Supplementary Materials for

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

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Other Supplementary Materials for this manuscript includes the following:

Materials and Methods

Reagents

The following reagents were purchased: NGFβ, GM-CSF, human and murine M-CSF (all from Peprotech), Lipopolysaccharide (*Escherichia coli* 055:B5 ; LPS), lipoteichoic acid (LTA), enterotoxin B (EntB), enterotoxin A (EntA), peptidoglycan (PGN), α–hemolysin (αHL) and protein A (SpA) from *Staphylococcus aureus* (all from Sigma-Aldrich), monosodium urate crystals (Source Bioscience), alpha phenol soluble modulins (αPSMs)1-4 (*31*), Proteinase K (Roche) and the TrkA agonist, gambogic amide (GA) (Enzo Life Sciences). Recombinant human IL-18 and IL-1β were both from R&D Systems. For western blotting, we used antibodies against the following: Phospho-TrkA (Tyr490) and phospho-PKCα/β (Cell Signalling Technology), TRKA (Millipore), NGF (Peprotech), LC3 (Novus Biologicals), MyD88, β-actin and bovine serum albumin (all Abcam). Allophycocyanine (APC)-labelled anti-TRKA (R&D Systems) and phycoerythrin (PE)-labelled anti-p75 (BD Pharmingen) were used for flow cytometry. For confocal experiments, LC3 antibody was from Nanotools. Unless otherwise stated, all other chemicals and reagents were purchased from Sigma-Aldrich.

Mice

The following knock-out mice were used: *Tnfr1*-/- and *Tnfr1*+/+ (*32*), *Nod2-/-* (kind gift of Professor A. Segal, UCL, UK), *Nox2*-/- (*33*), *Nlrp3*-/-, *Nlrc4*-/-, *Casp1/11*-/-, *Asc*-/- (a kind gift from Professor K. Fitzgerald UMass Medical School, Worcester, USA), *MyD88-/-* and *Trif-/-* cells (a kind gift from Professor S. Akira, Osaka University, Japan)

Cell lines and primary cells

Cell lines: The human monocytic cell line THP-1 and mouse macrophage cell line RAW264.7 were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (Hyclone), penicillin (100 U/ml) and streptomycin (100 μg/ml). THP-1 cells stably over-expressing human TRKA were cultured in the same medium containing 1 μg/ml puromycin. Differentiation of THP-1 into a macrophage-like cell was achieved by culturing in 5 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 2 days. HEK-293T and HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich) supplemented as above.

Primary cells: Human monocyte-derived macrophages were generated as described previously (*34,35*) and all samples were obtained from consented adults covered by local Research Ethics Committee approval. Human neutrophils were isolated from peripheral blood collected in EDTA, layered onto Polymorphprep (Axis-Shield) and centrifuged to yield a neutrophil-rich layer, which was resuspended following red cell ammonium chloride lysis in IMDM supplemented with GlutaMAX and 1% autologous serum. To generate mouse bone marrow derived macrophages (BMDM), femurs were flushed using DMEM

to extract the bone marrow. A single cell suspension, produced by gentle passage through a needle and syringe, was then cultured in DMEM supplemented with 20% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50μM β-ME and 200 ng/ml recombinant murine M-CSF (Peprotech) for 6 days to generate macrophages. Cells were used for experiments between days 7 - 21.

SiRNA knockdown: Day 6-8 human macrophages were transfected with 50 nM siRNA using HiPerFect (Qiagen) according to the manufacturer's instructions. The following siRNAs (Dharmacon) were used: ON-TARGET plus SMARTpool siRNAs targeting ATG12 (cat: L-010212-00); siGENOME SMARTpool siRNAs targeting NOX2 (CYBB; cat: M-011021-01-0005) or LC3 (MAP1LC3B, cat: M-012846-01) and non-targeting siRNA (cat: D-001206-14-20). Knockdown efficacy was assessed by immunoblotting at 48h post-transfection (ATG12, LC3) or by functional assay (NOX2).

