Bim and Bmf Synergize To Induce Apoptosis in Neisseria Gonorrhoeae Infection

Oliver Kepp1, Kathleen Gottschalk1, Yuri Churin1, Krishnaraj Rajalingam1, Volker Brinkmann2, Nikolaus Machuy1, Guido Kroemer3, Thomas Rudel1,4

1 Department of Molecular Biology, Max Planck Institute for Infection Biology, Berlin, Germany, 2 Core Facility for Microscopy, Max Planck Institute for Infection Biology, Berlin, Germany, 3 INSERM U848, Institut Gustave Roussy, Université Paris Sud, Paris, France, 4 Biozentrum, University of Würzburg, Department of Microbiology, Würzburg, Germany

Abstract

Bcl-2 family proteins including the pro-apoptotic BH3-only proteins are central regulators of apoptotic cell death. Here we show by a focused siRNA miniscreen that the synergistic action of the BH3-only proteins Bim and Bmf is required for apoptosis induced by infection with Neisseria gonorrhoeae (Ngo). While Bim and Bmf were associated with the cytoskeleton of healthy cells, they both were released upon Ngo infection. Loss of Bim and Bmf from the cytoskeleton fraction required the activation of Jun-N-terminal kinase-1 (JNK-1), which in turn depended on Rac-1. Depletion and inhibition of Rac-1, JNK-1, Bim, or Bmf prevented the activation of Bak and Bax and the subsequent activation of caspases. Apoptosis could be reconstituted in Bim-depleted and Bmf-depleted cells by additional silencing of antiapoptotic Mcl-1 and Bcl-XL, respectively. Our data indicate a synergistic role for both cytoskeletal-associated BH3-only proteins, Bim, and Bmf, in an apotic pathway leading to the clearance of Ngo-infected cells.

Introduction

Infection with various pathogens results in the inhibition or activation of apoptotic cell death [1]. Whereas viral pathogens frequently inhibit host cell apoptosis, many bacteria kill immune or epithelial cells by apoptosis allowing them to subvert immune reactions or to invade tissues, respectively. The obligate human specific bacterium Neisseria gonorrhoeae (Ngo), the causative agent of the sexually transmitted disease gonorrhea, induces apoptosis in genital epithelia. Since induction of apoptosis requires the firm attachment of the gonococci to host cells [2], exfoliation of infected epithelial cells covered with adherent bacteria has been suggested as the immediate cellular responses against infection [3,4]. This detachment-associated apoptosis of infected cells resembles anoikis, a special form of apoptosis that is induced by absent or inappropriate cell–matrix interactions [5].

Bcl-2 family proteins control mitochondrial outer membrane permeabilization (MOMP), which is the critical step in many forms of apoptosis [6,7]. The Bcl-2 family consists of pro- and antiapoptotic members that share homologies within their Bcl-2 homology domains (BH). The antiapoptotic Bcl-2 family proteins harbor BH1-4 domains and presumably act by sequestering and inhibiting proapoptotic Bcl-2 members [8]. Proapoptotic Bcl-2 family proteins can be further subdivided into the branch of pore forming, multidomain BH1-3 proteins (like Bak and Bax) and the BH3-only branch (including Bim, Bmf, Bid, Bad, Noxa and Puma) [9,10]. Active BH3-only proteins cause conformational changes within Bak and Bax, which subsequently homooligomerize and form pores in the outer mitochondrial membrane [11,12]. MOMP culminates in the release of proapoptotic proteins like cytochrome c, leading to the activation of caspases and caspase-independent death effectors [13].

The mechanisms through which BH3-only proteins activate Bak or Bax are not fully understood. BH3-only proteins may release Bak and Bax from inhibition by anti-apoptotic Bcl-2 protein [14]. Alternatively, the group of BH3-only proteins may include two subgroups, namely survival antagonists that neutralize BH1-4 proteins, and death agonists that activate BH1-3 proteins [15,16]. A competition of death agonists and survival antagonists for the binding to BH1-4 proteins has been reported [17]. Upon binding of survival antagonists, death agonists are released from their sequestration by BH1-4 proteins and hence freed to act directly on BH1-3 proteins.

Cytotoxic stimuli activate BH3-only proteins by a variety of distinct mechanisms such as p33-dependent transcriptional regulation (Puma and Noxa [18,19]), proteolytic cleavage (Bid [20]), dephosphorylation (BAD [21]) or phosphorylation (Bim and Bmf). Under normal circumstances, Bim and Bmf are sequestered via dynein light chains (DLC) to the actin and tubulin cytoskeleton, respectively, which prevents them from activating...
Results

Exfoliation of epithelial cells has previously been described to be caused by N. gonorrhoeae [3,4,26]. Since this process resembles anoikis,[4] we further investigated the connection between N. gonorrhoeae-infected cell detachment and apoptosis. HEK293 cells were infected with N. gonorrhoeae strain VPI (N242), a clinical isolate and morphological changes were correlated with the activation of caspases. Detachment from the culture support was visible as soon as 6 to 9 h post-infection (Figure 1A) concurrent with the proteolytic maturation of caspase 3 (Figure 1B). To test whether caspase activity is required for exfoliation, cells were infected in the absence or presence of the pan-caspase inhibitor Z-VAD-fmk and then were analyzed by electron and fluorescence microscopy. In the presence of Z-VAD-fmk, N. gonorrhoeae-infected cells continued to detach yet remained otherwise intact and hence failed to disintegrate by apoptosis (Figure 1C and 1D, Video S1) while the activation of caspases 3 and 7 was blocked during the entire duration of the experiment (Figure 1E and 1F). Detachment was further analyzed by acquiring z stacks of infected cells by laser scanning confocal microscopy and subsequent 3-dimensional remodeling (Figure 1G and Video S2). The detachment and induction of apoptosis is not a general response of these cells to infection stress since the same cell line exhibits marked apoptosis resistance as consequence of infection with C. trachomatis [27,28]. These results demonstrate that caspases are required for the apoptotic disassembly of N. gonorrhoeae-infected cells but not for their detachment.

