IL-4 Protects the Mitochondria Against TNFα and IFNγ Induced Insult During Clearance of Infection with *Citrobacter rodentium* and *Escherichia coli*

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*Citrobacter rodentium* is a murine pathogen that serves as a model for enteropathogenic *Escherichia coli*. *C. rodentium* infection reduced the quantity and activity of mitochondrial respiratory complexes I and IV, as well as phosphorylation capacity, mitochondrial transmembrane potential and ATP generation at day 10, 14 and 19 post infection. Cytokine mRNA quantification showed increased levels of IFNγ, TNFα, IL-4, IL-6, and IL-12 during infection. The effects of adding these cytokines, *C. rodentium* and *E. coli* were hence elucidated using an *in vitro* colonic mucosa. Both infection and TNFα, individually and combined with IFNγ, decreased complex I and IV enzyme levels and mitochondrial function. However, IL-4 reversed these effects, and IL-6 protected against loss of complex IV. Both *in vivo* and *in vitro*, the dysfunction appeared caused by nitric oxide-generation, and was alleviated by an antioxidant targeting mitochondria. IFNγ−/− mice, containing a similar pathogen burden but higher IL-4 and IL-6, displayed no loss of any of the four complexes. Thus, the cytokine environment appears to be a more important determinant of mitochondrial function than direct actions of the pathogen. As IFNγ and TNFα levels increase during clearance of infection, the concomitant increase in IL-4 and IL-6 protects mitochondrial function.

Infection with the attaching and effacing (A/E) murine pathogen *Citrobacter rodentium* is used as a model for studying the effects of other A/E pathogens that cause human diseases, such as enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC)1–3. *C. rodentium* infection causes colitis characterised by crypt hyperplasia, goblet cell depletion and the presence of transmural inflammatory infiltrate4. In concert with these features, enhanced crypt epithelial cell death is also observed both in *C. rodentium* infected colon and in *E. coli* infected human epithelial cells5–7.

Mitochondria play pivotal roles in cell function, providing most of the cell's energy and participating in the Ca2+, redox and pH homeostasis8,9. Thus, major mitochondrial dysfunction is likely to make the cells more susceptible to factors leading to cell death. Several inter-related mitochondrial pathways regulate cell death processes, mainly by disrupting the mitochondrial respiratory chain resulting in a decrease in adenosine triphosphate (ATP) production; opening the mitochondrial permeability transition pore causing dissipation of membrane potential; release of cytochrome c; alteration of the cell's redox status; and overproduction of reactive oxygen species8,9.

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C. rodentium uses the same machinery as A/E E. coli to infect the host, attaching to the surface of intestinal epithelial cells through formation of a type III secretion system (T3SS)\textsuperscript{1–3}. These bacteria use the T3SS to inject effectors, including the mitochondrion-associated protein (Map) and several virulence factors like EspF, EspG and EspH into host cells\textsuperscript{5,10–12}. EspF and Map are known to translocate into the host mitochondria and are involved in the disruption of normal cellular physiological functions\textsuperscript{11,13–15}. Previous studies have shown that in murine C. rodentium infection, EspF targets mitochondria to initiate the host cell death pathway by alteration of membrane potential and release of cytochrome c into the cytoplasm\textsuperscript{11,13,14,16}. Six days after C. rodentium infection, Map was found co-localised with host mitochondria, concurrent with a decrease in immunohistological staining for succinate dehydrogenase (SDH, complex II)\textsuperscript{15}. However, the effects of C. rodentium on the other mitochondrial respiratory complexes involved in the electron transport chain, complexes I, III and IV, have not been examined.

Direct attachment of bacteria or injection of bacterial effector proteins can thus cause mitochondrial dysfunction of luminal epithelial cells\textsuperscript{13,14,16}, but mitochondrial pathway mediated cell death has also been observed in basal crypt epithelial cells, even though C. rodentium are rarely found at the bottom of the crypt\textsuperscript{5}. This observation raises the possibility that cytokines upregulated during infection play a role in these responses, since cytokines influence mitochondria in other pathological conditions\textsuperscript{17}.

The aim of the present study was to examine the status of mitochondrial enzymes and function during infection and clearance in the murine C. rodentium infection model, and delineate the role of the bacteria per se versus cytokines induced during different time points of the infection, using an in vivo like polarised in vitro epithelial mucosal surface that secretes a mucus layer\textsuperscript{18}. We found mitochondrial dysfunction in the murine colonic epithelial cells following C. rodentium infection, in particular inhibition of complex I and IV of the mitochondrial respiratory chain, and loss of mitochondrial membrane phosphorylation capacity, membrane potential and ATP generation. The in vitro experiments indicated that the mechanism behind the mitochondrial dysfunction involved interferon gamma (IFN\(\gamma\)), tumour necrosis factor alpha (TNF\(\alpha\)) and C. rodentium decreasing complex I and IV quantity and activity through activation of the nitric oxide (NO) pathway. IL-4, overexpressed only during the infection clearance phase, partially abrogates the mitochondrial dysfunction by reducing enhanced NO production, signifying the beneficial role IL-4 might play during infection clearance.

**Results**

**Infection with C. rodentium induced colitis and cell death.** We have previously shown that in C. rodentium infected C57BL/6 mice, the highest pathogen density in the feces is reached around day 10, then starts to decrease at day 14 and finally the infection is cleared (i.e. less than 100 CFU C. rodentium/g feces) around day 19\textsuperscript{19}. We therefore focused on these three time points. Infection with C. rodentium produced features typical of colitis in wild type C57BL/6 (WT) mice (\(P<0.001\), Fig. 1A). On day 10, infected mice had mild overall colitis (Fig. 1A), but marked goblet cell depletion (Fig. 1E). On day 14 and 19 post-infection, there was an increase in crypt length, presence of neutrophils in the lamina propria and goblet cell depletion (Fig. 1B–H). In line with previous studies demonstrating cell death and sloughing of cells during C. rodentium infection\textsuperscript{19}, the presence of the active cleaved form of caspase-3, indicative of apoptosis, increased in both the luminal surface and in the crypts (Fig. 11). The caspase-9-caspase-3 cascade is activated by pro-apoptotic molecules such as cytochrome c released from mitochondria\textsuperscript{20,21}.

