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Commensal microbiota stimulate systemic neutrophil migration through induction of Serum amyloid A

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Summary

Neutrophils serve critical roles in inflammatory responses to infection and injury, and mechanisms governing their activity represent attractive targets for controlling inflammation. The commensal microbiota is known to regulate the activity of neutrophils and other leucocytes in the intestine, but the systemic impact of the microbiota on neutrophils remains unknown. Here we utilized *in vivo* imaging in gnotobiotic zebrafish to reveal diverse effects of microbiota colonization on systemic neutrophil development and function. The presence of a microbiota resulted in increased neutrophil number and myeloperoxidase expression, and altered neutrophil localization and migratory behaviours. These effects of the microbiota on neutrophil homeostasis were accompanied by an increased recruitment of neutrophils to injury. Genetic analysis identified the microbiota-induced acute phase protein serum amyloid A (Saa) as a host factor mediating microbial stimulation of tissue-specific neutrophil migratory behaviours. *In vitro* studies revealed that zebrafish cells respond to Saa exposure by activating NF- κ B, and that Saa-dependent neutrophil migration requires NF- κ B-dependent gene expression. These results implicate the commensal microbiota as an important environmental factor regulating diverse aspects of systemic neutrophil development and function, and reveal a critical role for a Saa-NF- κ B signalling axis in mediating neutrophil migratory responses.

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Introduction

Leucocytes such as neutrophils and macrophages are key mediators and effectors of inflammatory stimuli and represent attractive therapeutic targets for controlling acute and chronic inflammation. The complex community of microorganisms residing within the intestine (gut microbiota) has been identified as an important environmental factor regulating leucocyte function within the intestinal compartment (Abt and Artis, 2009). However, the presence of microbiota appears to also have profound systemic effects on leucocytes. Peripheral neutrophils collected from germ-free (GF) rodents display reduced phagocytosis, microbicidal activity, and production of superoxide anion and nitric oxide compared with ex-GF animals colonized with normal microbiota (conventionalized or CONVD) (Ohkubo *et al.*, 1990; 1999; Clarke *et al.*, 2010). Similarly, macrophages collected from peritoneal exudate in GF animals display reduced superoxide anion production and microbicidal activity, and impaired chemotaxis compared with CONVD controls (Jungi and McGregor, 1978; Morland and Midtvedt, 1984; Czuprynski and Brown, 1985; Mitsuyama *et al.*, 1986; Oliveira *et al.*, 2005). The importance of the microbiota on systemic inflammation is further underscored by reports that multiple rodent models of spondyloarthritis do not develop disease when raised under GF conditions (Taurog *et al.*, 1994; Rehakova *et al.*, 2000), but disease can be initiated upon gut colonization with specific bacteria (Rath *et al.*, 1996; Sinkorova *et al.*, 2008).

Although recent research has yielded an abundance of new information about the impact of gut microbiota on intestinal leucocyte biology and immunity (Abt and Artis, 2009), gut microbiota effects on systemic leucocyte biology remain relatively unresolved. Our current information of the systemic effects of microbiota on neutrophils is largely derived from *ex vivo* experiments conducted on neutrophils collected from peripheral blood or bone marrow from GF and CONVD mammals (Ohkubo *et al.*, 1990; 1999; Clarke *et al.*, 2010). However, studies generated from *ex vivo* neutrophils may not be representative of the systemic population and do not fully recapitulate the native physiologic context of live tissues. Finally, mammals are not amenable to the high-resolution *in vivo* microscopy required to comprehensively define the systemic impact of microbiota on neutrophils. As a result, the specific aspects of systemic neutrophil activity affected by microbiota are not fully understood.

The zebrafish has several features that make it an attractive model to study the roles of commensal microbiota on systemic neutrophil biology. First, zebrafish are optically transparent from fertilization through early adulthood, permitting high-resolution imaging of host-microbe interactions in the intact physiologic context of a living vertebrate (Rawls *et al.*, 2007; Kanther *et al.*, 2011). Second, the zebrafish has innate and adaptive immune systems that share extensive homology with those of humans and other mammals (Kanther and Rawls, 2010). Likewise, the digestive tracts of zebrafish and mammals are similar, including an intestine, pancreas, liver and gall bladder (Ng *et al.*, 2005; Wallace *et al.*, 2005). Third, we have developed methods for rearing zebrafish under GF conditions and colonizing GF zebrafish with members of the commensal microbiota (Pham *et al.*, 2008).

Previous analyses of gnotobiotic zebrafish and mice have revealed that the presence of a microbiota causes extensive alterations in diverse aspects of host immunity and physiology. Reciprocally, host-mediated mucosal factors such as antimicrobial proteins, IgA, mucins, and inflammation alter microbial community composition and function (reviewed in Abt and Artis, 2009; Kanther and Rawls, 2010; Hooper *et al.*, 2012; Tremaroli and Backhed, 2012). This complex interplay between host and microbial factors is central to the maintenance of homeostasis. However the host signalling pathways that mediate microbial cues to regulate systemic leucocyte responses remain unresolved.

Serum amyloid A (Saa) is a circulating HDL-associated apolipoprotein and acute phase protein. The Saa gene family (3 in humans, 4 in mice, 1 in zebrafish) is conserved across vertebrates (Fig. S1), suggesting important biological roles. *Saa* genes are expressed by multiple tissues including liver, intestinal epithelium (Eckhardt *et al.*, 2010), and macrophages (Meek *et al.*, 1992) and are markedly induced by diverse inflammatory stimuli (Uhlir and Whitehead, 1999) including gut microbiota (Hooper *et al.*, 2001; Rawls *et al.*, 2006; Ivanov *et al.*, 2009; Kanther *et al.*, 2011). Serum Saa protein level is a salient biomarker for inflammatory disorders including IBD (Okahara *et al.*, 2005; Noble *et al.*, 2008), necrotizing enterocolitis, sepsis (Ng *et al.*, 2010), and chronic obstructive pulmonary disease (Bozinovski *et al.*, 2008). The precise roles of Saa in inflammation remain elusive because both pro- and anti-inflammatory actions have been reported. Reported pro-inflammatory roles for Saa include stimulation of extracellular matrix (ECM)-degrading enzymes such as MMP9 (Lee *et al.*, 2005), recruitment of neutrophils and monocytes (Badolato *et al.*, 1994; Su *et al.*, 1999; Connolly *et al.*, 2010), suppression of neutrophil apoptosis (Christenson *et al.*, 2008), stimulation of granulocytosis (He *et al.*, 2009), opsonization of Gram-negative bacteria (Shah *et al.*, 2006), Nlrp3 inflammasome activation (Ather *et al.*, 2011; Niemi *et al.*, 2011), and stimulation of pro-inflammatory cytokines such as IL1 β (Patel *et al.*, 1998; Lee *et al.*, 2005; Cheng *et al.*, 2008; Niemi *et al.*, 2011). In contrast, numerous reports cite anti-inflammatory effects of Saa on neutrophils, including inhibition of MPO production (Renckens *et al.*, 2006), oxidative burst (Linke *et al.*, 1991; Gatt *et al.*, 1998), and migration (Gatt *et al.*, 1998), and induction of IL10 expression (Shah *et al.*, 2006; Cheng *et al.*, 2008; De Santo *et al.*, 2010). These diverse effects may be due to Saa's ability to stimulate signalling events through multiple transmembrane receptors, including formyl peptide receptor 2 (Fpr2/Fpr11) (Su *et al.*, 1999), receptor for advanced glycosylation end-products (RAGE) (Cai *et al.*, 2007), scavenger receptor class B type I (Scarb1/CLA-1) (Baranova *et al.*, 2005), and Toll-like receptor 2 (Tlr2) (He *et al.*, 2009). The signal transduction pathways that act downstream of Saa to regulate gene expression include extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38, c-Jun N-terminal kinase (JNK), Akt and NF- κ B (Baranova *et al.*, 2005; Jijon *et al.*, 2005; Cheng *et al.*, 2008). However, the relationship between these pathways and the distinct immune cellular responses evoked by Saa remain unclear. Importantly, *in vivo* genetic analysis of Saa has been complicated by the fact that the human and mouse genomes encode 3 and 4 paralogous *Saa* genes respectively (Fig. S1). We previously showed that colonization with a normal microbiota in zebrafish results in NF- κ B-dependent induction of *saa* expression in the distal intestine, liver and swim bladder (Kanther *et al.*, 2011). However, the *in vivo* roles of Saa in systemic neutrophil biology, as well as neutrophil requirements for NF- κ B in these