Synergistic action of BH3-only proteins during apoptosis

Since infection of HEK293 cells with N242 caused the most prominent effects, we focused on this system to further investigate the mechanisms underlying infection-induced apoptosis. We have previously demonstrated that infection with Neisseria induced the activation of Bak and Bax and finally apoptotic cell death [32]. To delineate the signaling pathway leading to the activation of Bak and Bax, we systematically depleted BH3-only proteins in a RNA interference miniscreen. The knockdown of the siRNAs was validated by quantitative real-time PCR (>75% knockdown at the mRNA level) and immunoblot analysis (>75% knockdown at the protein level) (Figure 2A and 2B). Knockdown of Bim and Bmf (but not that of Bid or Bad) resulted in a significant reduction of effector caspase activity, as measured with a fluorogenic caspase 3/7 substrate or by immunochemical detection of proteolytically mature caspase 3 (Figure 2C and 2D, and Figure S4). Bim and Bmf knockdown specifically inhibited the caspase activation induced by N. gonorrhoeae (Figure 2C and 2D, and Figure S4), yet had no effect on caspase activation induced by the genotoxic agent cisplatin (Figure S5A). N. gonorrhoeae infection failed to induce Puma, Noxa and any of the tested mRNAs for BH3 only proteins (Figure 2E, and Figure S6), although cisplatin was able to activate the transcription of both the Puma and Noxa genes (Figure S3B and Figure S3C). Accordingly, the depletion of Puma or Noxa did not affect caspase-3 activation in N. gonorrhoeae-infected cells (Figure 2F). These data demonstrate that Bim and Bmf are specifically required for the N. gonorrhoeae-triggered activation of caspases.

Release of Bim and Bmf from the cytoskeleton during infection

In healthy cells, Bim and Bmf are sequestered to the cytoskeleton by binding to dynein light chains. In response to cytotoxic stimuli, that induce cytoskeletal rearrangements, Bim

Acknowledgments

The authors would like to thank the Swiss National Science Foundation (SNF) for financial support (grant number 31003A_129088).
Bim and Bmf may act as stress sensors in thus far that they are released from the cytoskeleton and induce the activation of Bak and Bax [22,23]. Accordingly, cytoskeleton fractions obtained from Ngo-infected cells generally contained less Bim and Bmf than those from non-infected control cells (Figure 2G and Figure S7A). A similar result was obtained when cytoskeleton and cytosol were separated by sucrose gradient centrifugations. Bim and Bmf from infected samples shifted from heavier cytoskeleton containing to lighter fractions (Figure S7B), indicating a release of these proteins from the cytoskeleton in infected cells. Addition of Z-VAD-fmk did not prevent the release of Bim and Bmf from the cytoskeleton (Figure 2G), demonstrating that this phenomenon occurs independently of caspase activity.

JNK-dependent activation of Bim and Bmf

Active JNK-1 is reportedly sufficient for the release of Bim and Bmf from the cytoskeleton [24]. We have previously shown that JNK-1 is activated already 30 minutes after infection with Neisseria, leading to NFκB activation and proinflammatory responses [33]. Although the short-term effects of JNK-1 activation can be cytoprotective, prolonged JNK activation induces apoptotic cell death [34]. Phosphorylated, active JNK-1 could be detected for the whole period of Ngo infection up to 15 h (Figure 3A), correlating with a reduced electrophoretic mobility of Bim and Bmf at later timepoints (Figure 3B and Figure S8). ERK seems not to be involved in the signaling as there was no activation upon infection (Figure S9). Silencing of JNK-1 with validated siRNAs (Figure 3C) prevented the shift in the size of Bim and Bmf (Figure 3B, 3C, and 3D), indicating a role of JNK-1 in post-translational modification of Bim-L and Bmf in Ngo-infected cells.

The caspase activity of Ngo-infected cells depleted of JNK was reduced to the same level as that of cells subjected to the knockdown of Bim or Bmf (Figure 3E). Moreover, the frequency of cells with apoptotic chromatin condensation was reduced in JNK-1-depleted as compared to control cells (Figure 3F). JNK-1 depletion also partially inhibited the Ngo-induced release of Bim and Bmf from the cytoskeleton (Figure 3G). In addition inhibiting JNK-1 by means of a chemical inhibitor partially reduced a...
Bim and Bmf induced apoptosis synergistically in a manner, with Bak acting upstream of Bax [32,37]. The activation of Bak involves its release from antiapoptotic Bcl-2 analogues such as Mcl-1 [38]. Combined silencing of Mcl-1 and Bim, but not that of Mcl-1 and Bmf or Mcl-1 knockdown alone reestablished the apoptotic program triggered by Ngo infection [Figure 5C], suggesting that Bim acts as a specific Mcl-1 antagonist in this system. Combined silencing of Bcl-XL plus Bmf, but not that of Bcl-XL and Bim or Bcl-XL alone also reestablished Ngo-induced apoptosis (Figure 5C). In contrast, Bcl-2 co-silencing had no apoptosis-sensitizing effect on either Bim- or Bmf-depleted cells. Potential off target effects within the same protein family could be excluded by systematic cross analysis. In particular, Bim- and Bmf silencing did not cause deregulated expression of anti-apoptotic members of the Bcl-2 family (Figure S10), ruling out an indirect effect of Bim and Bmf depletion by overexpression of apoptosis inhibitors. We concluded from these data that Bim and Bmf activate apoptotic pathways by functionally sequestering Mcl-1 and Bcl-XL, respectively.

