A non-death function of the mitochondrial apoptosis apparatus in immunity

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Abstract

Apoptosis is a frequent form of programmed cell death, but the apoptotic signaling pathway can also be engaged at a low level, in the absence of cell death. We here report that such sub-lethal engagement of mitochondrial apoptosis signaling causes the secretion of cytokines from human epithelial cells in a process controlled by the Bcl-2 family of proteins. We further show that sub-lethal signaling of the mitochondrial apoptosis pathway is initiated by infections with all tested viral, bacterial, and protozoan pathogens and causes damage to the genomic DNA. Epithelial cells infected with these pathogens secreted cytokines, and this cytokine secretion upon microbial infection was substantially reduced if mitochondrial sub-lethal apoptosis signaling was blocked. In the absence of mitochondrial pro-apoptotic signaling, the ability of epithelial cells to restrict intracellular bacterial growth was impaired. Triggering of the mitochondrial apoptosis apparatus thus not only causes apoptosis but also has an independent role in immune defense.

Keywords apoptosis; cell-autonomous immunity; immune recognition; infection; minority MOMP

Subject Categories Autophagy & Cell Death; Immunology

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Introduction

The function of apoptosis is cell-autonomous killing (Vaux *et al*, 1994). Apoptosis mostly proceeds through mitochondria: The outer mitochondrial membrane is permeabilized, releasing cytochrome *c* to activate cytosolic caspases, which execute apoptosis through proteolysis of numerous substrates (Hengartner, 2000). Mitochondrial apoptosis is determined by the Bcl-2 protein family. Two Bcl-2

family members, Bax and Bak, act as effectors that upon activation cause the release of cytochrome *c*. The anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-X_L inhibit the activation of Bax/Bak (Chipuk *et al*, 2010).

Two recent observations have expanded our understanding of apoptosis. First, a cell undergoing apoptosis can react to the release of mitochondrial components. Especially mitochondrial DNA (mtDNA) can be released during later stages of apoptosis (McArthur *et al*, 2018; Riley *et al*, 2018), and mammalian cells can react to this release with the secretion of cytokines. However, this is only seen if caspase activity is prevented, suggesting that caspases can turn cell activation off (Rongvaux *et al*, 2014; White *et al*, 2014; Giampazolias *et al*, 2017). Since caspases are generally activated upon mitochondrial permeabilization, the relevance of this is not obvious.

Secondly, apoptosis signaling may be initiated at a low level, in the absence of cell death. Only few mitochondria are permeabilized, and small amounts of cytochrome *c* are released, causing only limited caspase activation. Apoptosis here appears to be triggered but then aborted before the point of no return. The process has been termed minority MOMP (Ichim *et al*, 2015), and the cell stays alive and can presumably repair any damage caused (Sun *et al*, 2017).

To us, these two independent observations indicated the possibility that low-level (sub-lethal) “apoptosis” signaling (minority MOMP) can trigger cytokine secretion, causing inflammation and immune alert. Full apoptosis activates caspases that counteract this immune function. The small amounts of caspase activated during sub-lethal signaling following minority MOMP may however be too low to turn the signal off, resulting in cytokine secretion and immune activation. We tested this possibility and report here that indeed human cells can react to low-level apoptosis induction with cytokine secretion. We then show that minority MOMP (or a process akin to minority MOMP) occurs during microbial infection with viruses, intracellular bacteria, and a protozoan parasite. Infection-associated minority MOMP contributed to cytokine secretion as well as to cell-autonomous immunity. The results suggest that mitochondria have, in addition to their functions in metabolism and apoptosis, a function in immune alert, which is orchestrated by the mitochondrial apoptosis pathway.

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Results

A small pro-apoptotic stimulus can cause the secretion of cytokines from human cells through activation of mitochondrial apoptosis signaling

We first tested whether sub-lethal apoptosis could cause the secretion of cytokines using the small-molecule Bcl-2 family antagonist, ABT-737 (Oltersdorf *et al*, 2005). Because it inhibits not all anti-apoptotic Bcl-2-like proteins (in particular sparing Mcl-1), ABT-737 is only a weak pro-apoptotic stimulus but can induce minority MOMP (Ichim *et al*, 2015). Starting at 48 h of stimulation, secretion of IL-6 was observed (Fig EV1A), and substantial amounts of IL-6 were detected after 72 h (Fig 1A). Cytokine secretion was not seen in HeLa cells lacking the two pro-apoptotic Bcl-2 family members Bax and Bak, essential mediators of mitochondrial apoptosis (Figs 1A and EV1A). Over-expression of the inhibitor of mitochondrial apoptosis, Bcl-X_L, which is poorly inhibited by ABT-737 in intact cells (Merino *et al*, 2012), likewise blocked IL-6 secretion (Fig 1A). Bax/Bak-dependent secretion of the chemokines IL-8 and CXCL1 as well as fibroblast growth factor 2 (FGF-2) was also observed (Fig 1B–D). At higher concentrations, ABT-737 induced apoptosis in some cells, but little apoptosis occurred between 48 and 72 h of treatment (Fig EV1B), when most cytokine was produced, suggesting that the secreting cells did not undergo apoptosis. Non-apoptotic cell death was not observed during ABT-737 treatment (Fig EV1D). To test whether dead cells stimulated IL-6 secretion from bystander cells, we incubated HeLa cells with lysates generated by freeze-thawing HeLa cells. No secretion of IL-6 was detected under these circumstances (Fig EV1E). Treatment with the Mcl-1 inhibitor S63845 (Kotschy *et al*, 2016) also induced Bax/Bak-dependent secretion of IL-6 (Fig 1E). ABT-737 also triggered IL-6 secretion from human melanoma cells (Figs 1F and EV1C) at concentrations where apoptosis induction was low (Fig 1G). Higher concentrations of ABT-737 induced apoptosis in 1205Lu melanoma cells, and less IL-6 secretion was observed (Fig 1F and G); this is consistent with the model that higher amounts of active caspases counteract cytokine secretion. Little IL-6 secretion was seen in melanoma cells where apoptosis signaling was inhibited by Bcl-X_L (Fig 1F and G). Mitochondrial DNA (mtDNA) may, when released into the cytosol (McArthur *et al*, 2018), trigger a cytokine response through the cyclic GMP-AMP synthase (cGAS) and the stimulator of interferon genes (STING) (Rongvaux *et al*, 2014; White *et al*, 2014). We therefore generated a HeLa cell line where mtDNA was degraded upon tamoxifen-regulated expression of a viral DNase (HSV-1 UL 12.5) targeted to mitochondria (Corcoran *et al*, 2009) (Fig 1H). When these cells were treated with ABT-737, they produced significantly less IL-6 (Fig 1I), implicating mtDNA in cellular activation. Further, STING-deficient cells were unable to produce IL-6 (Fig 1A) when treated with ABT-737. This suggests that ABT-737, very likely in the absence of cell death, induces the secretion of cytokines from human cells in a process that depends on mtDNA and signaling through STING.