responses, remain unclear. In this study, we took advantage of the fact that the zebrafish genome encodes only a single Saa gene to define the requirement for Saa in microbiota-induced neutrophil responses. Our results reveal novel roles for the microbiota on systemic neutrophil biology including increased number and migratory behaviour and suggest that Saa-dependent neutrophil migration requires NF- κ B signalling.

Results

Microbiota promotes increased neutrophil number and pro-inflammatory gene expression

To investigate the impact of the commensal microbiota on zebrafish myeloid lineages, we queried results from a microarray-based functional genomic comparison of gene expression in whole zebrafish at 6 days post fertilization (dpf) that had been raised GF or conventionalized since 3dpf (CONVD). Functional categorization of the resulting list of microbiota-regulated transcripts revealed enrichment for genes involved in leucocyte development and function (Kanther *et al.*, 2011). CONVD zebrafish displayed relative increases in transcript levels for 17 genes known to be specifically expressed by myeloid leucocytes, including the zebrafish homologue of mammalian myeloperoxidase *mpx* (also called *mpo*; Table 1) (Rawls *et al.*, 2004; 2006; Kanther *et al.*, 2011). Our previous whole-mount *in situ* hybridization analysis of *mpx* mRNA in GF and CONVD zebrafish suggested that this increase in *mpx* transcript level could be due to increased neutrophil number or increased *mpx* mRNA levels in individual neutrophils (Kanther *et al.*, 2011). To test if these transcript differences were associated with alterations in neutrophil number, we used transgenic *Tg(mpx:GFP)* zebrafish that robustly express GFP specifically in neutrophils (Renshaw *et al.*, 2006). Stereomicroscopic evaluation of GFP(+) neutrophil number and localization in whole 6dpf GF and CONVD zebrafish revealed a qualitative increase in neutrophil number throughout the animal (Fig. 1A). Flow cytometry of GFP(+) neutrophils from dissociated 6dpf *Tg(mpx:GFP)* fish confirmed a significant increase in total steady-state number of neutrophils per animal in CONVD compared with GF animals (Fig. 1B). Quantitative RT-PCR in flow-sorted neutrophils from GF and CONVD larvae revealed significant increases in *mpx* mRNA in sorted neutrophils from CONVD animals (Fig. 1C). Colonization with a commensal microbiota therefore results in significant increases in neutrophil *mpx* expression together with increases in steady-state neutrophil number.

Microbiota regulates tissue distribution and migration of neutrophils

We imaged whole 6dpf GF and CONVD *Tg(mpx:GFP)* zebrafish to evaluate the effect of microbiota on neutrophil localization and migration. Consistent with our previous *mpx* RNA whole-mount *in situ* hybridization results (Kanther *et al.*, 2011), CONVD zebrafish displayed increased GFP expression in the kidney, a site of definitive haematopoiesis (Fig. 1A). Since the intestine harbours microbial communities that are markedly denser than that of the surrounding water, we analysed the frequency and distribution of GFP(+) neutrophils associated with intestines dissected from *Tg(mpx:GFP)* GF and CONVD larvae. We observed increased numbers of GFP(+) neutrophils in the intestines of CONVD *Tg(mpx:GFP)* zebrafish compared with GF controls, most significantly in the proximal region (segment 1) of the intestine (Fig. 1D). To determine if these changes in localization were associated with altered neutrophil migratory behaviours, we used confocal microscopy

to quantify migration of individual neutrophils in live 6dpf GF and CONVD *Tg(mpx:GFP)* fish. Compared with GF controls, neutrophils in CONVD animals displayed significantly elevated migration velocity and decreased meandering (i.e. increased directional migration) compared with GF controls (Fig. 2, Movies S1 and S2). These results indicate that the microbiota regulates systemic neutrophil localization and migratory activity.

Microbiota promotes neutrophil recruitment to extra-intestinal injury

To determine if the observed effects of microbiota on neutrophil number, localization, and migration have functional consequences, we used a well-established injury model in which a portion of the tail fin in larval zebrafish is resected and the recruitment of leucocytes to the wound is quantified over time (Renshaw *et al.*, 2006; Yoo and Huttenlocher, 2011). Although early (1 h) GFP(+) neutrophil recruitment to the wound was slightly higher in GF animals compared with CONVD controls, later evaluation at 3, 6 and 15 h after injury revealed significantly more neutrophils recruited to the wound in CONVD animals (Fig. 3). These results confirm that colonization with a microbiota augments the host's capacity for recruiting neutrophils to extra-intestinal injury.

Saa is required for increases in neutrophil migration and suppression of inflammatory biomarkers following colonization with a microbiota

We next sought to define the role of zebrafish *saa* on neutrophil responses to the microbiota. Injection of zebrafish embryos with morpholino antisense oligonucleotides (MO) targeting *saa* resulted in partial knockdown of *saa* transcript through 6dpf (Fig. S2A). By comparing 6dpf GF and CONVD *Tg(mpx:GFP)* zebrafish injected with a MO targeting *saa* (*saa*-MO) or a standard negative control MO (ctrl-MO), we found that *saa* knockdown did not qualitatively alter the effects of the microbiota on neutrophil tissue distribution (Fig. 4A). Using a computational approach to quantify GFP(+) neutrophil number in whole live zebrafish (Ellett and Lieschke, 2012), we observed that the microbiota-induced increases in neutrophil number (Fig. 4B) and *mpx* mRNA levels (Fig. 4C) were also not affected by *saa* knockdown. In contrast, *in vivo* imaging of 6dpf GF and CONVD *Tg(mpx:GFP)* fish revealed striking tissue-specific *saa*-dependent alterations in microbiota-induced neutrophil migration behaviour (Movies S3–5 and S6). In ctrl-MO animals, neutrophils associated with the CHT, intestine, and fin displayed significantly increased migration velocity in the presence of a microbiota. In *saa*-MO animals, the microbiota caused a similar increase in fin neutrophils but failed to increase neutrophil migration velocity in the CHT and intestine (Fig. 4F). The presence of a microbiota in ctrl-MO animals caused a significant reduction in the meandering index of neutrophils associated with the CHT but not those in the intestine or fin. In *saa*-MO animals, the effect of the microbiota on CHT neutrophil meandering was significantly attenuated (Fig. 4G). Strikingly, neutrophils associated with the intestine in *saa*-MO animals displayed a microbiota-dependent decrease in meandering that was not observed in ctrl-MO controls (Fig. 4G). These results suggest multiple novel *in vivo* roles for Saa in regulating tissue-specific neutrophil migratory behaviours. These alterations in microbiota-induced neutrophil migration in *saa*-MO fish were accompanied with significant increases in microbiota-dependent induction of inflammatory biomarkers *ncf1* and *illb* (Fig. 4D and E). These results reveal a potential anti-inflammatory role for *saa* in suppressing

inflammatory gene expression and complex tissue-specific roles in neutrophil migration responses to commensal microbiota.

