Role of Pro-apoptotic BH3-only proteins in *Listeria monocytogenes* infection

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Abbreviations

Lm: *Listeria monocytogenes*

DKO: Double knockout

MOMP: Mitochondrial outer membrane permeabilization

NETs: Extracellular DNA traps

ROS: Reactive oxygen species

MPO: Extracellular myeloperoxidase (MPO)

LLO: listeriolysin O

PMA: Phorbol myristate acetate

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HKLM: Heat-killed Lm

Here, we show that BimNoxa DKO mice are resistant to lethal doses of *Listeria*. We found that they are able to clear bacterial infection more efficiently than WT mice. This effect is mainly mediated by downregulation of TNF α as well as increased ROS production and NET release.

Abstract

The ability of pathogens to influence host cell survival is a crucial virulence factor. *Listeria monocytogenes* (Lm) infection is known to be associated with severe apoptosis of hepatocytes and spleen cells. This impairs host defense mechanisms and thereby facilitates the spread of intracellular pathogens. The general mechanisms of apoptosis elicited by Lm infection are understood, however, the roles of BH3-only proteins during primary Lm infection have not been examined. To explore the roles of BH3-only proteins in Lm-induced apoptosis, we studied *Listeria* infections in mice deficient in Bim, Bid, Noxa or double deficient in BimBid or BimNoxa. We found that BimNoxa double knockout mice were highly resistant to high dose challenge with *Listeria*. Decreased bacterial burden and decreased host cell apoptosis were found in the spleens of these mice. The ability of the BH3-deficient mice to clear bacterial infection more efficiently than WT was correlated with increased concentrations of reactive oxygen species, neutrophil extracellular DNA trap release and downregulation of TNF α . Our data show a novel pathway of infection-induced

apoptosis that enhances our understanding of the mechanism by which BH3-only proteins control apoptotic host cell death during *Listeria* infection.

Introduction

Apoptotic cell death is thought to play a critical role in the development of immune cells, in the maintenance of tissue homeostasis [1], and in the contraction of an immune response following infection. Apoptosis is regulated via three major pathways: the intrinsic or mitochondrial pathway, the death receptor pathway and the perforin/granzyme pathway.

The intrinsic apoptotic pathway can be activated by developmental cues, cytokine withdrawal and diverse cytotoxic stress signals. This pathway involves mitochondrial outer membrane permeabilization (MOMP) caused by the activation of Bax and Bak. MOMP, in turn, is responsible for the release of apoptogenic factors, such as cytochrome c, which promotes activation of the initiator caspase-9. After activation, caspase 9 cleaves and activates the effector caspases 3 and 7, which then cause cell demolition [1, 2].

The intrinsic pathway is controlled by the interactions between anti-apoptotic and pro-apoptotic members of the B cell lymphoma 2 (Bcl-2) family of proteins [3, 4]. The anti-apoptotic members, including Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and A1, share four Bcl-2 homology domains (BH) and are essential for cell survival. The BH3-only subgroup, including Bim, Bid, Puma, Bad, Bmf, Bik, Hrk and Noxa, is essential for initiation of apoptosis and thought to bind and activate the pro-apoptotic effector proteins Bax/Bak either directly or indirectly by neutralizing the Bcl-2 pro-survival proteins[5].

The ability of pathogens to influence host cell survival can be a crucial factor underlying their pathogenicity [6]. Bacterial pathogens can elicit diverse cell stresses, such as ER stress or

stress related to the production of reactive oxygen species (ROS). These stress factors trigger the intrinsic pathway of apoptosis[7]. It was reported that Bim and Bmf are critical for host cell apoptosis upon infection with *Neisseria gonorrhoeae* [8].