**Loss of immunohistological (IHC) staining intensity for mitochondrial respiratory enzyme complex I, II and IV in infected WT mice.** Electron microscopy has previously shown that the mitochondria are located uniformly in non-goblet cells of the colon\textsuperscript{22}. In the full goblet cells, the mucin granulae displaces most of the mitochondria to the rim of the cells, and evacuation of the mucin droplets discloses a rich content of mitochondria spread throughout the cytoplasm\textsuperscript{22}. In line with this, the IHC staining patterns of all four complexes (complex I–IV) were relatively uniform in the majority of the epithelial surface cells, whereas the full goblet cells displayed pale areas where the mucin granulae are present (Fig. 2). During infection (day 10, 14 and 19) with C. rodentium, the intensity of the immunohistochemical staining for complex I, II and IV decreased in the epithelial cells (\(P<0.05\), Fig. 2A,B and D). However, no loss of staining intensity for complex-III was observed (Fig. 2C).

**Infection with C. rodentium caused dysfunction of mitochondrial respiratory enzyme complexes I and IV in infected WT mice.** Next, we investigated if the decrease of staining intensity of mitochondrial complexes reflected their activity. Complex-I activity decreased by 43% during the mid-infection time point at 10 days post-infection, further decreased by day 14 (–59%) and remained low through to day 19 (–61%, \(P<0.001\), Fig. 3A). Similarly, complex-IV activity was reduced during infection (day 10: –37%, day 14: –40%, day 19: –46%, \(P<0.05\), Fig. 3C). No loss of enzymatic activity was observed at any time points for complex-II-III activity (Fig. 3B). In addition to the unchanged complex III protein levels and activity, we did not detect any loss in citrate synthase activity with infection (\(p = 0.6\); control mice: 1.146 ± 0.10 U/mg protein, infected mice day 10: 1.046 ± 0.12 U/mg protein, infected mice day 14: 1.02 ± 0.026 U/mg protein). Together, this indicates that the amount of mitochondria do not decrease\textsuperscript{23}, but that a loss of mitochondrial functionality occurs.
**C. rodentium** infection caused a reduction of phosphorylation capacity, mitochondrial transmembrane potential and ATP generation in infected WT mice. The mitochondrial phosphorylation capacity was decreased by 60% at day 14 post infection, and by 45% at day 19 ($P < 0.01$, Fig. 3D). The mitochondrial transmembrane potential was decreased by at least 40% at all time points of infection ($P < 0.001$, Fig. 3E). Thus, infection impaired most factors important for mitochondrial respiration, and indeed, the ATP generation ability also decreased by up to 47% ($P < 0.01$, Fig. 3F).

Both pro- and anti-inflammatory cytokines are expressed in vivo during **C. rodentium** infection. In order to identify the cytokines that may be impacting mitochondrial function we used an RT-PCR array of Th1/Th2 related genes to examine how the cytokine profile differed between day 10, 14 and 19 post **C. rodentium** infection. IFN-$\gamma$ and IL-12 mRNA were upregulated at all time points, whereas TNF-$\alpha$ and IL-4 upregulation started at day 14 and IL-6 mRNA only increased at day 19 post infection (Table 1). The increased levels of TNF-$\alpha$, IFN-$\gamma$ and IL-12 are in line with a previous study using different time points$^{24}$, and the increased levels of IL-4, TNF-$\alpha$ and IFN-$\gamma$ at day 19 post infection was confirmed using individual RT-PCRs (fold increase mean [range] IL-4 5.9 [2.4–12.6], TNF-$\alpha$ 5.3 [1.3–9.7], IFN-$\gamma$ 5.9 [1.8–9.6]).

**In vitro** treatment with TNF-$\alpha$, individually and in combination with IFN-$\gamma$, caused loss of complexes I and IV, which was alleviated by IL-4. We recently developed a semi-wet interface culture method that in combination with mechanical and chemical stimulation creates an *in vitro* mucosal...
surface with polarised cells, functional tight junctions, a three-dimensional architecture and a mucus layer\textsuperscript{18}. We treated this surface with cytokines for 96 h to mimic the extended period of elevated cytokine stimulus that occurred during the infection (Table 1). Immunohistochemical staining indicated that the levels of complexes I-IV remained largely unaffected by IL-4, IL-6, IL-12 and IFN\(\gamma\) (Fig. 4). Furthermore, no difference in intensity of complex II and complex III staining was observed in any of the other cytokine treatments performed (Fig. 4B,C). In contrast, TNF\(\alpha\) caused a marked loss of complex-I and IV staining intensity (\(P < 0.01\) and \(P < 0.001\), Fig. 4A,D). Combining treatments of TNF\(\alpha\) and IFN\(\gamma\), in analogy with the in vivo cytokine expression during day 14 and 19 post infection, further decreased the intensity of the complex-I and IV staining, but this loss was alleviated by simultaneous treatment with IL-4 (\(P < 0.01\) vs \(P < 0.001\), Fig. 4A,D).

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**Figure 2. Tissue localization and semi-quantification of the mitochondrial respiratory enzyme complexes in the murine distal colon during C. rodentium infection.** Immunohistochemical staining using antibodies for (A) MTND6 (complex I) (B) SDHA (complex-II) (C) CYC1 (complex-III) (D) CCO-Vlc (complex-IV). Statistics: unpaired t test. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\) vs. control, n = 5–6 mice/group. The infection experiments were performed twice, and each time point contain results pooled from 5–6 mice. Scale bar 100\(\mu\)m, magnification \(\times\) 200.
In vitro reduction of the protein levels of complexes I and IV caused by C. rodentium or ETEC infection was alleviated by IL-4. The transepithelial resistance of the in vitro mucosal surface remained unaffected after infection with C. rodentium (pre infection: 226 ± 22 Ω, 24h post infection: 233 ± 23 Ω), indicating that the membranes were intact, although some bacteria had translocated across the membrane and were found in the basolateral compartment. Infection caused loss of staining for complex I and IV (P < 0.001 vs P < 0.001) but not for complex II and III (Fig. 4). IL-4 treatment reversed the infection-induced loss of staining for complex I and IV (P < 0.05, Fig. 4A, D), and IL-6 provided protection against loss of complex IV (P < 0.05, Fig. 4D). To investigate if other intestinal pathogens could have similar effects, we infected the in vitro mucosal surface with enterotoxigenic E. coli (ETEC), a human pathogen that lacks the type III secretion system and do not cause A/E lesions. ETEC infection decreased the transepithelial resistance of the in vitro mucosal membranes (pre infection: 210 ± 51 Ω, 24h post infection: 134 ± 12 Ω), but still very similar results were obtained when ETEC was used as the infecting agent instead (Fig. 5). Together, these results indicate that infection, IFN-γ and TNF-α have negative effects on mitochondrial respiration, which is alleviated by IL-4, while IL-6 afforded some protection.