Saa-dependent induction of neutrophil migration requires NF- κ B activity

Saa has been shown to promote neutrophil migration in mammals (He *et al.*, 2009; Connolly *et al.*, 2010) and we have shown that SAA activates NF- κ B signalling in mammalian cells (Jijon *et al.*, 2005). Because the NF- κ B transcription factor has been linked to neutrophil migration (Penzo *et al.*, 2010), we hypothesized that increased Saa levels in response to microbiota might activate NF- κ B and NF- κ B-dependent immune cell migration. To test this, we turned to cell culture where the cell autonomous roles of SAA and NF- κ B could be readily evaluated. Culture methods for purified zebrafish neutrophils have not been established, therefore we first tested the effects of SAA on the PAC2 zebrafish fibroblast cell line. Western blot analysis showed that SAA induced phosphorylation of the NF- κ B protein inhibitor I κ B α , a key process for canonical NF- κ B activity (Fig. 5A). Immunofluorescence analysis showed that SAA promoted nuclear translocation of the NF- κ B transcriptional subunit RelA/p65 (data not shown). To confirm that SAA functionally impacts NF- κ B signalling, we investigated transcriptional activity using pikbaa:Luc gene reporter system (Kanther *et al.*, 2011). Luciferase activity increased \sim 3-fold in pikbaa:Luc-transfected PAC2 cells following SAA stimulation, a level similar to LPS stimulation (Fig. 5B). Importantly, increased NF- κ B activity was associated with SAA-induced accumulation of NF- κ B target genes *mmp9* and *ikbaa* mRNA (Fig. 5C and D). These findings demonstrate that SAA induces NF- κ B signalling and expression of NF- κ B target genes in zebrafish cells. We next sought to directly test the functional impact of NF- κ B signalling in Saa-induced neutrophil migration using mouse peritoneal neutrophils. Using a transwell migration assay, we observed that peritoneal neutrophil motility in response to SAA increased by \sim 2-fold (Fig. 6A and B). Neutrophil migration was reduced by 82% when the NF- κ B inhibitor Bay 11-7082 (BAY) was co-incubated with SAA (Fig. 6A and B). Treatment with the protein synthesis inhibitor cycloheximide (CHX) strongly attenuated SAA-induced neutrophil transmigration (73%), suggesting that NF- κ B-mediated gene expression is necessary for neutrophil movement (Fig. 6A and B). These findings implicate microbiota-induced SAA expression as an important host response regulating neutrophil behaviour.

Discussion

The majority of microbes on the human body reside in the intestine, where they are known to contribute significantly to intestinal physiology and mucosal immunity. There is, however, increasing evidence that the influence of the microbiota extends beyond the confines of the intestine to other tissues and their pathologies (McFall-Ngai *et al.*, 2013). Therefore, the identification of the cellular and molecular mechanisms by which the microbiota shapes the systemic physiology of animal hosts is an important research objective. An improved understanding of the microbiota's impact on systemic leucocyte function is particularly needed due to the implication of the microbiota in the aetiology of inflammatory diseases in intestinal and extra-intestinal compartments. Previous analysis of the microbiota's impact on neutrophil biology in mammals has been limited to *ex vivo* comparisons of neutrophils collected from peripheral blood or bone marrow (Ferencik *et al.*,

1985; Ohkubo *et al.*, 1990; 1999; Clarke *et al.*, 2010). Here we have utilized the transparency of the zebrafish to provide the first comprehensive view of the microbiota's systemic impact on *in vivo* neutrophil function. Our results reveal diverse consequences of microbiota colonization on neutrophil homeostasis and behaviour, as well as recruitment of neutrophils to injury. We also show a mechanistic role for a SAA-NF- κ B signalling axis in microbiota-dependent neutrophil migration. These findings underscore the potential of the microbiota to influence the systemic physiology of animal hosts and provide an important new conceptual framework for understanding the microbiota's roles in inflammatory diseases.

Our observations of increased systemic neutrophil number in CONVD compared with GF zebrafish larvae reveal a novel role for the microbiota in defining the steady state neutrophil population. Systemic neutrophil number might be influenced by differences in microbiota composition or husbandry practices, because a recent comparison of starved GF and conventionally raised zebrafish larvae in a different zebrafish facility did not reveal differences in neutrophil number (Galindo-Villegas *et al.*, 2012). Inflammatory stimuli can regulate the hematopoietic compartment in zebrafish, as injection with LPS induces myelopoiesis (Liongue *et al.*, 2009) and bacterial infection or tail wounding induces mobilization of neutrophils from the CHT (Yoo and Huttenlocher, 2011; Deng *et al.*, 2013). Our study using commensal microbial colonization adds a novel aspect to bacteria-host interaction by showing that microbial cues regulate myelopoietic programs.

In vivo imaging of GFP(+) neutrophils in gnotobiotic zebrafish revealed significant influences of microbiota on neutrophil localization and migratory behaviour. We detected a quantitative increase in neutrophil localization in the intestines of CONVD versus GF zebrafish, with the most marked increases in the proximal intestine. This is consistent with a previous study by Bates and colleagues that reported an increase in MPO(+) cell number in the distal intestine of CONVD versus GF zebrafish (Bates *et al.*, 2007). These microbiota-associated increases in intestinal neutrophil number may be due to tissue-specific alterations in neutrophil recruitment or retention, or may simply reflect the observed overall increase in systemic neutrophil number. Sites of haematopoiesis are dynamic during zebrafish development, and occur in the CHT and kidney at the larval stages under study here (for review see Kanther and Rawls, 2010). We observed salient qualitative increases in GFP(+) neutrophil localization to the kidney region in CONVD zebrafish. The significance of this microbiota-induced neutrophil localization remains unknown, and could be indicative of altered granulopoiesis in the kidney hematopoietic tissue or linked to microbiota-induced NF- κ B activation in the adjacent swim bladder (Kanther *et al.*, 2011). Time-lapse *in vivo* microscopy revealed that microbiota-induced alterations in neutrophil localization were accompanied by significantly increased neutrophil migration velocity in all evaluated tissues and decreased neutrophil meandering in the CHT region. These observed tissue-specific influences of the microbiota on neutrophil behaviour underscore the utility of *in vivo* imaging in the zebrafish for defining the regional impact of microbial colonization status.