Minority MOMP is induced by microbial infection

Low-level activation of Bax or Bak, causing minority MOMP, can thus activate a cell to secrete cytokines even in the presence of

caspases. Strong pro-apoptotic stimuli will not induce cytokine secretion because sufficient caspase activity is generated to counteract this cell activation. However, small injuries to a cell may cause only minority MOMP, and the cell will respond with cytokine secretion. This observation opens the possibility that the stimulatory potential of mitochondria may have a physiological function. There are several potential biological functions of minority MOMP-associated cell stimulation. The mitochondrial apoptosis system can be triggered by numerous stimuli and changes in condition. Minority MOMP, with the associated cell activation, may therefore be a sensitive reporter of cellular stress or injury. One potentially important function is in pathogen recognition. Many pathogens first infect non-professional immune cells such as epithelial cells or fibroblasts, which have the capacity to alert and attract professional immune cells through soluble mediators. High levels of apoptosis are not typically seen when human cells are infected with pathogens. However, many pathogens have some pro-apoptotic potential (Hacker, 2017). It therefore seemed a plausible hypothesis that pathogens may cause sub-lethal activation of the apoptosis machinery, resulting in minority MOMP and cell stimulation, detectable as cytokine secretion.

Apoptosis involves the activation of caspases. Using standard assays, we detected caspase activity in only few cells during infection of HeLa cells with various pathogens (see below). This lack of pronounced caspase activation indeed seems a general feature of minority MOMP (Ichim *et al*, 2015) (Fig EV1B). However, DNA-damage (induced by the caspase-activated DNase, CAD) has been found to be a marker of the activation of the apoptosis system not only in apoptosis (Enari *et al*, 1998) but also in minority MOMP (Ichim *et al*, 2015). The apoptosis inducer staurosporine induced the marker of a DNA-damage response, γ H2AX, in control but not in Bax/Bak- or CAD-deficient HeLa cells nor when caspases were inhibited (Fig EV2A). Titrating staurosporine, we found that the detection of γ H2AX was indeed more sensitive than the detection of caspase activity (Fig EV2B and C). We therefore used the detection of DNA damage and of the γ H2AX-DNA-damage response in a Bax/Bak-, caspase-, and CAD-dependent fashion, but in the absence of pronounced caspase activation and cell death, to define minority MOMP during infection.

The poxvirus modified vaccinia virus Ankara (MVA) induced the detectable activation of caspase-3 in a few (about 7%) infected cells (Fig 2A). MVA-infected cells showed a strong γ H2AX signal indicative of a DNA-damage response. This signal was almost completely absent when caspases were inhibited, when cells were lacking Bax/Bak or CAD, or when Bcl-X_L was over-expressed (Fig 2B). Mouse embryonic fibroblasts (MEFs) also showed a γ H2AX signal upon MVA infection, which was absent in cells deficient in Bax and Bak (Fig EV3A). γ H2AX accumulated in the typical nuclear foci (Fig EV4A and B). Assessment of DNA damage by single-cell gel electrophoresis identified DNA damage upon infection with MVA in almost the entire population of cells. This DNA-damage was almost abrogated by caspase inhibition and much reduced in the absence of CAD and Bax/Bak, or in cells over-expressing Bcl-X_L (Figs 2C and EV3B, Appendix Fig S1A; the response to the DNA-damaging agent etoposide is shown as a control in Appendix Fig S1A). Thus, the apoptosis system causes DNA damage in the majority of cells infected with MVA, but most cells do not undergo apoptosis.

Figure 1.

Figure 1. Treatment with Bcl-2 family inhibitors causes the secretion of cytokines from human cells.

- A–E HeLa cell lines were treated with the small-molecule Bcl-2/Bcl-X_L inhibitor ABT-737 (A–D) or the Mcl-1 inhibitor S63845 (E) for 72 h. Cytokines were measured by ELISA. CTRL, non-targeting control gRNA; Bax/Bak, both Bax and Bak had been deleted by CRISPR/Cas9; Bcl-X_L, over-expressing Bcl-X_L; STING, deletion of STING. (A) Concentrations of IL-6 were determined (CTRL, *n* = 9; Bax/Bak, *n* = 6; Bcl-X_L, *n* = 3; STING, *n* = 3). (B) Concentrations of IL-8 were determined (CTRL, *n* = 7; Bax/Bak, *n* = 4). (C) Concentrations of CXCL1 were determined (CTRL, *n* = 8; Bax/Bak, *n* = 5). (D) Concentrations of FGF-2 were determined (CTRL, *n* = 7; Bax/Bak, *n* = 4). (E) HeLa CTRL cells and Bax/Bak-deficient cells were treated with the Mcl-1 inhibitor S63845. Concentrations of IL-6 in supernatants are shown (CTRL, *n* = 8; Bax/Bak, *n* = 7).
- F 1205Lu human melanoma cells or 1205Lu melanoma cells over-expressing Bcl-X_L were treated with ABT-737. Concentrations of IL-6 in the supernatants were measured after 48 h (*n* = 5).
- G 1205Lu human melanoma cells or 1205Lu melanoma cells over-expressing mouse Bcl-X_L were treated with various concentrations of ABT-737. To some aliquots, the caspase inhibitor zVAD-fmk (50 μM) was added. Relative numbers of apoptotic cells were determined as in Fig EV1B after 48 h. Data are means/SEM of at least three independent experiments (1205Lu, *n* = 4; 1205Lu 5 μM ABT-737, *n* = 3; 1205Lu/Bcl-X_L and 1205Lu + zVAD, *n* = 3).
- H HeLa-UL12.5 cells untreated or treated with tamoxifen (100 nM, 24 h) were stained with PicoGreen (3 μl/ml) to detect mitochondrial DNA. Scale bar, 10 μm.
- I HeLa CTRL cells and HeLa-UL12.5 cells were treated with ABT-737 (10 μM). Concentration of IL-6 in the supernatant was measured after 72 h (*n* = 3).
- Data information: **P* < 0.05 for treated versus untreated (solid lines) or for modified cell lines versus CTRL (unpaired two-tailed *t*-test for different cell lines and paired two-tailed *t*-test for treated against untreated conditions in the same cell line).