Figure 3. Mitochondrial function in the murine distal colon after C. rodentium infection. (A) complex I activity (B) complex II-III activity (C) complex IV activity (D) mitochondrial phosphorylation capacity (E) mitochondrial Membrane potential (F) mitochondrial ATP generation. Values are mean ± S.E.M. Statistics: ANOVA with Student Newman-Keuls Multiple Comparison post hoc test: *P < 0.05, **P < 0.01, ***P < 0.001 vs. control. (n = 4–9 mice/group). The infection experiments were performed twice, and each time point contains results pooled from 4–9 mice.
Table 1. Changes in mRNA level of cytokines in wildtype and IFNγ knockout mice infected with C. rodentium. mRNA from two sets of two mice in each group were pooled for the time points of day 0 and day 10 (i.e. data are representative of four mice in each group), whereas the time points day 14 and 19 contained mRNA pooled from three mice in each group. Data are presented as fold change compared to uninfected control mice of the same genotype. Data were normalized by the RT2 Profiler PCR Array data analysis software (QIAGEN) using the housekeeping genes Gusb, Hprt1, Hsp90ab1, Gapdh and Actb. Fold changes ≥2.5 were accepted as upregulation.

| Cytokine | Day 10 wt | Day14 wt | Day19 wt | Day10 IFNγ−/− |
|----------|-----------|----------|----------|---------------|
| IFNγ     | 8.3       | 13.7     | 72.5     | NA            |
| TNFα     | 1.5       | 4.7      | 11.4     | 2.5           |
| IL-12     | 2.7       | 22.7     | 42.9     | 2.2           |
| IL-4      | 1.1       | 6.9      | 4.1      | 2.8           |
| IL-6      | 2.0       | 2.2      | 6.5      | 20.8          |

Figure 4. Semi-quantification of mitochondrial respiratory enzyme complexes of an in vitro intestinal model treated with cytokines and C. rodentium infection. Immunohistochemical staining using antibodies for (A) MTND6 (complex I) (B) SDHA (complex-II) (C) CYC1 (complex-III) (D) CCO-VIc (complex-IV). Values are mean ± S.E.M. Statistics: ANOVA with Student Newman-Keuls Multiple Comparison post hoc test: *P < 0.05, **P < 0.01, ***P < 0.001.
but only against loss of complex-IV. For further mitochondrial functional studies we therefore focused on C. rodentium infection, IFN\(\gamma\), TNF\(\alpha\) and IL-4.

**Effects on enzymatic activity of the mitochondrial respiratory complexes I and IV caused by C. rodentium infection, TNF\(\alpha\) and IFN\(\gamma\), was alleviated by IL-4.** In line with the immunohistochemistry results, complex I-IV activities remained unaffected by IL-4 and IFN\(\gamma\), and complex II and III activities were also not affected by TNF\(\alpha\) and IFN\(\gamma\) treatments (Fig. 6). In contrast, TNF\(\alpha\) reduced complex I and IV activity (−36% and −39%, \(P<0.01\), Fig. 6A,C). Combining treatments of TNF\(\alpha\) and IFN\(\gamma\), in analogy with the in vivo cytokine expression during day 14 and 19 post infection, further decreased complex I and IV activity (−58% and −52%, \(P<0.001\), Fig. 6A,C). IL-4 reversed the combined inhibitory impact of TNF\(\alpha\) and IFN\(\gamma\) on complex I and IV activity (−58% to −28%, \(P<0.01\), Fig. 6A and −52% to −22%, \(P<0.05\), Fig. 6C).

Infection with C. rodentium alone led to decreases in complex I and IV activities that were counteracted by IL-4 (−58% to −13%, \(P<0.05\), and −55% to −14%, \(P<0.001\), Fig. 6A,C). Infection did not exacerbate the reduction of complex I and IV enzymatic activity caused by TNF\(\alpha\) alone or in combination...
Figure 6. Mitochondrial function of an in vitro mucosal intestinal model treated with cytokines and C. rodentium infection. (A) complex-I activity (B) complex-II-III activity (C) complex-IV activity (D) mitochondrial phosphorylation capacity (E) mitochondrial membrane potential (F) mitochondrial ATP generation. Values are mean ± S.E.M. Statistics: ANOVA Student Newman-Keuls Multiple Comparison post hoc test: *P < 0.05, **P < 0.01, ***P < 0.001.
with IFNγ, and IL-4 provided similar protection against the detrimental effects of this combination in the presence of infection (from −68% to −41%, P < 0.05, and −49% to −15%, P < 0.01, Fig. 6A–C). Infection did, however, decrease the enzymatic activity of both of these complexes in cells treated with IFNγ (P < 0.01, Fig. 6A and P < 0.05, Fig. 6C). Complex II–III activity was not affected by infection with or without cytokine treatment.