Discussion

Neisseria gonorrhoeae is a highly adapted human pathogen that utilizes multiple adhesins to interact with host cell receptors to trigger cytoskeletal reorganization, invasion or phagocytic uptake, intraphagosomal accommodation, nuclear reprogramming of host cells, cytokine/chemokine release and finally host cell apoptosis [39]. By investigating the apoptotic pathway involved in the infection-induced activation of Bak and Bax, we discovered an unexpected connection between pathogen-induced cytoskeletal reorganization and apoptosis. Attachment of bacteria initiated the activation of Rac-1 leading to rearrangement of the cytoskeleton (which is presumably required for exfoliation) and the activating phosphorylation of the stress kinase JNK-1. JNK-1 then participated in the activation of the BH3-only proteins Bim and Bmf that together facilitate Bak- and Bax-dependent apoptosis.

Besides the well characterized isolate N242 [29], several other clinical isolates induced exfoliation and apoptosis indicating that gonococci trigger similar pathways leading to cell death. Our preliminary data on the initial trigger of cell detachment leading to cell death unveiled a role of specific adhesins – receptor interactions. N242 induced exfoliation and cell death in different cell lines tested. These effects very likely depend on the interaction of one or more of the expressed Opa proteins with a yet uncharacterized receptor. Although derivatives of strain MS11 failed to induce apoptosis in HeLa cells, a similar efficient response as with N242 was observed with derivative N1163 (Opa57; PorBIA) upon infection of HeLa-CEACAM1 but not in HeLa-CEACAM3. Interestingly, CEACAM-1 has been shown to be upregulated in primary ovarian surface epithelial cells by gonococcal infection suggesting that the interaction with this receptor has in vivo relevance [40]. Moreover, the specificity for one CEACAM-recombinant cell line over the other is interesting, because both have been demonstrated to be susceptible for infection with Opa57-expressing gonococci [41]. It is therefore likely that particular adhesin-receptor interactions determine the detachment and apoptosis induction as consequence of this cell – pathogen interaction. This assumption would be in agreement with several reports on the inhibition of apoptosis by gonococcal infection [42]. In one of these studies, Bim was downregulated upon infection of epithelial cells with a pilated gonococcal derivative [43], supporting a central role of Bim in life-death decisions as consequence of gonococcal infections.

Numerous bacterial pathogens induce the reorganization of the host cell cytoskeleton, often initiating the active uptake of bacteria translocation of Bim and Bmf from heavier to lighter fractions in sucrose gradients (Figure S7C). In conclusion, JNK-1 depletion and Bmf prior to Ngo infection prevented the activation of both Bak and Bax (Figure 5A and 5B and Figure S4), underlining the essential need of both Bim and Bmf in this pathway.

| Strain Number | Isolate       | Adherence* | Detachment* | Caspase-3† |
|---------------|---------------|------------|-------------|------------|
| VP1 (N242)    | n.d.          | ++         | +           | +          |
| MZ155/04 Blood| ++            | ++         | +           | +          |
| MZ359/05 Urethra| +         | ++         | +           | +          |
| MZ441/05 Urethra| −           | −          | −           | −          |
| MZ308/06 Cervix| +           | +          | ++          | +          |
| MZ452/06 Vagina| −           | −          | −           | −          |
| MZ489/06 n.d. | +            | +          | +           | +          |
| MZ552/06 n.d. | +            | +          | +           | +          |
| MZ38/07 Urethra| −           | −          | −           | −          |
| MZ114/07 Urine| ++           | +          | +           | +          |
| MZ245/07 n.d. | +            | +          | +           | +          |

*Adherence was determined by plating assays of Saponine lysed infected cells. Detachment was determined by microscopic assays. Infection induced p17/p19 active caspase-3 fragments and PARP cleavage determined by immunoblotting. †Adherence similar as VP1; ++, adherence at least 10-fold more efficient as VP1; −, no significant adherence; n.d., not documented.
Nevertheless, the activation of apoptosis is not a common outcome of such bacterial infections. Our data suggest that downstream of cytoskeletal reorganization, the prolonged activation of JNK-1 is required for lethal signaling. It is interesting to note that short term activation of JNK induces antiapoptotic and proinflammatory responses in the host cell infected with Ngo [33,45]. JNK may therefore exert a dual function during Ngo infection, first by protecting the cell for a short period post-infection and then by triggering the exfoliation of the infected cells.

We show here that JNK was required for Bim- and Bmf-dependent apoptosis during infection, consistent with the previously described JNK-specific phosphorylation of Bim and Bmf within their dynein binding domains [24]. Accordingly, the release of Bim and Bmf from the cytoskeleton as well as their reduced electrophoretic mobility was reduced in JNK-1-depleted cells (Figure 3G).

The exact mode of BH3-only activity is still being discussed. Here we show that both Bim and Bmf are essential to induce Bak and Bax activity for Ngo-triggered apoptosis. As the double knockdown of Bim and Mcl-1 re-sensitized cells for apoptosis, the action of Bmf alone seems to trigger apoptosis efficiently in the absence of Mcl-1. Likewise, apoptosis could be rescued in the absence of Bmf by co-knockdown of Bcl-XL, suggesting that the action of Bim alone suffices to induced apoptosis in the absence of Bcl-XL. In this scenario, both Bim and Bmf need to be activated for efficient induction of cell death due to their joint capacity to inhibit two different anti-apoptotic Bcl-2 homologues (see model in Figure 6). As a result, this study furnishes yet another example for the complex relationship between antagonizing pro- and anti-apoptotic Bcl-2 family proteins.

