The Drosophila Baramicin polypeptide gene protects against fungal infection

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Abstract

The fruit fly Drosophila melanogaster combats microbial infection by producing a battery of effector peptides that are secreted into the haemolymph. Technical difficulties prevented the investigation of these short effector genes until the recent advent of the CRISPR/CAS era. As a consequence, many putative immune effectors remain to be formally described, and exactly how each of these effectors contribute to survival is not well characterized. Here we describe a novel Drosophila antifungal peptide gene that we name Baramicin A. We show that BaraA encodes a precursor protein cleaved into multiple peptides via furin cleavage sites. BaraA is strongly immune-induced in the fat body downstream of the Toll pathway, but also exhibits expression in other tissues. Importantly, we show that flies lacking BaraA are viable but susceptible to the entomopathogenic fungus Beauveria bassiana. Consistent with BaraA being directly antimicrobial, overexpression of BaraA promotes resistance to fungi and the IM10-like peptides produced by BaraA synergistically inhibit growth of fungi in vitro when combined with a membrane-disrupting antifungal. Surprisingly, BaraA mutant males but not females display an erect wing phenotype upon infection. Here, we characterize a new antifungal immune effector downstream of Toll signalling, and show it is a key contributor to the Drosophila antimicrobial response.

Author summary

The ways that animals combat infection involve complex molecular pathways that are triggered upon microbial challenge. While a great deal is known about which pathways are key to a successful defence response, far less is known about exactly what elements of that response are critical to combat a given infection. Using the fruit fly—a genetic workhorse of Biology—we recently showed that a class of host-encoded antibiotics called “antimicrobial peptides” are essential for defence against bacterial infection, but do not contribute as strongly to defence against fungi. However a number of fly immune peptides remain uncharacterized, possibly explaining this gap in our understanding of the fly antifungal defence. Here we describe a novel antifungal peptide gene of fruit flies, and show that it is a major contributor to the fly antifungal defence response. We also found that
this gene seems to regulate a behaviour that flies perform after infection, paralleling exciting recent findings that these genes are involved in neurological processes. Collectively, we clarify a key part of the fly antifungal defence, and contribute an important piece to help explain the logical organization of the immune defence against microbial infection.

Introduction

The innate immune response provides the first line of defence against pathogenic infection. This reaction is usually divided into three stages: i) the recognition of pathogens through dedicated pattern recognition receptors, ii) the activation of conserved immune signalling pathways and iii) the production of immune effectors that target invading pathogens [1,2]. The study of invertebrate immune systems has led to key observations of broad relevance, such as the discovery of phagocytosis [3], antimicrobial peptides (AMPs) [4], and the implication of Toll receptors in metazoan immunity [5]. Elucidating immune mechanisms, genes, and signalling pathways has greatly benefited from investigations in the fruit fly Drosophila melanogaster, which boasts a large suite of molecular and genetic tools for manipulating the system. One of the best-characterized immune reactions of Drosophila is the systemic immune response. This reaction involves the fat body (an analog of the mammalian liver) producing immune effectors that are secreted into the haemolymph. In Drosophila, two NF-κB signalling pathways, the Toll and Imd pathways, regulate most inducible immune effectors: the Toll pathway is predominantly activated in response to infection by Gram-positive bacteria and fungi [5,6], while the immune-deficiency pathway (Imd) responds to the DAP-type peptidoglycan most commonly found in Gram-negative bacteria and a subset of Gram-positive bacteria [7]. These two signalling pathways regulate a transcriptional program that results in the massive synthesis and secretion of humoral effector peptides [6,8]. Accordingly, mutations affecting the Toll and Imd pathways cause extreme susceptibilities to systemic infection that reflect the important contribution of these pathways to host defence. The best-characterized immune effectors downstream of these pathways are antimicrobial peptides (AMPs). AMPs are small and often cationic peptides that disrupt the membranes of microbes, although some have more specific mechanisms [9]. Multiple AMP genes belonging to seven well-characterized families are induced upon systemic infection [10]. However transcriptomic analyses have revealed that the systemic immune response encompasses far more than just the canonical AMPs. Many uncharacterized genes encoding small secreted peptides are induced to high levels downstream of the Toll and Imd pathways, pointing to the role for these peptides as immune effectors [11]. In parallel, MALDI-TOF analyses of the haemolymph of infected flies revealed the induction of 24 peaks—mostly corresponding to uncharacterized peptides—that were named "IMs" for Immune-induced Molecules (IM1-IM24) [8]. Many of the genes that encode these components of the immune peptidic secretome had remained unexplored until recently. This is mainly due to the fact that these IMs belong to large gene families of small genes that were not typically disrupted using random mutagenesis [10,12].

The CRISPR/Cas9 gene editing approach now allows the necessary precision to delete small genes, singly or in groups, providing the opportunity to dissect effector peptide functions. In 2015 a family of 12 related IM-encoding genes, unified under the name Bomanins, were shown to function downstream of Toll. Importantly, a deletion removing 10 out of the 12 Bomanins revealed their potent contribution to defence against both Gram-positive bacteria and fungi [13]. While Bomanins contribute significantly to Toll-mediated defence, their molecular functions are still unknown and it is unclear if they are directly antimicrobial [14]. Two other IMs
encoding IM4 and IM14 (renamed Daisho1 and Daisho2, respectively) were shown to contribute downstream of Toll to resistance against Fusarium fungi. Interestingly, Daisho peptides bind to fungal hyphae, suggesting direct antifungal activity [15]. Finally, a systematic knock-out analysis of Drosophila AMPs revealed that they play an important role in defence against Gram-negative bacteria and some fungi, but surprisingly little against Gram-positive bacteria [16]. An unforeseen finding from these recent studies is the high degree of AMP-pathogen specificity: this is perhaps best illustrated by the specific requirement for Diptericin, but not other AMPs, in defence against Providencia rettgeri [16,17]. Collectively, these studies in Drosophila reveal that immune effectors can be broad or specific in mediating host-pathogen interactions. Understanding the logic of the Drosophila effector response will thus require a careful dissection of the remaining uncharacterized immune induced peptides.

Previous studies identified an uncharacterized Toll-regulated gene (CG18279/CG33470), which we rename “BaraA” (see below), that encodes several IMs, indicating a role in the humoral response. Here, we have improved the annotation of IMs produced by BaraA to include: IM10, IM12 (and its sub-peptide IM6), IM13 (and its sub-peptides IM5 and IM8), IM22, and IM24. Using a BaraA reporter, we show that BaraA is not only immune-induced in the fat body, but also expressed in the head, and nervous system tissue including the eyes, and ocelli. Importantly, we show that flies lacking BaraA are viable but susceptible to specific infections, notably by the entomopathogenic fungus Beauveria bassiana. Consistent with this, the IM10-like peptides produced by BaraA inhibit fungal growth in vitro when combined with the antifungal Pimaricin. Surprisingly, BaraA deficient males also display a striking erect wing behaviour upon infection. Collectively, we identify a new antifungal immune effector downstream of Toll signalling, improving our knowledge of the Drosophila antimicrobial response.

Results

BaraA is regulated by the Toll pathway

A previous microarray study from De Gregorio et al. [11] suggested that BaraA (CG18279/CG33470) is primarily regulated by the Toll pathway, with a minor input from the Imd pathway (Fig 1A). Consistent with this, we found several putative NF-κB binding sites upstream of the BaraA gene (guided by previous reports [18–20]). Notably there are two putative binding sites for Relish, the transcription factor of the Imd pathway and three putative binding sites for the Dif/Dorsal transcription factors acting downstream of Toll (S1A Fig and S1). We challenged wild-type flies and Imd or Toll pathway mutants (RelE20 and spzrm7 respectively) with the yeast Candida albicans, the Gram-negative bacterium Escherichia coli, or the Gram-positive bacterium Micrococcus luteus. RT-qPCR analysis confirms that BaraA is abolished in spzrm7 flies similar to the Toll-regulated BomBc3 gene (Fig 1B), but remains highly inducible in RelE20 flies (S1B Fig). Collectively, the expression pattern of BaraA is reminiscent of the antifungal peptide gene Drosomycin with a primary input by the Toll pathway and a minor input from the Imd pathway [10,21].