The same was observed during infection with influenza A virus (IAV, an RNA virus). Almost no IAV-infected cells with detectable caspase-3 activation were recorded (Fig 2A), but a strong, Bax/Bak- and CAD-dependent DNA-damage response and DNA damage were seen (Figs 2D and E, EV3C and EV4C, and Appendix Fig S1A). Herpes simplex virus 1 (HSV-1) is known to cause a DNA-damage response in human cells. HSV-1 induced a γH2AX response in infected cells irrespective of the presence of Bax/Bak (HeLa or MEFs) or CAD (Figs 2F and EV3D and EV4D). Detectable caspase-3 activation was under 3% of cells (Fig 2A). A DNA-damage response to herpesviruses including HSV-1, which replicate in the nucleus of the infected cell, has been noted several times (Luftig, 2014). We found however that HSV-1 also induced actual DNA damage. However, DNA damage was again not seen in cells deficient in either CAD or Bax/Bak (Fig 2G and Appendix Fig S1A), despite the γH2AX signal. Thus, during HSV-1 infection, a γH2AX response is generated very likely by viral nuclear replication (Luftig, 2014), and this response does not require actual DNA damage. However, HSV-1, like the other viruses tested here, also induces DNA damage through minority MOMP, but this DNA damage requires CAD and Bax/Bak.

We tested two species of human-pathogenic bacteria, *Chlamydia trachomatis* (obligate intracellular) and *Salmonella Typhimurium* (which invades and replicates in human cells). *C. trachomatis* has been reported to induce a DNA-damage response (Chumduri *et al*, 2013). We confirmed this and further found that this DNA-damage response was blocked by caspase inhibition, required CAD, and did not occur when mitochondrial apoptosis was blocked (Figs 2H and EV4E and F). The requirement for Bax/Bak was confirmed in MEFs (Fig EV3E). No caspase-3 activation was detected upon infection with *C. trachomatis* (Fig 2A; see also Fig EV5G, below). *Salmonella Typhimurium* also caused a DNA-damage response in HeLa cells, dependent on CAD and mitochondrial apoptosis regulators (Figs 2I and EV4G), but in the absence of detectable caspase-3 activation (Fig 2A). The requirement for Bax or Bak was confirmed in MEFs (Fig EV3F) and in HCT116 human colorectal carcinoma cells (Fig EV3G). Finally, the protozoan parasite *Toxoplasma gondii* also induced a DNA-damage response that depended on the presence of CAD and of Bax/Bak (Figs 2J and EV4H) in the absence of caspase-3 activation strong enough to be detected (Fig 2A). Comparative efficiency of the infections in the various cells is shown in Appendix Fig S1A. These results are evident that very diverse

infectious agents all trigger the activation of the mitochondrial apoptosis apparatus. The activity is mostly too low to cause caspase-3 activation to the extent that is seen during apoptosis or apoptotic cell death but high enough to activate CAD and to cause DNA-damage and a DNA-damage response. This event thus meets criteria of minority MOMP. The current definition of minority MOMP includes the release of small quantities of cytochrome *c* (Ichim *et al*, 2015). We tested this for the infection with *C. trachomatis* and found evidence for small-scale cytochrome *c* release (see below). We will therefore refer to the process here as minority MOMP.

Infection-associated minority MOMP has an immune function

The physiological function of this mechanism may be to alert the cell to the presence of the pathogen. We tested whether minority MOMP during cellular infection also initiated cytokine secretion. HeLa cells produced IL-6 upon infection with MVA, which was significantly lower from cells deficient in Bax/Bak or over-expressing Bcl-X_L (Fig 3A). No difference in IL-6 secretion was found upon infection with HSV-1 (Fig 3B). HeLa cells infected with *C. trachomatis* produced both IL-6 and IL-8, and the secretion of both was reduced in cells lacking Bax/Bak or over-expressing Bcl-X_L (Fig 3C and D). *S. Typhimurium* infection caused production of IL-6 and CXCL-1, and secretion of both was reduced in cells deficient in Bax/Bak (Fig 3E and F); reduced IL-6 secretion was also observed in infected Bax/Bak-deficient MEFs (Fig EV5A). HeLa cells infected with *T. gondii* secreted IL-6, IL-8, CXCL-1, and FGF-2; all of these cytokines were significantly, but in some cases dramatically reduced in supernatants from infected Bax/Bak-deficient HeLa cells (Fig 3G–J).

To obtain information of signaling events during infection-associated minority MOMP, we tested for the contribution from other components. Digestion of mtDNA through expression of the mitochondrial DNase UL 12.5 reduced the secretion of IL-6 during MVA infection but had no effect on IL-6 produced during infection with *S. Typhimurium* (Fig EV5B). Conversely, the absence of CAD had no effect on IL-6 secretion upon MVA infection but was associated with reduced secretion of IL-6 from HeLa cells infected with either *S. Typhimurium* or *C. trachomatis* (Fig EV5D and E). IL-6 secretion during infection with MVA, *S. Typhimurium*, or *C. trachomatis* was diminished from cells lacking STING (Fig EV5C–E). This suggests that STING signaling is required for a full cytokine response during

Figure 2.