These systemic impacts of the microbiota on neutrophil development are predicted to have diverse functional consequences on host immunity and inflammation. In support, we found that the presence of a microbiota is associated with significant alterations in inflammatory

gene expression in neutrophils. Our previous genomic comparison of whole GF and CONVD zebrafish larvae revealed that transcripts encoding multiple myeloid markers including neutrophil-specific *mpx* were increased in the presence of the microbiota (Kanther *et al.*, 2011). Moreover, we previously identified individual bacterial species sufficient to induce *mpx* in gnotobiotic zebrafish (Rawls *et al.*, 2004; 2006; Kanther *et al.*, 2011) and showed that this response requires functional bacterial flagella (Rawls *et al.*, 2007). Here we found that *mpx* mRNA levels were increased in sorted neutrophils from CONVD compared with GF zebrafish larvae. This is consistent with previous analysis of MPO levels in neutrophils from gnotobiotic mammals (Ferencik *et al.*, 1985), suggesting an evolutionarily conserved role for microbiota in controlling neutrophil gene expression programs. The breadth and impact of microbiota-induced alterations in neutrophil transcription remain to be defined. However, a recent genetic analysis in zebrafish revealed that *mpx* functions to downregulate H₂O₂ gradients established at sites of injury and thereby contributes to the resolution of inflammation (Pase *et al.*, 2012). This data suggests that increased *mpx* expression in neutrophils in the presence of the microbiota might serve as an anti-inflammatory response to commensal microbial colonization. Consistent with a previous study (Galindo-Villegas *et al.*, 2012), we also found that the presence of a microbiota significantly increased the number of neutrophils recruited to a fin injury. This could be due to increased recruitment or retention of neutrophils in the wound in the presence of a microbiota, or could reflect the observed overall increase in systemic neutrophil number in those animals. Although GF neutrophils express less *mpx* transcript, other microbiota-induced responses must cause this wound recruitment phenotype because *mpx*-deficient neutrophils develop normally and migrate normally to a fin wound (Pase *et al.*, 2012). Notably, GF fish recruited more neutrophils exclusively at an early time point (1 h) after injury, suggesting neutrophils might be slower to arrive but accumulate in greater numbers in the wound of colonized animals. Together, these results establish that the microbiota regulates neutrophil function as well as development, and suggest that identification of the underlying molecular mechanisms could provide potential therapeutic targets for controlling inflammation.

Our results identify a novel role for Saa in regulating neutrophil migratory behaviour in response to microbiota colonization. SAA proteins are known to be produced by multiple vertebrate tissues and cell types in response to various stimuli including gut microbiota. Indeed, we showed that zebrafish *saa* transcript levels in distal intestine, liver, and swim bladder are elevated upon colonization with a microbiota via *myd88*-dependent and NF- κ B-dependent mechanisms (Kanther *et al.*, 2011). However the functional consequence of commensal microbiota-induced SAA on neutrophil activity remained unknown. The existence of a single Saa orthologue in zebrafish allowed us to test the requirement of *saa* function on neutrophil responses to the microbiota using MO knockdown. Microbiota-induced alterations in neutrophil number, localization and *mpx* levels were unaffected by *saa* knockdown, suggesting that these host responses do not require *saa*. In contrast, neutrophil migratory responses to the microbiota were strongly affected by *saa* knockdown. Microbiota-induced increases in neutrophil velocity were attenuated in the CHT and intestines of *saa*-MO fish but not in the fin. This reveals novel tissue-specific roles for *saa* in neutrophil migration, and suggests that neutrophils located in these tissue compartments

might have different sensitivities or accessibility to Saa protein. The ability of the microbiota to induce a lower meandering index in the CHT was also attenuated following *saa* knockdown, suggesting that this host response also requires Saa. Knockdown of *saa* additionally caused an unexpected reduction in meandering index in the intestine, consistent with an increased inflammatory tone in that tissue. In support of this notion, *saa* knockdown was associated with increased expression of inflammatory biomarkers *illb* and *ncfl*, reminiscent of increased susceptibility to DSS colitis in mice deficient for *Saa1* and *Saa2* (Eckhardt *et al.*, 2010). Importantly, MO injection resulted in only a partial knockdown of wild-type *saa* transcript. Therefore *saa* may have additional functions that could be revealed by stronger loss of function approaches.

Saa induces neutrophil migration in mammals as well as zebrafish. Saa is known to activate NF- κ B signalling in mammalian cells, and NF- κ B has also been linked to mammalian neutrophil migration. To test whether Saa induces neutrophil migration by activating NF- κ B, we turned to cell culture platforms where the cell autonomous effects of Saa can be readily evaluated. We find that zebrafish fibroblasts, like mammalian cells, respond to Saa exposure by inducing the NF- κ B signalling pathway and downstream transcriptional targets. Transwell migration assays using murine peritoneal neutrophils revealed that Saa-dependent induction of neutrophil migration requires NF- κ B-dependent gene expression. We previously found that the ability of the microbiota to induce zebrafish *saa* required NF- κ B (Kanther *et al.*, 2011), indicating that the NF- κ B pathway is involved at multiple steps in this process. Together, our data support a model in which the presence of a microbiota results in NF- κ B-dependent induction of *saa* in multiple tissues, which leads to systemic NF- κ B-dependent increases in neutrophil migration. In parallel, the microbiota causes altered neutrophil number, localization, and *mpx* expression using *saa*-independent mechanisms. Since the NF- κ B pathway regulates gene transcription in multiple tissues and cell types (Kanther *et al.*, 2011), *in vivo* analysis of the cell-autonomous roles of NF- κ B in neutrophil behaviour will require new approaches for controlling the NF- κ B pathway specifically in the neutrophil lineage. Although this study focused exclusively on neutrophils, we anticipate that the microbiota might have similar effects on other leucocyte lineages. Additional studies are needed to determine the similarities and differences between leucocyte responses to colonization by commensal microbiota and infection with pathogenic microbes. Of particular interest are the mechanisms by which gut microbes might regulate aspects of haematopoiesis and mobilization of immune cells to distinct target tissues such as the gut. An improved understanding of how commensal and pathogenic microbes control systemic neutrophil function could lead to the development of new probiotic, antibiotic and pharmacologic approaches for controlling neutrophil activity and inflammation to reduce incidence and severity of human IBD and other inflammatory diseases.

Experimental procedures

Animal husbandry

All experiments using zebrafish and mice were performed using protocols approved by the Animal Studies Committee of the University of North Carolina at Chapel Hill. Conventionally raised wild-type (TL strain) and *Tg(BACmpx:GFP)ⁱ¹¹⁴* [hereafter referred to

as *Tg(mpx:GFP)*] (Renshaw *et al.*, 2006) zebrafish were maintained as described (Flynn *et al.*, 2009; Kanther *et al.*, 2011). Production using *in vitro* fertilization methods, colonization, maintenance and sterility testing of GF zebrafish was performed as described (Pham *et al.*, 2008). GF and CONVD animals were reared at an average density of 1.3 animals per ml in sterile vented tissue culture flasks (Cellstar) housed in an air incubator at 28.5°C on a 14 h light cycle. Wild-type 8- to 12-wk-old C57BL/6 mice were maintained under specific pathogen free conditions.

***In vivo* imaging**

For time-lapse imaging, zebrafish were anesthetized in 4× Tricaine (MS-222; Sigma-Aldrich), and then mounted in 1% low melting point agarose containing 1× Tricaine on glass bottom dishes (MatTek Corporation). Solidified agarose containing fish was then covered in sterile GZM containing 2× Tricaine. Time-lapse movies were captured using a Zeiss 510 Meta Laser Scanning Confocal Microscope at a rate of 1 frame every 15 s for 5 or 15 min. For live whole animal imaging, zebrafish were anesthetized as described above, mounted in 3% methylcellulose, and imaged using a Leica M205 FA stereomicroscope.