**Materials and Methods**

**Cell culture, bacterial strains, and infection**

HeLa cells (human cervix carcinoma, later diagnosed as adenocarcinoma) ATCC CCL2 and HeLa cell lines expressing recombinant CEACAM receptors [31] were grown in RPMI 1640 (Gibco) supplemented with 10% heat inactivated FCS in the presence of 5% CO2. The cells were routinely passaged every 2–3 days and the passage number never exceeded 20 passages before a new batch with a low passage number was used. Cells were seeded 24 h before infection and were washed several times.
with RPMI before infection. HEK 293T cells (immortalized human embryonic kidney) ATCC CRL-11268 for the production of virus and HEp-2 (ATCC CCL-23) were grown in DMEM (Gibco) supplemented with 10% heat inactivated FCS. ME-180 (HTB-33) were grown in McCoys 5a supplemented with 10% heat inactivated FCS respectively under the same conditions.

Figure 3. JNK mediates Bim and Bmf activity. (A) JNK phosphorylation and post-translational modification of Bim at the indicated time points during the course of infection were visualized using the indicated antibodies. (B) Modification of Bim and Bmf 15 h post-infection with and without JNK was studied by SDS PAGE and Western blot analysis with the respective antibodies. Arrows indicate the two detected forms of Bim and Bmf. (C,D) The JNK-dependent modification of Bim and Bmf was analyzed using siRNA-mediated knockdown validated by Western blot and qRT-PCR. (E) An effect of JNK knockdown on caspase activation upon infection was shown by the use of an antibody specific for the large subunit of mature caspase-3. siRNAs against Bim, Bmf, and Bid were used as positive and negative controls, respectively. (F) The effect of JNK-1-knockdown on apoptosis induction was analyzed upon siRNA treatment. The fraction of cells showing condensed/fragmented chromatin was counted in five microscopical fields and three independent experiments using Hoechst 33342. (G) Cytoskeletal extracts were analyzed for Bim and Bmf in JNK knockdowns, infected, and uninfected controls using the indicated antibodies.

doi:10.1371/journal.ppat.1000348.g003

The following *N. gonorrhoeae* strains and derivatives were used in this study: The clinical isolate Ngo strain VP1 (N242; PorBIA; P2; Opa27; Opa27.2; Opa27.5; Opa28; Opa29; Opa30; LPS type L1) [29]; Ngo strain MS11 derivatives N302 (PorBIB; P2; Opa2), N920 (PorBIA; P2; Opa2), a PorBIA derivative of N917 (PorBIB; P2; Opa2) and N927 (PorBIA; P2; Opa2) have been described [46,47]. MS11 derivative N1163 (PorBIA; P2; Opa57) is a PorBIA derivative of

Figure 4. Rac-1 required for cytoskeletal changes and apoptotic signaling. (A) Shown are phase contrast pictures depicting the respective cytoskeletal phenotype of untreated control cells, cells transfected with siJNK-1, siRac1, and treated with the Rac inhibitor NSC23766 15 h post-infection. (B) Inhibition of apoptosis in infected, NSC23766 pretreated cells was quantified by determining the ratio of cells with condensed and normal chromatin by fluorescent microscopy. (C,D) The effect of Rac inhibitor NSC23766 on JNK activation and subsequent caspase cleavage was determined by Western blot using the indicated antibodies.

doi:10.1371/journal.ppat.1000348.g004
strain N313 [41]. Clinical gonococcal isolates from Germany were obtained anonymously from the strain collection of the National Reference Laboratory for Meningococci hosted by the Institute for Hygiene and Microbiology at the University of Würzburg. Species confirmation for those strains was obtained at the Reference Laboratory by standard biochemical tests and partial 16S rRNA sequencing. Gonococci were grown on GC agar base plates (Becton Dickinson, Difco and Remel) supplemented with Proteose Pepton Nr. 3 (Difco) and 1% vitamin mix for 14–20 h at 37°C in 5% CO2 in a humidified atmosphere. Infections were routinely performed in the absence of FCS at a multiplicity of infection (MOI) of 1. If not indicated else wise the respective assays were carried out after 15 h of infection with N242.

For the inhibition of caspases, cells were pre-incubated with 50 μM Z-VAD-fmk (Bachem) for 15 min prior to infection and throughout the experiment. Cisplatin was used at a concentration of 50 μM for 20 h in supplemented media.

**Western blot**

5×10^5 cells per sample were harvested in 100 μl loading buffer (60 mM Tris-HCl pH 8.0, 6% SDS, 10 mM DTT, 6% β-mercaptoethanol, 40% glycerol, and 0.1% bromophenol blue) and 20 μl of the protein lysates were separated and transferred as described before [32]. The following antibodies and sera were used in this study: anti-β-Actin (Sigma); anti-Bad (Cell Signaling); anti-Bak NT (Upstate); anti-Bak (Ab-1) (Millipore); anti-Bax (6A7) (BD Pharmingen); anti-Bid (Cell Signaling); anti-Bim (Sigma); anti-Bmf (Cell Signaling); anti-cleaved Caspase-3 (Cell Signalling); anti-JNK-1 (Santa Cruz); anti-pJNK-1 (Cell Signalling) and anti-Mcl-1 (BD Pharmingen).