Figure 2. Pathogen infection induces DNA damage and a DNA-damage response through the mitochondrial apoptosis pathway.

HeLa cell derivatives were infected with viral, bacterial, or protozoan pathogens. CTRL, cells carrying a non-targeting gRNA; CAD and Bax/Bak, cell lines deficient in CAD or Bax and Bak (CRISPR/Cas9 mutants); Bcl-X_L, cells over-expressing Bcl-X_L.

- A The caspase-3 reporter cell line (labeled DEVD) and the control cell line (DEVG; see Materials and Methods) were infected and analyzed at various time points post-infection by flow cytometry (*Chlamydia trachomatis* (Ctr; MOI = 1, 30 h); *Salmonella* Typhimurium (Sty; MOI = 50, 24 h); *Toxoplasma gondii* (MOI = 5, 16 h); MVA (MOI = 10, 16 h); influenza A virus (IAV; MOI = 1, 16 h); herpes simplex virus 1 (HSV-1; MOI = 1, 16 h)). Staurosporine-treated cells (1 μ M, 3 h) were used as a positive control. Percentages of cells positive for caspase-3 activation (FRET loss) were determined. Data are means/SEM of three independent experiments ($n = 3$).
- B, C Cells were infected with modified vaccinia virus Ankara (MVA) (MOI = 10). The caspase inhibitor zVAD-fmk was used at 100 μ M; cells were analyzed 16 h post-infection.
- D, E Cells were infected with influenza A virus (IAV) (MOI = 1; analysis was 16 h post-infection). D, detection of viral M2 protein was used as infection control.
- F, G Cells were infected with herpes simplex virus 1 (HSV-1) (MOI = 1; analysis was 16 h post-infection).
- H Cells were infected with *Chlamydia trachomatis* (Ctr) (MOI = 1; analysis was 30 h post-infection).
- I Cells were infected with *Salmonella* Typhimurium (Sty) (MOI = 50; analysis was 24 h post-infection).
- J Cells were infected with the protozoan parasite *Toxoplasma gondii* (MOI = 5; analysis was 16 h post-infection).

Data information: (B, D, F, H–J) Cells were tested for the DNA-damage response marker γ H2AX by Western blotting. Western blots are representative of at least three independent experiments. (C, E, G) Cells were subjected to single-cell gel electrophoresis (Comet assay). Each dot depicts one cell. Three experiments per condition were performed and are shown (150 cells per condition). Bars show means. The experiments in (D and F) were done in parallel, and the uninfected controls shown are the same.

Source data are available online for this figure.

infection with diverse pathogens. The upstream signaling however may involve mtDNA in some cases and CAD-dependent DNA-damage in others. The cytosolic protein Apaf-1, which is required for caspase activation in the mitochondrial apoptosis pathway upon release of cytochrome *c*, was also required for the full secretion of IL-6 from HeLa cells infected with MVA or with *S. Typhimurium* (Fig EV5C and E).

We then tested for the limited release of cytochrome *c* from mitochondria of HeLa cells infected with *C. trachomatis*. Indeed, co-staining of infected cells with antibodies to cytochrome *c* and a mitochondrial marker (Tom20) identified parts of the mitochondrial network devoid of staining for cytochrome *c* in some cells (Fig EV5H). We quantified this loss by staining infected HeLa cells for mitochondrial cytochrome *c*, followed by flow cytometric quantification (Waterhouse & Trapani, 2003). We observed a shift in most of the cell population toward lower levels of cytochrome *c* between 15 and 18 h post-infection, but no further loss of cytochrome *c* until 22 h post-infection (which is around the time point of analysis we used for most experiments with this bacterium). No effector caspase activity was detected as before (Fig EV5G). The reduction in cytochrome *c* staining by MOMP (induced by a combination of BH3 mimetics) was much stronger and appeared near-complete (Fig EV5F), and this was accompanied by caspase activation (Fig EV5G). These results suggest that indeed small quantities of cytochrome *c* are released during infection with *C. trachomatis*, adding a key criterion of minority MOMP.

Epithelial cells are frequent targets of infectious pathogens. An important ability of these cells is to signal to recruit immune cells, and minority MOMP-associated cytokine secretion is likely to serve this purpose. Most cells however also have some capacity for self-defense, referred to as cell-autonomous immunity (Randow *et al*, 2013). We tested whether mitochondrial pro-apoptotic signaling contributes to the ability of epithelial cells to restrict growth of *C. trachomatis*. The bacteria indeed showed better growth in HeLa cells deficient in Bax/Bak than in control cells, as assessed by flow cytometry and by microscopy (Fig 4A, Appendix Fig S1A, and Movie EV1). The same effect was seen in a second cell line (Fig 4B). Analysis of bacterial replication (measuring bacterial genome equivalents by quantitative PCR) showed a similar effect in HeLa cells

(Fig 4C). Intriguingly, no difference in chlamydial growth was observed in HeLa cells lacking Apaf-1, CAD, or STING (Fig 4C). When HeLa cells were infected with *S. Typhimurium*, intracellular growth was also enhanced in cells deficient in Bax/Bak or over-expressing Bcl-X_L (Fig 4D and E). In the case of *S. Typhimurium* infection, the loss of Apaf-1 did result in enhanced intracellular replication (Fig 4E). The data suggest that minority MOMP and associated cell stimulation can contribute not only to the secretion of cytokines and thereby the initiation of inflammation but also to the cellular containment of intracellular bacteria by epithelial cells.

Discussion

These results identify a link between minority MOMP and the cell stimulatory potential of mitochondria. Such stimulatory potential had been described earlier, but it had been confined to the experimental situation where caspases were absent or inhibited. Our study suggests that minority MOMP has stimulatory potential even in the absence of additional experimental manipulation. Indeed, in an ABT-737-sensitive cell, high concentrations of the drug were less efficient than low concentrations at inducing the secretion of IL-6, supporting the model that higher levels of caspase activity turn off the stimulation that is seen during minority MOMP. The requirement for STING suggests that DNA recognition is involved. Depending on the infectious agent used, either mtDNA or CAD was required for full cytokine secretion, suggesting different pathways to STING activation.