**In vivo**, IL-4 counteracted the decreases in mitochondrial phosphorylation capacity, transmembrane potential and ATP generation caused by *C. rodentium* infection, TNFα and IFNγ. Reflecting the loss of complex I and IV activity, the mitochondrial phosphorylation capacity was hampered by TNFα and IFNγ both in the absence (−50%, P < 0.01) and presence (−60%, P < 0.001) of *C. rodentium* infection (Fig. 6D). IL-4 alleviated this impairment (P < 0.05) to a degree that it was not statistically different from non-treated mucosal membranes (Fig. 6D). Infection per se, and also in combination with TNFα and IFNγ, caused a reduction in mitochondrial phosphorylation capacity (Fig. 6D). IL-4 alleviated the impairment of the mitochondrial phosphorylation caused by the cytokines and infection, together or alone, to a degree similar to non-treated mucosal membranes (Fig. 6D). The impact of cytokines and *C. rodentium* infection on mitochondrial membrane potential (Fig. 6E) and ATP generation (Fig. 6F) followed a very similar pattern. Thus, IL-4 alleviated the detrimental effect of TNFα and IFNγ on ATP generation in both uninfected (from −38% to −11%, P < 0.05) and infected (from −60% to −14%, P < 0.001, Fig. 6F) conditions, and negated the direct impact of *C. rodentium* infection, reviving the mitochondrial ATP generation from 58% to 85% (P < 0.05).

**In vivo**, the levels of complex I and IV are more affected by the cytokine environment than by pathogen density. To elucidate the role of the cytokine environment versus the direct actions of *C. rodentium in vivo*, we studied IFNγ−/− mice, as IFNγ increased early in infection, concomitantly with the mitochondrial dysfunction (in contrast to TNFα). IFNγ−/− mice had a similar *C. rodentium* burden to that of the WT mice at day 10 post infection (mean ± SEM: Log 6.5 ± 0.2 CFU/g feces for IFNγ−/− and Log 6.6 ± 0.3 CFU/g feces for WT, n = 7) while at day 14 post infection the density was slightly higher in the IFNγ−/− mice (P < 0.05, Log 4.8 ± 0.3 CFU/g feces) than in the WT (mean Log 3.4 ± 0.3 CFU/g feces) mice. The cytokine environment during the course of infection was different in IFNγ−/− compared to WT mice, mainly with regards to that IL-4 and IL-6 were upregulated already by day 10 post infection (3-fold vs 20-fold, Table 1).

All four complexes (complex I–IV) were present relatively uniformly in the majority of the epithelial cells in the colon of IFNγ−/− mice, with a similar tissue location and staining intensity as in the WT mice (compare the non-infected controls in Figs 2 and 7). In contrast to the loss of staining intensity of subunits of complex I and IV found in colons from WT mice after infection (Fig. 2), there was no statistically significant loss of any of the four complexes in IFNγ−/− mice (Fig. 7A–D). Thus, it appears that *in vivo*, the cytokine environment is a more important determinant of mitochondrial complex levels than the direct actions of the pathogen. This is further supported by the observation that in the WT mice, the mitochondrial respiratory chain remained impaired even at day 19 post infection, when the pathogen burden had subsided, but the expression of TNFα and IFNγ remained elevated (Figs 2 and 3). Although the caspase-3 levels in the colonic tissue from IFNγ mice were slightly elevated at day 14 post infection (3-fold vs 20-fold, Table 1), these results are not surprising since the higher level of the NO 2− generated in WT mice in all time points post-infection (P < 0.001, Fig. 8B). However, the levels of 3-NT were higher in WT mice compared to IFNγ−/− mice, with the day 14 and 19 timepoints in the WT having intensity scores twice as high as the IFNγ−/− mice (P < 0.05, Fig. 8B). Since IFNγ−/− mice had a similar pathogen burden, but a cytokine environment without IFNγ but higher in IL-4 and IL-6, this suggests that the cytokine environment, and not the pathogen burden, is the main cause of the NO generation *in vivo*.

**In vivo**, the cytokine environment, and not the *C. rodentium* density, determine the level of 3-Nitrotyrosine. Infection with *C. rodentium* resulted in an increase in immunostaining intensity for 3-Nitrotyrosine (3-NT), a marker for oxidative damage, in both infected WT and IFNγ−/− mice at all time points post-infection (P < 0.05–0.0001, Fig. 8B). However, the levels of 3-NT were higher in WT mice compared to IFNγ−/− mice, with the day 14 and 19 timepoints in the WT having intensity scores twice as high as the IFNγ−/− mice (P < 0.05, Fig. 8B). Since IFNγ−/− mice had a similar pathogen burden, but a cytokine environment without IFNγ but higher in IL-4 and IL-6, this suggests that the cytokine environment, and not the pathogen burden, is the main cause of the NO generation *in vivo*.

**In vivo** and *in vitro*, NO generation increased during *C. rodentium* infection and increased levels of TNFα and IFNγ, which was counteracted by IL-4. In line with the above results, infection with *C. rodentium* resulted in increased generation of NO 2−, another index for oxidative damage, in WT mice in all time points post-infection (P < 0.001, Fig. 8C). In the non-infected *in vitro* mucosal membranes, the combined action of IFNγ and TNFα led to the highest generation of NO 2− (P < 0.001, Fig. 8D), and this was alleviated by IL-4 treatment (P < 0.05, Fig. 8D). *In vitro* infection further increased the generation of NO 2−, and IL-4 alleviated both the NO 2− generation-induced by the bacteria alone (P < 0.01, Fig. 8D) and by the combined actions of infection, IFNγ and TNFα (P < 0.01, Fig. 8D). Although the higher level of the NO 2− generation in the infected *in vitro* membranes may at first glance appear to contradict the *in vivo* results demonstrating that the cytokine environment is a more important determinant of NO-levels than the pathogen density *in vivo*, these results are not surprising since...
in vitro, C. rodentium multiplies unhindered, and the bacterial density after 24 h of co-culture is higher than in vivo.

