Activated macrophages utilize glycolytic ATP to maintain mitochondrial membrane potential and prevent apoptotic cell death

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We have previously analysed the bioenergetic consequences of activating J774.A1 macrophages (MΦ) with interferon- γ (IFN- γ) and lipopolysaccharide (LPS) and found that there is a nitric oxide (NO)-dependent mitochondrial impairment and stabilization of hypoxia-inducible factor (HIF)-1 α , which synergize to activate glycolysis and generate large quantities of ATP. We now show, using tetramethylrhodamine methyl ester (TMRM) fluorescence and time-lapse confocal microscopy, that these cells maintain a high mitochondrial membrane potential ($\Delta\Psi_m$) despite the complete inhibition of respiration. The maintenance of high $\Delta\Psi_m$ is due to the use of a significant proportion of glycolytically generated ATP as a defence mechanism against cell death. This is achieved by the reverse functioning of F₀F₁-ATP synthase and adenine nucleotide translocase (ANT). Treatment of activated MΦ with inhibitors of either of these enzymes, but not with inhibitors of the respiratory chain complexes I to IV, led to a collapse in $\Delta\Psi_m$ and to an immediate increase in intracellular [ATP], due to the prevention of ATP hydrolysis by the F₀F₁-ATP synthase. This collapse in $\Delta\Psi_m$ was followed by translocation of Bax from cytosol to the mitochondria, release of cytochrome *c* into the cytosol, activation of caspases 3 and 9 and subsequent apoptotic cell death. Our results indicate that during inflammatory activation 'glycolytically competent cells' such as MΦ use significant amounts of the glycolytically generated ATP to maintain $\Delta\Psi_m$ and thereby prevent apoptosis.

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Activation of murine macrophages (MΦ) with interferon- γ (IFN- γ) and lipopolysaccharide (LPS) leads to a mitochondrial defect that is dependent on the release of large quantities of nitric oxide (NO) produced by the inducible NO synthase (iNOS). This NO-dependent mitochondrial defect results in the complete arrest of mitochondrial ATP synthesis by oxidative phosphorylation (OXPHOS). In this situation, macrophages upregulate glycolysis by several fold to generate more ATP. In spite of this increase, the demand for energy of activated MΦ is such that the cellular [ATP] is reduced by approximately 40% after 12 h. However, at this time there is no difference in the viability of control and activated MΦ.¹

We have previously shown in Jurkat cells that after inhibition of respiration by exogenous NO, or by endogenous NO generated after treatment with anti-Fas antibody, there is upregulation of glycolysis and hyperpolarization of the mitochondrial membrane potential ($\Delta\Psi_m$).^{2,3} In subsequent experiments we showed that such hyperpolarization occurs only in cells capable of upregulating glycolysis, such as astrocytes, but not in neurons that are unable to do so.⁴ In the latter cells there is a rapid and progressive decline of $\Delta\Psi_m$ and increased apoptotic cell death. It is known that the onset of

apoptosis is associated with a collapse in $\Delta\Psi_m$ and an alteration in mitochondrial matrix configuration,^{5–7} leading to the release of pro-apoptotic proteins into the cytosol.^{8–12}

Mitochondrial membrane hyperpolarization has been observed in cells of the immune system in which apoptosis has been induced by a variety of agents, including withdrawal of growth factor,¹³ Fas signalling,^{2,3,14} hypoxia,¹⁵ T-cell activation^{16,17} and staurosporine treatment.¹⁸ Increases in $\Delta\Psi_m$ were not observed, however, when other apoptosis-inducing agents, such as permeabilization of the outer mitochondrial membrane,^{5,19} H₂O₂²⁰ and dexamethasone,^{21–23} were used. Thus it is possible that mitochondrial membrane hyperpolarization is part of a defence mechanism, which precedes apoptosis, developed by glycolytically competent cells provided that the mitochondria are not the primary target of the apoptosis-inducing agent. Such might be the case in activated lymphocytes from patients with systemic lupus erythematosus that have hyperpolarized mitochondria and are resistant to apoptosis.²⁴ In such a situation, apoptosis would follow the failure of the cells to maintain $\Delta\Psi_m$.

To analyse whether the fate of cells is critically dependent on the regulation of the mitochondrial status, especially $\Delta\Psi_m$, we decided to study the sequence of events that follows the

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Abbreviations: MΦ, macrophages; IFN- γ , interferon- γ ; LPS, lipopolysaccharide; NO, nitric oxide; HIF-1 α , hypoxia-inducible factor-1 α ; TMRM, tetramethylrhodamine methyl ester; ANT, adenine nucleotide translocase; $\Delta\Psi_m$, mitochondrial membrane potential; OXPHOS, oxidative phosphorylation; iNOS, inducible NO synthase; SEITU, S-ethyl isothiourrea; FCCP, p-trifluoromethoxy carbonyl cyanide phenyl hydrazone; CMXRos, chloromethyl-X-rosamine; DMEM, Dulbecco's modified Eagle's medium; PI, propidium iodide; FITC, fluorescein isothiocyanate; H₂O₂, hydrogen peroxide

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increase, and later collapse, of $\Delta\Psi_m$ in J774.A1 MΦ activated for a period longer than the 12 h used previously.¹ In this study we show that activated MΦ use significant amounts of glycolytically generated ATP to maintain the $\Delta\Psi_m$ of defective mitochondria that are unable to generate ATP. This is achieved through F_0F_1 -ATP synthase and adenine nucleotide translocase (ANT) acting in reverse mode. Disruption of such a mechanism by inhibiting either of these enzymes leads to a collapse in $\Delta\Psi_m$, translocation of the pro-apoptotic Bcl-2 protein Bax to the mitochondria, release of cytochrome c into the cytosol, subsequent activation of pro-apoptotic caspases and apoptotic cell death.

