated the next day after overnight incubation at 37°C. Values were standardized to levels of colonization in control cell preparations.

Ucp2 knockdown

NIH/3T3 cells were nucleofected according to the manufacturer's manual (Kit R, U-030, amaxa) with minor modifications. 1.0×10^6 cells were nucleofected with 6 picomoles/sample control or *Ucp2* siRNA (Dharmacon *Ucp2* smart pool) and plated on 3 wells of the 6-well plate. 24 hours after nucleofection, the cells were trypsinized and 2.0×10^4 cells were replated in the 12-well plate. 2 days after nucleofection, the cells were incubated with 1.0×10^6 TAMRA-stained apoptotic SCI or Jurkat cells in DMEM supplemented with 10 % FBS and 1 % penicillin-streptomycin-glutamine at 37 °C for desired times.

Measurement of mitochondrial membrane potential

To detect mitochondrial membrane potential, cells treated with the indicated experimental conditions were stained with Mitotracker Deep Red FM (invitrogen), whose accumulation in mitochondria is dependent on mitochondrial membrane potential. Mitochondrial membrane potential was also measured using TMRE (invitrogen) or a dual emission potential-sensitive probe, JC-1 dye (SIGMA-Aldrich), according to the manufacturer's protocol. For TMRE staining, cells were incubated with 50 nM of TMRE in culture medium in the 5 % CO₂ incubator at 37°C for 30 minutes and fluorescence coming from cells was measured by flow cytometry. For JC-1 staining, cells in a 24-well plate were incubated with 1 ml of mitochondrial staining solution containing the 50 % medium used for cell growth and 2.5 µg/ml of JC-1 dye in the 5 % CO₂ incubator at 37 °C for 20 minutes. The cells were then washed twice with warm PBS and trypsinized. Overflow of green fluorescent signal to red fluorescence was compensated and the intensity of red fluorescence coming from JC-1 aggregates was detected using the FACSCalibur instrument (Becton Dickinson).

Measurement of intracellular ATP

Intracellular ATP levels were measured using CellTiter-Glo Luminescent Cell Viability Assay kit (Promega) according to the manufacturer's manual. Cells were treated with experimental conditions and trypsinized. 2.5×10^4 cells/50 µl were added into a well of the 96-well plate. After that 50 µl of CellTiter-Glo Reagent was added to the well containing the cells. The cells were incubated on orbital shaker for 2 minutes and followed by incubation at room temperature for 10 minutes. Luminescence was measured using MicroBeta TriLux (EG&G WALLAC).

Analysis of cell clearance in the thymus

Five to six week-old wild type and *Ucp2* deficient mice were intraperitoneally injected with 300 µl of PBS containing 250 µg dexamethasone dissolved in ethanol (10 mg/ml). 6 hours after injection, the mice were sacrificed, and thymi were extracted and smashed. For quantification of thymic cellularity, thymocytes were resuspended with HBSS supplemented with 5 % FBS. The cells were diluted and mixed with 50µl of quantification beads (Spherotech Inc). The mixture was subjected to flow cytometry analysis to allow for the quantification thymocytes. To monitor the presence of TUNEL positive uncleared apoptotic

cells, 6 hours after injection, the thymi from the mice were extracted and embedded in O.C.T. compound. The O.C.T compound embedded thymi then were frozen in liquid nitrogen. After that, thymic cryo-sections on glass slides were stained using the In Situ Cell Death Red kit according to manufacture's instructions (Roche). Sections were mounted with Prolong Antifade + DAPI medium and analyzed by Axio imager 2 with Apotome (ZEISS). To monitor ex vivo apoptosis of thymocytes, 1.0×10^7 thymocytes from 5~6-week-old Ucp2 wild type and deficient littermate mice were incubated with 50 μ M dexamethasone at 37 °C for 4 or 6 hours. The cells were washed with PBS once and then stained with annexin V and propidium iodide (Annexin V: FITC apoptosis detection kit II, BD).

