

Published in final edited form as:

Science. 2014 October 31; 346(6209): 641–646. doi:10.1126/science.1258705.

A Spaetzle-like role for Nerve Growth Factor β in vertebrate immunity to *Staphylococcus aureus*

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Abstract

Many key components of innate immunity to infection are shared between *Drosophila* and humans. However, the fly *Toll* ligand *Spaetzle* is not thought to have a vertebrate equivalent. We have found that the structurally related cystine-knot protein, Nerve Growth Factor β (NGF β), plays an unexpected *Spaetzle*-like role in immunity to *Staphylococcus aureus* infection in chordates. Deleterious mutations of either human NGF β or its high-affinity receptor TRKA were associated with severe *S. aureus* infections. NGF β was released by macrophages in response to *S. aureus*

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exoproteins through activation of NLRP3 and NLRP4, and enhanced phagocytosis and superoxide-dependent killing, stimulated pro-inflammatory cytokine production, and promoted calcium-dependent neutrophil recruitment. *TrkA* knockdown in zebrafish increased susceptibility to *S. aureus* infection, confirming an evolutionarily conserved role for NGF β -TRKA signaling in pathogen-specific host immunity.

Staphylococcus aureus causes a range of serious infections including skin ulceration, osteomyelitis, pneumonia and septicaemia (1, 2). Several evolutionarily conserved components of anti-staphylococcal immunity have been identified using *Drosophila* as a model organism (3,4). One of the key mediators of immunity to Gram-positive bacteria in *Drosophila* is the soluble protein *Spaetzle* which, when activated by *Spaetzle* processing enzyme (SPE) upon infection, triggers effector immunity in an autocrine and paracrine manner through *Toll* receptor activation (3, 5-8). To detect potential vertebrate equivalents of *Spaetzle*, we searched the human proteome using a relatively tolerant PROSITE pattern (C-X(35,45)-C-X(10,15)-C-X(25,32)-C-X-C; modified from ref. 9) to identify 166 soluble proteins potentially containing a >10 membered cystine knot domain (See Supplementary Material). We identified the neurotrophin Nerve Growth Factor β (NGF β) as a possible vertebrate orthologue of *Spaetzle* (Figure 1A, B). NGF β regulates the survival, differentiation and function of central and peripheral neurons (10, 11), predominantly through activation of its high affinity receptor, tropomyosin-related kinase receptor A (TRKA). Like *Spaetzle*, NGF β is generated by enzymatic cleavage of a precursor pro-protein to form a biologically active cystine knot dimer (Ref. 11; Figure 1C). Because NGF β is implicated in the modulation of inflammation in non-neuronal cells (12 – 14), we asked whether NGF β could play a *Spaetzle*-like role in co-ordinating vertebrate immunity to *S. aureus*.

Deleterious biallelic mutations in the genes encoding NGF β (*NGF*; Ref. 15, 16) or *TRKA* (*NTRK1*; Ref. 17) lead to a profound congenital sensory and autonomic neuropathy (termed *Hereditary Sensory and Autonomic Neuropathy* (HSAN) 4 and 5). We found that these individuals also had frequent severe *S. aureus* infections of skin, teeth, joints and bone (Figure S1) suggesting a pathogen-specific immune defect. To further explore the role of NGF β in staphylococcal immunity, we measured its release from primary human macrophages obtained from healthy individuals. Infection of cells with live, but not killed, *S. aureus* stimulated de novo synthesis and secretion of both pro-NGF and mature NGF β (Figure 1D-F). We found considerable variation in NGF β stimulation by clinical isolates of *S. aureus*. Clones triggering lower levels of NGF β were associated with increased all-cause patient mortality (Figure S1); again suggesting a protective role for NGF β during *S. aureus* infection. The exact mechanisms generating mature NGF β remain unclear but it is likely that endogenous and *exogenous* host proteases (such as furins (18), matrix metalloproteinase (MMP) 7 and plasmin (19)) as well as bacterial proteases (Figure S1) combine to cleave Pro-NGF during *S. aureus* infection, suggesting similarities with the regulation of *Spaetzle* processing (20).