Results

Effect of activation of MΦ on their $\Delta\Psi_m$. Mitochondrial oxygen uptake (respiration) of IFN- γ and LPS-treated (activated) MΦ was almost completely ($\geq 95\%$) inhibited within the first 12 h (not shown), an effect that was maintained

throughout the experimental period of 72 h (Figure 1a). To distinguish the NO-dependent and NO-independent components of inflammation, some activated macrophages were treated with the NO synthase inhibitor, S-ethyl isothiourea (SEITU; 500 μ M), at the time of activation. In activated + SEITU-treated MΦ, respiration was higher than in controls (62.3 ± 6.6 versus 51.4 ± 2.5 pmol O_2 /s/ 10^6 cells), but in both groups it could be reduced by approximately 70% after treatment with oligomycin, indicating that the mitochondria are coupled to the same extent (Figure 1a). Furthermore, treatment with p-trifluoromethoxy carbonyl cyanide phenyl hydrazine (FCCP) led to a maximal respiration rate that was similar in both groups (104 ± 6.8 versus 97.6 ± 7.2 pmol O_2 /s/ 10^6 cells for control and for activated + SEITU-treated cells, respectively).

Despite the total inhibition of respiration, the $\Delta\Psi_m$ of activated MΦ, measured by tetramethyl rhodamine methyl ester (TMRM) fluorescence, was significantly increased from 742 ± 75 a.u. (of controls) to 910 ± 69 a.u. (Figures 1b and c). The maintenance of $\Delta\Psi_m$ by the mitochondria of activated

Figure 1 Activated MΦ maintain $\Delta\Psi_m$ despite NO-dependent inhibition of respiration. J774.A1 MΦ were activated with IFN- γ and LPS for 72 h in the presence or absence of 500 μ M SEITU. Mitochondrial oxygen uptake (respiration) was measured in the presence and absence of 2 μ g/ml oligomycin (to identify mitochondrial-dependent oxygen uptake) and presented as mean $\pm$ S.D. of $n = 5$, *Significantly different from appropriate control, §significant difference between activated and activated plus SEITU-treated MΦ, ANOVA and Tukey's *post hoc* test ($P < 0.05$) (a). Representative confocal images of control (b), activated (c) and activated + SEITU-treated MΦ (d) stained with 20 nM TMRM to indicate $\Delta\Psi_m$ (red) and counterstained with 2 μ g/ml Hoechst 33342 to identify the nuclei (blue). The $\Delta\Psi_m$ values shown are mean $\pm$ S.D. of 12–15 cells per experiment ($n = 3$)

MΦ, whose respiration is completely inhibited by NO, points to the involvement of the reverse function of F_0F_1 -ATP synthase. The $\Delta\Psi_m$ of activated + SEITU-treated MΦ was also significantly higher than that of controls (1095 ± 85 a.u.; Figure 1d).

Dependence of $\Delta\Psi_m$ on F_0F_1 -ATP synthase in activated MΦ. Three treatment groups of cells (control, activated, and activated plus SEITU-treated) were incubated for 72 h and then treated with oligomycin ($2 \mu\text{g/ml}$) or bongkreikic acid ($10 \mu\text{M}$) to inhibit F_0F_1 -ATP synthase or ANT, respectively, while recording $\Delta\Psi_m$ using time-lapse confocal microscopy. Treatment with either compound led to a rapid and significant increase in $\Delta\Psi_m$ in the control and in the activated + SEITU-treated MΦ (Figures 2a and b). This effect was maintained for at least 12 h (not shown). In contrast, treatment of activated MΦ with either compound led to an immediate fall in $\Delta\Psi_m$ to approximately 70% (Figures 2a and b). This was followed by a progressive decline to approximately $39 \pm 5\%$ within the next 60 min and to $15 \pm 4\%$ of the original values within 24 h (not shown). The effect of oligomycin and bongkreikic acid on the $\Delta\Psi_m$ of activated MΦ could be observed from 12 h activation onwards, when respiration was almost completely ($>95\%$) inhibited. The TMRM fluorescence in all cell groups could be abolished by treatment with FCCP, indicating its specificity to $\Delta\Psi_m$ (Figures 2a and b). We also used aurovertin B and atractyloside to inhibit F_0F_1 -ATP synthase and ANT, respectively, and found that they gave similar results as oligomycin and bongkreikic acid (not shown).

In a separate group of experiments the three groups of cells were treated with antimycin A, rotenone or KCN. These respiratory chain inhibitors did not affect the $\Delta\Psi_m$ of activated MΦ but significantly reduced that of both control and of activated + SEITU-treated groups (see Figure 2c for result using antimycin A). Subsequent treatment with oligomycin or bongkreikic acid led to an immediate fall in $\Delta\Psi_m$ to approximately 65–70% in all three groups (Figure 2c); this was followed by a progressive decline to 34–39% of the initial values within 60 min. Further incubation for 24 h resulted in the reduction of $\Delta\Psi_m$ to 10–17% of the initial values (not shown).

Effect of MΦ activation on ATP concentration. Three treatment groups of cells (control, activated, and activated plus SEITU-treated) were incubated for 12 h before treatment with oligomycin or bongkreikic acid to determine the relationship between MΦ activation, $\Delta\Psi_m$ and cellular [ATP]. Control MΦ had a steady-state cellular [ATP] of 10.1 ± 1.51 nmol ATP/ 10^6 cells that fell to 5.7 ± 0.59 nmol ATP/ 10^6 cells within 2 min of treatment with oligomycin (Figure 3a). After a further 12 h, however, the [ATP] was restored to a value of 9.6 ± 0.84 nmol ATP/ 10^6 cells (Figure 3b). This was associated with upregulation of glycolysis, as showed by lactate accumulation in the medium during this period (Figure 3c). Treatment of control cells with antimycin A had a similar effect on [ATP] and upregulation of glycolysis. Before oligomycin or antimycin A treatment, the [ATP] of MΦ activated for 12 h was 5.4 ± 0.38 nmol ATP/ 10^6 cells (i.e., less than that in control cells); however, in contrast with control MΦ, treatment with

Figure 2 Effect of inhibitors of complex III, F_0F_1 -ATP synthase and ANT on $\Delta\Psi_m$. Control, activated and activated plus SEITU-treated cells were treated with oligomycin (a), bongkreikic acid (b) or antimycin A followed by oligomycin (c), and TMRM fluorescence was monitored as an indicator of $\Delta\Psi_m$. FCCP completely abolished the TMRM fluorescence in all treatments. Whole-cell TMRM fluorescence of 12–15 cells, mean $\pm$ S.D., $n = 3$

oligomycin led to a significant increase of [ATP] to 7.7 ± 0.73 nmol ATP/ 10^6 cells within 2 min; this was maintained for the following 12 h. Antimycin A treatment did