Transient transfection: For transient expression of TRKA or TRKA-G517E for calcium imaging, HeLa cells seeded onto 35 mm glass-bottom culture dishes were co-transfected with 2 μg of GCaMP3 and pCDNA3.1-NTRK1 or pCDNA3.1-NTRK1-G517E plasmid DNA using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's recommendations and used for experiments after 24h. The mutation G517E was introduced into human NTRK1 cloned into pcDNA3.1(+) by overlap polymerase chain reaction (PCR) extension method using the Quickchange Multi Site-Directed Mutagenesis Kit (Stratagene).

Stable transfection: To generate stable TRKA-expressing THP-1 cells, the NTRK1 gene was cloned into the puromycin resistant pHRsin-pGK lentiviral vector (kind gift of Prof P. Lehner, CIMR, UK) by means of *Eco*RI sites, viral particles generated and cells transduced and then antibiotic selected. Surface TRKA expression was confirmed by flow cytometry. To stably knockdown MyD88, THP-1 cells were transduced with a lentiviral plasmid (pLKO.1) expressing shRNA targeting human MyD88 (Thermo Scientific). Protein knockdown was confirmed by western blotting.

Bacterial strains, culture and infection

The following *S. aureus* strains were used as previously described: Newman, 8325-4 SH1000, RFP-tagged SH1000 (*36*), *kata ahpC* double mutant (*37*),*SpA*-deficient, *agrB*-, *SaeR*-, *SaeS*-, *lukAB*- and *hlA*-mutants (*38*), *S. aureus* LAC and LAC *psmα* and *psmβ* deletion mutants (*39*). Antibiotics were used at the following concentrations: chloramphenicol 30 μg/ml, erythromycin 5 μg/ml, lincomycin 25 μg/ml and tetracycline 5 μg/ml. Killed *S. aureus* was prepared either by autoclaving at 120˚C for 20 min or by incubation with 4% paraformaldehyde for 30 min at RT followed by washing in PBS. Bacterial numbers were assessed by counting colony forming units (c.f.u) after serial dilution on LB-agar. Other bacterial species used were: *S. pneumoniae* TIGR4 (kind gift from Prof. Jerry Brown, UCL), clinical strains of *E. coli* and *S. epidermidis*, *Enterococcus faecalis* OG1RF, *M. bovis* BCG, *M. abscessus* (ATCC reference strain 19977) and *Salmonella enteric* (kind gift from Prof Vassilis

Koronakis). For secretion experiments, bacterial infections were performed for 1h at 37˚C, 5% CO_2 at MOI of 0.1:1, unless otherwise stated.

Clinical isolates: S. aureus isolates from blood culture were obtained from hospital inpatients (n = 71) with no known immunodeficiency or immunosuppression. Differentiated THP-1 cells on 24-well plates at 2 x 10^4 cells/well were infected with each isolate at MOI 0.1:1 in triplicate and supernatants analysed for secreted NGF by ELISA after 24h as described above.

Nebraska transposon mutagenesis library (NTML): The first 1,920 (NE1-NE1920) of the 1, 952 mutant derivatives of *S. aureus* USA300-JE2 comprising the NTML (University of Nebraska Medical Centre and NARSA; http://app1.unmc.edu/fgx/) were thawed on ice and a multipoint replicator (V&P Scientific) was used to innoculate wells containing 120 µl of Tryptic Soy Broth (TSB; Sigma Aldrich) containing erythromycin and lincomycin in 96-well plates. The plates were incubated at 37°C, 5% CO_2 for 20h and then 1 µl of culture was transferred to 100 µl of fresh TSB with antibiotics and incubated for a further 1h at 37°C, 5% CO_2. One µl of this culture was then used to infect THP-1 cells seeded at 7 x 10^5 cells/well in RPMI medium supplemented with 10% FCS on 96-well plates and incubated for 24h at 37°C, 5% CO_2. Supernatants were collected and assayed for secreted NGF as described. Validation of hits was performed by bacteriophage transduction into an independent background strain as described elsewhere (*40*), using Φ11 as the transducing phage. Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) and transposon integration confirmed by PCR using KOD HotStart DNA Polymerase (Novagen) and primers flanking the insertion site.