To further characterize the expression of BaraA, we generated a BaraA-Gal4 transgene in which 1675bp of the BaraA promoter sequence is fused to the yeast transcription factor Gal4. Monitoring GFP in BaraA-Gal4>UAS-mCD8-GFP flies (referred to as BaraA>mGFP) confirms that the BaraA reporter is highly induced in the fat body after infection by M. luteus, but less so by E. coli (Fig 1C). This result is consistent with a recent time course study that found Toll-regulated genes (including BaraA) were rapidly induced after injection stimulating the Imd pathway, but this principally Imd-based induction resolves to nearly basal levels within 48 hours [22] (and see S1B and S1C Fig). Additionally, larvae pricked with M. luteus show a robust GFP signal primarily stemming from the fat body when examined 2hpi (S1D Fig). We
also observed a constitutive GFP signal in the headcase of adults (Fig 1D), including the border of the eyes and the ocelli (Fig 1E). Dissection confirmed that the BaraA reporter is expressed in brain tissue, including posterior to the central brain furrow in adults and at the posterior of the ventral nervous system in larvae. Other consistent signals include GFP in the wing veins and subcutaneously along borders of thoracic pleura in adults (Fig 1F and 1G), and in spermatheca of females (S1E Fig). There was also sporadic GFP signal in other tissues that included the larval hindgut, the dorsal abdomen of developing pupae, and the seminal vesicle of males.
These expression patterns largely agree with data reported in FlyAtlas1 (wherein BaraA is called “IM10”) [23].

**Baramicin A encodes a precursor protein cleaved into multiple peptides**

Previous studies using bioinformatics and proteomics have suggested that four highly immune-induced peptides (IM10, IM12, IM13, and IM24) are encoded in tandem as a single polypeptide precursor by CG33470 (aka IMPPP/BaraA) [8,24]. Some less-abundant sub-peptides (IM5, IM6, and IM8) are also produced by additional cleavage or degradation of IM12 and IM13 [24]. Using a newly generated null mutant (“ΔBaraA,” described below and design shown in Fig 2A), we analyzed haemolymph samples of wild-type and ΔBaraA flies infected with a bacterial mixture of *E. coli* and *M. luteus* by MALDI-TOF analysis. We confirmed the loss of the seven immune-induced peaks corresponding to IMs 5, 6, 8, 10, 12, 13, and 24 in ΔBaraA flies (Fig 2A). We also noticed that an additional immune-induced peak at ~5975 Da was absent in our BaraA mutants. Upon re-visiting the original studies that annotated the *Drosophila* IMs, we realized this peak corresponded to IM22, whose sequence was never determined [8,24] (see S1 Text and S2 Data for details). We subjected haemolymph from infected

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**Fig 2. The BaraA gene structure.** A) MALDI-TOF analysis of haemolymph from iso *w^{1118}* wild-type and iso ΔBaraA flies 24 hours post-infection (hpi) confirms that BaraA mutants fail to produce the IM10-like and IM24 peptides. iso ΔBaraA flies also fail to produce an immune-induced peak at ~5975 Da corresponding to IM22 (the C-terminal peptide of BaraA, see S1 Text). A diagram of the ΔBaraA^SW1 mutation that replaces the N-terminal gene region with a DsRed construct is shown in the bottom right. B) The BaraA gene encodes a precursor protein that is cleaved into multiple mature peptides at RXRR furin cleavage sites. The sub-peptides IMs 5, 6, and 8 are additional minor cleavage products of IM12 and IM13. IM22 is additionally cleaved following its GIND motif (S2 Fig and S3A). C) There is a BaraA locus duplication event present in the Dmel_R6 reference genome. This duplication is not fixed in laboratory stocks and wild-type flies [25]. The ΔBaraA mutation was generated in a background with only one BaraA copy.  

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flies to LC-MS proteomic analysis following trypsin digestion and found that in addition to the known IMs of BaraA (IMs 5, 6, 8, 10, 12, 13, and 24), trypsin-digested fragments of the BaraA C-terminus peptide were also detectable in the haemolymph (S2 Fig). The range of detected fragments did not match the full length of the C-terminus exactly, as the first four residues were absent in our LC-MS data (a truncation not predicted to arise via trypsin cleavage). The BaraA C-terminus lacking these four residues has a calculated mass of 5974.5 Da, exactly matching the observed mass of the IM22 peak absent in BaraA mutant flies. Furthermore in other Drosophila species these four residues are absent, and instead the C-terminus directly follows an RXRR furin cleavage motif (S3A Fig). Therefore IM22 cleavage in other species, even by an alternate cleavage process, should result in the same matured IM22 domain as found in D. melanogaster. Taken together, we conclude that IM22 is the mature form of the BaraA protein C-terminus.

Thus, a single gene, BaraA, contributes to one third of the originally described Drosophila IMs. These peptides are encoded as a polypeptide precursor interspersed by furin cleavage sites (e.g. RXRR) (Fig 2B). We note that the IM10, IM12 and IM13 peptides are tandem repeats of related peptides, which we collectively refer to as “IM10-like” peptides (S3B Fig). The IM22 peptide also contains a similar motif as the IM10-like peptides (S3A and S3B Fig), suggesting a related biological activity. We name this gene “Baramicin A” (symbol: BaraA) for the Japanese idiom Bara Bara (バラバラ), meaning “to break apart;” a reference to the fragmenting structure of the Baramicin precursor protein and its many peptidic products.

A BaraA duplication is present in some laboratory stocks
Over the course of our investigation, we realized that IMPPP (CG18279) was identical to its neighbour gene CG33470 owing to a duplication event of the BaraA locus present in the D. melanogaster reference genome. The exact nature of this duplication is discussed in a separate article [25]. In brief, the duplication involves the entire BaraA gene including over 1kbp of 100% identical promoter sequence, and also the neighbouring sulfatase gene CG30059 and the 3’ terminus of the ATP8A gene region (Fig 2C). We distinguish the two daughter genes as BaraA1 (CG33470) and BaraA2 (CG18279). Available sequence data suggests the BaraA1 and BaraA2 transcripts are 100% identical. In a separate study, we analyzed the presence of the BaraA duplication using a PCR assay spanning the junction of the duplicated region (also see S3 Data). Interestingly, BaraA copy number is variable in common lab strains and wild flies, indicating this duplication event is not fixed in D. melanogaster [25].

Over-expression of BaraA improves the resistance of immune deficient flies
Imd, Toll deficient flies are extremely susceptible to microbial infection as they fail to induce hundreds of immune genes, including antimicrobial peptides [11]. It has been shown that over-expression of even a single AMP can improve the resistance of Imd, Toll deficient flies [26]. As such, immune gene over-expression in Imd, Toll immune-compromised flies provides a direct assay to test the ability of a gene to contribute to defence independent of other immune effectors. We applied this strategy to Baramicin A by generating flies that constitutively express BaraA using the ubiquitous Actin5C-Gal4 driver (Act-Gal4) in an immune-deficient RelE20, spzrm7 double mutant background (S4A Fig). In these experiments, we pooled results from both males and females due to the very low availability of homozygous Rel, spz adults when combined with Act-Gal4. Overall, similar trends were seen in both sexes, and separate male and female survival curves are shown in S4 Fig.
Ubiquitous BaraA expression marginally improved the survival of Rel, spz flies upon infection with *M. luteus* bacteria, however there was no effect upon infection with *E. coli* (S4B and S4C Fig). On the other hand, ubiquitous expression of BaraA provided a more pronounced protective effect against infection by a variety of fungal pathogens. This was true upon pricking with *C. albicans* (Fig 3A), or upon natural infections using *Aspergillus fumigatus* or *Neurospora crassa* filamentous fungi (Fig 3B and 3C). This over-expression study reveals that BaraA alone can partially rescue the susceptibility of *Imd, Toll* deficient flies to infection, and points to a more prominent role for BaraA in antifungal defence.