Figure 3 Effect of oligomycin and antimycin A on cellular [ATP] and lactate release in control, activated and activated plus SEITU-treated cells. After 12 h activation with IFN- γ and LPS in the presence or absence of SEITU, cells were treated with 2 μ g/ml oligomycin or 1 μ M antimycin A, and cellular [ATP] was determined after 2 min (a) and 12 h (b) of incubation. The change in lactate concentration in the medium between these two time points was shown (c) as a measure of glycolysis. *Significant difference between untreated and oligomycin- or antimycin A-treated cells, §significant difference between oligomycin- and antimycin A-treated cells, ANOVA and Tukey's *post hoc* test ($P < 0.05$), mean $\pm$ S.D., $n = 3-5$

Figure 4 Activated M Φ rely on glycolytic ATP to prevent cell death. J774.A1 M Φ activated for 12 h with IFN- γ and LPS in the presence or absence of 500 μ M SEITU were treated with 2 μ g/ml oligomycin (a) or 1 μ M antimycin A (b) or transferred to a medium with galactose as the sole glycolytic substrate (c) and cell viability was determined by the Trypan blue exclusion method. The incubation medium was replaced every 24 h with fresh medium containing the corresponding treatments. Inhibition of F $_0$ F $_1$ -ATP synthase with oligomycin led to a gradual death of both activated and activated plus SEITU-treated M Φ (a), whereas antimycin A reduced the viability of activated plus SEITU-treated M Φ without affecting activated M Φ (b). Substitution of the glycolytic substrate glucose with galactose in the medium led to a rapid reduction in viability in activated M Φ but to a very gradual reduction in viability in the activated plus SEITU-treated M Φ (c). Mean $\pm$ S.D., $n = 5-7$

not change the [ATP] of activated MΦ. Glycolysis in these MΦ was already upregulated during the 12 h activation and was not further increased by treatment with oligomycin or antimycin A (Figure 3c). Before oligomycin or antimycin A treatment, the [ATP] in activated + SEITU-treated MΦ was comparable to that in control MΦ (9.7 ± 1.10 nmol/ 10^6 cells) and was reduced by treatment with oligomycin or antimycin A to 5.6 ± 0.66 and 4.5 ± 0.56 nmol/ 10^6 cells, respectively. However, unlike in control MΦ, the [ATP] of activated + SEITU-treated cells remained low during the following 12 h (Figure 3b), despite an increase in glycolysis (Figure 3c). Bongkreikic acid treatment produced similar effects to oligomycin in all three groups of cells (not shown).

$\Delta\Psi_m$ and cell viability. Three treatment groups of cells (control, activated, and activated plus SEITU-treated) were incubated for 12 h before treatment with oligomycin, bongkreikic acid or antimycin A. Control MΦ had a viability of $\geq 98\%$ throughout the experimental period and treatment with oligomycin or bongkreikic acid had no significant effect on cell viability (Figure 4a). Activation of MΦ reduced viability to approximately 64% within 72 h. Treatment of activated MΦ with oligomycin reduced the viability to 35% by 48 h and to 0% by 72 h (Figure 4a). Activated + SEITU-treated MΦ had a viability of approximately 86% at 72 h. Oligomycin treatment reduced the viability of these MΦ to 12% by 48 h and to 0% by 72 h (Figure 4a). The effects of bongkreikic acid treatment were similar to those of oligomycin in all treatment groups (not shown). Antimycin A treatment of activated + SEITU-treated MΦ reduced the viability to 10% by 48 h and 0% by 72 h but had no effect on the viability of activated MΦ (Figure 4b).

To assess the extent of dependence of activated MΦ on glycolytically generated ATP for survival, we switched to a medium in which galactose was the sole source of carbohydrate, after 12 h activation. The conversion of galactose to pyruvate does not yield net glycolytic ATP; thus, cells need functional mitochondria to survive in such a medium. The viability of control MΦ was not affected by replacement of glucose in the medium with galactose. In contrast, such treatment led to cell death ($> 98\%$) of activated MΦ within 8 h, whereas incubation of activated + SEITU-treated MΦ in this medium led to a gradual reduction of viability (Figure 4c).

Apoptosis and necrosis in activated MΦ after collapse of the $\Delta\Psi_m$. After 12 h activation, cells were treated with oligomycin, bongkreikic acid or antimycin A and $\Delta\Psi_m$ and cell viability were assessed for 60 h using mitotracker red chloromethyl-X-rosamine (CMXRos) and annexin-V staining and flow cytometry analysis. None of the drugs had any effect on the viability of control MΦ (Figure 5b). In activated MΦ, with no drug treatment, there was a progressive apoptotic cell death (Figures 5a–d). Treatment of activated

MΦ with oligomycin increased the number of apoptotic cells in a time-dependent manner, whereas antimycin A had no effect. In activated + SEITU-treated MΦ, with no drug treatment, approximately 90% of the cells were viable (mitotracker red positive, Figure 5b). However, treatment of these cells with oligomycin or antimycin A resulted in a progressive increase in both apoptosis and necrosis (Figures 5a, d and e).

MΦ activated for 12 h and transferred to medium with galactose as the sole carbohydrate source died rapidly by necrosis (approximately 85% of cells were annexin-V negative and propidium iodide (PI) positive at 8 h after transfer, not shown). Activated + SEITU-treated MΦ incubated in galactose medium died slowly by a combination of apoptosis and necrosis, and hence approximately 49% of cells were annexin-V and PI positive (apoptotic) and 16% were annexin-V negative and PI positive (necrotic) at 72 h (not shown).