Tail wounding assay

GF and CONVD 6dpf *Tg(mpx:GFP)* zebrafish were anesthetized as described above. Fish were mounted in 3% methylcellulose on a 35 mm Petri dish by placing only the anterior part of the fish into the methylcellulose. The posterior part of the fish was covered with sterile GZM. Fish were then observed under a LeicaS6E StereoZoom stereomicroscope with a Leica L2 cold light illuminator. Tail amputations were performed posterior to the end of the notochord using a scalpel. GF and CONVD animals were revived in sterile GZM containing antibiotics (AB-GZM) (Pham *et al.*, 2008), and kept at 28.5°C. Fish were collected at time points indicated and euthanized in 8× Tricaine. Larvae were fixed in 4% paraformaldehyde overnight at 4°C, and washed 3 times for 10 min and 3 times for 1 h in PBS + 0.2% Tween. Fish were then mounted in 3% methylcellulose and imaged using a Leica M205 FA stereomicroscope. Numbers of GFP(+) neutrophils posterior to the notochord were quantified in 8–10 fish for each condition.

Morpholino injections and validation

Zebrafish embryos at the 1–2 cell stage were injected with morpholinos (GeneTools LLC) targeting *saa* (*saa.i2e3*, 0.9 pmol per embryo; GTCCTTTGCACTTCAAAAATAGAGT), or standard control MO (0.9 pmol per embryo; Gene Tools LLC) using a Drummond Nanoject II microinjector. Efficacy of splice-blocking MOs was measured by RT-PCR. cDNA was prepared from pools of whole larvae at 6dpf as described (5–15 larvae per pool) (Rawls *et al.*, 2007), and 10 ng of cDNA was used as a template in PCR reactions using gene-specific primers (forward: CTTGCTGTGCTGGTGATGTT; reverse: AGTCTTCTGGGGT CATCTTC). We resolved PCR amplicons on 2% agarose gels to detect morphant transcripts (Fig. S2).

Flow cytometry analysis

Tg(mpx:GFP) zebrafish were reared under GF and CONVD conditions. GF and CONVD 6dpf larvae were pooled and killed (50 animals per condition per experiment). Excess media was removed and animals were finely chopped using sterile razor blades in a 10 cm Petri dish, diluted in 1.5 ml 5% fetal bovine serum in Hanks' balanced salt solution (FBS/HBSS), and then transferred to sterile Eppendorf tubes. Cells were pelleted at 1000 g for 5 min at 4°C, and cell pellets were washed with 1 ml FBS/HBSS and pelleted again at 1000 g for 5 min at 4°C. Cell pellets were then treated with 500 µl 10 mg ml⁻¹ collagenase/dispase liberase (Roche) solution in FBSS/HBSS for 35 min at room temperature with vigorous shaking. Digestion was stopped using 500 µl stopping solution [100 µl 0.5 M EDTA (pH 8.0) in 9.9 ml FBS/HBSS]. Cells were pelleted at 1000 g for 5 min at 4°C. The cell pellets were resuspended in 500 µl FBS/HBSS and passed through 40 µm mesh filter (BD Falcon), and the mesh was washed twice with 250 µl FBSS/HBSS. Cells were then brought to final volume of 1 ml of FBS/HBSS prior to sorting using a MoFlo sorting flow cytometer (Beckman Coulter). Conventionally raised non-transgenic and transgenic controls (pools of 50 fish per genotype) were prepared as above and used to define fluorescence and cell size gates. Single cell suspensions from GF and CONVD zebrafish were sorted for GFP fluorescence using Summit software. For RNA analysis GFP(+) neutrophils were collected in 1 ml cold FBSS/HBSS and stored at 4°C. These cells were then pelleted at 1000 g for 5 min at 4°C. The supernatant was then removed and replaced with 1 ml Trizol (Invitrogen). Cells were then prepared as described below for quantitative RT-PCR analysis.

Cell tracking analysis

Tracking analysis of EGFP-expressing neutrophils was performed in Volocity (Improvision, Perkin Elmer). The locations of individual cells were tracked in each frame of time-lapse images (5 or 15 min) captured as described above using Volocity's 'track objects manually' tool. Tracking was performed as described in the Volocity User Guide. 3–5 time-lapse movies per experimental condition were analysed. For tissue-specific analyses, tracked cells were categorized as either caudal hematopoietic tissue (CHT), intestine, or fin cells based on their location for the duration of the movie.

Zebrafish cell culture and stimulation

PAC-2 zebrafish embryonic fibroblast cells were grown at 28°C in Leibowitz L-15 medium supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) and penicillin G (50 U ml⁻¹; Life Technologies) in 0.5% CO₂. Cell lines were used between passages 15 and 35. Cells were grown to near confluency (90%) in 6-well plates (Costar), and were stimulated with LPS (10 µg ml⁻¹; from *Escherichia coli* 0111:B4; Sigma) or SAA (1, 4 µM; PeproTech) for the specified amount of time in media containing 1% FBS. Immunofluorescence assays for RelA/p65 were performed as described (Kanther *et al.*, 2011).

Western immunoblot analysis

PAC-2 cells were stimulated with LPS (10 µg ml⁻¹) or SAA (4 µM) at specified time points. Cells were harvested and lysed in 1× Laemmli buffer, and the protein concentration was

measured using a Bio-Rad quantification assay (Bio-Rad Laboratories). Western blot for I κ B α (S32; Cell Signaling) and actin (MP Biomedicals) was performed as described previously (Kanther *et al.*, 2011).

Transfection and luciferase activity assays

For transfections, PAC-2 cells were seeded into 12-well tissue culture plates ($\sim 5 \times 10^5$ cells per well) and grown in 1 ml medium with 1% FBS at 28°C to $\sim 70\%$ confluency. Transfections with 0.2 $\mu\text{g ml}^{-1}$ of the previously described pikbaa:Luc were performed using Lipofectamine 2000 (Invitrogen) as described by the manufacturer (Kanther *et al.*, 2011). After 24 h transfection, fresh medium was supplied and cells were stimulated with LPS (10 $\mu\text{g ml}^{-1}$) or SAA (4 μM) for 24 h. Cells were then lysed and luciferase activity was determined using an LMax luminometer microplate reader (Molecular Devices, Sunnyvale, CA, USA). Results were normalized for extract protein concentrations measured with the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

Quantitative RT-PCR analysis

To isolate RNA from whole zebrafish larvae, 6dpf larvae (5–30 larvae per group) were anesthetized in 4 \times Tricaine (Sigma-Aldrich), placed in 1 ml Trizol (Invitrogen) taking care to remove any excess media, and then repeatedly passed through a 25-gauge needle (BD Syringe) until homogenized. Samples were vortexed for 30 s, incubated at room temperature for 5 min, supplemented with 0.2 ml chloroform per sample, vortexed for 30 sec, and incubated at room temperature for 2 min. The samples were then centrifuged at 12 000 g for 15 minutes at 4°C, and then the colourless upper phase containing the RNA was transferred to a new RNase-free tube. An equal volume of 70% RNase-free ethanol was added to each tube containing the upper phase, and then RNA was isolated using PureLink™ RNA Mini Kit (Ambion) following the manufacturer's specifications. To isolate RNA from primary sorted zebrafish neutrophils or cultured zebrafish PAC2 fibroblasts, we used Trizol (Invitrogen) following manufacturer's specifications. Total RNA was used in reverse transcription and quantitative PCR assays using gene-specific primers for *I8S* (forward: CACTTGTCCTCTAAGAAGTTGCA; reverse: GGTT GATTCCGATAACGAACGA), *mpx* (forward: TCCAAAGCTATG TGGGATGTGA; reverse: GTCGTCCGGCAAACACTGAA), *ncf1* (forward: TTCATCTCGCCGTCAGACTCGTTT; reverse: TGTAC ACATAGTGCTGGCTGGGAA), *il1b* (forward: TGGACTTCG CAGCACAAAATG; reverse: GTTCACTTCACGCTCTTGGATG), *ikbaa* (forward: GCCGTGCAGATCATCAAAC; reverse: CCGC TG TAGTTAGGGAAGGT), and *mmp9* (forward: CATCACTG AAATCCAGAAGGAGCTT; reverse: GTTACCATTGCCTGA GATCTTC) as described (Kanther *et al.*, 2011).