**IM10-like peptides display antifungal activity in vitro**

*The Baramicin A* gene encodes a polypeptide precursor that ultimately produces multiple mature peptides. However the most prominent BaraA products are the 23-residue IM10, 12, and 13 peptides (collectively the "IM10-like" peptides); indeed three IM10-like peptides are produced for every one IM24 peptide (Fig 2B), and IM22 also bears an IM10-like motif (S3 Fig). This prompted us to explore the in vitro activity of the BaraA IM10-like peptides as potential AMPs.
We synthesized IM10, IM12, and IM13 and performed in vitro antimicrobial assays with these three IM10-like peptides using a 1:1:1 cocktail with a final concentration of 300 μM (100 μM each of IM10, IM12, and IM13). We monitored the microbicidal activity of this peptide cocktail using a protocol adapted from Wiegand et al. [27]. We did not detect any killing activity of our IM10-like peptide cocktail alone against *Pectobacterium carotovora Ecc15* (hereafter “Ecc15”), *Enterococcus faecalis*, or *C. albicans*. Previous studies have shown that the microbicidal activities of Abaecin-like peptides, which target the bacterial DNA chaperone DnaK, increase exponentially in combination with a membrane disrupting agent [28–30]. Inspired by this approach, we next assayed combinations of the IM10-like cocktail with membrane-disrupting antibiotics relevant to tested microbes that should facilitate peptide entry into the cell. We again found no activity of IM10-like peptides against *Ecc15* or *E. faecalis* when co-incubated with a sub-lethal dose of Cecropin or Ampicillin respectively, indicating IM10-like peptides likely do not affect *Ecc15* or *E. faecalis* either alone or in combination with membrane-disrupting antibiotics. However, we observed a synergistic interaction between IM10-like peptides and the commercial antifungal Pimaricin against *C. albicans* (Fig 3D). Co-incubation of the IM10-like cocktail with Pimaricin significantly improved the killing activity of Pimaricin at 16 and 32 μg/mL relative to either treatment alone. While not statistically significant, the combination of IM10-like cocktail and Pimaricin also outperformed either the IM10-like cocktail alone or Pimaricin alone across the entire range of Pimaricin concentrations tested.

We next co-incubated dilute preparations of *B. bassiana* strain R444 spores under the same conditions as used previously with *C. albicans*, plated 2 μL droplets, and assessed the diameters and corresponding surface area of colonies derived from individual spores after 4 days of growth at 25°C to assess growth rate. We found that neither the IM10-like cocktail nor Pimaricin alone significantly affected surface area relative to a PBS buffer control (Tukey’s HSD: p = 0.656 and 0.466 respectively). However in combination, the IM10-like cocktail plus Pimaricin led to significantly reduced colony size compared to either treatment alone, corresponding to a 19–29% reduction in surface area relative to controls (Fig 3E, Tukey’s HSD: p < .01 in all cases). This indicates that incubation with IM10-like peptides and Pimaricin synergistically inhibits *B. bassiana* mycelial growth, revealing an otherwise cryptic antifungal effect of the BaraA IM10-like peptides in vitro.

Overall, we found that IM10-like peptides alone do not kill *C. albicans* yeast or impair *B. bassiana* mycelial growth in vitro. However, IM10-like peptides seem to synergize with the antifungal Pimaricin to inhibit growth of both of these fungi.

**BaraA deficient flies broadly resist like wild-type upon bacterial infection**

To further characterize BaraA function, we generated a null mutation of BaraA by replacing the ‘entire’ BaraA locus with a dsRed cassette using CRISPR mediated homology-directed repair with fly stocks that contain only one BaraA gene copy (BDSC #2057 and BL51323) (Fig 2A). After isolation, this mutation (BaraA\textsuperscript{SW1}) was then backcrossed once to a lab strain of *w*\textsuperscript{1118} (used in [13–15]) to remove a second site mutation (see Materials and methods). The resulting *w*\textsuperscript{1118}; BaraA\textsuperscript{SW1} flies are hereon referred to as “*w;ΔBaraA*.” As a consequence of this backcrossing event, *w;ΔBaraA* flies are a mixed genetic background, which we arbitrarily compare to OR-R as representative wild-type flies. Finally, the *BaraA\textsuperscript{SW1}* mutation was isogenized by seven rounds of backcrossing into the *w*\textsuperscript{1118} DrosDel isogenic genetic background (iso *w*\textsuperscript{1118}) [31] as described in Ferreira et al. and are hereon referred to as “iso ΔBaraA” [32]. Relevant to this study, both our OR-R and DrosDel iso *w*\textsuperscript{1118} wild-type lines contain the BaraA duplication and thus have both BaraA1 and A2 genes, while *w;ΔBaraA* and iso ΔBaraA flies...
lack BaraA entirely. In the following experiments, we compare the immune response of both w; ΔBaraA and iso ΔBaraA to wild-type flies, and focused on phenotypes that were consistent in both genetic backgrounds.

We validated these mutant lines by PCR, qPCR and MALDI-TOF peptidomics (Fig 2A and S3 Data). BaraA-deficient flies were viable with no morphological defects. Furthermore, ΔBaraA flies have wild-type Toll and Imd signalling responses following infection, indicating that BaraA is not required for the activation of these signaling cascades (S5A–S5C Fig). BaraA mutant flies also survive clean injury like wild-type (S5D Fig), and have comparable lifespan to wild-type flies (S5E Fig). We next challenged BaraA mutant flies using our two genetic backgrounds with a variety of pathogens. We included susceptible Imd deficient flies, Toll deficient spzΔ flies and Bomanin deficient BomΔ55C flies as comparative controls. We observed that BaraA null flies have comparable resistance as wild-type to infection with the Gram-negative bacteria Ecc15 and Providencia rhizodecanarea (S6A and S6B Fig), or with the Gram-positive bacterium Bacillus subtilis (S6C Fig). In contrast, we saw a mild increase in the susceptibility of w; ΔBaraA flies to infection by the Gram-positive bacterium E. faecalis (HR = +.73, p = .014). We also saw an early mortality phenotype in iso ΔBaraA flies (at 3.5 days, p < .001), although this was not ultimately statistically significant (S7A Fig; p = .173). This trend of a mild susceptibility was broadly consistent in deficiency crosses and flies ubiquitously expressing BaraA RNAi (S7B and S7C Fig), though none of these sets of survival experiments individually reached statistical significance. Overall, the susceptibility of BaraA mutants to E. faecalis is mild, but appears consistent using a variety of genetic approaches.

**BaraA mutant flies are highly susceptible to Beauveria fungal infection**

Entomopathogenic fungi such as Metarhizium and Beauveria represent an important class of insect pathogens [6]. They have the ability to directly invade the body cavity by digesting and crossing through the insect cuticle. The Toll pathway is critical to survive fungal pathogens as it is directly responsible for the expression of Bomanin, Daisho, Drosomycin and Metchnikowin antifungal effectors [13,15,16,33,34]. The fact that i) BaraA is Toll-regulated, ii) BaraA IM10-like peptides display antifungal activity in vitro, and iii) BaraA overexpression improves the resistance of Imd, Toll deficient flies against fungi all point to a role for BaraA against fungal pathogens.