Cell migration assay

2×10^6 cells/ml THP-1 cells were resuspended with RPMI containing 5 % FBS, 1 % penicillin, streptomycin and glutamine and 10 mM HEPES and were pre-incubated with the desired concentration of FCCP for 1 hour. 500 μ l of apoptotic supernatant or MCP-1 (25 ng/ml) was added to lower chamber of a 24-well plate with 5 μ m pore-size transwell (5 μ m pore size, Corning) and 100 μ l of cells (2×10^6 cells/ml) was loaded on the upper chamber of the plate and then incubated at 37°C for 1 hour. The percentage of migrated cells was determined by FACS with 5.1- μ m AccuCount beads and compared to input cells. For BMDMs migration, 4.0×10^5 cells/ml were resuspended in RPMI containing 1 % BSA, 1 % penicillin, streptomycin and glutamine and 10 mM HEPES. 500 μ l of apoptotic supernatant or Sdf-1 (50 ng/ml) was added to lower chamber of the 24-well transwell plate and 100 μ l of BMDMs was added on upper chamber and incubated at 37°C for 3 hours. The number of BMDMs migrated on the underside of the membrane was determined by Diff-Quick staining and counted using microscopy.

Thymus staining

Tissue sections were blocked for 15 min with 2% normal goat serum and then were stained for 60 min at 25 °C with PE-conjugated F4/80 (eBioscience) and FITC-conjugated anti pancytokeratin (Sigma). After incubation, slides were washed three times in PBS and then mounted with GelMount (Molecular Probes, invitrogen).

Fatty acid oxidation (FAO) assay

Phagocytes were seeded in a 12-well plate at a density of 2×10^5 cells per well on the day prior to the FAO assay. Next day, the phagocytes were incubated with 2×10^7 apoptotic thymocytes or 2 μ m carboxylate-modified beads for the desired times. After incubation, each well was washed five times with PBS. Rates of FAO were determined over the next two hours as follows: A 0.2 mL PCR tube with 50 μ L 1M NaOH was leaned diagonally in each well prior to addition of 300 μ L FAO buffer (Krebs Ringer phosphate buffer pH 7.4 containing 1% fatty acid free BSA (Sigma Aldrich), 5 mM glucose (Sigma Aldrich), 125 μ M palmitate (Sigma Aldrich), 1mM carnitine (Sigma Aldrich), and 6 μ Ci 1- 14 C-palmitate (Perkin Elmer)). The wells were immediately sealed with masking tape and incubated at 37 °C for 2 hours before 100 μ L of 2 M perchloric acid was injected through the tape to stop the reaction and release 14 CO₂ trapped as bicarbonate. The plate was incubated for 2 hours at room temperature to trap the released 14 CO₂ as bicarbonate in NaOH. The tape was removed and NaOH from each tube was diluted with 5 mL scintillant and counted with a Beckman LS6500 scintillation counter. Partially oxidized metabolites of 14 C-palmitate were recovered from the FAO buffer precipitate by extraction in chloroform:methanol (2:1). Acid soluble 14 C-metabolites were recovered from the aqueous phase, diluted in scintillant, and counted. Rates of total FAO were determined by summing 14 CO₂ and 14 C labeled acid soluble metabolite disintegration rates and normalizing to the specific activity of the FAO buffer, cell number, and the duration of incubation.

Measurement of superoxide

LR73s were plated at a density of 1.0×10^5 cells/well in a 12-well plate in alpha-MEM supplemented with 10 % FBS and 1 % penicillin, streptomycin and glutamine. The cells were treated with indicated drugs for four hours and incubated with 2 μ M MitoSox (invitrogen) for 30 minutes. The cells were trypsinized and analyzed by flow cytometry.