The murine model of systemic *Listeria monocytogenes* (Lm) infection has proven to be an excellent experimental system to study innate and adaptive immune responses to intracellular bacterial pathogens [9]. Lm is a facultative, intracellular, Gram-positive bacterium that replicates within the cytosol of both phagocytic and non-phagocytic cells [10, 11]. During the early phase of an Lm infection, macrophages, monocytes, neutrophils and NK cells secrete pro-inflammatory cytokines, such as TNF α , IL-6, IL-12 and IFN- γ , which slow the spread of the bacteria and facilitate the development of a protective immune response against the pathogen [12-15]. The production of ROS or nitric oxide (NO) also contributes to pathogen clearance in vivo [16, 17]. In addition to these mechanisms of bacterial killing, the activation of neutrophils causes the release of extracellular DNA traps (NETs) with proteolytic activity that can trap and kill the pathogens [18, 19]. The adaptive immune response, which takes several days to develop, plays no significant role in the early phase of the infection. However, a recent study has implicated an important role for memory CD8⁺ T cells in the early immune response to Lm infection. [20].

Lm infection is known to be associated with substantial apoptosis of thymocytes, hepatocytes, and spleen cells [21, 22]. It has been hypothesised that apoptosis of infected cells serves to limit the spread of the bacteria [23]. However, apoptosis of immune cells would be expected to diminish host defense and thus facilitate the spread of intracellular pathogens.

There is some insight into the mechanisms of apoptosis triggered by Lm infection and its contribution to the host defense. Death receptor induced apoptosis, mediated through the interaction between Fas ligand-Fas, does not play a major role during primary listeriosis [24].

One study reported that TRAIL, known to induce apoptosis of certain tumor cells but not most normal cell [25, 26], plays a role in *Listeria* induced apoptosis and listeriosis. TRAIL deficient mice were shown to be partially resistant to primary Lm infection [27]. However, the roles of BH3-only proteins in apoptosis during primary Lm infection have not yet been examined.

We therefore studied listeriosis in mice deficient in BimBid, BimNoxa, Bim, Bid or Noxa. We found that all BimNoxa DKO mice survived a high dose Lm challenge whereas all wild type mice died. Other gene targeted mice showed a range of improved survival Bid KO (80% survival), Bim KO (70% survival) > Bim 70% survival), BimBid DKO and Noxa KO mice (both 35% survival). Increased animal survival was associated with upregulation of ROS and NETs and down-regulation of TNF α .

Results

BimBid mice and BimNoxa double knockout mice are resistant to *Listeria* infection

BimBid and BimNoxa DKO (double KO) and WT mice were infected intravenously with a lethal dose of Lm (10^4) and animal survival was monitored. WT mice started dying on day 3 post-infection, and all WT mice succumbed to infection by day 6 (Figure 1A). In striking contrast, all BimNoxa DKO mice survived a lethal Lm dose (Figure 1A). The BimBid DKO mice also showed substantially increased, albeit not complete, resistance to this infectious challenge. Interestingly, the Bid KO mice were significantly more resistant to Lm infection compared to WT, Bim KO, Noxa KO mice and curiously even compared to BimBid DKO mice (Figure 1B). Collectively, these results show the following order of resistance to Lm infection: BimNoxa DKO > Bid KO > Bim KO > BimBid DKO = Noxa KO > WT.

To dissect the mechanism responsible for the survival advantage, particularly in BimNoxa DKO mice, we performed in vitro assays with spleen cells from infected animals. Interestingly, spleen cells from infected BimNoxa DKO mice showed dramatically reduced *Listeria* burdens on day 4 of culture (Figure 1C). Moreover, immunofluorescence histological analysis revealed higher bacterial load in spleens of Lm-infected WT mice compared to the BimNoxa DKO mice on day 4 post-infection (Figure 1D). These findings were extended by performing TUNEL analysis to identify the extent of cell death in tissue sections from spleens of infected mice. Large numbers of TUNEL positive cells (cells undergoing apoptosis) were detected in the spleens of WT mice on day 3 after Lm infection, but this was substantially reduced in the BimNoxa as well as BimBid DKO mice (Figure 1E).

Taken together, these results suggest that the BH3-only proteins Bim, Bid and Noxa have a critical role in the apoptosis of Lm infected cells and that their absence conferred protection from otherwise lethal infection. The combined loss of BimNoxa conferred complete protection (synergy), whereas single loss of Bim or Bid conferred better protection than combined loss of Bim and Bid (negative genetic interaction).