---

**Figure 5. Bim-specific and Bmf-specific targeting of Mcl-1 and Bcl-XL.** (A,B) The activation of Bak and Bax upon siRNA-mediated knockdown was visualized 15 h post-infection by immunoprecipitation with conformation-specific antibodies followed by SDS-PAGE and immunodetection with the indicated antibodies. (C) The network of Bcl-2 family proteins was analyzed by caspase activation assays after single or double knockdowns, 15 h post-infection. Shown are the means±SD of three independent experiments. (D,E) Knockdowns were validated by Western blot using the indicated antibodies (D) and qRT-PCR (E).

doi:10.1371/journal.ppat.1000348.g005

**Figure 6. Model of Bim-dependent and Bmf-dependent apoptosis during Ngo-induced apoptosis.** Ngo infection leads to a Rac-dependent activation of JNK-1 and a concurrent alteration of the cytoskeletal morphology. Upon JNK-1–mediated phosphorylation, the cytoskeleton–attached proapoptotic proteins Bim and Bmf are released. Subsequently, the antiapoptotic effects of Mcl-1 and Bcl-XL are abrogated by Bim and Bmf, respectively, leading to the activation of Bak and Bax and cell death.

doi:10.1371/journal.ppat.1000348.g006
Equal loading was routinely confirmed by appropriate loading controls.

**FACS assays**

Caspase 3 and 7 activities were measured by CaspACE assay. Control and infected cells were collected and stained with 10 μM CaspACE (Promega) in growth media at 37°C, 5% CO₂ for 20 min. After staining, cells were washed twice with PBS and immediately subjected to FACS analysis.

**Immunoprecipitation**

Immunoprecipitation was carried out as described earlier [32]. Solubilized cells were precleared and incubated with 2 μg of anti-Bax (6A7) or anti-Bak (Ab1) antibody at 4°C for 2 h. Immunoprecipitates were collected by incubating with protein G-Sepharose (Amersham) for 2 h. The pellets were washed intensively with lysis buffer and resuspended in sample buffer before analysis by Western blotting using anti-Bax NT and anti-Bak NT antibodies as described above.

**RNA interference**

5 × 10⁵ HeLa cells were transfected with 1 μg of siRNA (Qiagen) using RNAiFect transfection kit (Qiagen) according to the manufacturer’s instructions. The gene silencing was routinely validated by real time PCR as previously described [48] and by Western blot analysis 72 h post-transfection. The sequence targeted to silence Bim by shRNA expression: AGAGTTTGTGGACTCCTTTA; siRac-1 were: ACGAGTTTGTGGACTCCTTTA; TGGAGAATATA and GAGCTTTGAACAGGTAGTGAA respectively.

**Generation of stable shRNA-expressing HeLa**

Stable shRNA-expressing HeLa cells were generated as described before in Kepp et al., 2007. The DNA coding for the shRNA was cloned into pLVTH-M vectors from which it integrated upon viral transfer into the genome of target cells. GFP was used as a marker to select for stable clones. The following sequence was targeted to silence Bim by shRNA expression: TAAGATAACCGTCTGTTGG. The efficiency of gene silencing was validated by Western blot analysis and quantitative realtime PCR. The cells transduced with the empty vector were used as controls.

**Immunofluorescence microscopy**

Cells seeded on coverslips were infected for 15 h, fixed in 3.7% PFA and permeabilized using 0.1% Triton X-100. Nonspecific binding was blocked by using 1% goat serum. The samples were stained using anti-actin (Sigma) and anti-tubulin (molecular probes) followed by detection with fluorochrome-coupled secondary antibodies [Jackson Immuno Research] using a Leica confocal microscope with TCS software or a Zeiss immunofluorescence microscope with ACT software. 3-dimensional remodeling was performed using Metamorph and Imaris software.

For apoptosis quantification, fixed cells were stained with 1 μg/ml Hoechst 33342 (Invitrogen) for 10 min followed by intense washing with PBS. A minimum of 5 fields per slide was analyzed for chromatin condensation using a Zeiss immunofluorescence microscope.

**Transmission electron microscopy**

Control, infected and infected zVAD treated cells were fixed 15 h post-infection with 2.5% glutaraldehyde, post-fixed with 0.5% osmium tetroxide and contrasted using tannic acid and uranyl acetate. Specimens were dehydrated in a graded ethanol series and embedded in Polybed. Ultrathin sections were analyzed in a Leo 906E transmission electron microscope (Leo GmbH).

**Cytoskeletal preparation**

To analyze cytoskeleton-associated proteins 1 × 10⁶ cells were incubated for 15 min in HBSS (Gibco). All lipidic membranes were destabilized for 5 min at 4°C by incubation with 5 ml high detergent containing Buffer M (1 mM EGTA, 4% PEG 6000, 100 mM PIPES pH 6.9) containing 0.5% Triton X-100. The cytoplasmic and compartmental proteins containing supernatant was removed and the cytoskeleton was washed with cold Buffer M. The remaining proteins were collected in sample buffer and analyzed by Western blotting.

**Statistical analysis**

The averages and standard errors of the mean as well as the t-tests have been calculated using MS Excel. Significance is indicated with ** p < 0.01 and * p < 0.05. If not indicated else wise in the figure legend the data represents at least 3 independent experiments.