We next examined whether other bacterial species were also able to stimulate NGF β release from macrophages. Although a low level response was seen with some other bacteria (such

as *Enterococcus faecalis*), only *S. aureus* effectively triggered NGF β release (Figure S1). Indeed, the closely-related skin commensal *Staphylococcus epidermidis* was unable to stimulate significant NGF β production, suggesting that macrophages can discriminate between pathogenic and non-pathogenic staphylococcal species. Furthermore macrophages only secreted NGF β and not other neurotrophins (BDNF, NT3 and NT4) in response to infection (Figure S1). Thus, NGF β may act as a specific and sensitive signal for *S. aureus* infection in man, potentially explaining the clinical phenotype of patients with HSAN 4 and 5 and suggesting a non-redundant and pathogen-specific role for NGF β in innate immunity.

We then explored the cellular pathways triggering NGF β generation. Rather than involving conventional surface pattern recognition receptors, *S. aureus* elicits NGF β production through activation of NOD-like receptors (NLRs; Figure S2), a well-recognised consequence of infection with this bacteria (21), and suggests an additional potential role for NGF β during tissue damage.

To define the bacterial components responsible for NGF β release from macrophages, we screened the Nebraska library of *S. aureus* transposon mutants (22) for their ability to stimulate NGF β release from THP-1 cells. This identified a number of genes involved in bacterial cell wall synthesis, macromolecular transport, metabolism and cellular regulation (Figure S3; Additional Data Table S1) including the *saeR/saeS* 2 component gene system and autolysin, which regulate exoprotein and peptidoglycan release, respectively (23,24). As expected, a number of purified *S. aureus*-derived exoproducts (protein A, peptidoglycan and α -haemolysin) were able to stimulate NGF β release in a proteinase K-dependent manner (Figure S3). Because most single exoprotein deletion mutants were still capable of stimulating NGF β release suggesting redundancy (Figure S4), we turned to comparative mass spectroscopy of conditioned media from wild type and *saeS*- mutant *S. aureus* to define further bacterial components mediating NGF β release (Figure S4) and identified alpha phenol soluble modulins (α -PSMs), a recently described family of secreted peptides capable of membrane rupture (25), as putative factors (Figure S4). Thus, multiple *S. aureus* exoproteins can stimulate NGF β release from macrophages. We asked whether this regulatory mechanism might be evolutionarily conserved to control *Spaetzle* production in *Drosophila*. Intriguingly, although the regulation of *Spaetzle* activity has focused on its SPE-mediated activation (26), pro-*Spaetzle* levels in *Drosophila* phagocytes (S2 cells) were stimulated by wild type but not *saeR*- *S. aureus*, by conditioned media and by peptidoglycan (Figure S4), mirroring our results with NGF β .

We then evaluated the effects of NGF β on macrophage function. Primary human macrophages, which have constitutively high surface expression of TRKA but not the low affinity NGF receptor p75, responded to NGF β with sustained calcium signaling (Figure 2A), which could be reconstituted in HeLa cells expressing wild type TRKA but not the HSAN5-associated mutation G517E (Figure 2B). TRKA signaling in macrophages also triggered rapid activation of calcium-dependent PKC isoforms (Figure 2C) as well as other recognised components of TRKA signaling observed in neuronal cells (Additional Data Table S2). Since TRKA is thought to continue signaling following internalisation, thereby permitting signal transmission along axons (27), we examined whether phagosomal TRKA activation might occur and found persistent tyrosine phosphorylation of TRKA within *S.*

aureus-containing phagosomes (Figure 2D). Functionally, TRKA activation led to enhanced phagocytosis (Figure 2E), pro-inflammatory cytokine release from uninfected cells (Figure 2F) and increased *S. aureus*-induced phagosomal superoxide generation (Figure 2G). TRKA activation also enhanced intracellular killing of *S. aureus* in human and mouse macrophages (Figure 2H) and in TRKA-transfected, but not control, THP-1 cells (Figure 2I). This increased killing was dependent on intact receptor signaling (since it was not observed in cells from HSN4 patients), and was principally mediated through enhanced superoxide generation (Figure S5) and autophagy (Figure S6). TRKA-dependent effector responses also depended on intact TLR signaling, because intracellular killing in *S. aureus*-infected cells and cytokine production in uninfected cells were abrogated in *Myd88*^{-/-} and *Trif*^{-/-} macrophages (Figure S7), suggesting an evolutionarily conserved interaction between cystine knot proteins and *Toll* family receptors.