Testicular Torsion

This work was conducted in accordance with the *Guiding Principals of the Care and Use of Research Animals* promulgated by the Society for the Study of Reproduction. Adult male C57BL/6 mice were anesthetized with an intraperitoneal injection of 0.01 mg/g sodium pentobarbital and the testis was exteriorized through a low midline laparotomy, the gubernaculum was divided, and the testis was freed from the epididymo-testicular membrane. The testis was rotated 720° for 2 hr, during which time it remained in the abdomen with a closed incision. Following the 2 hr torsion, the incision was reopened, the testis was counter-rotated to the natural position, the gubernaculum was rejoined, and the testis was reinserted into the scrotum via the inguinal canal. Testes were examined at the time of repair for the degree of ischemia and reperfusion. Sham operated animals were treated identically except that upon completion of the torsion maneuver the testis was immediately counter-rotated.

Statistical analysis

Data are shown as the mean $\pm$ standard deviation (SD). For analysis of statistical difference of experiments involving two groups, Student's two-tailed t test was applied. A one-way ANOVA was applied for statistical analysis of three or more groups. Significance was defined when p values were < 0.05 .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Ucp2 is upregulated in phagocytes during engulfment and affects uptake of apoptotic cells

a, Increase in mitochondrial membrane potential in engulfing phagocytes. LR73 cells (phagocytes) were stained with JC-1 and then incubated with cypHer5E-stained apoptotic thymocytes or 2 μm carboxylate-modified beads as targets. To focus on the mitochondrial membrane potential specifically within engulfing and non-engulfing phagocytes within the same population (based on cypHer5E and pacific blue signal) was compared. LR73 cells treated with oligomycin (1 hour) served as a positive control for the increased mitochondrial membrane potential.

b, LR73 cells were incubated with cypHer5E-stained apoptotic thymocytes for 3 hours. After extensive washing to remove unbound and unengulfed apoptotic cells, phagocytes were further incubated for the indicated time and stained with JC-1 dye and analyzed as above.

c, LR73 cells were incubated with apoptotic thymocytes or 2 μ m carboxylate-modified beads for the indicated times and the intracellular ATP level was determined by a luciferase-based assay.

d, Upregulation of Ucp2 protein during engulfment. LR73 cells were incubated with either apoptotic thymocytes from Ucp2 knockout mice or 2 μ m carboxylate-modified beads for the indicated times. Mitochondrial fractions were prepared and immunoblotted for endogenous Ucp2. The mitochondrial membrane protein porin was used as an internal loading control. **e**, *Ucp2* mRNA levels in control or *Ucp2* siRNA transfected NIH/3T3 cells were determined by qPCR and normalized to *Hprt*. Inset, samples were also analyzed by PCR/agarose-gel electrophoresis (negative image shown).

f, Mitochondrial membrane potential in control or *Ucp2* siRNA transfected NIH/3T3 (2-day post-transfection) was determined via the JC-1 dye and flow cytometry.

g, h, NIH/3T3 cells transfected with control or *Ucp2* specific siRNA were incubated with TAMRA stained apoptotic thymocytes (**g**) or 2 μ m carboxylate-modified beads (**h**) for the indicated times. Engulfment of targets by NIH/3T3 phagocytes is shown as the percentage of cells engulfing targets (left) and the amount of targets engulfed per phagocyte assessed via the mean fluorescence intensity of engulfing phagocytes (MFI, right). Data are shown as mean $\pm$ SD and are representative of at least two independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 2. Ucp2 overexpression promotes continued uptake of apoptotic cells

a, Appropriate localization of Ucp2^{Flag} in transfected cells. Mitochondria isolated from LR73 cells transfected with Ucp2^{Flag} or the control vector were immunoblotted with antibodies to Flag, Porin or Erk2.

b, LR73 cells transfected with GFP or Ucp2 were assessed for their ability to engulf TAMRA-labeled apoptotic thymocytes or 2 μm carboxylate-modified beads.

c, LR73 cells expressing GFP or Ucp2 were incubated with TAMRA-stained apoptotic thymocytes for the indicated times. LR73 cells ingesting targets are shown as the percentage