**NO generation inversely correlated with mitochondrial function.** In C. rodentium infected mice, the level of NO\textsubscript{2}\textsuperscript{−} inversely correlated with complex I and complex-IV activities (Pearson product-moment correlation coefficient $r^2 = -0.823$, $p < 0.01$ and $r^2 = -0.714$, $p < 0.01$, respectively), mitochondrial phosphorylation ($r^2 = -0.846$, $p < 0.01$), mitochondrial membrane potential ($r^2 = -0.735$, $p < 0.01$) and ATP generation ($r^2 = -0.669$, $p < 0.01$, compare Figs 8C and 3). Similarly, after in vitro cytokine treatment and C. rodentium infection, the level of NO\textsubscript{2}\textsuperscript{−} inversely correlated with complex I and complex-IV activities ($r^2 = -0.851$, $p < 0.01$ and $r^2 = -0.733$, $p < 0.01$ respectively), mitochondrial phosphorylation ($r^2 = -0.744$, $p < 0.01$), mitochondrial membrane potential ($r^2 = -0.782$, $p < 0.01$) and ATP generation ($r^2 = -0.829$, $p < 0.01$, Fig. 8D compared with Fig. 6).

**In vivo and in vitro, mitoquinone (MitoQ) alleviated the damaging impact on mitochondrial function during C. rodentium infection.** MitoQ is an antioxidant that accumulates within mitochondria, and that has been used in clinical trials in humans\textsuperscript{25}. Treating mice with established infection with MitoQ (from day 5 to 14) restored the complex-I and complex-IV activities, mitochondrial phosphorylation, membrane potential and ATP generation (Fig. 9A,B). Furthermore, MitoQ treatment reverted the infection-induced 3-nitrotyrosine staining ($p < 0.05$, Fig. 9C). In line with these results, MitoQ also alleviated the damaging influence of infection, TNF\textsubscript{α} and IFN\textsubscript{γ} on these parameters in vitro (Fig. 10A–F).

Although treatment with MitoQ restored all of the mitochondrial parameters, not all features of the disease were improved. Crypt architecture and tissue damage improved ($P < 0.05$ for both, Fig. 9D), the caspase-3 levels decreased to an extent where it was not statistically different from uninfected controls, and the goblet cell depletion trended towards a decrease ($p = 0.077$, Fig. 9C–F). However, the number of C. rodentium in feces and spleen was similar to that of infected mice without MitoQ treatment (Fig. 9G).

**Discussion**

In the present study, we demonstrate for the first time that infection reduces mitochondrial complex I and IV protein levels and enzymatic activity and also phosphorylation capacity, transmembrane potential
Figure 8. Caspase-3, 3-NT staining scores and nitrite measurements after C. rodentium infection. (A) The caspase-3 staining scores based on immunohistochemical staining and corresponding representative photos of the caspase-3 tissue localization in the distal colon of WT and IFN-γ−/− mice. Values are mean ± S.E.M. Statistics: ANOVA with Newman-Keuls Multiple Comparison post hoc test, *P < 0.05 vs. corresponding non-infected genotype control, unpaired t test **P < 0.01 compared to WT of same infection period (n = 4–9 mice/group). Scale bar 50μm, magnification × 400. (B) Immunohistochemical staining scores and photos representing the tissue localization of the 3-NT residues in the murine distal colons of WT and IFN-γ−/− mice during infection. Scale bar 50μm, magnification × 400. Statistics: ANOVA with Student Newman-Keuls Multiple Comparison post hoc test: #P < 0.05 vs. corresponding genotype control, unpaired t test: *P < 0.05, **P < 0.01, ***P < 0.001 vs. WT at the same time point of infection, n = 4–9 mice/group. (C) Nitrite concentration in the murine distal colon after C. rodentium infection. Statistics: ANOVA with Student Newman-Keuls Multiple Comparison post hoc test: *P < 0.05, **P < 0.01, ***P < 0.001 vs. control. (D) Nitrite concentration in the in vitro mucosal intestinal model treated with cytokines and C. rodentium infection. Statistics: ANOVA with Student Newman-Keuls Multiple Comparison post hoc test: *P < 0.05, **P < 0.01, ***P < 0.001.
and ATP generation throughout infection and clearance of the pathogen. While previous studies have shown that bacteria can affect mitochondrial function, the endpoints examined were a loss of mitochondrial membrane potential or involvement in apoptosis induction; only one study went further and looked at complex II expression after infection. Using an in vivo-like in vitro mucosal surface, we identified that infection per se, as well as TNFα, individually and more severely in combination with IFNγ, caused the same effects as seen in vivo. Co-treatment with IL-4, however, reversed these responses, and IL-6 also protected against loss of complex IV. The negative effects on mitochondria were caused largely by NO generation, and were reversed by mitochondrial antioxidant treatment. IFNγ−/− mice, which had a similar pathogen burden as infected WT mice, but a colonic cytokine environment with higher levels of IL-4 and IL-6, displayed no loss of any of the four complexes, demonstrating that the effects on mitochondria found in vivo were largely cytokine driven. Thus, as the concentration of IFNγ and TNFα increase during the latter time points of infection and clearance in WT mice, the concomitant increase of IL-4 and IL-6 appears to protect the mitochondrial functions of the colonic epithelium.

Of the four multimeric complexes involved in mitochondrial respiratory phosphorylation, inhibition in both levels and activities of complex I and complex IV were observed at all post-infection time points. Although the extraction process may stress the mitochondria and therefore possibly alter their function,