We next determined the role of NGFβ-TRKA in human neutrophils, which are critical components of the host response to *S. aureus* infection (28). Neutrophils constitutively expressed TRKA (Figure 3A) and released NGFβ in response to live *S. aureus* and peptidoglycan (Figure 3B). As seen in macrophages, NGFβ stimulated neutrophils to generate superoxide (Figure 3C), secrete pro-inflammatory cytokines (Figure 3D) and enhanced intracellular killing of *S. aureus* (Figure 3E). NGFβ also stimulated chemokinesis and chemotaxis in a TRKA- and calcium-dependent manner (Figure 3F,G; Movie S1; Figure S8) suggesting that NGFβ may be an important chemotactic signal for neutrophil recruitment to sites of *S. aureus* infection.

To establish whether NGFβ-TRKA signaling represents a critical, evolutionarily conserved component of vertebrate immunity to *S. aureus* infection, we examined its role during in vivo infection of zebrafish. Effective morpholino knockdown of *trkA* was confirmed by immunohistochemistry, where we observed the expected loss of *trkA* protein in the forebrain and nose of zebrafish larvae (Figure 4A). Knockdown of *trkA* had a major effect on the host response to *S. aureus*: *trkA* morphants were more susceptible to *S. aureus* infection than controls; a phenotype that could be rescued by concomitant injection of morpholino-resistant *trkA* RNA (Figure 4B) and was only partially rescued in a transgenic line expressing *trkA* specifically in macrophages (Figure S9), suggesting the critical importance of *trkA* signaling in other cells (such as neutrophils). Bacterial counts in *trkA*-deficient fish rose faster and remained significantly higher than in controls (Figure 4C). We then explored the relationship between the ability of bacteria to stimulate NGFβ release from macrophages and the in vivo impact of silencing *trkA* expression during infection (Figure 4D). We observed a greater effect of *trkA* knockdown in fish infected with wild type (SH1000) *S. aureus* compared to animals infected with bacteria less able to trigger NGFβ release from macrophages: the *saeR*- *S. aureus* mutant (causing a mild infection) and *Enterococcus* (causing a severe infection). Furthermore *trkA* knockdown compromised neutrophil migration to sites of *S. aureus* infection (Figure 4E,F) as well as sterile inflammation (Figure 4G,H) supporting a role for NGFβ as an 'alarmin' for both *S. aureus* infection and for non-specific tissue damage.

In summary, our results indicate a critical role for NGFβ-TRKA signaling in controlling vertebrate innate immunity during *S. aureus* infection. It is also conceivable that other

vertebrate cystine-knot proteins might play similar roles to NGF β for other bacterial pathogens. The recent finding that *Spaetzle* also functions as a neurotrophin in *Drosophila* (29) suggests an evolutionarily conserved dual function for cystine knot proteins in both nerve development and anti-staphylococcal immunity and may explain stimulation of aberrant nerve growth by soft tissue infection by *S. aureus* (30). Our findings reveal pleotropic effects of the NGF β -TRKA pathway that may particularly influence innate immunity to *S. aureus* infection suggesting that, potentially, person-to-person variability in phagocyte secretion of, or response to, NGF β may influence vulnerability to *S. aureus* infection and may provide opportunities for therapeutic intervention, particularly in multi-drug resistant disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank S. Clegg and E. Henderson for help with patient samples, R. Mifsud and D. Cusens for initial phylogenetic and functional analysis, A. Segal for provision of *Nod2*^{-/-} mouse bone marrow and the aquarium staff of the Bateson Centre, University of Sheffield for zebrafish husbandry. This work was supported by The Wellcome Trust (Senior Clinical Research Fellowship to RAF (084953), project grant to SJF/SAR (089981), The Medical Research Council, UK (Research centre grant (G0700091), Senior Clinical Fellowship to SAR (G0701932)), Papworth Hospital and NIHR Cambridge Biomedical Research Centre, the Intramural Research Program of the National Institute of Allergy and Infectious Diseases (NIAID), U.S. National Institutes of Health (NIH).