Neutrophil isolation and migration assay

Mice were injected intraperitoneally with 2.5 ml of 3% Fluid Thioglycollate Medium (Difco Laboratories) previously auto-claved for 15 min under 15 psi. Mice were euthanized with CO₂ intoxication and neutrophils in the peritoneal cavity were retrieved by lavage with 10 ml of ice-cold HBSS supplemented with 1.5 mM ethylene diamine-tetraacetic acid (EDTA). Neutrophils were resuspended in 0.5% FBS RPMI 1640 medium and pre-treated for 1 h with

the NF- κ B inhibitor BAY-11-7082 (25 μ M; Calbiochem), or cycloheximide (50 μ g ml⁻¹; Sigma). Cells were plated at $\sim 2 \times 10^6$ per insert in 6-well Transwells (Corning) with 3 μ m pore in the presence of SAA (25 μ g ml⁻¹) and incubated at 37°C and 5% CO₂ for 2.5 h. Neutrophils were imaged and counted as previously described (Sun *et al.*, 2012).

Phylogenetic analysis

Protein sequences were exported from GenBank into the Cipres Science Gateway v3.2 (Miller *et al.*, 2010) and aligned using Muscle. Phylogenetic trees were inferred using maximum likelihood in RAxML HPC2 7.3.1 on XSEDE using a GAMMA model and a BLOSUM62 protein substitution matrix. The best-scoring ML tree was identified and prepared using Dendroscope v1.4. Rapid bootstrap resampling (1000 replicates) was used to test the robustness of inferred topologies. Multiple sequence alignments were prepared using Boxshade.

Statistical methods

Statistical analysis was performed using unpaired two-tailed Student's t-test, or one-way analysis of variance (_{ANOVA}) followed by Tukey's post-test. Values were calculated using GraphPad Prism software, and $P < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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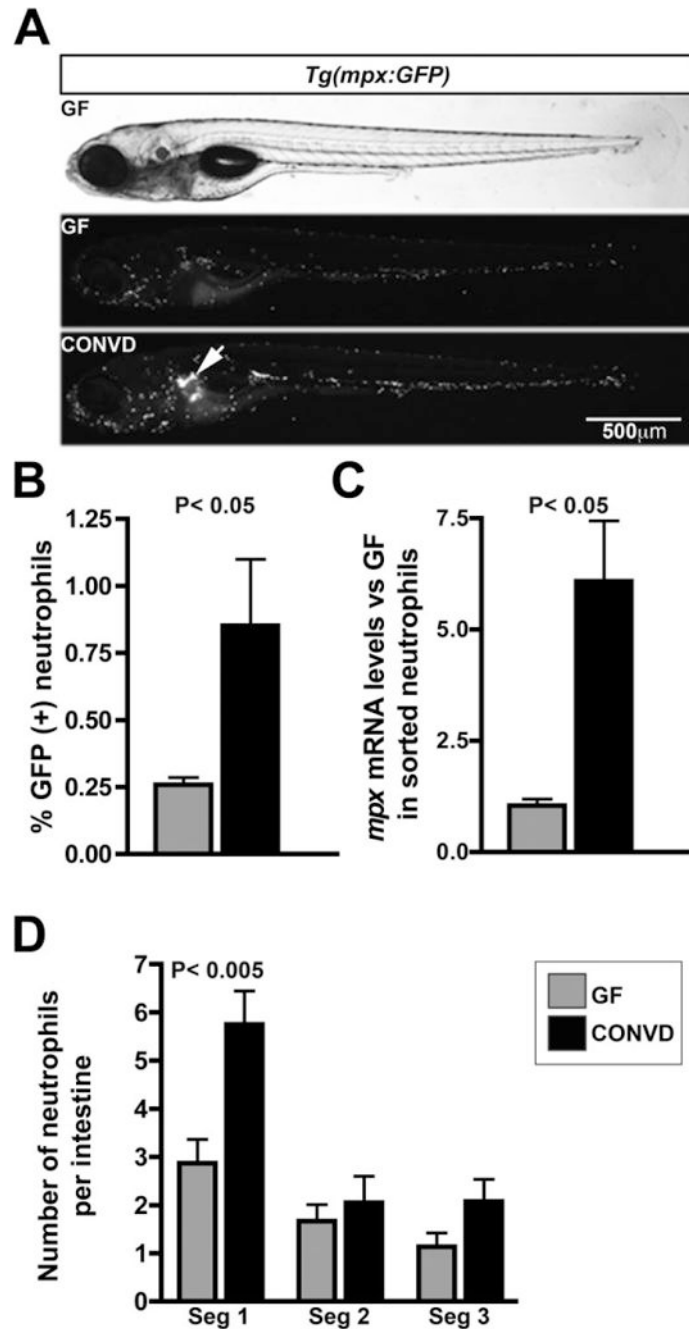


Fig. 1. Microbiota regulates neutrophil localization, number and stimulates inflammatory biomarkers. A. Live 6dpf *Tg(mpx:GFP)* zebrafish show GFP(+) neutrophil localization as a function of microbial status. Note the increased neutrophil localization in the kidney (white arrow) in CONVD animals. B. Flow cytometry analysis reveals that the percent frequency of GFP(+) neutrophils in dissociated 6dpf *Tg(mpx:GFP)* zebrafish is higher in CONVD compared with GF animals. C. qRT-PCR for *mpx* mRNA levels in sorted neutrophils. D. Quantification of mean total number of GFP(+) neutrophils associated with dissected

intestines by segment. Data are representative of 8–10 guts per microbial condition from two biological replicates. Significant Student's *t*-test *P*-values are shown.