Role of caspases in apoptosis of activated MΦ after collapse of $\Delta\Psi_m$. Apoptosis can be triggered by extrinsic (caspase 8-mediated) and/or intrinsic (caspase 9-mediated) pathways, both of which can lead to activation of caspase 3 and result in the degradation of cellular proteins. MΦ activation led to a slight increase in the enzymatic activities of caspases 3, 8 and 9. Treatment of 12 h activated MΦ with oligomycin to collapse the $\Delta\Psi_m$ triggered a rapid and pronounced activation of caspases 3 and 9, but not of caspase 8 (Figures 6a–c). A similar effect was obtained using bongkreikic acid (not shown). Antimycin A treatment, which had no effect on the $\Delta\Psi_m$ of activated MΦ, did not increase their caspase activity. There was little caspase activity in 12 h activated + SEITU-treated MΦ; however, treatment with either oligomycin or antimycin A resulted in a delayed and gradual activation of caspases 3, 8 and 9 (Figures 6d–f). In activated MΦ that were treated with oligomycin, the activation of caspases coincided with translocation of the pro-apoptotic Bcl-2 protein Bax to the mitochondria and the release of cytochrome c to the cytosol within 6 h of addition of oligomycin (Figures 6g and h). In the activated + SEITU treated MΦ, however, this happened to a lesser extent and after a long delay (Figure 6h).

Activated MΦ incubated in galactose medium died by necrosis and there was no significant activation of any of the caspases analysed (not shown). In contrast, in activated + SEITU-treated MΦ incubated in galactose medium, there was an approximately threefold activation of caspases 3 and 8, but no significant activation of caspase 9 at 72 h (not shown).

Discussion

We have previously shown that in murine MΦ activated with IFN- γ and LPS, respiration is completely inhibited by NO

Figure 5 Relationship between $\Delta\Psi_m$ and apoptosis in activated MΦ. Cells were activated with IFN- γ plus LPS $\pm$ SEITU for 12 h and treated with oligomycin or antimycin A. After a further incubation for 36, 48 and 60 h, cells were stained with mitotracker red CMXRos and annexin V-Alexa Fluor488 and analysed by flow cytometry as described in Materials and Methods. Representative dot plots are shown for the 48-h time point (a) and data from the other dot plots are presented as graphs in b–e. Cells stained with mitotracker red but not with annexin-V (top left quarters in the dot plots) are viable cells with no exposure of phosphatidylserine (b). Cells stained with both dyes (top right quarters) are early apoptotic cells that have exposed phosphatidylserine but not yet lost $\Delta\Psi_m$ (c). Cells stained with annexin-V but not with mitotracker red (lower right quarter) are apoptotic cells that have lost $\Delta\Psi_m$ (d). Cells that are not stained with either dye (lower left quarter) are necrotic (e). Values are mean $\pm$ S.D. of $n = 3$, and the S.D. values are omitted in the graphs for clarity

within 12 h and that this inhibition can be prevented by blocking iNOS activity with SEITU. Despite inhibition of respiration, activated M Φ maintain high cell viability by switching to glycolytic metabolism.¹ We now report that the $\Delta\Psi_m$ is maintained at a higher level in activated M Φ than in control M Φ , and that this protects the cells from apoptosis. In activated + SEITU-treated M Φ the mitochondria are functional and the basal respiratory rate and $\Delta\Psi_m$ are significantly higher than those of control M Φ .

Treatment of activated M Φ with respiratory chain inhibitors (antimycin A, KCN or rotenone) had no effect on $\Delta\Psi_m$, showing its independence of respiration in these cells. Such treatment did, however, reduce the $\Delta\Psi_m$ of control and of activated + SEITU-treated M Φ . Treatment of activated M Φ with oligomycin or bongkreikic acid led to the collapse of their $\Delta\Psi_m$, indicating its dependence on a reversed function of F_oF₁-ATP synthase and ANT. In contrast, treatment of cells with respiring mitochondria (i.e., control and activated plus SEITU-treated M Φ) with oligomycin or bongkreikic acid increased the $\Delta\Psi_m$. Pre-treatment of control and of activated + SEITU-treated M Φ with antimycin A induced a decrease in $\Delta\Psi_m$ in response to oligomycin or bongkreikic acid, similar to that in activated M Φ .

Our observations can be explained by the fact that the NO-mediated inhibition of respiration in activated M Φ abolishes the translocation of H⁺ across complexes I, III and IV, leading to a transient drop in $\Delta\Psi_m$. In response, the F₁ subunit of F_oF₁-ATP synthase starts hydrolyzing mitochondrial ATP and drives the Fo-rotor to pump H⁺ out of the matrix.²⁵ The resulting reduction in mitochondrial ATP/ADP ratio favours reversal of the ANT function.²⁶ The mitochondria of activated M Φ thus become consumers, rather than generators of ATP, further increasing the energetic demand of these cells.

Oligomycin or bongkreikic acid-induced inhibition of ATP hydrolysis by mitochondria of activated M Φ led to a 30% increase in the steady-state cellular [ATP], suggesting that mitochondria use this quota of glycolytic ATP to maintain $\Delta\Psi_m$. Treatment with antimycin A had no effect on the [ATP] of activated M Φ , as in these cells respiration was already inhibited by an NO-dependent mechanism. In contrast, treatment of control and of activated + SEITU-treated M Φ with antimycin A or oligomycin led to an immediate fall in the cellular [ATP]. Although glycolysis was upregulated in the following 12 h in both cell groups, the steady-state cellular [ATP] was restored only in control M Φ after treatment with antimycin A. Thus, in activated + SEITU-treated cells glycolysis cannot fully compensate for the antimycin A- or oligomycin-induced loss of mitochondrial function because of the higher energetic demand incurred by their activation. Similarly, in activated + SEITU-treated M Φ mitochondrial

ATP generation cannot compensate for the loss of glycolysis, as showed by the results of our experiments using galactose as the sole carbohydrate source in the medium. In the absence of glycolytic ATP generation, control M Φ were able to fulfil their energetic requirements by OXPHOS, whereas activated + SEITU-treated M Φ gradually died, as their increased ATP demand could not be fulfilled by OXPHOS alone. Activated M Φ , whose mitochondria were already inhibited by NO, died by necrosis more rapidly when incubated in galactose medium.

Activated M Φ underwent a basal level of apoptotic cell death that was associated with slight activation of caspases 3 and 8, showing that apoptosis in these cells was mainly triggered by the extrinsic pathway (for review see Kroemer *et al.*¹¹). Apoptosis was increased dramatically by collapsing $\Delta\Psi_m$ with oligomycin or bongkreikic acid; this was associated with activation of caspases 3 and 9, indicating activation of a mitochondria-dependent apoptotic pathway.