Minority MOMP, as defined by low-level caspase activation depending on the integrity of the mitochondrial apoptosis apparatus in the absence of apoptosis, was detected with all infectious agents tested. This suggests that minority MOMP is a very common occurrence during infection with at least intracellular pathogens. We have tested cytochrome *c* release during infection with *C. trachomatis* and found small-scale release, which appeared to affect part of the mitochondrial network or a small number of mitochondria. Although we have not tested this for the other pathogens, this suggests that indeed the recently defined process of minority MOMP operates during infection. All pathogens tested appear to have some

Figure 3. The mitochondrial apoptosis pathway contributes to cytokine secretion during infection.

HeLa cell lines with an intact (CTRL) or a deficient (Bax/Bak-double-deficient or Bcl-X_L-over-expressing) mitochondrial apoptosis apparatus were infected with pathogens; supernatants were analyzed for cytokines.

A Infection with MVA (MOI = 10 for 16 h; IL-6).

B Infection with HSV-1 (MOI = 1 for 16 h; IL-6).

C, D Infection with *Chlamydia trachomatis* (Ct; MOI = 1 for 30 h; IL-6, IL-8).

E, F Infection with *Salmonella Typhimurium* (STy; MOI = 50 for 24 h; IL-6, CXCL-1).

G–J Infection with *Toxoplasma gondii* (MOI = 5 for 16 h; IL-6, IL-8, CXCL-1, FGF-2).

Data information: Data are means/SEM of three independent experiments. *P < 0.05 between control and mutant cells (unpaired two-tailed t-test).

Figure 4.

Figure 4. The mitochondrial apoptosis apparatus contributes to contain intracellular bacterial growth.

- A, B (A) HeLa cell lines or (B) AGS cell lines with an intact (CTRL, carrying a non-coding gRNA) or inactive (Bax/Bak-double-deficient) mitochondrial apoptosis apparatus were infected with *Chlamydia trachomatis* expressing GFP (MOI = 0.2). Cells were analyzed by flow cytometry at the indicated time points. Cells were gated on the GFP-positive (infected) host cell population, and mean fluorescence intensity was recorded. Data are means/SEM of six (A) or 4–5 (B) experiments. (A) Growth of *C. trachomatis* was measured in HeLa cells as increase in GFP fluorescence between 18 and 38 h. The data show means/SEM of six independent experiments (genotype effect (significance of the difference in GFP expression between the cell lines overall, $P = 0.08$)). Red line indicates the fold change calculation of the individual different time points and cell lines (significance tested (one sample t-test) for each time point for a difference between Bax/Bak versus CTRL). (B) Growth of *C. trachomatis* was measured in AGS cells as increase in GFP fluorescence. The data show means/SEM of 4–5 independent experiments (genotype effect: $P < 0.0001$; time–genotype interaction: $P < 0.0001$) (12 and 38 h, $n = 4$; 18 h, $n = 5$).
- C Chlamydial genomes per cell culture (expressed as chlamydial DNA in 50 ng of total DNA, determined by quantitative PCR). * $P < 0.05$ between control and mutant cells ($n = 3$).
- D CTRL, Bax/Bak-deficient or Bcl-X_L-over-expressing HeLa cells were infected with *Salmonella* Typhimurium (MOI = 50). Extracellular bacteria were killed using non-cell-permeable antibiotic. At the indicated time points, cells were lysed, and aliquots were plated on agar plates. Recovered colony-forming units were calculated. Data are means/SEM of four independent experiments. Significant genotype effects were found between mutant and control cells (Bax/Bak $P < 0.0001$; Bcl-X_L $P < 0.0001$).
- E CTRL, Bax/Bak-deficient or Apaf-1-deficient HeLa cells were infected with *Salmonella* Typhimurium (MOI = 50). CFU/well was calculated as in (D). $n = 3$.
- Data information: For (A, B, and D), the significance was tested as described in Materials and Methods. For (C and E), significance was calculated by unpaired two-tailed t-test. * $P < 0.05$ between control and mutant cells.

pro-apoptotic activity, which is not strong enough to kill the infected cell (or does so only rarely) but that is sufficient to induce minority MOMP. The primary recognition of pathogens is known to occur through pattern recognition receptors (PRRs). For intracellular pathogens, RIG-like helicases (RLH, recognizing RNA), cGAS/STING (recognizing DNA), and NOD proteins (recognizing bacterial cell-wall components) are probably the important receptors. Intriguingly, most PRRs have been reported to have pro-apoptotic potential, including RLH (Besch *et al*, 2009), STING (Gulen *et al*, 2017), and NOD1 (Inohara *et al*, 1999). Signaling through PRR has mostly been tested in myeloid cells such as macrophages. In these situations, PRR stimulation with their cognate ligands normally does not induce apoptosis. However, this potential can be unmasked in many cases. For STING, it appears to be a simple question of signal strength, with strong stimuli inducing mitochondrial apoptosis (Gulen *et al*, 2017). The activity of RLH has been tested more thoroughly in MVA infection. MVA normally has anti-apoptotic potential in epithelial cells but taking away a Bcl-2-like, anti-apoptotic gene (F1L) renders MVA pro-apoptotic, and MVA-ΔF1L has been reported to induce apoptosis through signals requiring RLH (Eitz Ferrer *et al*, 2011). TLR4, the receptor for LPS, normally causes cell activation, but if NF-κB activity is blocked experimentally, it induces macrophage apoptosis (Ruckdeschel *et al*, 2004). The signaling pathways originating from PRR thus appear to be quite different, as are the downstream effects, for instance, with regard to the activation of interferon genes versus NF-κB (Pandey *et al*, 2014). Similarly, the upstream signals appear to be different but may in all cases show the possibility of linkage to mitochondrial apoptosis. Normally, PRR stimulation does not induce apoptosis. We speculate that not apoptosis but minority MOMP and cell activation is the physiological purpose of this signaling that links PRR to mitochondria. Physiologically, the induction of minority MOMP upon pathogen recognition may be an outcome that benefits the organism.