Conditioned medium: Conditioned culture medium was prepared by growing *S. aureus* strains in RPMI supplemented with 0.5% or 10% FCS and selection antibiotics, as appropriate, for 16h at 37˚C before centrifuging at 930 *g* for 15 min and sterile-filtering through a 0.2 µM filter. Protease digestion was performed for 1h at 55˚C using 100 µg/ml Proteinase K in 50 mM Tris-HCl (pH 7.4) with 5 mM $CaCl_2$, and the enzyme inactivated using 2 mM EGTA or by heating at 95˚C for 5 min.

Intracellular bacterial killing: Primary macrophages or differentiated THP-1 monolayers on 24-well plates at 2.5 x 10^5 cells/well were washed once with PBS and blocked for 30 min at 37˚C using RPMI containing 10% BSA. Cells were pretreated with NGF or TRKA agonist (gambogic amide) at the indicated concentrations for 1h and then infected with *S. aureus* (MOI: 0.1:1) or E.coli (MOI: 10:1), centrifuged for 3 min at 750 *g* and incubated for a further 1h at 37˚C to allow phagocytosis of bacteria. Monolayers were then washed twice with PBS and incubated for 20 min with medium (DMEM or RPMI with 10% FCS) containing 200 µg/ml gentamicin and 10 µg/ml penicillin to remove extracellular bacteria. For *S. aureus* killing assays, this was followed by 30 min with medium containing 10 µg/ml lysostaphin from *Staphylococcus staphylolyticus*. The cells were then washed once with PBS and incubated in medium containing 0.75 µg/ml vancomycin and NGF or TRKA agonist as appropriate for 1, 2 or 4h at

37˚C, 5% CO_2. For neutrophil killing assays, freshly isolated cells at 1 x 10^6 cells/well in RPMI containing 10% FCS were seeded onto 24-well plates in 0.4 ml/well and pre-treated with 500 μl/well of 2X concentrated NGF or TRKA agonist for 1h. The cells were then infected with 100 μl/well of 1 x 10^6 CFU/ml *S. aureus* (final MOI: 0.1:1) for 1h, and subsequently incubated for the indicated times at 37˚C, 5%CO_2. Neutrophils were pelleted by centrifugation at 400 *g* for 10 min and monolayers washed once with PBS. Cells were lysed in 250μl/well water containing 2% saponin and aliquots of the lysates, or dilutions thereof, plated onto LB-agar. Agar plates were incubated overnight at 37˚C and the viable bacteria enumerated. All treatments were performed in triplicate.

Proteomics

We initially found, by fractionation using size exclusion filters (Sartorius), that the 100-300 kDa *S. aureus*-conditioned medium fraction had all NGFβ-stimulating activity. Samples of conditioned medium fractions prepared from *S. aureus* wild-type (SH1000) and *SaeS*-mutant strains were run a short distance into a 4-12% pre-cast polyacrylamide gel and stained. Lanes were excised and cut into 6 approximately equal slices; proteins reduced, alkylated and digested in-gel. Tryptic peptides were analysed by LC-MSMS using an Orbitrap XL (Thermo) coupled to a NanoAcquity (Waters). Peptides were eluted using a gradient rising from 8 to 25% MeCN by 27 min and 40% MeCN by 35 min. MS spectra were acquired at 60,000 fwhm between 300-1650 m/z. MSMS spectra were acquired using Top 6 DDA triggering at 1000 counts. Raw files were processed using Maxquant 1.3.0.5 against the Uniprot *S. aureus* NCTC-8325 database (downloaded 040214, 2,889 entries). Protein and peptide FDR was set to 1% and label free quantitation (LFQ) enabled. Post-processing analysis was performed in Perseus 1.4.1.3.