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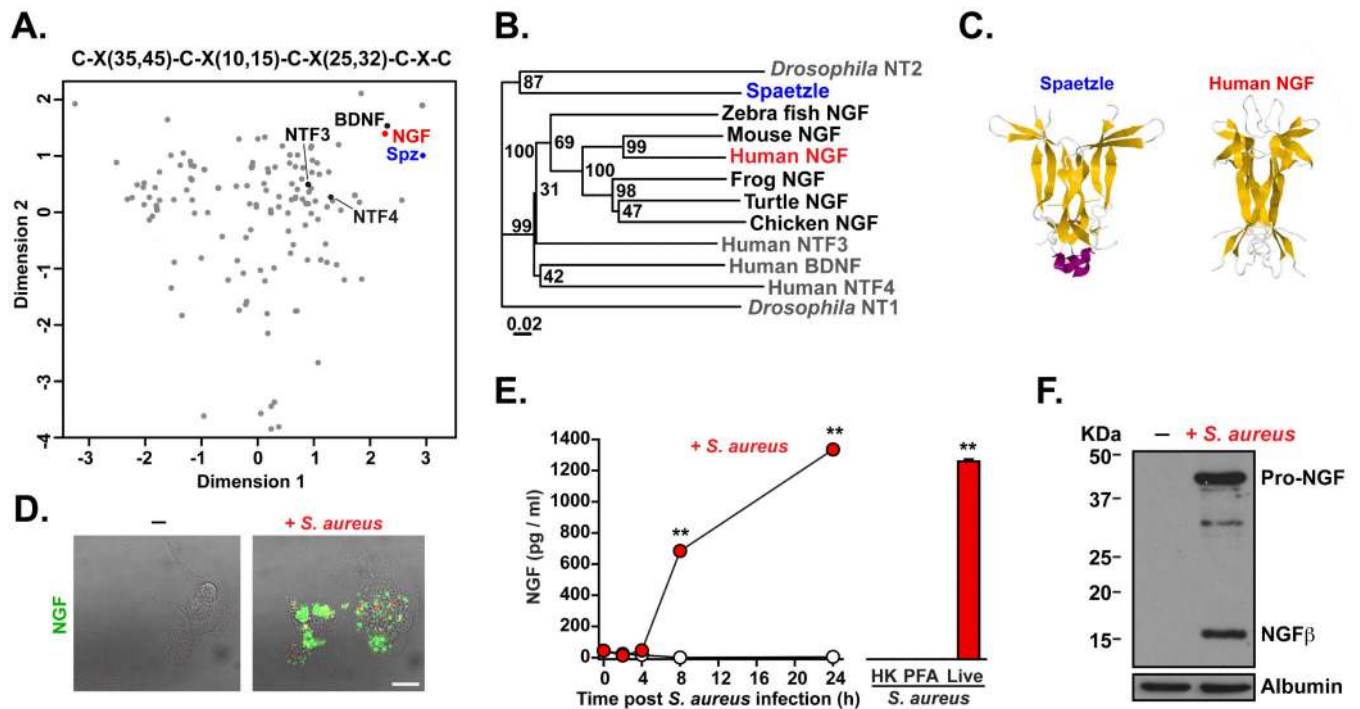


Figure 1. NGFβ is implicated in anti-staphylococcal immunity and is released from macrophages following *S. aureus* infection

A. Bioinformatic identification of potential human orthologues of *Spaetzle*. The human proteome was searched using a PROSITE pattern to find soluble proteins potentially containing a >10 membered cystine knot domain, which were then subjected to multifactorial analysis, incorporating structural prediction of disulphide bond formation with other structural and sequence parameters (See Supplementary Methods for details) to identify Nerve growth factor (NGF; red) as the closest human orthologue to *Spaetzle* (Spz; blue). The other human neurotrophins (brain-derived growth factor (BDNF), neurotrophic factor 3 (NTF3) and 4 (NTF4); black) are also highlighted. **B.** Phylogenetic alignment of vertebrate neurotrophic factors (NGF, BDNF, NTF 3 & 4), *Drosophila* neurotrophin (NT) 1 & 2 and the *Drosophila* immune regulator *Spaetzle*, with bootstrap values. **C.** Dimeric protein structures (from PDB) of *Spaetzle* and human NGFβ. **D.** Intracellular staining of NGFβ (green) in primary human macrophages uninfected (top) or infected with *S. aureus* (SH1000) (red; bottom) and then treated with monensin for 14h to prevent secretion. Scale: 5 μm. **E.** Time course of NGFβ release from primary human macrophages following infection with *S. aureus*. NGFβ secretion requires live bacteria since heat-killed (HK) or paraformaldehyde (PFA)-killed *S. aureus* do not trigger NGFβ release. **F.** Release of Pro-NGF and NGFβ from differentiated THP-1 cells upon infection with *S. aureus* for 12 h. * denotes $p \leq 0.05$; ** denotes $p \leq 0.005$. All experiments were carried out in at least triplicate and are representative of at least 3 independent repeats.