Decreased numbers of splenic neutrophils in Lm-infected BimNoxa DKO mice

We next examined the impact of *Listeria* infection on the numbers of the various splenic cell subsets in the WT as well as the KO and DKO mice. We found that 3 days after infection, the total numbers of cells in the spleen had doubled in all mouse strains (Figure 2A). In the uninfected DKO mice the percentages and total numbers of all cell types were comparable to the WT animals with the exception of fewer macrophages and more CD4 and CD8 T cells in BimBid DKO mice (Supporting Information Figure 1). Following infection, the numbers of CD11b⁺DC cells increased in the BimNoxa DKO mice while the number of monocytes remained unchanged in all strains. Importantly, the numbers of neutrophils were reduced in the infected BimNoxa DKO mice compared to the WT and BimBid DKO mice. To investigate whether accelerated migration to the liver or reduced recruitment from the bone marrow contributed to the neutrophil reduction seen in the spleens of infected BimNoxa DKO mice, we quantified neutrophil numbers in both liver and bone marrow. We found no significant changes in these compartments (data not shown). In addition, upon infection we observed increased numbers of CD4⁺ and CD8⁺ T cells in both the BimNoxa and BimBid DKO mice compared to the WT animals (Figure 2B).

Increased levels of ROS and NETs found in Lm-infected DKO mice

We next investigated the abundance of factors known to be critical for control of bacterial infection, such as ROS, in Lm infected mice of the different genotypes. Spleens of single and double deficient and WT mice infected with the high dose of *Listeria* were harvested and ROS levels measured using flow cytometry. As shown in Figure 3A, neutrophils and

monocytes from Lm infected BimNoxa and BimBid DKO mice exhibited higher ROS levels compared to their WT counterparts. At later time points (3 h post-infection) these cells from BimBid and BimNoxa DKO mice still maintained higher ROS levels than cells from all the other strains. We next analyzed cell death in parallel to ROS production in neutrophils to test if there was a connection. Both the BimNoxa and BimBid DKO neutrophils showed significantly improved survival in comparison with cells from WT mice with the highest protection seen in the BimNoxa DKO mice (Supporting Information Figure 2).

The production of ROS is thought to be essential for NETs formation and both together are known to be critical for bacterial killing. We therefore measured the amount of DNA in the serum of mice on day 3 post-infection. As shown in Figure 3B, the levels of DNA in the serum were significantly higher in the Lm infected BimNoxa and BimBid mice compared to the WT controls.

To extend these observations further, we isolated neutrophils from the spleens of these animals and stimulated them with *Listeria* to examine NET formation in vitro. NETs were identified by the detection of extracellular localization of DNA and myeloperoxidase (MPO) using confocal microscopy. Consistent with previous studies [18, 19], treatment of neutrophils with PMA (Figure 3c) caused an increase in extracellular DNA fluorescence within 3 h. Compared with WT neutrophils, BimNoxa and BimBid DKO neutrophils produced markedly more NETs when stimulated with *Listeria* (Figure 3C). Giemsa-stained blood smears from the BimNoxa and BimBid DKO mice confirmed the release of DNA from neutrophils (Figure 3D).

Depletion of neutrophils reduces the resistance of BimNoxa DKO mice to *Listeria* infection

Previous studies suggested that neutrophils play an essential role in the early immune defense against high dose Lm infection [28, 29]. To examine whether the resistance of the BimNoxa DKO mice was dependent on neutrophils, we treated WT and DKO animals with the specific neutrophil-depleting monoclonal antibody anti-Ly6G prior to *Listeria* infection. We found that after neutrophil depletion, WT mice showed no change in bacterial burden and died on day 2. (Figure 4A, B). Interestingly, the BimNoxa DKO mice rapidly succumbed to Lm infection after neutrophil depletion, with a striking increase in bacterial burden (Figure 4A, B). This indicates that neutrophils are essential for effective immune defense against *Listeria* in these animals. In the neutrophil depleted BimBid DKO mice, bacterial burden increased to a lesser extent than in the BimNoxa DKO animals. The death of the BimBid DKO mice was also accelerated by neutrophil depletion, but to a lesser extent compared to the BimNoxa DKO animals (Figure 4A, B), with all BimBid mice having succumbed by day 11. These results indicate that other cells, in addition to neutrophils, may also be important for the protection from *Listeria* infection in the BimBid DKO mice.