We infected BaraA mutant and wild-type flies using a septic injury model of Metarhizium rileyi strain PHP1705 (Andermatt Biocontrol). spzΔ flies and BomΔ55C mutant flies were highly susceptible to M. rileyi septic injury. Likewise, both w; ΔBaraA and iso ΔBaraA mutants showed a significant susceptibility to M. rileyi septic injury (Fig 4A, HR ≥ 1.0 and p < .05 in both cases). We next rolled flies in sporulating B. bassiana strain 802 petri dishes. Strikingly, both w; ΔBaraA and iso ΔBaraA flies displayed a pronounced susceptibility to natural infection with B. bassiana (HR = +2.10 or +0.96 respectively, p < .001 for both) (S8A Fig). An increased susceptibility to fungi was also observed using flies carrying the BaraA mutation over a deficiency (S8B Fig) or that ubiquitously express BaraA RNAi (S8C Fig). Moreover, constitutive BaraA expression (Act-Gal4>UAS-BaraA) in an otherwise wild-type background improves survival to B. bassiana 802 relative to Act-Gal4>OR-R controls (HR = -0.52, p = .010) (S8D Fig). We next used a preparation of commercial B. bassiana R444 spores (Andermatt Biocontrol) to perform controlled systemic infections by septic injury with a needle dipped in spore solution. In these experiments we monitored both survival and fungal load using qPCR primers specific to the B. bassiana 185 rRNA gene [35]. As seen with natural infection, BaraA mutants were highly susceptible to Beauveria systemic infection (Fig 4B), and suffered increased fungal load by 48 hours after infection (Fig 4C). We also compared the effect of
BaraA in defence against *B. bassiana* to the effect of deleting two classical antifungal peptide genes of *Drosophila*: *Metchnikowin* (Mtk) and *Drosomycin* (Drs). Use of infection models with very different virulence (septic injury vs. natural infection) suggests that BaraA contributes far more strongly to defence against *B. bassiana* compared to the combined effect of Mtk and Drs (S8E Fig), while Mtk and Drs did not greatly affect resistance relative to wild-type (HR = +0.15, p >0.10).

Finally, we combined the ΔBaraA mutation with both a UAS-BaraA construct on the 2nd chromosome or our BaraA-Gal4 driver on the 3rd chromosome to rescue the susceptibility of BaraA deficient flies. Supplementing ΔBaraA flies with BaraA expressed via the BaraA-Gal4>UAS-BaraA method restores resistance almost to wild-type levels (Fig 4D). Collectively, our survival analyses point to a role for BaraA in defence against entomopathogenic fungi, including *M. rileyi* and especially *B. bassiana*. Consistent with a direct effect of BaraA on fungi, BaraA mutant susceptibility is correlated with increased proliferation of *B. bassiana*, and heterologous expression of BaraA via the Gal4/UAS system rescues the susceptibility of mutants, confirming that mutant susceptibility is caused by the loss of BaraA.

**BaraA contributes to antifungal defence independent of Bomanins**

Use of compound mutants carrying multiple mutations in effector genes has shown that some of them additively contribute to host resistance to infection [16]. Compound deletions of
immune genes can also reveal contributions of immune effectors that are not detectable via single mutant analysis [16,36,37]. Recent studies have indicated that Bomanins play a major role in defence against fungi [13,14], though their mechanism of action is unknown. It is possible that Bomanin activity relies on the presence of BaraA, or vice versa. This prompted us to investigate the interaction of Bomanins and BaraA in defence against fungi. To do this, we recombined the BomΔ55C mutation (that removes a cluster of 10 Bomanin genes) with ΔBaraA. Furthermore, we used low-virulence models of infection that allowed some Bomanin mutant flies to survive, so as to ensure additional mutation of BaraA had an opportunity to affect survival if relevant. While natural infection with Aspergillus fumigatus did not induce significant mortality in BaraA single mutants (S6D and S6E Fig), we observed that combining ΔBaraA and BomΔ55C mutations increases fly susceptibility to this pathogen relative to BomΔ55C alone (HR = -0.46, p = .003; Fig 5A). We next exposed these ΔBaraA, BomΔ55C, double mutant flies to a low dose natural infection with 30mg of commercial spores of B. bassiana R444 as this dose allows some Bomanin mutant flies to survive. This is equivalent to approximately 60 million spores added to a vial containing 20 flies, many of which are removed afterwards upon fly grooming. When using this infection method, we found that BaraA mutation markedly increases the susceptibility of BomΔ55C mutant flies (HR = -0.89, p < .001), approaching spzrm7 susceptibility (Fig 5B).

If BaraA and Bom peptides relied on each other for activity, we would expect no increased susceptibility of double mutants. However BaraA, Bom double mutation results in increased susceptibility relative to Bom mutation alone. We conclude BaraA acts independently of Bomanins, agreeing with the ability of heterologous overexpression of BaraA to rescue Toll, Imd double mutant flies that are similarly deficient in Bomanin production (Fig 3A–3C). Alongside a more prominent activity of BaraA in defence against B. bassiana compared to Drs and Mtk (S8D Fig), these results suggest BaraA improves survival against fungi independent of other effectors of the systemic immune response also using effector mutant analysis, consistent with a direct effect on invading fungi.
ΔBaraA males display an erect wing phenotype upon infection

While performing natural infections with *A. fumigatus*, we observed a high prevalence of BaraA mutant flies with upright wings (Fig 6A and S9A Fig), a phenotype similar to the effect of disrupting the gene encoding the “erect wing” (ewg) transcription factor [38]. Curiously, this erect wing phenotype was most specifically observed in males. Upon further observation, erect wing was observed not only upon *A. fumigatus* infection, but also upon infections with all Gram-positive bacteria and fungi tested, and less so upon clean injury or using Gram-negative bacteria (S1 Table and S9B and S9C Fig). We eventually pursued this striking phenotype further using an *E. faecalis* septic injury model. A greater prevalence of erect wing flies was observed upon infection with live *E. faecalis* (Fig 6B). Strikingly, even injury with heat-killed *E. faecalis* is sufficient to induce erect wing (Fig 6C), collectively indicating that this phenotype is observed in BaraA mutants upon Toll pathway stimulation, but does not require a live infection.

Such a phenotype in infected males has never been reported, but is reminiscent of the wing extension behaviour of flies infected by the brain-controlling “zombie” fungus *Entomophthora muscae* [39]. Intrigued by this phenotype, we further explored its prevalence in other genetic backgrounds. We next confirmed that this phenotype was also observed in other

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Fig 6. ΔBaraA males display an erect wing phenotype upon infection. A) ΔBaraA males displaying erect wing six days after *A. fumigatus* natural infection. B-C) spz/+Δ and ΔBaraA males, but not BomΔ55C or RelE20 flies display the erect wing phenotype upon septic injury with live (B) or heat-killed *E. faecalis* (C). D) The presentation of erect wing in ΔBaraA flies is rescues by c564-Gal4 ubiquitous expression of BaraA. Barplots show the percentage of flies displaying erect wing following treatment, with individual data points reflecting replicate experiments. Asterisks indicate one-way ANOVA significance relative to reference w; ΔBaraA flies (*,* and *** = p < .01, and.001 respectively). Erect wing frequency after additional challenges are shown in S9 Fig and S1 Table.

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BaraA-deficient backgrounds such as Df(BaraA)/ΔBaraA; however the penetrance was variable from one background to another (S1 Table). Erect wing was also observed in ΔBaraA/+ heterozygous flies (Df(BaraA)/+ or ΔBaraA/+), indicating that the lack of BaraA on one chromosome was sufficient to cause the phenotype (S9D Fig), independent of overall susceptibility to E. faecalis (S7B Fig). Moreover, spz rm7 flies that lack functional Toll signalling phenocopy ΔBaraA flies and display erect wing, but other immune-deficient genotypes such as mutants for the Toll-regulated Bomanin effectors (BomAS5C), or RelE20 mutants that lack Imd signalling, did not readily display erect wing (Fig 6B and 6C and S1 Table). Thus the erect wing phenotype is not linked to susceptibility to infection, but rather to loss of BaraA alongside stimuli triggering the Toll immune pathway. This phenotype suggests an additional effect of BaraA on tissues related to the wing muscle or in the nervous system.