**Supporting Information**

[Figure S1](#) Activation of caspase 3 by clinical gonococcal isolates. Isolates MZ522/06; MZ155/04; MZ441/05; MZ489/06; MZ359/05; MZ38/07; MZ114/07; MZ308/06; MZ245/07; and MZ452/06 were used to infect HeLa cells for 15 h and active caspase 3, and cleaved caspase 3 substrate PARP was detected by immunoblotting (see Materials and Methods for details). Infection with N242 and N242 in the presence of the caspase inhibitor zVAD (N242 + zVAD) was used as positive and negative control, respectively. Actin was detected on the same blots as loading control. Found at: doi:10.1371/journal.ppat.1000348.s001 (0.39 MB TIF)

[Figure S2](#) N242-induced apoptosis in epithelia cells. HeLa, Me180, and Hep2 cells were infected with the indicated neisserial strains and the induction of apoptosis was analyzed by quantification of fragmented chromatin using Hoechst staining and fluorescence microscopy. The neisserial strain N242 caused significant apoptosis in all analyzed cell lines, whereas other strains failed to cause significant apoptosis in all cell lines. Found at: doi:10.1371/journal.ppat.1000348.s002 (0.16 MB TIF)

[Figure S3](#) Specific Opa-receptor interaction required for induction of cell detachment and apoptosis. (A) Fluorescence-activated cell sorting (FACS) analysis of CEACAM expression by HeLa-CEACAM1 and HeLa-CEACAM3. Control (HeLa) and recombinant cells expressing CEACAM1 (CEA1) or CEACAM3 (CEA3) were detached with accutase and incubated with mouse anti-ceacam antibody (clone D1HHD11) and anti-mouse Cy2 (red line). Isotope controls (black lines) were only incubated with anti-mouse-Cy2. (B) Induction of apoptosis depends on specific CEACAM-Opa interaction. Recombinant HeLa cell lines were either left uninfected (Ctr) or infected with N927 (Opa+;P-) or N1163 (Opa57;P-). Opa57 expressing bacteria adhered to both cell lines (not shown) as previously published by Bilkier et al., 2002. Apoptotic cells with condensed chromatin were quantified by microscopy. Shown is one typical example of several experiments.
with similar results. (C) Infected recombinant cell lines were analyzed for cell detachment by phase contrast microscopy. 

Found at: doi:10.1371/journal.ppat.1000348.s005 (0.68 MB TIF)

**Figure S4** Bim is necessary for Ngo-induced apoptosis. (A) Stable shRNA-expressing HeLa cells were treated with 20 μM cisplatin for 20 h, and the caspase activity was measured by FACS using CaspACE assay. The data represent the mean ± SD of three independent experiments. (B) Depletion of Bim prevented the activation of Bak and Bax as well as subsequent Caspase-activation upon Ngo infection. shBim and control cells harboring an empty vector were infected for 15 h, and the activity of Bak and Bax was analyzed by immunoprecipitation using conformation-specific antibodies. Caspase activity was shown by immunodetection of the cleaved forms of caspases 3. 

Found at: doi:10.1371/journal.ppat.1000348.s004 (0.13 MB TIF)

**Figure S5** DNA damage induced BH3-only signaling. (A) siRNA transfected cells were treated with 20 μM cisplatin for 20 h, and the caspase activity was measured by FACS using CaspACE assay. The data represent the mean ± SD of at least three independent experiments. (B) Puma and Noxa mRNA levels were examined by qRT-PCR and showed significant upregulation 20 h post-treatment. The data represent the mean ± SD of three independent experiments. (C) Protein levels were determined by Western blot with the indicated antibodies, demonstrating an increased protein level of both proteins 20 h after cisplatin treatment. 

Found at: doi:10.1371/journal.ppat.1000348.s005 (0.27 MB TIF)

**Figure S6** Neisserial infection causes no transcriptional regulation of BH3-only proteins. The effect of infection on the mRNA levels of the BH3-only proteins was analyzed by qRT-PCR and showed no significant changes in comparison with uninfected controls.

Found at: doi:10.1371/journal.ppat.1000348.s006 (0.06 MB TIF)

**Figure S7** Release of Bim and Bmf from their sequestration sites. (A) F-actin-enriched P1 and myosin-enriched P2 fractions were separated from 107 cells as described by Puthalakath et al. (Science 293: 1829–1832, 2001). The fractions were analyzed by Western blot, with the indicated antibodies, showing less Bmf and less Bim in P1 upon infection, whereas the level of Bim in P2 seems to stay unchanged. (B) Gradient centrifugation was carried out as described in Puthalakath et al., 2001. The 4 ml gradient was divided into 0.5 ml fractions, which were then analyzed by Western blot with the indicated antibodies. Bim and Bmf translocate upon infection from heavier fractions to lighter fractions of the gradient. (C) The inhibition of JNK-1 Inhibitor-1 (Calbiochem) at 1 μM during the experiment also inhibited the translocation of Bim and Bmf to lighter fractions of the gradient. (D) Same as under (C) using the Rac-1 inhibitor NSC23766. 

Found at: doi:10.1371/journal.ppat.1000348.s007 (0.99 MB TIF)

**Figure S8** Electrophoretic mobility of Bim changes at late time points. HeLa cells were infected, and samples were taken after the indicated time. The electrophoretic mobility of Bim was analyzed by Western blot, showing Bim phosphorylation no earlier than 9 h post-infection. 

Found at: doi:10.1371/journal.ppat.1000348.s008 (0.09 MB TIF)

**Figure S9** Exclusion of ERK-specific effects. ERK activation was analyzed in untreated and Ngo-infected samples in the presence and in the absence of the ERK-specific Inhibitor U0126. Western blot analysis showed no significant activation of ERK by infection with Ngo. 