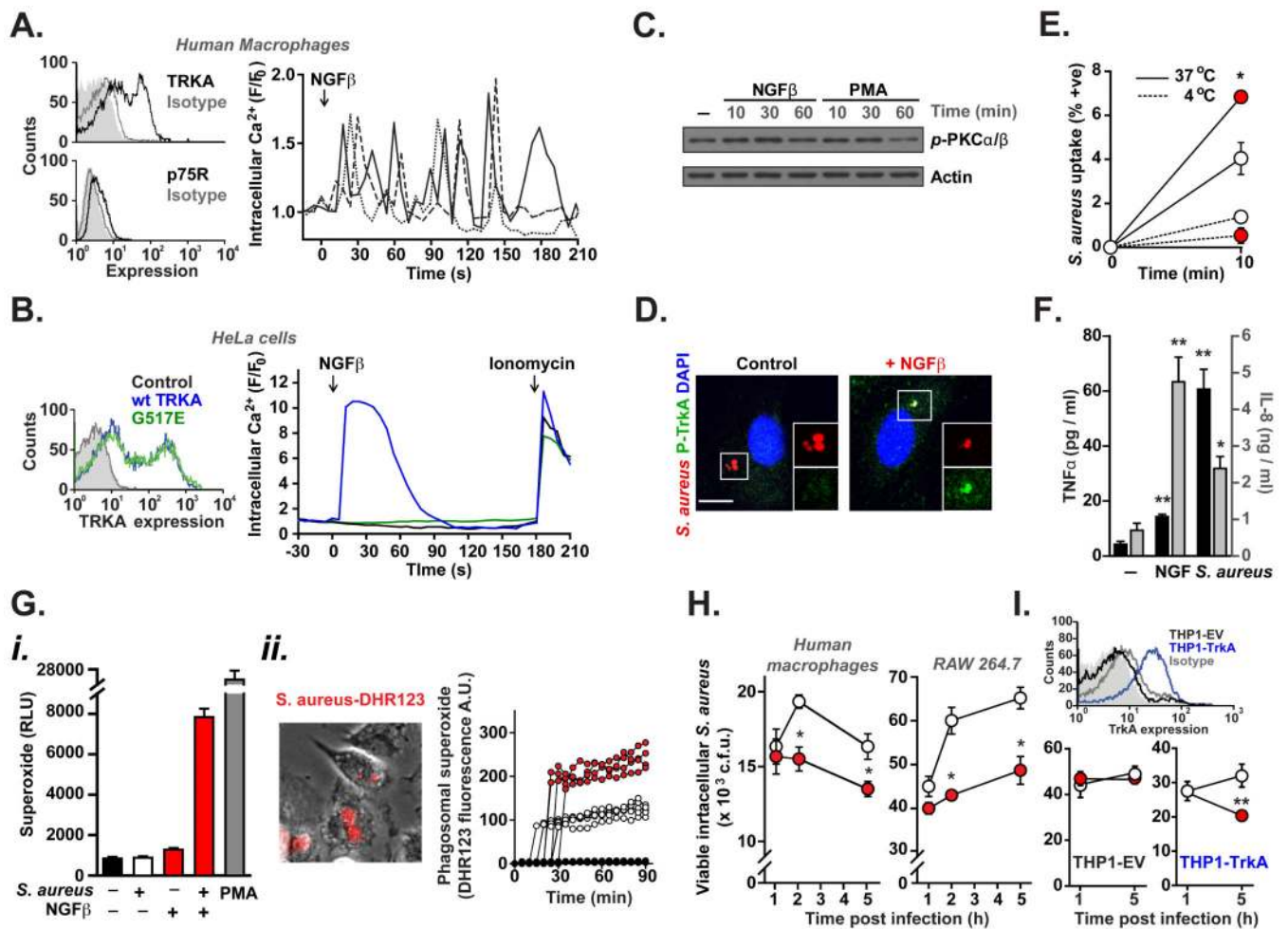


Figure 2. Effects of NGFβ-TRKA signaling in human macrophages

A. Addition of NGFβ (250 ng/ml; 9.25 μM) triggers sustained calcium oscillations in Fluo3-loaded primary human macrophages (detected by single cell confocal imaging). Three representative recordings normalised for starting fluorescence (F/F_0) are shown. *Inset:* Surface expression of TRKA and p75R (black) compared to isotype control (dark grey) or unstained cells (grey fill) on primary human macrophages. **B.** Single cell calcium signaling in GCamp3-expressing HeLa cells transfected with wild type TRKA (blue), HSN4-associated TRKA mutation (G517E; green) or empty vector (black) in response to NGFβ (250 ng/ml; 9.25 μM). *Inset:* Surface expression of TRKA in transfected HeLa cells. **C.** TRKA signaling in macrophages triggered rapid activation of calcium-dependent PKC isoforms. **D.** Co-localisation of intracellular phospho-TRKA (green) with RFP-labelled *S. aureus* (SH1000; red) in primary human macrophages treated for 30 min with 100 ng/ml (3.7 μM) NGFβ. Scale bar 10 μm. **E, F.** Addition of NGFβ to primary human macrophages increased (F) phagocytosis of RFP-labelled *S. aureus* and (G) release of TNFα and IL-8 (measured after 24h). **G. (i)** Luminol-based detection of superoxide in response to *S. aureus*, NGFβ or PMA. **(ii)** The generation of phagosomal superoxide, monitored by DHR123-labelled heat-killed *S. aureus*, is increased in cells treated with the TRKA-specific agonist gambogic amide (250 nM; red) compared to vehicle (white; $p < 0.005$) or bacteria without

cells (*black*). Four representative fluorescence traces from individual cells are shown for each group. **H,I**. TRKA activation (by gambogic amide; 250 nM) enhanced intracellular killing of *S. aureus* in (**H**) primary human macrophages (*left*) and the mouse macrophage cell line RAW 264.7 (*right*) and in (**I**) TRKA-transfected (*blue*), but not control (*black*) THP-1 cells. *Inset*: surface TRKA expression in THP-1 cells transfected with TRKA (*blue*) or empty vector (*black*) compared to isotype control (*grey*) and unstained cells (*grey fill*). All experiments were carried out in at least triplicate and are representative of at least 3 independent repeats.