Although SEITU protected activated M Φ from cell death, inhibition of respiration with oligomycin or antimycin A after 12 h activation induced significant cell death, and hence approximately 90% of these cells were dead after 48 h by a combination of apoptosis and necrosis. This was probably due to the inability of the glycolytic ATP supply to fulfil the increased energetic demand of these cells. Because of the absence of NO and hypoxia-inducible factor-1 α (HIF-1 α), the potent activators of glycolysis, the glycolysis of activated + SEITU-treated M Φ is upregulated only by approximately 2.5-fold, compared with approximately sixfold upregulation in activated M Φ .¹ Inhibition of glycolytic ATP generation by incubating these cells in galactose medium also led to gradual cell death, indicating that the increased ATP demand has to be fulfilled by OXPHOS and glycolysis. Inhibition of either glycolysis or respiration thus compromises the ATP supply and survival of these cells.

The exact mechanism by which the collapse in $\Delta\Psi_m$ leads to the release of pro-apoptotic proteins into the cytosol and induces apoptosis is not yet clear. Furthermore, whether the collapse in $\Delta\Psi_m$ or the induction of apoptosis occurs first also remains a subject of controversy.¹¹ Apart from OXPHOS, one of the functions of $\Delta\Psi_m$ is to facilitate the import of nuclear-encoded mitochondrial proteins, which are necessary for the maintenance of mitochondrial function and integrity.^{27,28} Thus, disruption of $\Delta\Psi_m$ may lead to loss of mitochondrial membrane integrity, release of pro-apoptotic proteins from the intermembrane space and subsequent activation of the caspase-mediated apoptotic pathway. In fact, it has been shown that the maintenance of $\Delta\Psi_m$ protects cells from death under apoptosis-inducing conditions.²⁹ Moreover, oligomycin-mediated inhibition of F_oF₁-ATP synthase in activated

Figure 6 Collapsing the $\Delta\Psi_m$ of activated M Φ leads to Bax translocation to the mitochondria, cytochrome c release and activation of caspases. Cells were activated in the presence or absence of SEITU for 12 h and then treated with oligomycin or antimycin A and samples were taken every 6 h to analyse the activities of caspase 3 (a, d), caspase 8 (b, e) and caspase 9 (c, f). Oligomycin and antimycin A treatment of control cells did not activate any of the caspases investigated (not shown). Treatment of activated M Φ with oligomycin led to an immediate activation of death caspases through the intrinsic pathway; antimycin A treatment, in contrast, had no effect (a–c). In activated + SEITU-treated M Φ , both of these treatments resulted in a gradual, slight activation of caspases through both the intrinsic and extrinsic pathways (d–f). Mean $\pm$ S.D. of 3–5 measurements. After 12 h activation with or without SEITU, cells were treated with oligomycin and incubated for a further 6 and 18 h and then harvested for the separation of cytosolic and mitochondrial fractions. The abundance of Bax and cytochrome c in both the cytosolic and mitochondrial fractions was evaluated by western blot (g, h). ATPase- β was used as a loading control for the mitochondrial fraction and also to check for possible contamination of the cytosolic fraction by mitochondria. α -Tubulin was used as a loading control for the cytosolic fraction. The blots are representatives of two experiments

MΦ leads to the collapse of $\Delta\Psi_m$ and accumulation of H^+ in the matrix, probably resulting in cytosolic alkalization and matrix acidification. It has been shown that alkalization of the cytosol leads to conformational changes in the pro-apoptotic Bcl-2 protein Bax and results in the translocation of this protein to the outer mitochondrial membrane^{30–33} and subsequent release of pro-apoptotic intermembrane space proteins into the cytosol.^{34,35} Our results have shown that within 6 h of inhibition of F_0F_1 -ATP synthase in activated MΦ, Bax is translocated to the mitochondria and cytochrome *c* is released into the cytosol, resulting in the activation of caspases.

In summary, we have shown that activated murine MΦ, in which mitochondria are inhibited, use a considerable proportion of their glycolytically generated ATP to maintain their $\Delta\Psi_m$ to prevent apoptotic cell death. This response suggests that activated MΦ, which are already engaged in defence, are also forced to defend themselves against their own mitochondria. However, it remains to be investigated whether diversion of energy for this purpose, which is also likely to occur in cells other than MΦ that express iNOS, favours or is detrimental to the successful outcome of an inflammatory reaction.

Materials and Methods

Reagents. The cell culture medium (Dulbecco's modified Eagle's medium, DMEM) supplemented with 4.5 g/l D-glucose, 2 mM L-glutamine (4 mM final) and 25 mM HEPES, penicillin, streptomycin and L-glutamine were from Invitrogen (Paisley, UK). Glucose-free DMEM was also obtained from Invitrogen, as were the potentiometric dye TMRM, the nuclear stain Hoechst 33342, the dead-cell nuclear stain PI, Vybrant Apoptosis Assay Kit containing annexin-V conjugated to the green-fluorescent dye Alexa Fluor488 for labelling apoptotic cells and MitoTracker Red CMXRos for labelling polarized mitochondria and ApoTarget colorimetric caspase assay sampler kit (for caspases 3, 8 and 9). Annexin V-conjugated to the green fluorescence dye fluorescein isothiocyanate (FITC) was from Biovision Inc (Mountain View, CA, USA). LPS of the bacterial strain *Staphylococcus typhosa* 0901 was from Difco (Surrey, UK), and murine IFN γ was from Insight Biotech (Wembley, UK). The lactate oxidase-based lactate assay kit was from Trinity Biotech (Bray, Ireland), the luciferase-based ATP assay kit ATPlite was from Perkin Elmer (Waltham, MA, USA). All other reagents were from Sigma-Aldrich (Dorset, UK).