Cytokine expression profiles during *Listeria* infection in DKO mice

To further explore the nature of the enhanced bacterial clearance in the BimNoxa and BimBid DKO mice, we next assessed the expression of cytokines in spleens and sera from Lm infected WT and DKO mice. Compared to WT controls, the Lm-infected BimBid DKO mice showed significantly increased serum IL-6 at 18 h and day 3 post-infection but decreased IFN- γ level at 18 h. The BimNoxa DKO mice also showed a significant reduction in IFN- γ

levels compared with WT mice at 18 h. Remarkably, the levels of TNF- α in the serum were significantly reduced in Lm-infected BimNoxa DKO mice compared to the WT and BimBid DKO animals on day 3, when WT mice started to succumb to infection (Figure 5A). The decreased levels of TNF- α were confirmed by intracellular staining and FACS analysis. Upon exposure to heat killed *Listeria*, cultures of spleen cells from BimNoxa DKO mice contained fewer TNF- α -producing cells compared to similarly treated spleen cells from WT mice (Figure 5B). Moreover, we found that both, infected neutrophils and monocytes from DKO mice produced less TNF- α compared to their WT counterparts (Figure 5C). Together, these results support the notion that BH3-only proteins may affect TNF- α secretion by controlling host cell survival when the bacterial load is high. We also analyzed IFN- γ production by CD4⁺ T cells on day 3 after infection (when WT mice started dying) but there was no difference between the different strains (Supporting Information Figure 3).

Neutralization of TNF α results in increased survival of WT mice

To investigate the possibility that overproduction of TNF- α was responsible for the premature death of WT mice during high dose Lm infection, we used neutralizing antibodies against TNF- α and assessed the survival of mice. TNF- α is required for clearance of Lm. Treatment with neutralizing antibody during the first 3 days following Lm infection therefore killed the WT mice. Interestingly, treatment with TNF- α neutralizing antibody on days 3 and 4 post infection resulted in increased survival of WT mice compared to Lm infected WT mice treated with an isotype matched control antibody. This shows that at this stage of *Listeria* infection the excess TNF- α causes liver damage with substantial morbidity and mortality (Figure 6A). The improved resistance to *Listeria* infection in WT mice after TNF-

α -neutralizing antibody treatment was associated with an improved clearance of *Listeria* (Figure 6B). Surprisingly, TNF- α neutralization had no effect on bacterial burden in BimBid DKO mice. These data indicate that neutralization of TNF- α at late stages of infection could rescue WT mice from rapid death following Lm infection and suggested that BH3-only proteins, through regulation of cell death, may impact on TNF- α production during bacterial infection.

Discussion

Over the past decade, our understanding of the ability of pathogens to manipulate host cell viability has increased substantially. Several studies have demonstrated that some intracellular pathogens delay host cell death to increase their replication, whereas others accelerate apoptosis as a pathogenic mechanism.

Several lines of evidence indicate that pathogens use multiple strategies to modulate apoptotic and survival signaling pathways in their host cells and that this impacts on disease progression. However, the nature of the molecules that control these processes are not well understood. In this study, we identified a previously unknown role of BH3-only proteins in *Listeria*-induced apoptosis and listeriosis. We found that mice deficient in Bim, Noxa and Bid are partially protected from high dose of Lm. We also describe a positive synergy in protection in BimNoxa DKO mice and a negative interaction in BimBid DKO mice. The DKO mice exhibited reduced numbers of apoptotic cells in their spleens and reduced bacterial load upon *Listeria* infection.