Found at: doi:10.1371/journal.ppat.1000348.s009 (0.07 MB TIF)

**Figure S10** Exclusion of off-target effects and cross-regulation. HeLa cells were transfected with siBim and siBmf. The knockdown of the specific gene products as well as potential off-target effects and cross-regulation within the Bcl-2 protein family was analyzed by Western blot with antibodies detecting the indicated proteins. No cross-regulation or off-target effects of the siRNA treatment could be detected. 

Found at: doi:10.1371/journal.ppat.1000348.s010 (0.16 MB TIF)

**Video S1** Infection-induced changes in cellular shape. Actin-GFP (Clonetech)-expressing cells were infected and a series of z-stacks was acquired every hour using an Olympus spinning disc microscope. The pictures were 3-dimensional remodeled using the Imaris software. Shown is one representative cell undergoing morphological changes.

Found at: doi:10.1371/journal.ppat.1000348.s011 (1.9 MB AVI)

**Video S2** Cytoskeletal changes prior to cell death. Cells were cultured in glass-bottom dishes and placed under an Olympus spinning disc microscope. Phase contrast pictures of the cells were taken every 10 min upon infection.

Found at: doi:10.1371/journal.ppat.1000348.s012 (2.3 MB AVI)

**Acknowledgments**

We thank Hans Thorn for help with the confocal microscopy and the live cell imaging, and Ulrich Vogel for providing clinical isolates of *N. gonorrhoeae*.

**Author Contributions**

Conceived and designed the experiments: OK YC KR TR. Performed the experiments: OK KG YC TR. Contributed reagents/materials/analysis tools: VB NM. Wrote the paper: OK KG TR.

**References**

1. Zychlinsky A, Sansonetti PJ (1997) Apoptosis as a proinflammatory event: what can we learn from bacteria-induced cell death? Trends Microbiol 5: 291–294.
2. Muller A, Gunther D, Dux F, Naumann M, Meyer TF, et al. (1999) Neisserial porin (PorB) causes rapid calcium influx in target cells and induces apoptosis by the activation of cysteine proteases. EMBO J 18: 339–352.
3. Apicella MA, Ketterer M, Lee FK, Zhou D, Rice PA, et al. (1996) The porin (PorB) causes rapid calcium influx in target cells and induces apoptosis by the activation of cysteine proteases. EMBO J 18: 339–352.
4. Agrawal DK, Kim SJ, Zychlinsky A, Sansonetti PJ (1996) Neisserial infection causes no transcriptional regulation of BH3-only proteins. The effect of infection on the mRNA levels of the BH3-only proteins was analyzed by qRT-PCR and showed no significant changes in comparison with uninfected controls.
5. Agrawal DK, Kim SJ, Zychlinsky A, Sansonetti PJ (1996) Neisserial infection causes no transcriptional regulation of BH3-only proteins. The effect of infection on the mRNA levels of the BH3-only proteins was analyzed by qRT-PCR and showed no significant changes in comparison with uninfected controls.
6. Agrawal DK, Kim SJ, Zychlinsky A, Sansonetti PJ (1996) Neisserial infection causes no transcriptional regulation of BH3-only proteins. The effect of infection on the mRNA levels of the BH3-only proteins was analyzed by qRT-PCR and showed no significant changes in comparison with uninfected controls.
7. Zychlinsky A, Sansonetti PJ (1997) Apoptosis as a proinflammatory event: what can we learn from bacteria-induced cell death? Trends Microbiol 5: 291–294.
8. Chen L, Willis SN, Wei A, Smith BJ, Fletcher JL, et al. (2005) Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol Cell 17: 393–403.
9. Cory S, Adams JM (2002) The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2: 647–656.
10. Letai AG (2008) Diagnosing and exploiting cancer’s addiction to blocks in apoptosis. Nat Rev Cancer 8: 121–132.
11. Wei MC, Lindsten T, Mootha VK, Weiler S, Gross A, et al. (2000) BID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. Genes Dev 14: 2060–2071.
12. Antounson B, Montesuitt S, Sanchez B, Martinou JC (2001) Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. J Biol Chem 276: 11615–11623.
13. Kroemer G, Galluzzi L, Brenner C (2007) Mitochondrial membrane permeabilization in cell death. Physiol Rev 87: 99–163.
31. McCaw SE, Liao EH, Gray-Owen SD (2004) Engulfment of Neisseria
30. Dehio C, Gray-Owen SD, Meyer TF (1998) The role of neisserial Opa proteins
29. Makino S, van Putten JP, Meyer TF (1991) Phase variation of the opacity outer
28. Rajalingam K, Sharma M, Lohmann C, Oswald M, Thieck O, et al. (2008) Mcl-
27. Rajalingam K, Oswald M, Gottschalk K, Rudel T (2007) Smac/DIABLO is
24. Lei K, Davis RJ (2003) JNK phosphorylation of Bim-related members of the
23. Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A (1999) The
21. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ (1996) Serine phosphorylation
20. Wang K, Yin XM, Chao DT, Milliman CL, Korsmeyer SJ (1996) BID: a novel
19. Nakano K, Vousden KH (2001) PUMA, a novel proapoptotic gene, is induced
18. Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, et al. (2000) Noxa, a BH3-
16. Cartron PF, Gallenne T, Bougras G, Gautier F, Manero F, et al. (2004) The first
15. Kuwana T, Bouchier-Hayes L, Chipuk JE, Bonzon C, Sullivan BA, et al. (2005)
14. Willis SN, Adams JM (2005) Life in the balance: how BH3-only proteins induce
13. Naumann M, Wessler S, Bartsch C, Wieland B, Meyer TF (1997) Neisseria
12. van Putten JP, Meyer TF (1993) Neisseria gonorrhoeae porin reduces invasion in
11. Bokoch GM (1998) p21-activated kinase
10. Cartron PF, Gallenne T, Bougras G, Gautier F, Manero F, et al. (2004) The first
9. Cuconati A, Mukherjee C, Perez D, White E (2003) DNA damage response and
8. Wang K, Yin XM, Chao DT, Milliman CL, Korsmeyer SJ (1996) BID: a novel
7. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ (1996) Serine phosphorylation
6. Rudel T, Zeke FT, Chuang TH, Bokoch GM (1998) p21-activated kinase
5. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ (1996) Serine phosphorylation
4. Lei K, Davis RJ (2003) JNK phosphorylation of Bim-related members of the
3. Naumann M, Rudel T, Meyer TF (1999) Host cell interactions and signalling
2. Pathalakath H, Villunger A, O'Reilly LA, Beaumont JG, Coultas L, et al. (2001)
1. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ (1996) Serine phosphorylation