Cell culture, activation and preparation of macrophages. The murine MΦ cell line J774.A1 (ATCC TIB 67) was maintained in suspension in stirrer bottles (Techne, Burlington, NJ, USA) in DMEM supplemented with 10% FCS, 100 units/ml penicillin and 100 μ g/ml streptomycin. The cell density was kept at $\leq 1 \times 10^6$ cells/ml to maintain viability at $\geq 98\%$. Cells were activated by resuspending them in stirrer bottles at a density of $0.5\text{--}0.8 \times 10^6$ cells/ml in fresh medium containing 10 U/ml murine IFN γ and 10 ng/ml LPS. In one group, the activity of iNOS was inhibited by administration of 500 μ M SEITU at the same time as IFN γ and LPS to distinguish the NO-dependent and NO-independent components of inflammation. In some experiments, oligomycin (2 μ g/ml), bongkreikic acid (10 μ M) or antimycin A (1 μ M) was added 12 h after activation with IFN γ and LPS and cells were incubated further. For experiments that involve incubation of activated cells in glucose-free medium, after 12 h of activation cells were spun down and resuspended in glucose-free DMEM supplemented with 20 mM galactose, 4 mM L-glutamine, 2 mM pyruvate, 10% FCS, 100 units/ml penicillin, 100 μ g/ml streptomycin and the corresponding treatments. The viability of activated cells incubated in galactose medium was determined every 2 h by the Trypan blue exclusion method.

As activated MΦ have a high glycolytic metabolism, we preserved optimal cell viability by changing the medium every 24 h to avoid nutrient depletion and medium acidification. For lactate and nitrite determination, 1 ml aliquot of the cell suspension was taken immediately after changing the medium and after 24 h, spun down and the supernatant was stored at 4°C until analysis.

Respirometry measurements and biochemical assays. The respiration of activated cells was measured by taking a cell suspension of approximately 3×10^6 cells from each treatment group at 12 and 24 h after activation and every 24 h thereafter. The cells were spun down, resuspended at 2×10^6 cells/ml in fresh medium and used in respirometry experiments after cell counting and viability determination. Intact cell respirometry, lactate, nitrite and ATP assays were performed as described previously.¹

Confocal imaging and determination of mitochondrial membrane potential.

A suspension of activated MΦ was removed from the stirrer bottles and seeded at a density of 0.1×10^6 cells/ 0.8 cm^2 growth area of a chambered coverglass (Lab-Tek, Nunc, Langensfeld, Germany) and incubated for 12 h before confocal imaging. The medium was aspirated, the cells were rinsed with Dulbecco's phosphate-buffered saline (DPBS), 200 μ l annexin-V binding buffer containing 10 μ l annexin V-FITC + 2 μ g/ml Hoechst 33342 + 20 nM TMRM was added and cells were incubated for 15 min in the dark. Confocal microscopic images were taken with the UltraVIEW ERS Live Cell confocal Microscope (Perkin Elmer) with a $\times 63$ oil immersion objective. The cells were kept at 37°C during the entire imaging period by a thermostatic chamber encasing the stage of the microscope. Data acquisition and further fluorescence analysis were carried out using UltraVIEW software. Whole cell fluorescence was analysed by marking individual cells in the field. Background fluorescence of a region without cells/cell debris was subtracted from the fluorescence values.

The role of the different respiratory chain complexes in maintaining $\Delta\Psi_m$ of activated and control MΦ was evaluated 72 h after activation by inhibiting the respiratory chain complexes I, III and IV with 1 μ M rotenone, 1 μ M antimycin A and 500 μ M KCN, respectively, in separate experiments. In addition, mitochondrial complex V and ANT were inhibited with 2 μ g/ml oligomycin and 10 μ M bongkreikic acid, respectively. In some experiments, as an alternative to oligomycin (an inhibitor of the F_0 subunit of F_0F_1 -ATP synthase) and bongkreikic acid (an inhibitor of ANT in its closed configuration) we also used 10 μ M aurovertin B (an inhibitor of the F_1 subunit of F_0F_1 -ATP synthase), and 500 μ M atractyloside (an inhibitor of ANT in its open configuration).

The kinetics of action of the various inhibitors on $\Delta\Psi_m$ were studied by recording the TMRM fluorescence in time-lapse experiments. In brief, the steady-state TMRM fluorescence was recorded for 60 s to obtain a stable fluorescence signal. Subsequently antimycin A was added and the signal was recorded for approximately 100 s until a relatively stable signal was obtained. Oligomycin or bongkreikic acid was added and the signal was recorded for approximately 180 s. The $\Delta\Psi_m$ was finally collapsed completely with 25 μ M FCCP. The FCCP concentration used here was approximately fivefold higher than that used to obtain maximal respiration, to ensure that the $\Delta\Psi_m$ was completely collapsed. To evaluate the roles of F_0F_1 -ATP synthase and ANT on $\Delta\Psi_m$ of activated MΦ without previous addition of other mitochondrial inhibitors, oligomycin or bongkreikic acid was added after recording the steady-state TMRM signal for 60 s, and the fluorescence was recorded for a further 150 s.

Flow cytometry analysis of mitochondrial membrane potential and cell death.

Activated MΦ were stained with the required fluorochromes and prepared for flow cytometry according to the manufacturer's recommendations. In brief, approximately 1×10^6 cells were removed from the stirrer bottles at different time points after activation, spun down and resuspended in 1 ml fresh medium containing 40 nM mitotracker red CMXRos, and incubated at 37°C for 30 min. Cells were then spun down, washed once with 10 ml DPBS and resuspended in 100 μ l annexin-V binding buffer that contained 5 μ l annexin V-Alexa Fluor488. They were then incubated at room temperature, in the dark, for 15 min and diluted with 400 μ l annexin-V binding buffer before measurement. Apoptotic and necrotic control cells were obtained after treatment of control MΦ with 1 μ M staurosporine and 2 mM H_2O_2 , respectively, for 4 h.

Analysis of the stained cells was carried out with a dual laser flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA, USA) and data were acquired with CELLQUEST software. Single fluorochrome-labelled cells were used to correct for the spectral overlap between the green and red fluorescence. Furthermore, cell size and granularity data were acquired using the forward (FSC) and side light scatter (SSC) filters, respectively. For each sample, 1×10^4 cells were analysed.

Caspase assay. Enzymatic activity of cysteine-aspartic acid proteases (caspases) in the cytosolic extracts of activated cells was determined spectrophotometrically by measuring the absorbance of free para-nitroaniline (pNA) as described by Taliani *et al.*³⁶ Samples for caspase assay were collected by removing 20 ml cell suspension immediately after oligomycin/antimycin

A treatment and every 6 h thereafter. Cells were spun down, washed once with DPBS, resuspended in 500 μ l chilled cell lysis buffer and incubated on ice (10 min) for complete cell lysis. After centrifugation (10 000 $\times$ g, 2 min), the supernatant was stored at -20°C until analysis.