Cytokine secretion upon infection was reduced in cells with defects in mitochondrial apoptosis in most tested cases, with the exception of HSV-1. Type and amount of detectable cytokine varied with the pathogen tested. The precise signaling events that make this difference are unclear. It seems likely that the triggering of different receptors by different pathogens may contribute to these differences. Intracellular bacteria, for instance, are probably largely

recognized by the peptidoglycan receptors NOD1/2 (Philpott *et al*, 2014), while viruses are mostly recognized by receptors of nucleic acids (Roers *et al*, 2016). While all pathogens tested activated minority MOMP, there are likely additional signals generated, and the combination of these signals will determine cytokine secretion.

It was interesting to observe that different signaling events appear to contribute to cytokine secretion and to bacterial containment. STING was required for full IL-6 secretion for all three pathogens tested. However, while MVA infection relied on mtDNA, bacterial infection (*C. trachomatis* and *S. Typhimurium*) showed CAD dependency. The latter may be linked to a recently discovered “alternative” pathway to STING activation, which has been observed upon experimental induction of DNA damage using the drug etoposide, and which involves formation of a complex containing STING, IFI16, TRAF6, and p53 (Dunphy *et al*, 2018). It seems conceivable that a similar complex is formed upon CAD-dependent DNA damage. Growth of both species of bacteria was enhanced in the absence of Bax and Bak. The loss of Apaf-1 enhanced *Salmonella* but not *Chlamydia* growth. Absence of CAD or STING also had no effect on the replication of *Chlamydia*. The signaling events determining this deserve in-depth study but are at this stage unclear. Numerous factors may be released during minority MOMP. During MOMP, unknown factors were released that triggered the loss of cIAP1 (Giampazolias *et al*, 2017), which in turn is known to activate NF-κB (Vince *et al*, 2007). NF-κB in turn activates numerous genes from many different functional systems, and these systems may work on containing intracellular bacteria.

The ability of cells to undergo apoptosis upon receipt of very diverse stimuli is almost notorious: Basically, any change to a cell’s environment seems to be able to trigger pro-apoptotic signaling. In addition to other functions of apoptosis (Strasser & Vaux, 2018), it also makes sense that this system, which can be activated in such promiscuous fashion, is used to detect infection, and perhaps cellular injury generally.

During minority MOMP, caspase activity was generated (since caspase inhibition blocked DNA damage) but was too low to be detected in standard assays; similar results have been described before for minority MOMP induced by ABT-737 (Ichim *et al*, 2015). A striking occurrence was however the appearance of damage to the genomic DNA, which depended on the activity and integrity of

mitochondrial apoptosis signaling, as it was abolished or much reduced by the loss of Bax/Bak, the over-expression of Bcl-X_L, the treatment with caspase inhibitor, and the deletion of CAD. This suggests that infection-associated damage to the genomic DNA is widespread indeed. Intriguingly, many infections are epidemiologically associated with human cancer (de Martel *et al*, 2012; Chumhuri *et al*, 2016). The link between infection and DNA damage that is established by minority MOMP may therefore be of substantial significance. DNA damage appears to be triggered by many or most infections. DNA damage will be repaired but may, in chronic or in repeated infections, with low frequency induce permanent genomic mutations and predispose to cancer. The results of this study therefore suggest that mitochondria have a function in the detection of microbial infection and cell-autonomous immunity, through the low-level, sub-lethal activation of the mitochondrial apoptosis apparatus. The results further identify DNA damage as a common occurrence during infection and indicate the possibility that infection-associated mutations, potentially leading to cancer, may be a side effect of this system of microbial detection.

Materials and Methods

Cell lines and culture conditions

HeLa229 cells (ATCC Cat# CCL-2.1) were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Gibco) with 10% FCS (Sigma-Aldrich, #F7524). Mouse embryonic fibroblast (MEF) cells transformed with SV40 large T-antigen were kindly provided by Dr David Huang, Melbourne, and were cultured in DMEM (Thermo Fisher Scientific) with 10% FCS and 50 µM 2-mercaptoethanol. HCT116 wild-type and Bax/Bak-deficient cells (Zhang *et al*, 2000) were cultured in DMEM/10% FCS. The metastatic melanoma cell lines 1205Lu and WM1158 (Dr Meenhard Herlyn, Wistar Institute, Philadelphia) were cultured in TU2% melanoma medium containing 80% (v/v) MCDB153 (Sigma-Aldrich, #M7403), 20% (v/v) Leibovitz's L-15, 2% (v/v) FCS (Thermo Fisher, Gibco), 5 µg/ml insulin (bovine, Sigma-Aldrich, #I4011), and 1.68 mM CaCl₂. AGS cells (ATCC CRL-1739; obtained from ECACC, Sigma-Aldrich) were maintained in Ham's F-12K medium (Gibco) with 10% FCS. HCT116 cells have been described (Zhang *et al*, 2000). 293FT cells (Invitrogen) were cultured in DMEM/10%FCS medium. All cells were incubated at 37°C with 5% CO₂. Gene-deficient cells were generated via CRISPR/Cas9 genome editing by transducing the cells with the lentiviral vector lentiCRISPR v2 (Addgene; Sanjana *et al*, 2014) and selection with puromycin (Invivogen). Guide RNAs were CTRL (non-targeting control) (ATCGTTTCCGCTTAACGGCG) or targeting Bax (CAAGCGCATCGGGGACGAAC), Bak (ACGGCAGCTCGCCATCATCG), CAD (TCGGCGTTGTCGGGAACACT), STING (GCTGGGA CTGCTGTAAACG), or Apaf-1 (TTCCTAAGGAACCTCCACA). Two monoclonal Bax/Bak-double-deficient AGS cell lines (clones C4 and G11) were generated by limiting dilution of polyclonal cells, which had been transduced simultaneously with both Bax and Bak targeting sgRNA constructs and selected with 0.75 µg/ml puromycin. Control gRNA (CTRL) was taken from the human GeckoV2 library (Sanjana *et al*, 2014). gRNA against human Bax, Bak, and CAD (DFFB, DFF40) was designed using the MIT server (<http://crispr.mit.edu/>), now discontinued. gRNA against STING