(left) or as mean fluorescence intensity of engulfing phagocytes, which reflects the ability to continue to ingest apoptotic cells (right).

d, Fluorescence microscopy of LR73 cells transfected with GFP alone or Ucp2^{Flag} were incubated with TAMRA stained apoptotic thymocytes for 4 hours. Arrowheads indicate engulfed apoptotic thymocytes. Scale bar, 20 μ m.

e, Phagocytes engulfing apoptotic cells in 40 randomly chosen fields from (**d**) were counted, and the percentage of engulfing phagocytes with the indicated number of internalized apoptotic cells is shown.

f, LR73 transfected with GFP or Ucp2 were incubated with 2 μ m carboxylate-modified beads for the indicated times. The percentage (left) and MFI (right) of cells ingesting synthetic targets is shown.

g, Mitochondrial membrane potential of LR73 cells transfected with Ucp2 or control vector was determined by staining with JC-1.

h, Fluorescence microscopy of NIH/3T3 cells transfected with Ucp2^{Flag} and stained with MitoTracker and anti-Flag antibody. Arrowheads indicate NIH/3T3 cells expressing Ucp2^{Flag} and arrows indicate non-transfected cells. Scale bar, 20 μ m.

i, LR73 phagocytes expressing GFP or Ucp2 were pretreated with vehicle alone (DMSO) or genipin (100 μ M) for 1 hour and incubated with TAMRA-labeled apoptotic thymocytes for 3 hours in the continued presence of DMSO or genipin. The percentage of phagocytes engulfing targets (left) and MFI of engulfing phagocytes (right) are shown. Note that the enhanced MFI of Ucp2 expressing cells was completely abolished by genipin.

j, LR73 cells transiently expressing wild type Ucp2 or proton leak mutant Ucp2^{D28N} were incubated with TAMRA-stained apoptotic thymocytes for 3 hours. The percentage of phagocytes engulfing targets (left) and MFI of engulfing phagocytes (middle) are shown. Right, expression levels of wild-type Ucp2 and Ucp2^{D28N} were determined by immunoblotting of mitochondrial fractions. Data are shown as mean $\pm$ SD and are representative of at least three independent experiments. *P<0.05, **P<0.01.

Figure 3. Ucp2 links mitochondrial membrane potential to engulfment of apoptotic cells

a, LR73 cells were pre-treated with synthetic uncouplers DNP (**a**, left), FCCP (**a**, right), or vehicle control for 1 hr and incubated with TAMRA-stained apoptotic thymocytes in the presence of vehicle or uncouplers for 2 hours. DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone.

b, Experiments similar to the uncouplers were performed using the electron transport chain inhibitor NaN_3 .

c, LR73 cells were pretreated with FCCP to decrease mitochondrial membrane potential for 1 hour and then incubated with TAMRA-stained apoptotic thymocytes for the indicated

times. The percentage of treated phagocytes engulfing targets (left) and the MFI of engulfing phagocytes (right) are shown.

d, LR73 cells stained with CellTracker (green) were pre-treated with ethanol or FCCP for 1 hr, and incubated with TAMRA-stained apoptotic thymocytes. Arrowheads indicate ingested thymocytes. Scale, 20 μ m.

e, Phagocytes engulfing apoptotic cells in 30 randomly chosen fields from (**d**) were counted and the percentage of phagocytes engulfing the indicated number of apoptotic cells is shown (n=3).

f, LR73 cells were pretreated with oligomycin to increase the mitochondrial membrane potential or vehicle control (ethanol) for 1 hour and then incubated with TAMRA-stained apoptotic thymocytes for 2 hours.

g, LR73 cells were cultured in RPMI medium with no or 2 mg/ml glucose for 6 hours and incubated for 2 hours with TMRA-stained apoptotic thymocyte in the continued absence or presence of 2 mg/ml glucose.