Caspase assays were performed according to the manufacturer's recommendation (Invitrogen). In brief, protein concentration in the cytosolic extract was determined using the bicinchoninic acid protein assay (BCA assay, Pierce, Rockford, IL, USA) and 200 μ g protein was used for the assay, in triplicate, in a 96-well microplate using 400 μ M caspase substrate. The reaction mixture was incubated for 2 h at 37°C and the absorbance of free pNA was measured at 405 nm. The reaction mixture with the corresponding caspase substrates was used as a blank. All samples were assayed with the substrates for caspases 3, 8 and 9.

Cell fractionation and detection of mitochondrial proteins. J774.A1 M Φ were grown in a suspension culture to approx. 0.8×10^6 cells/ml in four 500 ml stirrer bottles (Techne). Medium in the three bottles was renewed by spinning down cells and resuspending in a new medium 2 h before beginning the treatment. The cells in the fourth bottle were spun down and used for cytosol/mitochondrial fractionation of control cells. Cells were activated by adding 10 U/ml IFN- γ and 10 ng/ml LPS and incubated for 12 h. Cells in one bottle (12 h activation) were harvested by spinning down and those in the other two bottles were treated with 2 μ g/ml oligomycin and incubated for a further 6 h (18 h activation) and 18 h (30 h activation) and harvested accordingly. The same experimental setup was repeated with the activated + SEITU-treated cells. The cell pellets were lysed in isotonic buffer (70 mM sucrose, 200 mM mannitol, 1 mM EGTA, 10 mM Hepes, pH 7.4) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland) by Dounce homogenization. Unbroken cells, nuclei and heavy membrane particles were sedimented by spinning at 1000 $\times$ g and discarded. The supernatant, containing both the mitochondrial and cytosolic fractions, was spun at 12 000 $\times$ g for 20 min to pellet the mitochondrial fraction. The supernatant from this high-speed centrifugation (the cytosolic fraction) was stored at -80°C until needed. The pellet (mitochondrial fraction) was washed once with isotonic buffer and finally resuspended in RIPA lysis buffer (50 mM Tris-HCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4) supplemented with a protease inhibitor cocktail (Roche). These cytosolic and mitochondrial fractions were used to determine translocation of the pro-apoptotic Bcl-2 protein Bax into the mitochondria and efflux of cytochrome c into the cytosol after inhibiting F_0F_1 -ATP synthase with oligomycin.

Protein electrophoresis and western blotting. Protein concentration in the different samples was determined using the BCA Protein Assay Reagent. Sample aliquots were mixed with Laemmli buffer, boiled for 10 min, and 25 μ g total protein was fractionated using a precast 4–15% gradient SDS-PAGE gel electrophoresis (Bio-Rad, Hemel Hempstead, UK). Proteins were transferred to Hybond-P (PVDF) membranes (GE Healthcare, Amersham, UK) and subjected to immunoblot assays using mouse monoclonal antibodies against ATPase- β (Invitrogen), cytochrome c (BD-Pharmingen, San Jose, CA, USA), Bax (Sigma-Aldrich), α -tubulin (Abcam, Cambridge, UK) and horseradish peroxidase-conjugated goat antibody against mouse IgG (Dako, Glostrup, Denmark) at 1:2000 dilutions. The chemiluminescence signal was developed using ECL Plus western blot detection reagent (GE Healthcare).

Statistical analysis. Results are presented as mean $\pm$ S.D. Statistical significance tests were performed using paired sample *t*-test, or one-way ANOVA and the Tukey's *post hoc* test, as appropriate, using OriginPro 8.0 (Northampton, MA, USA). $P \leq 0.05$ was considered to be significant.

Conflict of interest

The authors declare no conflict of interest.

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- Garedeu A, Moncada S. Mitochondrial dysfunction and HIF1 α stabilization in inflammation. *J Cell Sci* 2008; **121**: 3468–3475.
- Beltran B, Mathur A, Duchon MR, Erusalimsky JD, Moncada S. The effect of nitric oxide on cell respiration: a key to understanding its role in cell survival or death. *Proc Natl Acad Sci USA* 2000; **97**: 14602–14607.