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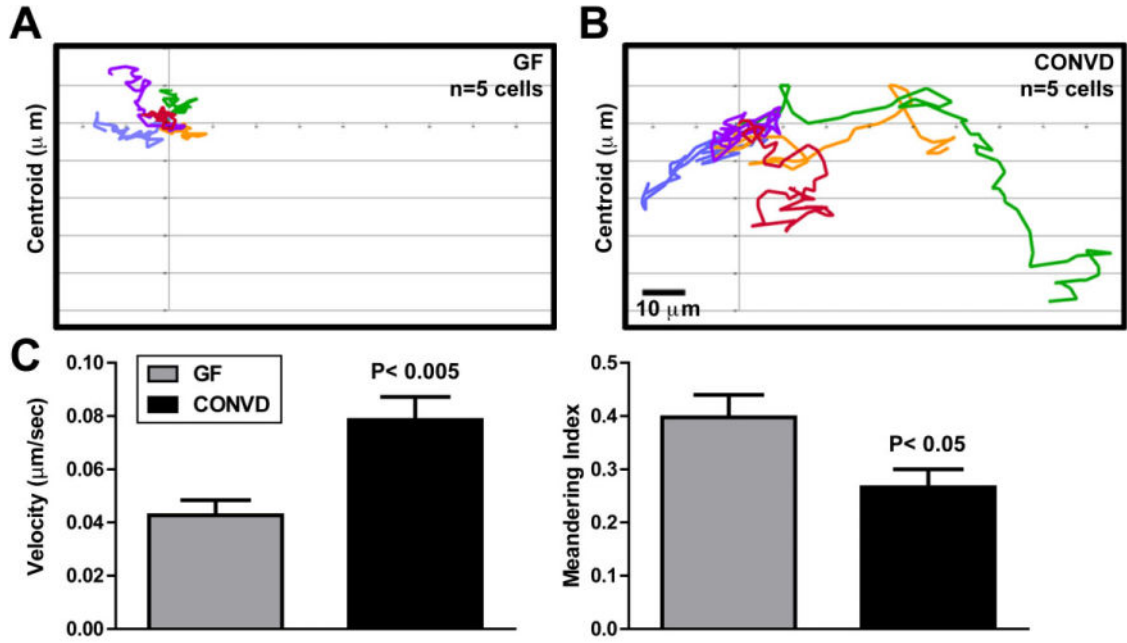


Fig. 2. Microbiota induces systemic neutrophil migration. Neutrophil tracking analysis of the CHT from 15-min movies of live 6dpf GF (A) and CONVD (B) *Tg(mpx:GFP)* zebrafish reveals increased migratory activity in CONVD. C. Quantification of neutrophil migration velocity and meandering index in 6dpf GF and CONVD zebrafish (calculated from 29 neutrophils per condition). Significant Student's *t*-test *P*-values are shown. See also Movies S1 and S2.

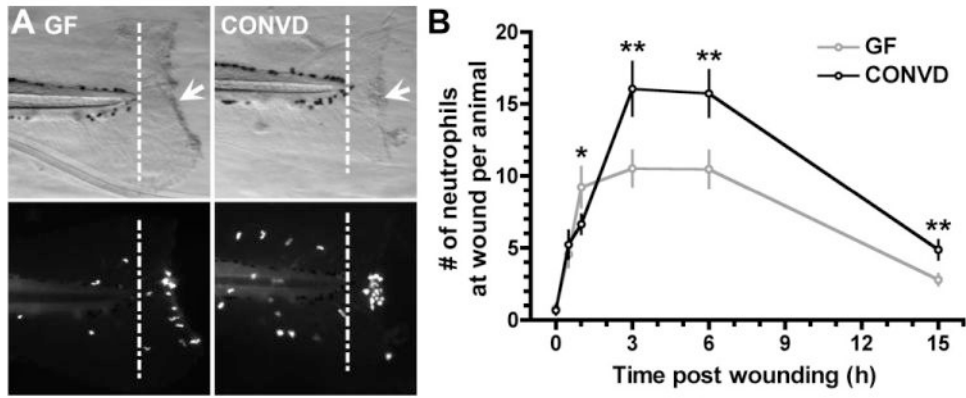


Fig. 3.

Microbiota promotes neutrophil recruitment to tail wounds in GF and CONVD

Tg(mpx:GFP) zebrafish. A. Brightfield and GFP fluorescence images of 6dpf GF and CONVD zebrafish tails 3 h post injury. B. Mean numbers of neutrophils recruited to wound site (posterior to the end of the notochord marked by white dashed line) at time points post injury as indicated. Data represents 8–10 fish per condition per time point. Significant Student's *t*-test *P*-values are shown: **P* < 0.05 and ***P* < 0.005 vs GF.

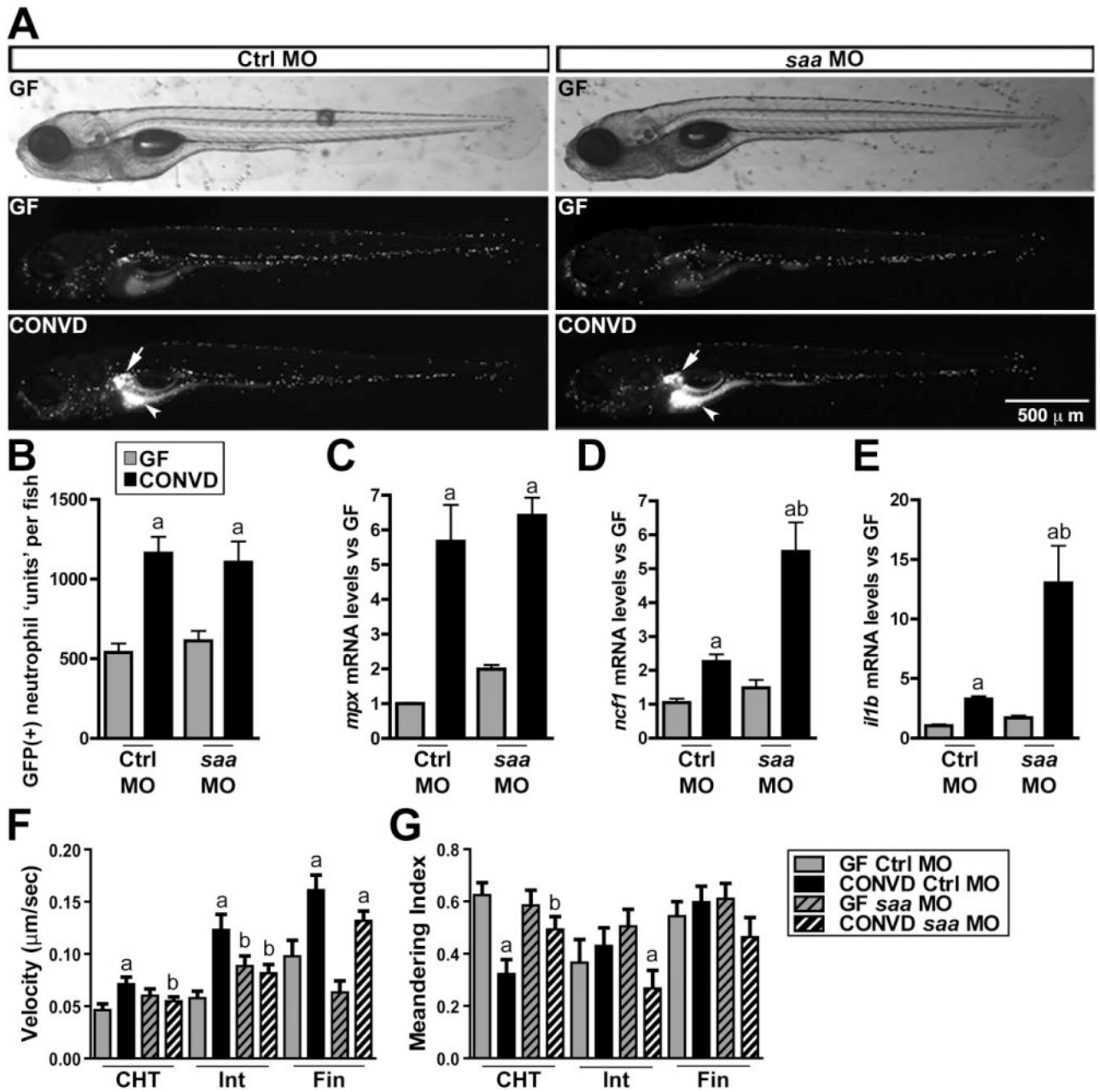


Fig. 4. *saa* mediates systemic neutrophil migration in response to the microbiota. Comparisons of 6dpf GF and CONVD *Tg(mpx:GFP)* zebrafish injected with either standard control (Ctrl MO) or a morpholino targeting *saa* (*saa* MO). A. Images of whole live 6dpf *Tg(mpx:GFP)* zebrafish show GFP(+) neutrophil localization including increased concentration of neutrophils in the kidney (white arrow) and intestine (white arrow head) in CONVD Ctrl MO and *saa* MO zebrafish. B. Neutrophil units quantified by GFP densitometry from whole animal images similar to those shown in A. C. qRT-PCR for *mpx* mRNA in sorted neutrophils. qRT-PCR for *ncf1* (D) and *il1b* (E) mRNA in whole 6dpf zebrafish. Quantification of neutrophil velocity (F) and meandering index (G) in the CHT, intestine