h, LR73 cells transfected with GFP or Ucp2 were incubated for 2 hours with TAMRA-stained apoptotic thymocytes in the presence or absence of 10 mg/ml glucose.

i, LR73 cells pre-treated with FCCP for 1 hour were incubated with TAMRA-stained apoptotic thymocytes in the continued presence of FCCP or vehicle control with/without 10 mg/ml glucose for 2 hours.

j,k, LR73 cells were incubated with either apoptotic thymocytes (**j**) or 2 μ m carboxylate-modified beads (**k**) for the indicated times and extensively washed to remove unengulfed targets. Rates of total palmitate oxidation were determined by summing $^{14}\text{CO}_2$ and acid soluble metabolite disintegration rates.

l, LR73 cells stably overexpressing vector control or Tim-4 were incubated with apoptotic thymocytes (left) or synthetic targets (right) for 3 hours and the levels of endogenous Ucp2 in the mitochondrial fractions is shown. The densitometric values indicating the fold increase in expression are shown.

m, Continued uptake of apoptotic cells by Tim-4 and Ucp2 overexpressing cells (compared to GFP transfected cells that reach a plateau) was determined by the MFI of phagocytes over a time course of engulfment with apoptotic thymocytes.

n, The increased uptake of apoptotic thymocytes by Tim-4 overexpressing cells was inhibited by genipin. Data are shown as mean $\pm$ SD and are representative of at least two independent experiments. *P<0.05, **P<0.01, ***P<0.001.

Figure 4. Impaired phagocytosis and clearance of apoptotic cells in $Ucp2$ deficient mice
a, Mitochondrial membrane potential of bone marrow-derived macrophages (BMDMs) from $Ucp2^{+/+}$ and $Ucp2^{-/-}$ mice were determined by JC-1 staining.
b, Engulfment of TAMRA-labeled apoptotic thymocytes by BMDM from $Ucp2^{+/+}$ and $Ucp2^{-/-}$ mice. Time course of engulfment by BMDMs displaying the percentage of phagocytes that engulf as well as the MFI of engulfing phagocytes. The less pronounced effect of these BMDM cultures could be due to compensatory $Ucp3$ upregulation (see Supplementary Fig. 11).

- c**, Comparable engulfment of synthetic targets (2 μ m carboxylate-modified beads) by BMDMs from *Ucp2^{+/+}* and *Ucp2^{-/-}* mice.
- d**, Top, Schematic of the assay for assessing clearance of apoptotic thymocytes *in vivo*. Bottom, photographs of representative thymi from *Ucp2^{+/+}* and *Ucp2^{-/-}* mice treated with vehicle or dexamethasone.
- e**, Total thymic cellularity of *Ucp2^{+/+}* and *Ucp2^{-/-}* mice treated with vehicle or dexamethasone (Dex) for 6 hr. $n=5\sim 8$ mice per group.
- f**, Cryosections of thymi from *Ucp2^{+/+}* and *Ucp2^{-/-}* mice treated with dexamethasone were stained with TUNEL (red) and DAPI (blue). Inset, TUNEL staining at higher magnification indicates apoptotic cells in the vehicle or Dex treated *Ucp2^{+/+}* and *Ucp2^{-/-}* mice.
- g**, Histogram of TUNEL staining from thymi sections in **f**. Data were averaged from 9 slices of thymi and 3 mice per group.
- h**, Representative pictures of detection of apoptotic cells in testes of six- to eight-week-old *Ucp2^{+/+}* and *Ucp2^{-/-}* mice after testicular torsion for 2 hours. Arrowheads, apoptotic cell.
- i**, Average number of apoptotic cells per tubule in (**h**). Staining of apoptotic nuclei in testes sections (specifically the seminiferous tubules), showing increased numbers of apoptotic germ cells in the *Ucp2^{-/-}* mice after testicular torsion. Average of data from 4 slices of each testes, and 2~3 mice per group. Data are shown as mean $\pm$ SD and are representative of at least two independent experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.