The expression profile of BaraA is complex and poorly defined in existing transcriptomic datasets, likely owing to the gene duplication of BaraA1 and BaraA2 complicating read mapping [22,25]. As BaraA is expressed in various tissues including the head/eye, crop, and fat body (Fig 1 and [23]), it is unclear if BaraA absence in the brain, neuromusculature, or non-neuronal tissues (such as the fat body) could underlie the predisposition to erect wing. To this end, we used stocks containing both the ΔBaraA mutation and either a UAS-BaraA construct, c564-Gal4 constitutive fat body driver, or BaraA-Gal4 driver, and performed genetic crosses to attempt to rescue the presentation of erect wing upon septic injury with heat-killed E. faecalis using the Gal4/UAS system. Surprisingly, constitutive BaraA expression in the fat body by c564-Gal4 rescued erect wing presentation to effectively zero levels (Fig 6D). On the other hand, ΔBaraA, BaraA-Gal4>UAS-BaraA flies displayed erect wing (exact genotype as in Fig 4E), similar to Df(BaraA)/+ and ΔBaraA/+ flies (S9D Fig). Indeed, qPCR of BaraA expression after infection shows that BaraA levels are lower than wild-type in both ΔBaraA, BaraA-Gal4>UAS-BaraA (S9E Fig) and ΔBaraA/+ transheterozygotes (S10 Fig).

Cumulatively, these experiments confirm that loss of BaraA results in the erect wing phenotype upon immune stimulus given BaraA deficiency, either by mutation or by loss of Toll signalling. This phenotype occurs independent of active infection, and is specifically tied to BaraA downstream of Toll signalling. A full transcriptional output of BaraA appears to be required to prevent erect wing after infection, as flies with less than wild-type BaraA expression are predisposed to displaying erect wing. However priming the hemolymph with BaraA peptides via constitutive expression in the fat body is sufficient to rescue the erect wing phenotype. Importantly, this rescue by fat body driven expression indicates that systemically secreted BaraA peptides mediate this phenotype, and not BaraA expression in e.g. neuronal tissue. Taken together, a wild-type induction of BaraA is required to prevent erect wing presentation following Toll activation, which can be ameliorated by priming the hemolymph with constitutive BaraA expression.

Discussion

Seven Drosophila AMP families were identified in the 1980s-1990s either by homology with AMPs characterized in other insects or owing to their abundant production and microbicidal activities in vitro [40]. In the 2000s, genome annotations revealed the existence of many additional paralogous genes from the seven well-defined families of AMPs [41,42]. At that time, microarray and MALDI-TOF analyses also revealed the existence of many more small immune-induced peptides, which may function as AMPs [8,24]. Genetic analyses using loss of function mutations have recently shown that some of these peptides do play an important role in host defence, however key points surrounding their direct microbicidal activities remain unclear. In 2015, Bomanins were shown to be critical to host defence using genetic approaches,
but to date no activity in vitro has been found [13,14]. The overt susceptibility of Bomanin mutants to most Gram-positive bacteria and fungi also suggests a generalist role in supporting the effectors of Toll, rather than a direct effect on microbes. In addition, two candidate AMPs, Listercin [43] and GNBP-like3 [44], have been shown to inhibit microbial growth upon heterologous expression using S2 cell lines or bacteria respectively. Most recently, Daisho peptides were shown to bind to fungal hyphae ex vivo, and are required for resisting Fusarium fungal infection in vivo [15]. However the mechanism and direct microbicidal activity of these various peptides at physiological concentrations has not yet been assessed.

In this study, we provide evidence from four separate experimental approaches that support adding BaraA products to the list of bona-fide antifungal peptides. First, the BaraA gene is strongly induced in the fat body upon infection resulting in abundant peptide production. BaraA is also tightly regulated by the Toll pathway, which orchestrates the antifungal response. Second, loss of function study shows that BaraA contributes to resistance against fungi. BaraA mutation increases susceptibility to M. rileyi and B. bassiana, and BaraA deficient flies suffer increased B. bassiana proliferation. Third, the antifungal activity of BaraA is independent of other key effectors. Overexpression of BaraA in the absence of Toll/Imd inducible peptides increased the resistance of compound Rel, spz deficient flies to various fungi including C. albicans, A. fumigatus, and N. crassa, and rescues the ΔBaraA mutant susceptibility to B. bassiana. Additionally, compound gene deletion of both BaraA and Bomanins causes greater susceptibility than Bomanin mutation alone after B. bassiana natural infection. Fourth, and lastly, a cocktail of the BaraA IM10-like peptides possesses antifungal activity against C. albicans and B. bassiana in vitro when co-incubated with the membrane disrupting antifungal Pimaricin.

While it is difficult to estimate the concentration of BaraA peptides in the haemolymph of infected flies, it is expected based on MALDI-TOF peak intensities that the IM10-like peptides should reach concentrations similar to other AMPs (up to 100μM) [10,21]; our in vitro assays used a peptide cocktail at the upper limit of this range. AMPs are often—but not exclusively—positively charged. This positive charge is thought to recruit these molecules to negatively charged membranes of microbes [10]. That said, the net charges at pH = 7 of the IM10-like peptides are: IM10 +1.1, IM12 +0.1, and IM13−0.9. Given this range of net charge, IM10-like peptides are not overtly cationic. However some AMPs are antimicrobial without being positively charged, exemplified by human Dermicidin [45] and anionic peptides of Lepidoptera that synergize with membrane-disrupting agents [46]. More extensive in vitro experiments with additional fungi and alternate membrane-disrupting antifungals (such as other insect or Drosophila antifungal peptides) should confirm the range of BaraA peptide activities. Furthermore, the potential activities of IM22 and IM24 should be addressed, which were not included in the present study. Future studies would benefit from testing different in vitro approaches, which might better mimic physiological conditions that could be relevant for BaraA peptide activity.

Our study also reveals that the Baramicin A gene alone produces at least 1/3 of the initially reported IMs. In addition to the IM10-like peptides and IM24 that were previously assigned to BaraA [24], we show IM22 is encoded by the C terminus of BaraA, and is conserved in other Drosophila species. The production of multiple IMs encoded as tandem repeats between furin cleavage sites is built-in to the BaraA protein design akin to a “protein operon.” Such tandem repeat organization is rare, but not totally unique among AMPs. This structure was first described in the bumblebee AMP Apidaecin [47], and has since also been found in Drosocin of Drosophila neotestacea [48]. In D. melanogaster, several AMPs are furin-processsed including Attacin C and its pro-peptide MPAC, wherein both parts synergize in killing bacteria [28]. Therefore, furin cleavage in Attacin C enables the precise co-expression of distinct peptides with synergistic activity. It is interesting to note that IM10-like peptides did not show
antifungal activity in the absence of membrane disruption by Pimaricin. An attractive hypothesis is that longer peptides encoded by BaraA such as IM22 and IM24 could contribute to the antifungal activity of BaraA by membrane permeabilization, allowing the internalization of IM10-like peptides. However rigorous experimentation is needed to determine the IM10-like mechanism of action. Indeed, the BaraA IM24 peptide is a short Glycine-rich peptide (96 AA) that is positively-charged (charge +2.4 at pH = 7). These traits are shared by amphipathic membrane-disrupting AMPs such as Attacins [10], however the precise role of the Baramicin IM24 domain is likely complex given the repeated evolution of neural-specific Baramicins that preferentially retain the IM24 domain [25].