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**Figure 9. Effects of MitoQ on mitochondrial functions, caspase-3, NO, colitis and C. rodentium translocation.** (A) complex I, II–III and IV activities and (B) mitochondrial phosphorylation capacity, membrane potential and mitochondrial ATP generation in infected and MitoQ treated mice. Values are mean ± S.E.M. Statistics: ANOVA with Student Newman-Keuls Multiple Comparison post hoc test: *P < 0.05, **P < 0.01, ***P < 0.001 (n = 4/group). (C) caspase-3 and 3-Nitrotyrosine immunostaining in infected and MitoQ treated mice. Values are mean ± S.E.M. Statistics: ANOVA with Student Newman-Keuls Multiple Comparison post hoc test: *P < 0.05 vs non-infected control, **P < 0.05 vs. infected control. (D) colonic scores of crypt architecture, tissue damage and goblet cell depletion, (E) Neutrophils in lamina propria and inflammatory cell infiltration, (F) crypt length and (G) C. rodentium counts in spleen of infected and MitoQ treated mice. Values are mean ± S.E.M. (n = 4 mice/group). Statistics: paired t test, *P < 0.05 compared to infected mice.
Figure 10. Effects of MitoQ on mitochondrial functions in the *in vitro* mucosal intestinal model treated cytokines with/without *C. rodentium* infection. (A) complex-I activity, (B) complex-II–III activity, (C) complex-IV activity, (D) mitochondrial phosphorylation capacity, (E) mitochondrial membrane potential, and (F) mitochondrial ATP generation. Values are mean ± S.E.M. Statistics: ANOVA with Student Newman-Keuls Multiple Comparison post hoc test: *P* < 0.05, **P** < 0.01, ***P** < 0.001.
we here provide two lines of evidence that support the results, whereof the first does not involve extraction: in situ quantification using immunohistochemistry (showing decreased levels of complex I and IV) and functional assays (demonstrating decreases in the activity of complexes I and IV, phosphorylation capacity, transmembrane potential and ATP generation). No inhibition in activity of complex II–III was noticed at any time-points. However, we did find a reduction in immunohistochemical staining intensity for complex II, which was in agreement with the earlier study21. This decrease in quantity while simultaneously retaining normal specific activity of succinate cytochrome c reductase (indicator for complex II–III activity) post-infection is puzzling. A reason for this discrepancy may be that the method adapted for measuring the activity of succinate cytochrome c reductase measures the enzymatic activity of both complex II and III, thus it is possible that normal complex III activity can mask the inhibition of complex II activity, although the method here is widely used and demonstrated to be suitable to detect complex II deficiency26. Another reason could be that a decrease in the level of enzyme can be compensated for by an increase in activity26.

Targeting of the host cell mitochondria appears to be a common strategy among many clinically important pathogens. Bacteria like Neisseria gonorrhoeae27, Neisseria meningitides28, Helicobacter pylori29 and Salmonella enterica serovar Typhimurium30, all target host cell mitochondria by translocating proteins that trigger cell death, mainly through apoptosis. Similarly, one report indicated involvement of C. rodentium effector proteins in causing cell death through affecting mitochondrial membrane potential and succinate dehydrogenase levels31. Cell death at the basal of the colonic crypts, where the presence of C. rodentium is unlikely, has also been reported32, and our caspase-3 staining results further confirmed a similar incidence of cell death both on the luminal surface and in the crypts during infection. As cytokines have the capability to regulate epithelial cell function irrespective of the position of the cells in the crypt, we investigated the impact of cytokines that changed expression after infection, on mitochondrial function. Treatment with TNFα in vitro decreased mitochondrial functional parameters and the levels and activity of the complex I and IV enzymes, and when combined with IFNγ had an even greater effect. Even though IFNγ is known to be an immunoregulatory cytokine promoting immune responses at the initiation of several bacterial infections33–34, the impact of IFNγ on mitochondrial function during infections has not been described previously. TNFα, alone as well as together with IFNγ, has previously been shown to affect mitochondrial function in non-intestinal tissues under different experimental conditions35–39.

The protective effects of IL-4 on complex I and IV activity and levels that we observed in vitro is consistent with its previously observed ability to abrogate cell death by maintaining mitochondrial membrane potential and anti-oxidant status in other systems, such as B-cells40. To conclusively prove that IL-4 and IL-6 provide the same function during infection in vivo is more complicated, as changing their levels alter a whole range of infection related parameters. Indeed, IL-6 deficient mice have been shown to have high mortality and 100-fold higher pathogen burdens compared to WT mice during C. rodentium infection41. However, our results from IFNγ−/− mice supports our proposal that IL-4 and IL-6 protects the mitochondria during infection as these mice, which had higher levels of IL-4 and IL-6 but a similar pathogen burden to WT mice, had no reduction of the levels of any of the four complexes during C. rodentium infection, and greatly reduced NO generation. As loss of mitochondrial ATP generation is the consequence of dysfunction of the mitochondrial respiratory chain, these results are in line with our observation in WT animals that there are high levels of caspase-3 staining, as well as high numbers of dead and sloughed off cells during clearance of infection even at day 19 post infection, when the pathogen burden is almost entirely absent42, but the expression of IFNγ and TNFα remains high. In WT mice, when the concentrations of IFNγ and TNFα increase during clearance of infection, the concurrent induction of IL-4 and IL-6 thus appears to protect the mitochondrial function of the colonic epithelium from further damage.

Our results suggest that NO generation caused by infection-induced TNFα and IFNγ was the main cause of mitochondrial dysfunction. The dysfunction was reversed by IL-4 treatment, indicating that IL-4 has a role in regulating NO production. That the NO pathway plays a substantial role in mitochondrial dysfunction is further verified by the protective effect of the antioxidant MitoQ, which is a scavenger for the peroxynitrite (ONOO−) that is generated when NO reacts with superoxide (O2−)43. Furthermore, a previous study has shown that C. rodentium infection results in up-regulation of iNOS in the colonic epithelium in vivo, and that iNOS−/− mice are protected from C. rodentium induced inflammation, including attenuated levels of TNFα and IFNγ44. Oxidative insult to the respiratory chain complexes can amplify and promote further oxidative damage45, and indeed mitochondrial complexes I and IV, which were most effected in our study, are encoded in the mitochondrial genome46 and thereby susceptible to mutations in mitochondrial DNA.

In spite of less epithelial damage after MitoQ treatment and similar levels of C. rodentium in colon, the number of C. rodentium in the spleen did not improve. However, the number of CFU that are found in the internal organs during this infection is rather low, and possibly the ones that enter do so through mechanisms other than direct translocation across a damaged epithelial membrane. A similar mitochondria targeting antioxidant (MitoTEMPO) was recently shown to inhibit superoxide induced E. coli translocation over mucosal membranes in vitro46. To elucidate if antioxidants with protective effects towards mitochondria could have a role in treating infections, an in vivo model where bacterial translocation over the epithelium plays a more prominent role in progression of disease needs to be utilised.
In conclusion, infection with this A/E pathogen induces mitochondrial dysfunction, which is largely caused by IFNγ and TNFα synergistically compromising complex I and IV levels and activity, via NO generation. In vitro, the pathogen per se also induces a similar effect, but in vivo, the cytokine environment appears to be the dominating factor governing mitochondrial enzyme levels. The presence of both IL-4 and IL-6 during the clearance phase of infection, when TNFα and IFNγ levels are exceedingly high, protects the colonic epithelial surface against more detrimental damage.