Lm has been shown to induce apoptosis of spleen cells in infected mice and this appears to be mediated mainly by the apoptogenic molecule, listeriolysin O (LLO) [30, 31]. Several

mechanisms have been implicated in LLO induced apoptosis. LLO has been shown to induce calcium flux [32]. Lm was reported to cause dramatic alterations in mitochondrial dynamics via LLO [33]. Moreover, LLO was shown to induce type I interferon expression and this sensitises lymphocytes to apoptosis and reduces resistance to *Listeria* infection [34, 35]. In line with this observation, our previous work revealed that poly IC induced apoptosis in dendritic cells and this was initiated by type I interferon signaling and was dependent on several pro-apoptotic BH3-only proteins [36]. A role for this cytokine in our present model still needs to be clarified. To our knowledge, there is no prior publication showing that LLO induces apoptosis through the activation of BH3-only proteins. Interestingly, the important role and impact of the intrinsic apoptotic pathway and BH3-only proteins has been described in other pathogen infections [37, 38].

The nature of the apoptogenic stimuli initiated by *Listeria* infection is still not resolved. Our data suggest that *Listeria* infection may kill host cells through activation of both the death receptor and intrinsic apoptotic pathways. Cross-talk between these two apoptotic pathways has been described in other models [39, 40]. However, the finding that combined loss of Bim and Noxa provided greater protection than loss of both Bim and Bid indicates a greater role for the intrinsic apoptotic pathway. We do not have a clear explanation for the increased protective effect of combined loss of Bim and Noxa compared to combined loss of Bim and Bid. One possible explanation is that Bim and Noxa are both activated in response to DNA damage; this is notable because infection with Lm was recently reported to induce DNA breaks and activate the DNA damage response pathways [41]. It is possible that Puma, which is also activated by DNA damage, may also contribute to apoptosis induced by *Listeria* infection [42, 43]. It would be interesting to examine the responses of BimPuma DKO and BimPumaNoxa triple KO mice to Lm infection.

Interestingly, loss of Bid provided greater resistance to Lm than combined loss of Bim and Bid. The synergy between loss of Bim and Noxa could be explained by the fact that both proteins are activated by the same upstream signals and that they both act in the same cell death pathway. The negative interaction between combined loss of Bim and Bid is more difficult to explain. One possibility is that initial protection from apoptosis in BimBid DKO cells may lead to the production of cytokines or stress conditions that induce apoptosis through (a) pathway(s) that require BH3-only proteins other than Bim or Bid, perhaps Puma and Noxa.

Our results demonstrated that production of extra-cellular NETs was elevated in BimNoxa DKO mice. Neutrophils are thought to constitute the first line of defense against bacteria. The increase in NETs and ROS levels may therefore reflect increased activation and/or survival of neutrophils. It is noteworthy that ROS are required for the formation of NETs [44] and NET production has been shown to be distinct from the classical form of cell death, such as apoptosis or necrosis [45]. The molecular pathways and the physiological role of NET formation are still only poorly understood. To our knowledge, this is the first report to show that neutrophils deficient in pro-apoptotic BH3-only proteins, and therefore resistant to apoptosis, are able to produce NETs following pathogen infection. This provides further evidence that NET release does not require activation of the intrinsic apoptotic pathway.

A potential mechanism for the improved survival of *Listeria* infected BimNoxa DKO mice is the impact of loss of these BH3-only proteins on the levels of cytokines. In this regard, TNF α is known to be critical for clearance of *Listeria* [46]. However, overproduction of TNF α can lead to shock and death. Therefore the TNF α produced during infection must be tightly regulated to allow for bacterial clearance without causing excess tissue damage and lethality [47, 48]. In this report we show that BH3-only proteins play an important role in

maintaining an appropriate level of TNF α during infection with high dose of Lm. Although the TNF α neutralization experiments that we performed strongly suggested that TNF α was a key mediator in the death of Lm infected WT mice, other cytokines and inflammatory mediators are likely to be involved as well. More studies are needed to understand how BH3-only proteins regulate TNF α production during Lm infection. Understanding of these processes may be useful for optimization of anti-TNF α therapies in treating a variety of diseases.