An unexpected observation of our study is the display of an erect wing phenotype by BaraA deficient males upon infection. Our study suggests that this phenotype relies on the activation of the Toll pathway in the absence of BaraA. Erect wing is also induced by heat-killed bacteria, and is not observed in Bomanin or Relish mutants, indicating that the erect wing phenotype is not a generic consequence of susceptibility to infection. The erect wing gene, whose inactivation causes a similar phenotype, is a transcription factor that regulates synaptic growth in developing neuromuscular junctions [38]. This raises the intriguing hypothesis that immune processes downstream of the Toll ligand Spaetzle somehow affect wing neuromuscular junctions, and that BaraA modulates this activity. Another puzzling observation is the sexual dimorphism exhibited for this response. Male courtship and aggression displays involve similar wing extension behaviours. Koganzewa et al. [49] showed that males deficient for Gustatory receptor 32a (Gr32a) failed to unilaterally extend wings during courtship display. Gr32a-expressing cells extend into the subesophageal ganglion where they contact mAL, a male-specific set of interneurons involved in unilateral wing display [49]. One possible explanation for the male specific effects of BaraA could be that BaraA mediates this effect through interactions with such male-specific neurons. Recent studies have highlighted how NF-κB signalling in the brain is activated by bacterial peptidoglycan [50], and that immune effectors expressed either by fat body surrounding the brain or from within brain tissue itself affect memory formation [44]. Moreover, an AMP of nematodes regulates aging-dependent neurodegeneration through binding to its G-protein coupled receptor, and this pathway is sufficient to trigger motor neuron degeneration following infection [51]. The ability of fat body-derived BaraA to rescue the erect wing phenotype suggests a similar interplay of the immune response with neuromuscular processes. Future studies characterizing the role of BaraA in the erect wing phenotype should provide insight on interactions between systemic immunity and host physiology more generally.

Here we describe a complex immune effector gene that produces multiple peptide products. BaraA encodes many of the most abundant immune effectors induced downstream of the Toll signalling pathway. We show that BaraA has a pronounced effect on survival after Beauveria fungal infection. Moreover, this gene regulates an erect wing behavioural response upon infection. How each peptide contributes to the immune response and/or erect wing behaviour will be informative in understanding the range of effects immune effectors can have on host physiology. This work and others also clarifies how the cocktail of immune effectors produced upon infection acts specifically during innate host defence reactions.

Materials and methods

Fly genetics and sequence comparisons

Sequence files were collected from FlyBase [52] and recently-generated sequence data [48,53] and comparisons were made using Geneious R10. Putative NF-κB binding sites were annotated using the Relish motif “GGRDNNHHBS” described in Copley et al. [19] and a manually
curated amalgam motif of “GGGHHNNDVH” derived from common Dif binding sites described previously [18,20]. Gene expression analyses were performed using primers described in S3 Data, and further microarray validation for BaraA expression comes from De Gregorio et al. [11]. The UAS-BaraA and BaraA-Gal4 constructs were generated using the TOPO pENTR entry vector and cloned into the pTW or pBPGUw Gateway vector systems respectively. The BaraA-Gal4 promoter contains 1675bp upstream of BaraA1 (but also BaraA2, sequence in S1 Text). The BaraA-Gal4 construct was inserted into the VK33 attP docking site (BDSC line #24871). The BaraA^{SW1} (ΔBaraA) mutation was generated using CRISPR with two gRNAs and an HDR vector by cloning 5’ and 3’ region-homologous arms into the pHD-dsRed vector, and consequently ΔBaraA flies express dsRed in their eyes, ocelli, and abdomen. ΔBaraA was generated using the Bloomingston stocks BL2057 and BL51323 as these backgrounds contain only one copy of the BaraA locus. The induction of the immune response in these flies was validated by qPCR and MALDI-TOF proteomics, wherein we discovered an aberrant Dso2 locus in these preliminary BaraA^{SW1} flies. We thus backcrossed the BaraA^{SW1} mutation once with a standard w^{1118} background (used in [13–15]) and screened for wild-type Dso2 before use in any survival experiments. As a consequence, w; ΔBaraA flies are considered an arbitrary genetic background with no appropriate wild-type control. We typically used Oregon-R (OR-R) flies as a representative wild-type that displays similar resistance to bacterial infections (S6 Fig). Of note, ΔBaraA was also isogenized into the DrosDel w^{1118} isogenic background for seven generations before use in isogenic fly experiments as described in Ferreira et al. [32]. We value the use of both genetic backgrounds to ensure that interpretation of mutant analysis is not biased by genetic background.

A full description of fly stocks used for crosses and in experiments is provided in S4 Data.

Microbe culturing conditions

Bacteria and C. albicans yeast were grown to mid-log phase shaking at 200rpm in their respective growth media (Luria Bertani, Brain Heart Infusion, or Yeast extract–Peptone–Glycerol) and temperature conditions, and then pelleted by centrifugation to concentrate microbes. Resulting cultures were diluted to the desired optical density at 600nm (OD) for survival experiments, which is indicated in each figure. The following microbes were grown at 37˚C: Escherichia coli strain 1106 (LB), Enterococcus faecalis (BHI), and Candida albicans (YPG). The following microbes were grown at 29˚C: Erwinia carotovora carotovora (Ecc15) (LB) and Micrococcus luteus (LB). For filamentous fungi and molds, Aspergillus fumigatus was grown at 37˚C, and Neurospora crassa and Beauveria bassiana strain 802 were grown at room temperature on Malt Agar in the dark until sporulation. Metarhizium rileyi strain PHP1705 and Beauveria bassiana strain R444 commercial spores were produced by Andermatt Biocontrol, products: Nomu-PROTEC and BB-PROTEC respectively. A summary of microbe strains is provided in S4 Data.

Survival experiments

Survival experiments were performed as previously described [16], with 20 flies per vial with 2–3 replicate experiments. 3–5 day old males were used in experiments unless otherwise specified. As Rel, spz double mutant flies and wild-type backgrounds differ drastically in their immune competence, we selected pathogens, infection routes, and temperatures to provide infection models that could best reveal phenotypes in these disparate genetic backgrounds. For fungi natural infections, flies were flipped at the end of the first day to remove excess fungal spores from the vials. Otherwise, flies were flipped thrice weekly. Statistical analyses were
performed using a Cox proportional hazards (CoxPH) model in R 3.6.3. We report the hazard ratio (HR) alongside p-values as a proxy for effect size in survival experiments. Throughout our analyses, we required $p < .05$ as evidence to report an effect as significant, but note interactions with $|HR|$ near or above 0.5 as potentially important provided p-value approached .05, and tamp down importance of interactions that were significant, but have relatively minor effect size ($|HR|$ less than 0.5) in our discussion of the data.

**Erect wing scoring**

The erect wing phenotype was scored as the number of flies with splayed wings throughout a distinct majority of the period of observation (30s); if unclear, the vial was monitored an additional 30s. Here we define splayed wings as wings not at rest over the back, but did not require wings to be fully upright; on occasion wings were held splayed outward at ~45˚ relative to the dorsal view, and often slightly elevated relative to the resting state akin to male aggressive displays. Sometimes only one wing was extended, which occurred in both thoracic pricking and fungi natural infections; these flies were counted as having erect wing. In natural infections, the typical course of erect wing display developed in two fashions at early time points, either: i) flies beginning with wings slightly splayed but not fully upright, or ii) flies constantly flitting their wings outward and returning them to rest briefly, only to flit them outward again for extended periods of time. Shortly after infection, some flies were also observed wandering around with wings beating at a furious pace, which was not counted as erect wing. However at later time points erect wing flies settled more permanently on upright splayed wings. Erect wing measurements were taken daily following infection, and erect wing flies over total flies was converted to a percent. Data points in Fig 6B–6D represent % with erect wing in individual replicate experiments with 20–25 flies per vial. Flies stuck in the vial, or where the wings had become sticky or mangled were not included in totals. S1 Table reports mean percentages across replicate experiments for all pathogens and genotypes where erect wing was monitored. Days post-infection reported in S1 Table were selected as the final day prior to major incidents of mortality. For *E. faecalis* live infections, *Bom*\(^{45SC}\) and *spz*\(^{rm7}\) erect wing was taken at 1dpi due to major mortality events by 2dpi specifically in these lines.