Methods

Animals. 6–8 weeks old, specific-pathogen-free, male C57BL/6 mice purchased from Taconic (England) or Charles River (Germany). IFNγ-deficient mice on a C57BL/6 background were bred and housed in ventilated cages under pathogen-free conditions at the Laboratory for Experimental Biomedicine (EBM), Sahlgrenska Academy, Gothenburg. Mice were fed ad libitum and monitored daily.

Ethics statement. All experimental procedures were approved, and performed in accordance with, the guidelines laid by the Göteborgs Djurförsöksstämman (Ethic No. 261/09) based on regulations from Djurförsöksförordningen DFS 2004:4.

Culture of the in vitro colonic mucosal model. For propagation, the human intestinal cell line HT29 MTX-E12 was cultured (at 37°C, 5% CO2 − 95% air) in RPMI containing 10% (v/v) FCS, 1% 5000 U (v/v) penicillin-streptomycin (Lonza). To form the in vitro colonic mucosal surface, 7.5 × 10^4 cells in 200 μl of RPMI containing 10% (v/v) FCS and penicillin-streptomycin were added to the apical side of Snapwell membranes (0.4 mm pores) with 12 mm diameter (Corning). When cells became confluent (4–6 days later) they were subjected to semi-wet interface culture with continuous rocking, with 2 ml media in the basolateral compartment and 50 μl of media in the apical compartment for 28 days. Basolateral media was refreshed every two days and for the first 6 days it was supplemented with 10 mM N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester (DAPT, Sigma-Aldrich).

Infection and treatments. Mice: C. rodentium strain ICC169 was grown on MacConkey agar (Oxoid) for 20 h at 37°C. Male C57BL/6 wild type and IFNγ−/− mice were infected with 100 μl of bacterial suspension (5 × 10^8 colony forming units (CFU) in Luria-Bertani (LB) broth) by oral gavage. Infection experiments were performed twice for each time point. MitoQ treatment: infected mice were administered MitoQ orally through drinking water (500 μM) from day 5 to 14 of infection period. This dose of MitoQ was chosen as it was not toxic in an earlier study. Mice were anaesthetised with isoflurane and killed by cervical dislocation at day 10, 14 and 19 post infection. The last 2.5 cm of colon, with cytokines for 96 h, starting on day 28 post confluency. The 96 h duration treatment was to mimic the guidelines laid by the Göteborgs Djurförsöksstämman (Ethic No. 261/09) based on regulations from Djurförsöksförordningen DFS 2004:4. For propagation, the human intestinal cell line HT29 MTX-E12 was cultured (at 37°C, 5% CO2 − 95% air) in RPMI containing 10% (v/v) FCS, 1% 5000 U (v/v) penicillin-streptomycin (Lonza). To form the in vitro colonic mucosal surface, 7.5 × 10^4 cells in 200 μl of RPMI containing 10% (v/v) FCS and penicillin-streptomycin were added to the apical side of Snapwell membranes (0.4 mm pores) with 12 mm diameter (Corning). When cells became confluent (4–6 days later) they were subjected to semi-wet interface culture with continuous rocking, with 2 ml media in the basolateral compartment and 50 μl of media in the apical compartment for 28 days. Basolateral media was refreshed every two days and for the first 6 days it was supplemented with 10 mM N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester (DAPT, Sigma-Aldrich).

In vitro cytokine treatments and infection: In vitro mucosal surfaces (described above) were treated with cytokines for 96 h, starting on day 28 post confluence. The 96 h duration treatment was to mimic physiological conditions during infection, as colon epithelial cells were exposed to elevated cytokine levels for days (table 1). The cultures were exposed individually and in combination with IFNγ (10 ng/ml), TNFα (10 ng/ml), IL-4 (1.5 ng/ml), IL-6 (15 ng/ml) and IL-12 (20 ng/ml); these concentrations were based on previous work. For MitoQ treatment, 50 nM was added to the basolateral side for 96 h, the dose was based on previous work. Antibiotic-free RPMI containing 10% FBS, cytokines and/or MitoQ was changed every 24 h. For infection, 10 μl of C. rodentium and enterotoxigenic E. coli (ETEC, strain E2265) suspensions with a respective OD of 2.0 and 0.1 at 410 nm (CFU: 10^7 and 5 × 10^4 CFU, corresponding to a multiplicity of infection of 10:1 and 0.5:1 ) in sterile PBS was added to the apical side of the membrane 24 h prior to the experimental end point. Although the epithelial surface is exposed to bacteria for days in vivo, this was not technically possible in vitro, due to overgrowth of the bacteria. To monitor the effects of infection on the membranes, Trans Epithelial Electrical Resistance (TEER) was measured using an EVOM2 meter and STX2 probe (World Precision Instruments, Sarasota, Florida, USA).

Histology For colitis analysis in infected mice, 5 μm sections of Carnoy’s fixed tissue were stained with haematoxylin/eosin, coded to blind the analysis, and the entire section was systematically scored for: aberrant crypt architecture (0–3), tissue damage (0–3), goblet cell depletion (0–3, confirmed by PAS/Alcian blue stain), lamina propria neutrophil counts (0–3), crypt abscesses (0–3) and inflammatory cell infiltration (0–3).