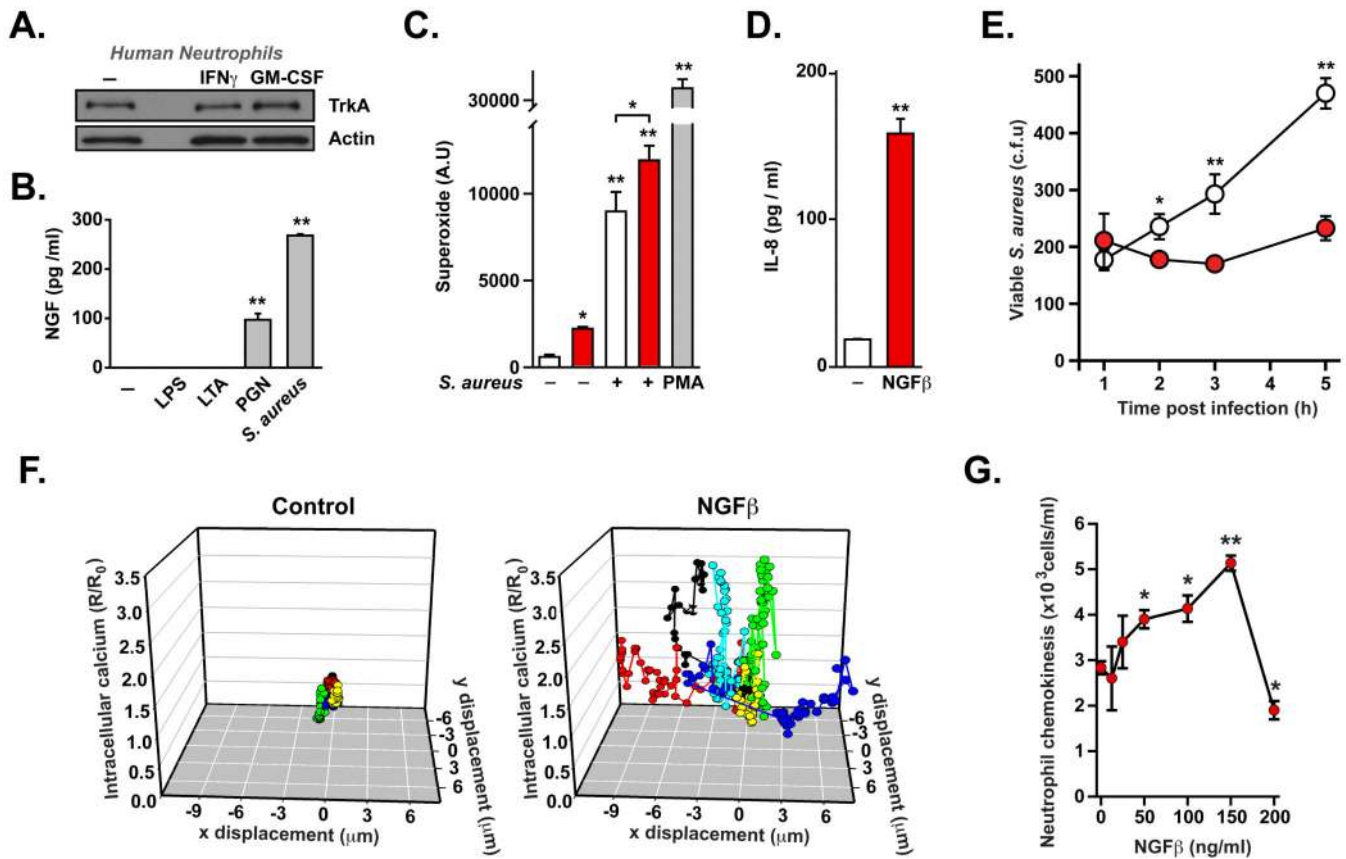


Figure 3. NGF β -TRKA signaling stimulates functional activation of neutrophils

A. TRKA expression on untreated, IFN- γ (10 ng/ml) or GM-CSF (100 ng/ml) -primed primary human neutrophils. **B.** Neutrophils secrete NGF β in response to live *S. aureus*, peptidoglycan (PGN) but not lipopolysaccharide (LPS; 100 ng/ml) or lipoteichoic acid (LTA; 5 μ g/ml). **C, D.** Neutrophils generate superoxide (C) and release IL-8 (D) in response to *S. aureus*, PMA and/or NGF β (red). **E.** Killing of *S. aureus* by human neutrophils is enhanced by treatment with NGF β (100 ng/ml; red) compared to control (white). **F.** Representative plots of x-y displacement and calcium levels in individual neutrophils following addition of vehicle (control) or NGF β . **G.** Chemokinesis of human neutrophils assessed using a transwell assay in response to increasing concentrations of NGF β . * denotes $p \leq 0.05$; ** denotes $p \leq 0.005$. All experiments were carried out in at least triplicate and are representative of at least 3 independent repeats.