- Beltran B, Quintero M, Garcia-Zaragoza E, O'Connor E, Esplugues JV, Moncada S. Inhibition of mitochondrial respiration by endogenous nitric oxide: a critical step in Fas signaling. *Proc Natl Acad Sci USA* 2002; **99**: 8892–8897.
- Almeida A, Almeida J, Bolanos JP, Moncada S. Different responses of astrocytes and neurons to nitric oxide: the role of glycolytically generated ATP in astrocyte protection. *Proc Natl Acad Sci USA* 2001; **98**: 15294–15299.
- Scorrano L, Ashiya M, Buttle K, Weiler S, Oakes SA, Mannella CA et al. A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. *Dev Cell* 2002; **2**: 55–67.
- Gottlieb E, Armour SM, Harris MH, Thompson CB. Mitochondrial membrane potential regulates matrix configuration and cytochrome c release during apoptosis. *Cell Death Differ* 2003; **10**: 709–717.
- Zamzami N, Marchetti P, Castedo M, Zanin C, Vayssiere JL, Petit PX et al. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death *in vivo*. *J Exp Med* 1995; **181**: 1661–1672.
- Marchetti P, Castedo M, Susin SA, Zamzami N, Hirsch T, Macho A et al. Mitochondrial permeability transition is a central coordinating event of apoptosis. *J Exp Med* 1996; **184**: 1155–1160.
- Spierings D, McStay G, Saleh M, Bender C, Chipuk J, Maurer U et al. Connected to death: the (unexpurgated) mitochondrial pathway of apoptosis. *Science* 2005; **310**: 66–67.
- Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ et al. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J* 1998; **17**: 1675–1687.
- Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. *Physiol Rev* 2007; **87**: 99–163.
- Chipuk JE, Green DR. Do inducers of apoptosis trigger caspase-independent cell death? *Nat Rev Mol Cell Biol* 2005; **6**: 268–275.
- Khaled AR, Reynolds DA, Young HA, Thompson CB, Muegge K, Durum SK. Interleukin-3 withdrawal induces an early increase in mitochondrial membrane potential unrelated to the Bcl-2 family. Roles of intracellular pH, ADP transport, and F(0)F(1)-ATPase. *J Biol Chem* 2001; **276**: 6453–6462.
- Banki K, Hutter E, Gonchoroff NJ, Perl A. Elevation of mitochondrial transmembrane potential and reactive oxygen intermediate levels are early events and occur independently from activation of caspases in Fas signaling. *J Immunol* 1999; **162**: 1466–1479.
- Gao Q, Wolin MS. Effects of hypoxia on relationships between cytosolic and mitochondrial NAD(P)H redox and superoxide generation in coronary arterial smooth muscle. *Am J Physiol Heart Circ Physiol* 2008; **295**: H978–H989.
- Nagy G, Koncz A, Fernandez D, Perl A. Nitric oxide, mitochondrial hyperpolarization, and T cell activation. *Free Radic. Biol Med* 2007; **42**: 1625–1631.
- Perl A, Gergely Jr P, Nagy G, Koncz A, Banki K. Mitochondrial hyperpolarization: a checkpoint of T-cell life, death and autoimmunity. *Trends Immunol* 2004; **25**: 360–367.
- Scarlett JL, Sheard PW, Hughes G, Ledgerwood EC, Ku HH, Murphy MP. Changes in mitochondrial membrane potential during staurosporine-induced apoptosis in Jurkat cells. *FEBS Lett* 2000; **475**: 267–272.
- Kinnally KW, Antonsson B. A tale of two mitochondrial channels, MAC and PTP, in apoptosis. *Apoptosis* 2007; **12**: 857–868.
- Gottlieb E, Vander Heiden MG, Thompson CB. Bcl-x $_L$ prevents the initial decrease in mitochondrial membrane potential and subsequent reactive oxygen species production during tumor necrosis factor alpha-induced apoptosis. *Mol Cell Biol* 2000; **20**: 5680–5689.
- Petit PX, Lecoeur H, Zorn E, Dauguet C, Mignotte B, Gougeon ML. Alterations in mitochondrial structure and function are early events of dexamethasone-induced thymocyte apoptosis. *J Cell Biol* 1995; **130**: 157–167.
- Sionov RV, Cohen O, Kfir S, Zilberman Y, Yefenof E. Role of mitochondrial glucocorticoid receptor in glucocorticoid-induced apoptosis. *J Exp Med* 2006; **203**: 189–201.
- Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T et al. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *J Exp Med* 1995; **182**: 367–377.
- Fernandez D, Perl A. Metabolic control of T cell activation and death in SLE. *Autoimmun Rev* 2009; **8**: 184–189.
- Kinosita K, Adachi K, Itoh H. Rotation of F1-ATPase: how an ATP-driven molecular machine may work. *Annu Rev Biophys Biomol Struct* 2004; **33**: 245–268.
- Klingenberg M. The ADP and ATP transport in mitochondria and its carrier. *Biochim Biophys Acta* 2008; **1778**: 1978–2021.
- Krayl M, Lim JH, Martin F, Guiard B, Voos W. A cooperative action of the ATP-dependent import motor complex and the inner membrane potential drives mitochondrial preprotein import. *Mol Cell Biol* 2007; **27**: 411–425.
- Martin J, Mahlike K, Pfanner N. Role of an energized inner membrane in mitochondrial protein import. $\Delta\psi$ drives the movement of presequences. *J Biol Chem* 1991; **266**: 18051–18057.
- Koya RC, Fujita H, Shimizu S, Ohtsu M, Takimoto M, Tsujimoto Y et al. Gelsolin inhibits apoptosis by blocking mitochondrial membrane potential loss and cytochrome c release. *J Biol Chem* 2000; **275**: 15343–15349.
- Khaled AR, Kim K, Hofmeister R, Muegge K, Durum SK. Withdrawal of IL-7 induces Bax translocation from cytosol to mitochondria through a rise in intracellular pH. *Proc Natl Acad Sci USA* 1999; **96**: 14476–14481.

31. Cartron PF, Oliver L, Mayat E, Meflah K, Vallette FM. Impact of pH on Bax α conformation, oligomerisation and mitochondrial integration. *FEBS Lett* 2004; **578**: 41–46.
32. Tafani M, Cohn JA, Karpnich NO, Rothman RJ, Russo MA, Farber JL. Regulation of intracellular pH mediates Bax activation in HeLa cells treated with staurosporine or tumor necrosis factor- α . *J Biol Chem* 2002; **277**: 49569–49576.
33. Belaud-Rotureau MA, Leducq N, Macouillard Poulletier de GF, Diolez P, Lacoste L, Lacombe F *et al*. Early transitory rise in intracellular pH leads to Bax conformation change during ceramide-induced apoptosis. *Apoptosis* 2000; **5**: 551–560.
34. Antonsson B, Conti F, Ciavatta A, Montessuit S, Lewis S, Martinou I *et al*. Inhibition of Bax channel-forming activity by Bcl-2. *Science* 1997; **277**: 370–372.
35. Cartron PF, Bellot G, Oliver L, Grandier-Vazeille X, Manon S, Vallette FM. Bax inserts into the mitochondrial outer membrane by different mechanisms. *FEBS Lett* 2008; **582**: 3045–3051.
36. Talanian RV, Quinlan C, Trautz S, Hackett MC, Mankovich JA, Banach D *et al*. Substrate specificities of caspase family proteases. *J Biol Chem* 1997; **272**: 9677–9682.

Retraction

Activated macrophages utilize glycolytic ATP to maintain mitochondrial membrane potential and prevent apoptotic cell death

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The authors wish to retract the above paper. We have recently identified errors affecting certain panels in Figure 6, in which data were processed in such a way that the figure panels do not reflect

the original data. AG regrets the inappropriate figure manipulations, of which his co-authors were completely unaware. Although we believe that the conclusions reached in this paper are essentially sound, we feel that the most responsible course of action is to retract the paper. We sincerely apologize to the scientific community for any confusion or adverse consequences resulting from the publication of these data.