Erect wing measurements were performed in parallel with survival experiments, which often introduced injury to the thorax below the wing possibly damaging flight muscle. It is unlikely that muscle damage explains differences in erect wing display. First: we noticed erect wing initially during natural infections with *A. fumigatus*, and observed erect wing upon *B. bassiana R444* and *Metarhizium rileyi PHP1705* natural infections (S1 Table). Second: only 1 of 75 total *iso w*\(^{111A}\)* males displayed erect wing across 4 systemic infection experiments with *E. faecalis*. For comparison: 19 of 80 total *iso ∆BaraA* and 48 of 80 *w; ∆BaraA* flies displayed erect wing (S1 Table). Future studies might be better served using an abdominal infection mode, which can have different infection dynamics [54]. However we find erect wing display to be robust upon either septic injury or natural infection modes.

**IM10-like peptide in vitro activity**

The 23-residue Baramicin peptides were synthesized by GenicBio to a purity of >95%, verified by HPLC. An N-terminal pyroglutamate modification was included based on previous peptidomic descriptions of Baramicins IM10, IM12, and IM13 [55], which we also detected in our LC-MS data (S2 Fig). Peptides were dissolved in DMSO and diluted to a working stock of 1200μM in 0.6% DMSO; the final concentration for incubations was 300μM in 0.15% DMSO. For microbe-killing assays, microbes were allowed to grow to log-growth phase, at which point they were diluted to ~50cells/μL (for *C. albicans* this was OD ≈ 0.01 in our hands).
Two μL of culture (~100 cells), and 1μL water or antibiotic was mixed with 1μL of a 1:1:1 cocktail of IM10, IM12, and IM13 peptides to a final concentration of 300μM total peptides; 1μL of water + DMSO (final concentration = 0.15% DMSO) was used as a negative control. These 4μL microbe-peptide solutions were incubated for 24h at 4°C. Microbe-peptide cultures were then diluted to a final volume of 100μL and the entire solution was plated on LB agar or BiGGY agar plates. Colonies were counted manually. For combinatorial assays with bacteria, C. albicans yeast, and B. bassiana R444 spores, peptide cocktails were combined with membrane disrupting antimicrobials effective against relevant pathogens beginning at: 10 μM Cecropin A (Sigma), 500μg/mL Ampicillin, or 250μg/mL Pimaricin (commercially available as “Fungin,” InVivogen), serially diluted through to 0.1 μM, 0.5μg/mL, and 4μg/mL respectively.

Beauveria bassiana R444 spores were prepared by dissolving ~30mg of spores in 10mL PBS, and then 4μL microbe-peptide solutions were prepared as described for C. albicans followed by incubation for 24h at 4°C; this spore density was optimal in our hands to produce distinct individual colonies. Then, 4μL PBS was added to each solution and 2μL droplets were plated on malt agar at 25°C. Colony diameters were measured 4 days after plating by manually analyzing colony diameters in Inkscape v0.92. Experimental batches were included as co-variates in one-way ANOVA analysis. The initial dataset approached violating Shapiro-Wilk assumptions of normality (p = 0.061) implemented in R 3.6.3. We subsequently removed four colonies from the analysis, as these outliers had diameters over two standard deviations lower than their respective mean (removed colonies: PBS 15mm, PBS 25mm, IM10-like+Pimaricin 21mm, and a second IM10-like+Pimaricin colony of 21mm); the resulting Shapiro-Wilk p-value = 0.294, and both QQ and residual plots suggested a normal distribution. Final killing activities and colony surface areas were compared by One-way ANOVA with Holm-Sidak multiple test correction (C. albicans) and Tukey’s honest significant difference multiple test correction (B. bassiana R444).

Gene expression analyses
RNA was extracted using TRIzol according to manufacturer’s protocol. cDNA was reverse transcribed using Takara Reverse Transcriptase. qPCR was performed using PowerUP master-mix from Applied Biosystems at 60°C using primers listed in S3 Data. Gene expression was quantified using the PFAFL method [56] with Rp49 as the reference gene. Statistical analysis was performed by one-way ANOVA with Holm-Sidak’s multiple test correction or student’s t-test. Error bars represent one standard deviation from the mean.

Proteomic analyses
Raw haemolymph samples were collected from immune-challenged flies for MALDI-TOF proteomic analysis as described in [15,16]. MALDI-TOF proteomic signals were confirmed independently at facilities in both San Diego, USA and Lausanne, CH. In brief, haemolymph was collected by capillary and transferred to 0.1% TFA before addition to acetonitrile universal matrix. Representative spectra are shown. Peaks were identified via corresponding m/z values from previous studies [8,24]. Spectra were visualized using mMass, and figures were additionally prepared using Inkscape v0.92.

Supporting information
S1 Fig. Supplemental BaraA expression patterns. A) 400bp of upstream sequence from BaraA annotated with putative Rel or Dif/dl binding sites (included in S1 Data). B) Expression of BaraA in wild-type and spzrm7 flies following injury with the Gram-negative bacterium
E. coli or the Gram-positive bacterium M. luteus. As seen in a previous microarray (Fig 1A), basal BaraA expression is depressed in RelE20 flies, but is nevertheless highly induced upon infection, likely representing the BaraA response to injury. C) In a separate set of experiments, BaraA returns to near-baseline levels of expression by 24hpi using E. coli. Meanwhile BaraA remained induced after pricking with M. luteus, mirroring the Toll-regulated BomBc3 but not the Imd-regulated DptA. D) The BaraA>mGFP reporter line shows a robust induction of GFP 2hpi upon pricking with M. luteus in larvae. E) Expression of BaraA>mGFP in the spermatheca of females (yellow arrow). Representative images shown.

S2 Fig. LCMS coverage of trypsin-digested and detected BaraA peptides aligned to the protein coding sequence. Detected peptide fragments (blue bars) cover the whole precursor protein barring furin site-associated motifs. Additionally, two peptide fragments are absent: i) the first 4 residues of the C-terminus ("GIND," not predicted a priori), and ii) the C-terminus peptide’s "RPDGR" motif, which is predicted as a degradation product of Trypsin cleavage and whose size is beyond the minimum range of detection. Without the GIND motif, the mass of the contiguous C-terminus is 5974.5 Da, matching the mass observed by MALDI-TOF for IM22 (Fig 2A). The N-terminal Q residues of IM10, IM12, IM13, and IM24 are pyroglutamate-modified, as described previously [24]. The asparagine residues of IM10-like peptides are sometimes deamidated, likely as a consequence of our 0.1% TFA sample collection method as "NG" motifs are deamidated in acidic conditions [58].

S3 Fig. Alignments of BaraA peptide motifs. A) Aligned IM22 peptides of Drosophila Baramicin A-like genes, with the IM10-like 'VWKRPDGRTV' motif noted. The GIND residues at the N-terminus are cleaved off in Dmel\BaraA by an unknown process, and this subsequent peptide is similarly cleaved following RXRR furin cleavage sites in subgenus Drosophila flies. As a consequence, the mature IM22 peptide is predicted to be the same across species even when different cleavage mechanisms are utilized. B) Alignment of the three IM10-like peptides of D. melanogaster BaraA with the "VXRPXRTV" motif noted. The residue 8 polymorphism of either G (IM12) or D (IM10, IM13) has evolved repeatedly in outgroup flies [25], indicating it is likely key for IM10-like peptide activity.