(TMEM173) was taken from the Brunello gRNA database (Doench *et al*, 2016). Deletion was confirmed by Western blotting [Appendix Fig S1A; Bax/Bak-deficient cells have already been used in an earlier study (Dudek *et al*, 2018)]. Cells over-expressing murine Bcl-X_L were generated by lentiviral transformation using the vector pEF1-GWPuro-mBcl-X_L [where mouse Bcl-X_L had been inserted into the described backbone (Weber *et al*, 2016)], followed by puromycin selection. Lentiviruses were produced in 293FT cells (Invitrogen) using the packaging vectors psPAX.2 and psMD2.G (Addgene Plasmids, #12260 and #12259; Dr Didier Trono). After harvesting and filtration of the supernatants, cell lines were infected in the presence of 1 µg/ml of polybrene (Millipore, #TR-1003-G).

Antibodies

Primary antibodies used were anti-γH2AX (anti-phospho-ser139-histone H2A.X, 2577, Cell Signaling), anti-GAPDH (MAB374, Millipore), anti-influenza A M2 (MA1-082, Thermo Fisher Scientific), anti-HSV-1 gD (sc-21719, Santa Cruz Biotechnology), anti-*Chlamydia trachomatis* Hsp60 (ALX-804-072-R100, Enzo Life Sciences), anti-*Salmonella*-LPS (sc-52223, Santa Cruz Biotechnology), anti-*Toxoplasma gondii* GRA7 (Hermanns *et al*, 2016), and anti-active caspase-3 (BD Biosciences 559565). Secondary antibodies were (Western blotting) anti-mouse IgG(H+L)-HRP (115-035-166, Dianova), anti-rabbit IgG(H+L)-HRP (A6667, Sigma-Aldrich), (flow cytometry and immunofluorescence) anti-mouse Ig(H+L)-Cy5 (715-175-151, Dianova), anti-mouse Ig(H+L)-DyLight 488 (115-485-062, Dianova), anti-rabbit IgG(H+L)-Alexa Fluor 647 (711-605-152, Dianova), anti-rabbit Ig(H+L)-DyLight 488(711-545-152, Dianova), anti-rat IgG(H+L)-Alexa Fluor 647 (712-605-150, Dianova), Tom20 (Santa Cruz Biotechnology (sc-11415)), and cytochrome c (Cell Signaling (12963)).

Reagents

ABT-737 (S1002) was from Selleck Chemicals, and the Mcl-1 inhibitor S63845 (A8737) was obtained from APEX BIO. Staurosporine and etoposide were from Sigma-Aldrich (S4400 and E1383). zVAD-fmk was from Bachem (N-1510.0025). qVDOPh was from Apex Bio (GEN1589978).

Infectious agents and infections

MVA stocks (wt and GFP-expressing MVA) were kindly provided by Dr Gerd Sutter, LMU Munich, and influenza A virus (A/WSN/1933, H1N1) and HSV-1 (strain McIntyre, ATCC) were kindly provided by Dr Georg Kochs, Institute of Virology, Freiburg. 3 × 10⁵ cells per well in 6-well plates were infected with MOI of 10 (MVA) or 1 (IAV, HSV-1). *Chlamydia trachomatis* LGV strain L2 (ATCC, strain 434) was kindly provided by Dr Agathe Subtil, Paris. 3 × 10⁵ cells per well in 6-well plates were seeded 1 day prior to infection with MOI = 1 or MOI as indicated. *Salmonella enterica* Typhimurium (an isolate from a patient with enteritis, University Medical Center Freiburg) was streaked on LB agar plates. One colony was used to inoculate LB medium. The overnight culture was diluted 1:30 in the culture medium of the target cells, and bacteria were grown for 3 h. This culture was then used for infection. After 20-min incubation, medium was replaced with medium containing gentamicin (50 µg/ml).

After 40 min, medium was again replaced with medium containing 5 µg/ml gentamicin, and culture was continued until harvesting. Tachyzoites of the *Toxoplasma gondii* strain RHΔ*hxp*rt (Roos et al, 1994) were cultivated in confluent monolayers of human foreskin fibroblasts (HS27, ATCC CRL-1634), harvested, and immediately used for infection of HeLa229 cells. 3×10^5 HeLa cells were seeded in 6-well plates and infected the next day with *T. gondii* at an MOI of 5.

Western blotting

Cells were washed once with PBS (14190169, Thermo Fisher Scientific), directly lysed by adding 200 µl sample buffer (Tris-HCl, pH 6.8, 40% glycerol, 6% SDS, 400 mM DTT, bromophenol blue), and harvested using a cell scraper. Samples were run on SDS-PAGE (EZ run 12.5% gel solution (BP7712-500, Thermo Fisher Scientific) or Novex™ WedgeWell™ 4-20% Tris-Glycine Mini Gels (XP04202BOX, Thermo Fisher Scientific)), and transferred to PVDF membranes (10600023, GE Healthcare). HRP-coupled secondary antibodies were used, and signals were detected with ECL substrate.

Caspase-3 reporter cells

Caspase-3 activity was detected in HeLa229 cells stably expressing a FRET reporter construct where GFP and RFP were linked by a peptide containing the DEVD caspase-3 recognition sequence (as described before using different fluorescent proteins; Tyas et al, 2000). Active caspase-3 was detected as FRET loss by flow cytometry. A cell line carrying the same construct but with the DEVG sequence (a poor substrate of caspase-3) was used as control. The expression constructs were made and kindly provided by Dr Jan Rohr, Freiburg.