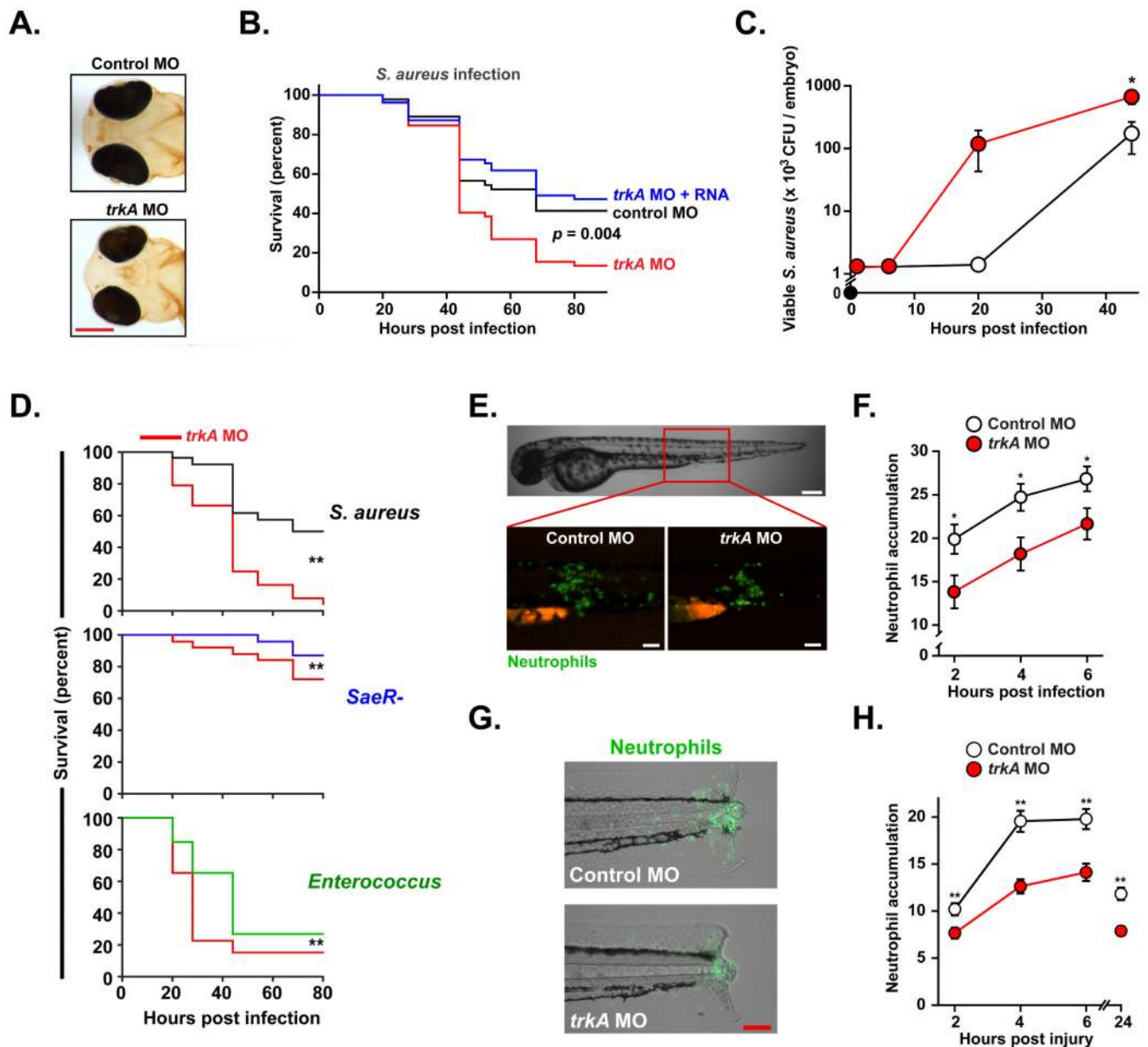


Figure 4. Disruption of NGF β -TrkA signaling compromises *S. aureus* immunity *in vivo*

A. Reduced TRKA protein expression (assessed by immunohistochemistry) in the forebrain and nose of 72 hpf zebrafish larvae injected with *trkA*-targeted (bottom) but not control (*top*) morpholinos. **B.** Kaplan-Meier survival curves of fish infected with *S. aureus*. *TrkA* morphants (*red*) were more susceptible to *S. aureus* infection than controls (*black*) and could be rescued by concomitant injection of morpholino-resistant *trkA* RNA (*blue*). N at least 45 per group, performed as three independent experiments. **C.** Numbers of viable *S. aureus* were significantly greater in *trkA* morphant (*red*) than control (*black*) fish assessed as colony forming units (c.f.u.) per embryo. **D.** Morpholino *trkA* knockdown (*red*) caused a greater effect on mortality in fish infected with wild type (SH1000) *S. aureus* (*black*) compared to animals infected with bacteria less able to trigger NGF β release from macrophages: the

saeR- *S. aureus* mutant (causing a mild infection; *blue*) and *Enterococcus faecalis* (causing a severe infection; *green*). **E-H**. Reduced migration of GFP-tagged neutrophils to sites of *S. aureus* infection (**E,F**) or sterile inflammation (**G,H**) in *trkA* morphants (*red*) compared to controls (*white*). Representative images at 4h post infection (E; scale bar: brightfield 200 μm , fluorescence 100 μm) or tail injury (G; scale bar 100 μm). N at least 32 per group, performed as 3 independent experiments. * denotes $p \leq 0.05$; ** $p \leq 0.005$ and *** $p \leq 0.0005$. Unless otherwise stated, data shown is representative of at least 3 independent experiments.