Bim and Bmf Induce Apoptosis Synergistically

48. Machuy N, Thiede B, Rajalingam K, Dimmler C, Thieck O, et al. (2005) A
47. Bauer FJ, Rudel T, Stein M, Meyer TF (1999) Mutagenesis of the Neisseria
46. Kupsch EM, Knepper B, Kuroki T, Heuer I, Meyer TF (1993) Variable opacity
45. Naumann M, Rudel T, Meyer TF (1999) Host cell interactions and signalling
44. Massari P, King CA, Ho AY, Wetzler LM (2003) Neisserial PorB is translocated
43. Howie HL, Shiflett SL, So M (2008) Extracellular signal-regulated kinase
42. Massari P, Naumann M, Meyer TF, Gray-Owen SD (2001) Pathogenic
41. Billker O, Popp A, Brinkmann V, Wenig G, Schneider J, et al. (2002) Distinct
40. Muenzner P, Naumann M, Meyer TF, Gray-Owen SD (2001) Pathogenic
39. Naumann M, Rudel T, Meyer TF (1999) Host cell interactions and signalling
38. Cuconati A, Mukherjee C, Perez D, White E (2003) DNA damage response and
37. Kupsch EM, Knepper B, Kuroki T, Heuer I, Meyer TF (1993) Variable opacity
36. Baas F, Groll M, van Deurs B, Ruegg C (2000) The role of the cytosolic
35. Kupsch EM, Knepper B, Kuroki T, Heuer I, Meyer TF (1993) Variable opacity
34. Billker O, Popp A, Brinkmann V, Wenig G, Schneider J, et al. (2002) Distinct
33. Naumann M, Wessler S, Bartsch C, Wieland B, Meyer TF (1997) Neisseria
32. Kopp O, Rajalingam K, Kinnum S, Rudel T (2007) Bak and Bax are non-
31. Makino S, van Putten JP, Meyer TF (1991) Phase variation of the opacity outer
30. Dehio C, Gray-Owen SD, Meyer TF (1998) The role of neisserial Opa proteins
29. Makino S, van Putten JP, Meyer TF (1991) Phase variation of the opacity outer
28. Rajalingam K, Sharma M, Lohmann C, Oswald M, Thieck O, et al. (2008) Mcl-
27. Rajalingam K, Oswald M, Gottschalk K, Rudel T (2007) Smac/DIABLO is
26. Rudel T, Zeke FT, Chuang TH, Bokoch GM (1998) p21-activated kinase
25. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ (1996) Serine phosphorylation
24. Lei K, Davis RJ (2003) JNK phosphorylation of Bim-related members of the
23. Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A (1999) The
22. Pathalakath H, Villunger A, O'Reilly LA, Beaumont JG, Coultas L, et al. (2001)
21. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ (1996) Serine phosphorylation
20. Wang K, Yin XM, Chao DT, Milliman CL, Korsmeyer SJ (1996) BID: a novel
19. Nakano K, Vousden KH (2001) PUMA, a novel proapoptotic gene, is induced
18. Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, et al. (2000) Noxa, a BH3-
17. Kim H, Raffadunn-Shah M, Tu HC, Jeffers JR, Zambrjiti GP, et al. (2006)
16. Cartron PF, Gallenne T, Bougras G, Gautier F, Manero F, et al. (2004) The first
15. Kuwana T, Bouchier-Hayes L, Chipuk JE, Bonzon C, Sullivan BA, et al. (2005)
14. Willis SN, Adams JM (2005) Life in the balance: how BH3-only proteins induce
13. Naumann M, Wessler S, Bartsch C, Wieland B, Meyer TF (1997) Neisseria
12. van Putten JP, Meyer TF (1993) Neisseria gonorrhoeae porin reduces invasion in
11. Bokoch GM (1998) p21-activated kinase
10. Cartron PF, Gallenne T, Bougras G, Gautier F, Manero F, et al. (2004) The first
9. Cuconati A, Mukherjee C, Perez D, White E (2003) DNA damage response and
8. Wang K, Yin XM, Chao DT, Milliman CL, Korsmeyer SJ (1996) BID: a novel
7. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ (1996) Serine phosphorylation
6. Rudel T, Zeke FT, Chuang TH, Bokoch GM (1998) p21-activated kinase
5. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ (1996) Serine phosphorylation
4. Lei K, Davis RJ (2003) JNK phosphorylation of Bim-related members of the
3. Naumann M, Rudel T, Meyer TF (1999) Host cell interactions and signalling
2. Pathalakath H, Villunger A, O'Reilly LA, Beaumont JG, Coultas L, et al. (2001)
1. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ (1996) Serine phosphorylation