(Int), and fin (calculated from 7–29 neutrophils per tissue per condition). Student's *t*-test *P*-values are indicated: a, *P* < 0.05 compared with GF condition in same genotype; b, *P* < 0.05 compared with Ctrl MO in same microbial condition. See also Movies S3–S6.

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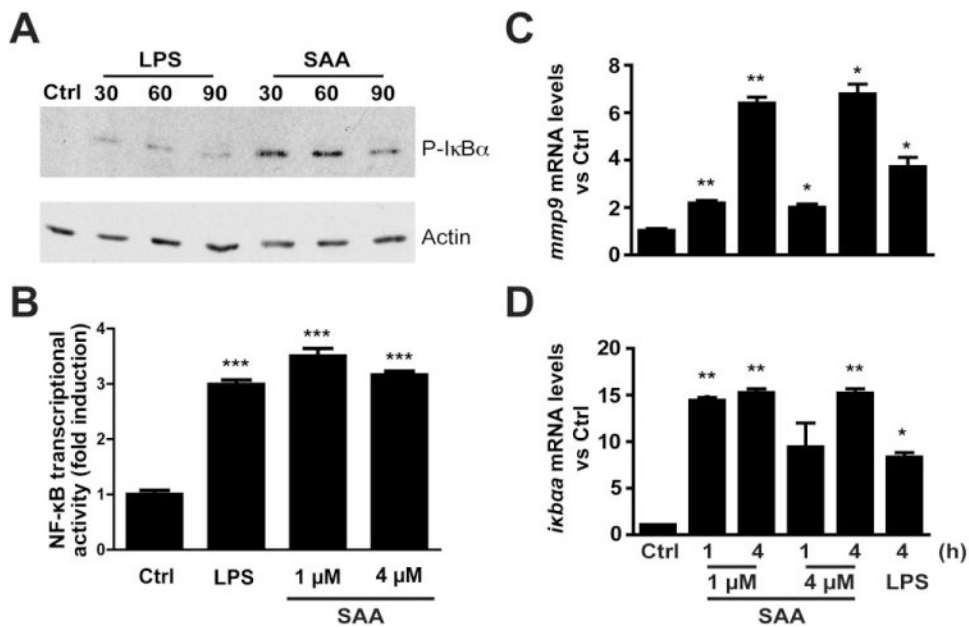


Fig. 5. SAA stimulation of a zebrafish cell line results in activation of the canonical NF-κB pathway and induces expression of NF-κB target genes. A. Western blot of zebrafish PAC-2 cells shows rapid phosphorylation of IκBα proteins after LPS (10 μg ml⁻¹) or SAA (4 μM) stimulation. B. Zebrafish PAC-2 cells transfected with *ikbaa*-luciferase gene reporter (*pikbaa*:Luc) show increased luciferase activity upon stimulation with LPS (10 μg ml⁻¹) or SAA (1, 4 μM). C and D. qRT-PCR using primers for *mmp9* and *ikbaa*, predicted NF-κB target genes, demonstrate induction upon stimulation of PAC-2 cells with LPS (10 μg ml⁻¹) or SAA (1, 4 μM) normalized to 18S ribosomal RNA [rRNA]). Data are expressed as mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

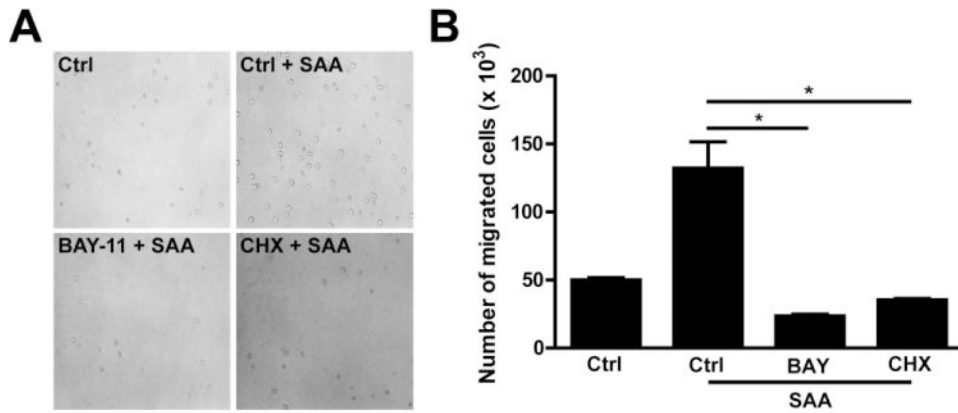


Fig. 6. SAA promotes neutrophil migration and requires NF- κ B and protein synthesis. A. Peritoneal isolated murine neutrophils were pretreated for 1 h with BAY 11-7082 (BAY, 25 μ M) or cycloheximide (CHX, 50 μ g ml⁻¹) and then plated into the top well of a Transwell system. The cells' migration in response to SAA (2.08 μ M, 25 μ g ml⁻¹) in the bottom well was enumerated after 2.5 h. Representative light images of neutrophils migrated into bottom wells. Magnification 200 \times . B. Quantitative measurements of migrated neutrophils. Data are expressed as mean \pm SEM. * P < 0.05. Results are representative of two independent experiments.

Table 1

Elevated transcript levels for myeloid lineage genes in CONVD compared with GF zebrafish larvae.

Gene name	FC ^a	Reference ^b
<i>myeloid-specific peroxidase (mpx)</i>	8.0	Bennett <i>et al.</i> , 2001; Lieschke <i>et al.</i> , 2001
<i>matrix metalloproteinase 9 (mmp9)</i>	7.2	Yoong <i>et al.</i> , 2007
<i>microfibrillar-associated protein 4 (mfap4)</i>	6.4	Zakrzewska <i>et al.</i> , 2010
<i>matrix metalloproteinase 13a (mmp13a)</i>	5.1	Qian <i>et al.</i> , 2005; Yoong <i>et al.</i> , 2007
<i>neutrophil cytosolic factor 1 (ncf1)</i>	3.0	Qian <i>et al.</i> , 2005
<i>lymphocyte cytosolic plastin 1 (lcp1)</i>	2.2	Bennett <i>et al.</i> , 2001
<i>CCAAT/enhancer binding protein, beta (cebpb)</i>	2.1	Thisse <i>et al.</i> , 2001
<i>coronin, actin binding protein, 1A (coro1a)</i>	2.0	Song <i>et al.</i> , 2004

^aTranscript fold change (FC) in 6dpf CONVD compared with GF zebrafish larvae from Kanther *et al.*, (2011).

^bReference establishing myeloid lineage expression for the respective zebrafish gene.

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