Finally, our findings suggest that inhibition of select BH3-only proteins, once specific drugs are discovered, might constitute an attractive therapeutic approach for controlling infection by intracellular pathogens.

Materials and Methods

Ethics statement

Animal experiments were performed in strict accordance to the Swiss Federal Regulations. The protocol was approved by the “Service de la consommation et des affaires vétérinaires du Canton de Vaud”, Switzerland (Permit Number: 1847.1). All efforts were made to minimize suffering and minimize the number of mice needed to assess statistical significance and experimental reproducibility.

Mice and *Listeria* Infection

The mice used were females or males aged at least 8 weeks. Mice were WT (C57BL/6), Bim, Noxa and Bid single KO or BimNoxa and BimBid DKO. *Listeria monocytogenes* was grown as previously described [49]. Mice were injected intravenously with 10^4 cfu of *Listeria*.

To quantitate *Listeria* in organs, small portions of the spleens were fixed in 10% formalin and stained with *Listeria* O Antiserum Poly Types 1 and 4 (BD Difco™) and Alexa Fluor® 647 anti-rabbit (Poly4064, BioLegend).

For *in vivo* neutrophil depletion, mice were injected i.p. with 500 µg 1A8 monoclonal anti-Ly6G antibody (BioXcell) one day prior to Lm infection. Control mice received isotype matched rat IgG (Sigma). For *in vivo* blockade of TNFα, mice were injected i.p. with 1 mg anti- TNFα antibody (Remicade, Merck Sharp&Dohme AG) on days 3 and 4 post infection. Control mice received Ig isotype matched monoclonal antibody (human IgG).

5.2 Antibodies

The following fluorochrome labeled antibodies were used. PE anti-CD3ε (145-2C11), eFluor®450 anti-CD3ε (eBio500A2), PerCP-Cy5.5 anti-CD8a (53-6.7), eFluor®450 anti-CD8a (53-6.7), FITC anti-CD4 (RM4-5), APC anti-CD4 (RM4-5), APC anti-Ly6C (HK1.4), APC eFluor®780 anti-CD11b (M1/70), Alexa Fluor® 700 anti-B220 (RA3-6B2) were purchased from eBioscience; while the PE-Cy7 anti-CD4 (RM4-5), PerCP-Cy5.5 anti-Ly6G (1A8), PE-Cy7 anti-CD11c (N418), FITC anti-CD24 (M1/69) were purchased from BioLegend.

TUNEL Assay

Spleens were collected and fixed in 10% buffered formalin and embedded in paraffin. Tissue sections were prepared and subjected to TUNEL staining (to reveal apoptotic cells) without blocking the endogenous peroxidase and visualized using a Leica DM IL LED microscope. The extent of apoptosis in the tissues was measured using the Image software (version 1.49h, National Institutes of Health, USA) and the data were presented as the number of apoptotic cells divided by the total area of the sections measured. A total of 10 tissue sections were analyzed for each animal.

Reactive Oxygen Species (ROS) Detection

Spleen cells were incubated with Heat Killed *Listeria* (HKLM) for 1 or 3 h. The intracellular ROS production was detected using the dye Dichloro-dihydro-fluorescein diacetate (*DCFH-DA*) according to the manufacturer's instructions (Molecular Probes Inc.) and analyzed by flow cytometry.

Detection of NETs by Immunostaining

The neutrophils were isolated from the bone marrow and inserted in Lab-Tek chamber slides (10^5 cells/well). The cells were incubated with live *Listeria monocytogenes* (MOI 100:1) for 3 h. Neutrophils were stimulated with phorbol myristate acetate (PMA) as a positive control for the formation of NETs. The slides were fixed for 10 min in PFA 4% and blocked for 45 min with 2% BSA in PBS. The cells were then incubated with rabbit polyclonal anti-MPO antibody (Dako) and Alexa fluor® 488 anti-rabbit (Life Technologies). To visualize DNA, cells were stained with Hoechst 33342 (Invitrogen). After washing three times with TBS, the sections were incubated with 1:250 dilutions of anti-mouse Alexa Fluor 488 and anti-rabbit