RT-PCR for cytokines. For the quantitative RT-PCR cytokine array, total RNA was extracted from distal colon using the RNaseq mini kit (QIAGEN), and cDNA prepared using the QuantiTect Reverse Transcription kit (QIAGEN). mRNA from two sets of two mice in each group were pooled for the time points of day 0 and day 10 (i.e. data representative of four mice in each group), while time points day...
In vivo mucosal surface. The method of Modica-Napolitano et al. 1989\(^\text{58}\) with minor modifications was used: Cells from 12 membranes, pooled into 3 replicates (4 membranes per sample) per combination
Assessing mitochondrial respiratory enzyme complex activities. The complex activities in the mitochondrial fractions were measured following standard protocols: NADH-ferricyanide reductase (complex-I) activity was measured following the method of Hatefi79 using ferricyanide as the electron acceptor in a system containing 0.17 mM NADH, 0.6 mM ferricyanide, and Triton X-100 (0.1% v/v) in 50 mM phosphate buffer, pH 7.4 at 30 °C. The reaction was initiated with addition of the mitochondrial suspension (10 μg protein), and the rate of oxidation of NADH was measured by the decrease in absorbance at 340 nm.

The activity of succinate cytochrome c reductase (complex II–III) was assayed by following the succinate supported reduction of ferricytochrome c to ferrocytochrome c at 550 nm in an assay mixture containing 100 mM phosphate buffer, 2 mM succinate, 1 mM KCN, 0.3 mM EDTA and 1.2 mg/ml cytochrome c (Sigma-Aldrich) in a total volume of 0.4 ml60. The reaction was initiated by adding mitochondrial suspension (10 μg protein) to the sample cuvette. The same assay was repeated with (10 μM) antimycin (Sigma-Aldrich) to determine the inhibitor sensitive rate and the results were expressed as nmoles of cytochrome c reduced/min/mg protein.

The activity of cytochrome c oxidase (complex IV) was assayed by measuring the rate of decrease of absorbance at 550 nm at room temperature following the oxidation of reduced cytochrome c (50 μM) in 10 mM phosphate buffer, pH 7.461. Ferricyanide (1 mM) was added to oxidize ferrocytochrome c in the blank cuvette and the reaction initiated in the sample cuvette by the addition of mitochondrial suspension (10 μg). The activity of the enzyme was expressed as nmoles of cytochrome c oxidised/min/mg protein.

Measurement of mitochondrial phosphorylation capacity. Phosphate utilization was assayed following a method published previously62. In a total volume of 250 μl, an aliquot of 25 μl mitochondrial suspension was diluted into a medium containing 125 mM KCl, 75 mM sucrose, 0.1 mM EGTA, 1 mM MgCl₂, 2 mM HEPES, 2 mM phosphate, 0.3% BSA, 0.5 mM ADP (Sigma-Aldrich), 5 mM pyruvate, 10 mM succinate and 10 mM glucose, followed by immediate addition of 5 units of hexokinase (Sigma-Aldrich) and incubation at 37 °C for 30 min. The reaction was terminated by addition of 5% ice cold trichloroacetic acid (TCA) and the amount of inorganic phosphate was measured spectrophotometrically.

ATP synthesis. ATP content was measured in aliquots of mitochondrial suspension by a colorimetric assay method using the phosphorylation of glycerol to generate a quantifiable product at 570 nm using a commercial ATP assay kit (ab83355, Abcam).

Measurement of mitochondrial transmembrane potential. Murine colon: aliquots of mitochondrial suspensions were incubated at 37°C for 30 min in isotonic buffer A containing 10 mM pyruvate, 10 mM succinate and 1 mM ADP with 5 μM JC-1 (5,5′,6,6′-tetraethylbenzimidazolylcarbocyanine iodide, CS0760, Sigma-Aldrich). After incubation, the dyed mitochondria were collected by centrifugation, washed with isotonic buffer A to remove excess dye and resuspended in the same buffer in appropriate dilution, followed by measurement of fluorescence intensity (λex 490 nm, λem 590 nm). In vitro mucosal surface: Mitochondrial transmembrane potential in intact HT29 MTX-E12 cells was measured using a tetramethylrhodamine ethyl ester (TMRE) mitochondrial membrane potential assay kit (ab113852, Abcam) following the manufacturer’s instructions.

Measurement of citrate synthase activity. The activity was determined spectrophotometrically at 30°C according to the method of Sere (1969)63. The assay medium consisted of 0.1 M tris-HCl (pH-8.5), 0.1 mM 5-dithiois-2- nitrobenzoic acid (DTNB), acetyl CoA, 500 μM oxaloacetic acid, and a mitochondrial suspension (10 μg protein) in a total volume of 1 ml. As citrate synthase irreversibly catalyzes the reaction CoA-SH + DTNB → TTNB + CoA-S-S-TNB, the readout product used was thionitrobenzoic acid (TNB) which can be quantified by absorption at 412 nm. One unit of enzyme is defined as 1 μmol of oxaloacetate utilized/min per mg of protein.
Measurement of total nitrite release. Nitrite levels were determined in the cell free supernatant obtained from cell and tissue homogenate (see mitochondria isolation section). Nitrate reductase (ab156629, Abcam) was used to convert nitrates to nitrites and the rest of the procedure was adapted from the Griess reagent kit (G-7921, Life Technologies).

Protein concentration. Estimated after solubilizing the samples in 1% SDS following the method of Lowry24.

Statistical analysis. All tests were performed using Prism (GraphPad Software, version 3.0) or SPSS statistics 18 (IBM). Values are expressed as mean ± S.E.M. Comparison of data between control and infected at a specific time-point was made using the unpaired t test. Differences were considered significantly different if P was <0.05. One-way analysis of variance (ANOVA) with Student Newman-Keuls Multiple Comparison test was used to compare data for more than 2 experimental groups. Normality was confirmed using the Kolmogorov-Smirnoff test, and homoscedasticity was confirmed using the Bartlett’s test. Only one data set failed the test (Fig. 2D), but passed these tests after Log10 transformation. For Figs 5 and 9, the number of n in each treatment group was too small (n=3) to perform these tests, however, overall the data followed a normal distribution, and in experiments with several treatments and balanced data, heterogeneous variances do not noticeably increase the risk of Type I error25. All aspects considered, we decided One-Way ANOVA followed by post hoc testing using Student Newman-Keuls test was the most appropriate way to treat the data since One-Way ANOVA is a robust statistical test if sample sizes are similar. The Pearson product-moment correlation coefficient was used for analyzing correlations.

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Author Contributions
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