Generation of HeLa-UL12.5 cells

The plasmid pSAK UL12.5, harboring HSV-1 UL12.5, was a kind gift from James R Smiley, University of Alberta. UL12.5 was PCR-amplified from pSAK UL12.5 and cloned into the lentiviral vector pF5UAS_MCS_SV40_Puro using BamHI and NheI digestion of both the PCR product and vector. HeLa229 cells were first transfected with a pFU-GEV16 construct (expression vector containing transcriptional activator). After selection with hygromycin, cells were subsequently infected with the lentivirus carrying the above UL12.5 sequence (under control of a tamoxifen-responsive element) and puromycin-resistant cells were selected. To induce UL12.5, cells were treated with 100 nM 4-hydroxytamoxifen for 24 h. Degradation of mitochondria DNA was assessed by PicoGreen staining (3 µl/ml).

Immunofluorescence

Cells (5×10^4) per well were seeded in 24-well plates with coverslips in each well and infected. Cells were fixed with 4% PFA for 15 min at room temperature. After three times of washing with PBS, ice-cold methanol was added. The samples were incubated at -20°C for at least 30 min. Slides were washed again three times and then blocked (PBS with 5% BSA and 0.3% Triton X-100) for 60 min. Antibodies were diluted in PBS/0.5% BSA and incubated with slides

for 2 h at room temperature (1 h for secondary antibodies). Further washing steps were done in PBS/1% BSA/0.3% Triton X-100. Nuclei were stained with Hoechst 33342 dye (Sigma-Aldrich #B2261). Images were acquired with a BZ 900 microscope (Keyence).

Flow cytometry

Cells were harvested by trypsinization (Trypsin, 25300096, Thermo Fisher) and were washed three times with PBS, fixed in 4% PFA for 15 min at room temperature followed by methanol fixation as above. Staining was done as described for immunofluorescence, and cells were analyzed by flow cytometry in a FACSCanto II or a FACSCalibur cytometer (Becton Dickinson).

ELISA

Cytokines in supernatants were measured using the following kits: murine IL-6 (88-7064-88, eBioscience), human IL-6 (430506, BioLegend), human IL-8 (431506, BioLegend), human CXCL1 (DY275-05, R&D Systems), and human FGF-2 (BLD434312, BioLegend) following the manufacturers' protocols.

Single-cell gel electrophoresis (Comet assay)

Infected cells were harvested and washed with PBS. The cell suspension was mixed with 0.7% low-melting agarose and poured on a slide pre-coated with 1% normal-melting agarose. Cells were lysed by incubation in buffer containing 2.5 M NaCl, 127 mM EDTA, 10 mM Tris-HCl, 1% (v/v) Triton X-100, 5% (v/v) DMSO (pH 10) for 1 h at 4°C . During lysis and the following electrophoresis, the slides were protected from ambient light. Samples were incubated for 20 min in alkaline buffer (300 mM NaOH, 1.3 mM EDTA, pH 13) to unwind the DNA. Electrophoresis was performed for 20 min at 25 V/300 mA. Slides were neutralized in Tris-HCl buffer (0.4 M, pH 7.5) and washed in distilled water. Samples were fixed in 100% ethanol and stored at 4°C . DNA was stained with Hoechst 33342 dye. Analysis was done using ImageJ and the plug-in OpenComet (<http://www.cometbio.org>).

Monitoring of bacterial growth

HeLa229 or AGS cells were seeded at a density of 2.5×10^5 cells per ml in a 12-well plate 8 h before infection. Cells were infected in triplicate with *C. trachomatis* L2 expressing RSGFP (L2pTK2GFP; strain L2/434/Bu transformed with plasmid p2TK2-SW2 IncDProm-RSGFP-IncDTerm (Agaisse & Derre, 2013)) at an MOI of 0.2. Samples were harvested at different time points (12/18/38 h) by trypsinization. Harvested cells were fixed with 3.6% PFA in PBS for 30 min, washed once with PBS, and analyzed by flow cytometry. Samples were gated on live, chlamydia-infected (GFP-positive) populations, and mean fluorescence intensity (MFI) of the GFP signal was determined. *Salmonella* Typhimurium infection of HeLa cells was done as described above. At the indicated time points, cells were washed three times with PBS and lysed in 1 ml of PBS containing 1% (v/v) Triton X-100 and 0.1% (v/v) SDS and scraped off the plate. The lysate was diluted serially (1:10 to 1:1,000), and 10 µl from each dilution was plated on LB plates. Following

incubation at 37°C overnight, colonies were counted and colony-forming units (CFU) per well were calculated. For statistical analysis of bacterial growth in HeLa and AGS cells, a linear mixed model (Brown & Prescott, 1999) was fitted with a random intercept (subject = experiment). The continuous response variable (MFI (for *Chlamydia*) or bacterial number (for *Salmonella*)) was modeled as a linear function of time, group (Bax/Bak C4, Bax/Bak G11 with reference CTRL for AGS), Bax/Bak, Bcl-X_L with reference CTRL for HeLa), and the time–group interaction as explanatory variables. If a significant interaction was detected, group comparisons were made separately for each time point. All computations were performed with the statistical software R system using the lme package.

Bacterial DNA was detected by quantitative PCR. We used the published primer specific for the *Chlamydia trachomatis* 16S gene [Ct16sF TCGAGAATCTTTGCAATGGAC and Ct16sR CGCCCTTTACGCCAATAAA (Goldschmidt et al, 2006)]. At the indicated time points of infection, we isolated the total DNA [PureLink Genomic DNA Isolation Kit, Invitrogen (K182002)] and used 50 ng for PCR. PCR products were detected using SYBR Select Master Mix [Thermo Fisher Scientific (#4472918)]. To calculate the amount of bacterial DNA, we used a serial dilution of pure bacterial DNA and performed a standard curve of the generated Ct-values.

Expanded View for this article is available online.

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Author contributions

DB, BD, AH, AW, SK, JV, AM, JH, TS, and IEG performed experiments and contributed data. GH designed the study. AW, SK, IEG, and GH supervised the study. DB and GH wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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