S4 Fig. Over-expression of BaraA partially rescues Rel, spz double mutant susceptibility to infection in both males and females. A) Validation of the UAS-BaraA construct in the Rel, spz background. Flies were unchallenged. B) Overexpressing BaraA did not improve the survival of Rel, spz flies upon E. coli infection. C) Overexpressing BaraA only marginally improves survival of Rel, spz females, but not males, upon M. luteus infections. Infections using a higher dose (OD = 100) tended to kill 100% of Rel, spz flies regardless of sex or expression of BaraA, suggesting that if BaraA overexpression does affect susceptibility to M. luteus, this effect is possible within only a narrow window of M. luteus concentration. D-F) Overexpressing BaraA improves survival of Rel, spz male and female flies upon injury with C. albicans (D) or natural infection with A. fumigatus (E) and N. crassa (F). P-values are shown for each biological sex in an independent CoxPH model not including the other sex relative to Rel, spz as a reference.

S5 Fig. RT-qPCR shows that the expression of BomBc3 (A) Drs (B) and DptA (C) is wild-type 18hpi in iso ΔBaraA flies. D) BaraA mutants survive clean injury like wild-type flies.
E) iso ΔBaraA flies have similar lifespan compared with the iso w^1118 wild-type (males + females, iso vs. iso ΔBaraA: HR = 0.26, p = .118)

S6 Fig. Additional survivals using ΔBaraA flies in two distinct genetic backgrounds upon infection by a diversity of microbes. A-B) No significant susceptibility of ΔBaraA flies to Ecc15 (A), P. burhodogranariea (B), or B. subtilis (C), bacterial infections. D-E) w; ΔBaraA males were slightly susceptible to A. fumigatus natural infection (HR > 0.5, p = .078), but not females, nor isogenic flies. Additional infections using ΔBaraA, Bom^ASSC double mutant flies reveals that BaraA mutation increases the susceptibility of Bom^ASSC flies in both males and females (cumulative curves shown in Fig 5A). Blue backgrounds = Gram-negative bacteria, orange backgrounds = Gram-positive bacteria, yellow backgrounds = fungi.

S7 Fig. Survival analysis suggests a minor contribution of BaraA to defence against infection by E. faecalis. A) w; ΔBaraA but not iso ΔBaraA flies are significantly susceptible to E. faecalis. However we note that iso ΔBaraA flies suffer an earlier mortality than iso w^1118 wild-type controls that is highly significant if the experiment is artificially censored at 3.5 days (dotted line and associated statistics). B) Crosses with a genomic deficiency (Df(BaraA)) leads to increased susceptibility in both the w background and isogenic DrosDel background, with Df (BaraA)/ΔBaraA flies suffering the greatest mortality in either crossing scheme. Both deficiency crosses yielded an earlier susceptibility in BaraA-deficient flies (shown with dotted black lines), however neither experiment ultimately reached statistical significance. C) BaraA RNAi flies (Act>BaraA-IR) suffered greater mortality than Act>OR-R or OR-R/BaraA-IR controls, but this was not statistically significant at α = .05; p-values reported are comparisons to Act>BaraA-IR flies.

S8 Fig. Additional survival analyses reveal a consistent contribution of BaraA to defence against infection by B. bassiana. A) BaraA mutants in both backgrounds are highly susceptible to natural infection with the entomopathogenic fungus B. bassiana 802. B) Crossing with a genomic deficiency (Df(BaraA)) leads to increased susceptibility of Df(BaraA)/ΔBaraA flies for both the w background and isogenic DrosDel background relative to wild-type controls (p < .05) upon B. bassiana 802 natural infection. C) Act>BaraA-IR flies were more susceptible than the OR-R wild-type (p = .008) and OR>BaraA-IR (p = .004), although not significantly different from our Act>OR-R control (p = .266). D) Overexpressing BaraA (Act>UAS-BaraA) improved survival against B. bassiana 802 relative to Act>OR-R controls (HR = -0.52, p = 0.010). E) BaraA alone contributes to survival against B. bassiana to a far greater extent than the two canonical antifungal peptide genes Mtk and Drs, which in fact had little effect on survival outcome.

S9 Fig. Frequency of erect wing display following additional challenges. A) Erect wing occurs in flies given natural infection with A. fumigatus, wherein flies do not readily succumb to infection (S6D Fig) and no thoracic injury was introduced. B-C) Erect wing frequencies 2dpi after clean injury (B), or Ecc15 septic injury (C). The erect wing frequencies of flies pricked by HK-E. faecalis (Fig 6C) are included in brown to facilitate direct comparison with the frequency observed upon Toll pathway activation. D) The frequency of erect wing display is increased following E. faecalis septic injury in ΔBaraA/+ or Df(BaraA)+/ flies. Data points are pooled from w; ΔBaraA and iso ΔBaraA crosses after E. faecalis infections shown in S7A Fig and data in S1 Table. E) C_7BaraA-C_7Rp49 (ΔC_7) non-normalized expression of the
BaraA-Gal4/UAS-BaraA method to better visualize expression level differences. This Gal4/UAS approach rescues BaraA expression in ΔBaraA flies, though not quite to wild-type levels. A very low level of expression was observed in ΔBaraA, UAS-BaraA/ΔBaraA flies without the Gal4 (indicating a tiny level of UAS leakiness), while BaraA was never detected in w; ΔBaraA flies. Differences in this ΔCt y-axis effectively equate to Log2 expression differences. The level of BaraA induction in these ΔBaraA, BaraA-Gal4/UAS-BaraA was ~3.3x the unchallenged state by 24hpi.

(TIF)

S10 Fig. ΔBaraA/+ transheterozygotes suffer significantly reduced BaraA expression. A) Schematic detailing the BaraA loci of genotypes used in transheterozygote crosses. B-C) BaraA (B) and BomBc3 (C) expression after B. bassiana pricking in BaraA homozygous or heterozygous flies. Transheterozygotes with one mutant locus have significantly reduced BaraA expression. Intriguingly, OR-R flies (homozygous for 2 gene copies) have higher BaraA expression levels compared to w1118 (1 gene copy) after infection (B), which appears to be unrelated to the activation of the Toll response generally as BomBc3 levels were comparable across genotypes (C). Instead, OR-R flies seemingly reach a slightly greater absolute expression (S9E Fig). Statistically significant differences at 24hpi are indicated by red letters, to facilitate complex multiple comparisons (one-way ANOVA with Holm-Sidak’s multiple test correction). Genotypes with the same letter group are not significantly different from each other. In all cases, no significant differences were observed amongst unchallenged flies.

(TIF)

S1 Table. Erect wing frequencies from various infection experiments. Following initial erect wing observations after A. fumigatus natural infection, we scored erect wing frequency in all subsequent survival experiments. Data represent the mean % of males displaying erect wing ± one standard deviation. n exp = number of replicate experiments performed, and dpi ewg taken = days post-infection where erect wing data were recorded. We additionally performed natural infections with Metarhizium rileyi that generally did not cause significant mortality even in ΔBaraA, Bomfassc double mutant males, but nevertheless induced erect wing specifically in ΔBaraA males and spzm7 controls. Bacterial infections were performed by septic injury, while fungal challenges were either natural infections (NI) performed by rolling flies in spores or septic injuries as indicated. Underlying data are included in S5 Data.

(XLSX)

S1 Text. Supplementary discussion of IM22 identification and BaraA-Gal4 construct.

(DOCX)

S1 Data. Putative NF-κB sites in the Baramicin promoter.

(XLSX)

S2 Data. Standard curves to calculate peptide masses in Uttenweiler-Joseph et al. [8] and this study to identify IM22, and charge characteristics of Baramicin peptides.

(XLSX)

S3 Data. Primers used in this study and annotation of BaraA copy number in a selection of wild-type strains.

(XLSX)

S4 Data. Fly stock and Microbe strain information.

(XLSX)
S5 Data. Complete erect wing data S1 Table. (XLSX)

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