Alexa Fluor 555 secondary antibodies (Molecular Probes), as well as DAPI (Invitrogen) at room temperature for 1 h. The sections were then washed with TBS, mounted with anti-fade mounting medium (Invitrogen), and examined under an IX81 Olympus confocal microscope. All images were processed using the FV10-ASW 3.0 viewer. The slides were examined under the confocal microscope Leica SP5 Tandem and the images were processed using the LAS AF software (Leica).

Serum NET Quantification

Blood samples were collected on day 3 post infection and sera were prepared. Serum NETs were quantified using the QuantiFluor® dsDNA Sample Kit (Promega) according to the manufacturer's instructions. Fluorescence was recorded in a fluorometer (SpectraMax GEMINIXS, Molecular Devices) with filter settings of 504 nm (excitation) and 531 nm (emission). DNA levels were calculated from a standard curve generated from a series of Lambda DNA standard provided by the manufacturer.

To stain blood smears we placed 35 µl droplets collected from heparin whole blood samples onto glass slides and gently spread the blood using the edge of a second glass slide. For histological staining, blood smears were air-dried, fixed in methanol, and stained by standard Giemsa stain protocol.

Flow Cytometry

Spleens from individual mice were harvested and digested with collagenase D. The spleen cells were stained with different combinations of cell surface antibodies. For the intracellular

staining, cells were stimulated with heat-killed Lm (HKLM) in the presence of Brefeldin A for 4 h. Intracellular TNF α staining was conducted using PE anti- TNF α monoclonal antibody (MP6-XT22). Cells were analysed in a LSRII flow cytometer (BD) and data examined using FlowJo software.

ELISA

The blood was taken at 4 h, 18 h and 3 days post-infection (p.i.) and incubated with DNase I (40 μ g/mL) at 37°C for 2 h. After centrifugation at 3'000g for 10 min, the serum was collected and analysed with the ELISA Kit for IL-12 p40 and IL-6 from BD Bioscience and for TNF- α , IFN- γ from eBioscience.

Statistical Analysis

Data were analysed using GraphPad PRISM software (version 5.0). Statistical analysis using the unpaired Student's *t*-test was performed where indicated. Mouse survival was analysed using Kaplan-Meier curves. Differences in survival were analysed using the log rank tests (GraphPad Prism, GraphPad Software, Inc.).

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Disclosures

All authors declare no financial or commercial conflict of interest.

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Legends

Figure 1. BH3-only protein deficient mice are resistant to *Listeria* infection

(A, B) Survival of mice infected with high dose of Lm i.v. (A), WT, BimBid DKO and BimNoxa DKO and (B), WT, Bim KO, Noxa KO and Bid KO mice were infected i.v. with Lm and their survival were monitored daily. Eight to 10 mice per group were used. Animal survival data are presented in a Kaplan-Meier plot and log rank tests were used to compare the susceptibility compared to WT mice (NS: not significant, ** $p < 0.01$, *** $p < 0.001$). Data shown are compiled from two independent experiments. (C) *Listeria* burden in CFUs per spleen of WT, BimBid and BimNoxa DKO mice on days 2 and 4 post infection with 10^4 Lm. Circles represent individual mice, bars represent mean. Five mice per group and time point are shown from one single experiment. This experiment was repeated twice with similar results. (D) Histological sections of spleens from WT and DKO mice infected with Lm. Spleens were stained for B cells (green), T cells (blue) and *Listeria* (red). One representative image from three independent experiments is shown. (E) Cell death and tissue injury were reduced in BimBid and BimNoxa DKO mice. Spleens were collected on day 4 post Lm infection and the extent of apoptosis was determined by TUNEL staining. Apoptotic cells are shown in dark brown. The percent apoptosis is presented as mean $\pm$ SEM of ten mice per strain and are pooled from two independent experiments (right). (D, E) Scale bars represent 100 μ m. Significance was determined in relation to WT group. *** $p < 0.001$ (two-tailed unpaired Student's t-test).

Figure 3. Increased levels of ROS and NET in *Listeria*-infected BimNoxa DKO mice

(A) Neutrophils and monocytes were isolated from WT, Bim KO, Bid KO, Noxa KO, BimBid DKO and BimNoxa DKO mice and stimulated ex vivo with *Listeria*. ROS production was measured by flow cytometry. (B) The concentrations of cell-free DNA in the blood of naïve and *Listeria*-infected mice 4 days after infection was measured with the QuantiFluor® dsDNA Sample Kit (Promega). (A-B) Data shown as mean ± SEM of n=10 and are pooled from two independent experiments. Significance was determined in relation to the WT group. *p<0.05, **p<0.01, ***p<0.001 (two-tailed unpaired Student's t-test). (C)

Neutrophils of the indicated genotypes were activated for 3 h with *Listeria* or PMA, stained for DNA (blue) and MPO (green) and visualised by confocal microscopy. NETs are identified by laminar extracellular DNA (DAPI positive) that colocalised with MPO immunostaining (red arrows). The scale bar represents 40 μ m. (D) Giemsa stained blood smears from Lm-infected mice. Images show neutrophils from DKO mice with NET-like structures appearing as seemingly disintegrated cells with a decondensed chromatin (red arrow) and intact neutrophils from WT mice delineated by their lobular and segmented nuclei (black arrow). The scale bar represents 50 μ m. (C-D) One representative image from three independent experiments is shown (3 mice per genotype in each experiment).

Figure 4. Neutrophils are essential for control of Lm infection in BimNoxa DKO mice

(A) WT, BimBid DKO, and BimNoxa DKO mice were treated with anti-Ly6G 1A8 antibody or isotype matched IgG control Ab 1 day before Lm infection. Animal survival data are presented as a Kaplan-Meier plot and log rank tests were used to compare the survival

between different strains of mice. Data are derived from 4-5 mice per group. One single experiment was performed. Significance was determined in relation to WT mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (two-tailed unpaired Student's t-test). (B) *Listeria* CFUs per spleen at day 4 post infection in WT and BimBid DKO mice. Mice were treated with isotype matched control Ab or Ly6G antibody. Circles represent individual mice, bars represent means. Three to five mice per group are shown, from a single experiment.

Figure 5. Cytokine pattern in mice during the early phase of *Lm* infection

(A) WT, BimBid DKO and BimNoxa DKO mice were infected i.v. with Lm. At the indicated time points, serum cytokine concentrations were assessed by ELISA. (B) Three days after infection, spleen cells from the indicated Lm-infected mice were restimulated in vitro with HKLM in the presence of brefeldin for 4 h and stained for intracellular TNF- α . The total number of TNF- α -producing spleen cells is shown-. (C) Total numbers of TNF- α -producing monocytes (Ly6^{high}) and neutrophils (Ly6^{intermediate}) were determined by flow cytometry. (A-C) Data are shown as mean $\pm$ SEM of 4-5 mice per group and pooled from two independent experiments. Significance was determined in relation to the WT group. *p<0.05, **p<0.01, ***,***p<0.001 (two-tailed unpaired Student's t-test).

Figure 6. TNF- α blockade prolongs survival of *Listeria*-infected WT mice

(A) WT mice were injected i.p. with 2 mg of anti-TNF- α monoclonal antibody or Ig isotype matched control Abs on days 3 and 4 after Lm infection. Animal survival data are presented as a Kaplan-Meier plot and log rank tests were used to compare the survival between different strains. Significance was determined in relation to IgG control treated mice (* $p < 0.05$). Data are compiled from two independent experiments (n=10). (B) *Listeria* CFUs per spleen at day 6 post infection in WT and BimBid DKO mice. Mice were treated with isotype-matched control Abs or anti-TNF- α antibody. Circles represent individual mice and bars represent means. Four to five mice per group from a single experiment are shown.

B)

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