Intracellular signaling

PKC assays: For detection of total and phospho-PKC-α/β, macrophages seeded at 1.5 x 10^6 cells on 6-well plates were serum-starved for 16h and then stimulated with 100 ng/ml NGFβ or PMA for the indicated times before washing twice with ice-cold PBS and lysing directly into Laemmli sample buffer containing protease inhibitors and β-mercaptoethanol. Protein expression was analysed by western blotting.

Antibody array: Primary human macrophages on 90 mm culture dishes were washed once with PBS and treated with 100 ng/ml NGF-β for 15 min at 37˚C. Cells were collected using Kinexus lysis buffer, quantified by Bradford Protein Assay (Biorad) and the expression levels and phosphorylation states of cell signalling proteins were monitored using the Kinex™ KAM850 Antibody Microarray Kit components and analysis service (Kinexus, Canada), according to the manufacturer's instructions.

Cytokine production: Human TNF-α and IL-8 levels were measured in 25μl of supernatant on a Luminex 200 instrument (Merck Millipore, UK) using the

reagents and protocol supplied with Milliplex MAP Human Cytokine/Chemokine kit (Merck Millipore, UK). Neat supernatants were quantified for murine TNF-α, IL-8 and IL-1β levels using Standard ELISA Development Kits (Peprotech), according to the manufacturer's instructions. Relevant tissue culture fluid was used as background control.

Superoxide measurements: Superoxide production was measured from macrophages plated in to Lumitrac 200 plates (Greiner Bio-One Ltd) at a concentration of 1 x 10^5 per 100µl in DMEM-10% FCS without phenol red, using the protocol supplied with the Diogenes Cellular Luminescence Enhancement System (Diogenes, National Diagnostics) on a GloMax 96 Microplate Luminometer (Promega Corporation). To measure phagosomal superoxide, primary macrophages, pre-chilled at 4˚C to synchronise uptake, were infected with heat-killed Dihydrorhodamine (DHR)123-labelled *S.aureus* SH1000 (MOI: 5:1). Cells were treated with TRKA agonist immediately prior to incubation in the 37˚C chamber of a Nikon Biostation IM-Q Microscope and phagosomal superoxide monitored by live imaging of DHR123 fluorescence at 5 min intervals. Acquired images were processed using Imaris software.

Intracellular Ca^{2+} measurements: Purified human neutrophils were resuspended in cell loading medium (HBSS with 2% BSA, 2 mM $CaCl_2$ and 3.9 mM glucose) at 1 x 10^7/ml. The cells were incubated for 1h with the FuraRed AM and/or Fluo-3 AM (1-2 μM, Molecular Probes, Invitrogen) with Pluronic F-127 and then washed twice and resuspended in cell-loading medium or BSA-free medium at 2 x 10^6 cells/ml and plated onto 35 mm glass-bottom culture dishes (MatTek Corporation) for imaging. For macrophages, cells plated onto culture dishes were loaded *in situ* with Fluo-3 AM in cell loading medium containing 0.5 mM probenecid. Live cell imaging was performed using Zeiss 510 Confocal microscope as previously described (*41*).

Chemotaxis and chemokinesis

Neutrophils were isolated as described and neutrophil chemotaxis and chemokinesis experiments performed using ChemoTx 96-well transwell plates with a 5 nm pore size polyvinylpyrrolidone (PVP)-treated filter (Neuro Probe Inc.). Medium (IMDM + 1% autologous serum), fMLP (0.001 – 1000 nM) or NGFβ (12.5 – 200 ng/ml) was placed in the upper and/or lower chamber and 2.5 x 10^5 neutrophils were added to the test site on top of the filter and the plate incubated for 90 min at 37˚C. The transmigrated cells were collected from the lower chamber and counted manually. In some experiments, neutrophils were pre-treated for 30 min with 20 μM BAPTA-AM (Molecular Probes, Invitrogen), TRKA Inhibitor (Calbiochem) or vehicle control. All migration measurements were performed in triplicate on at least 3 separate occasions.

Neurotrophin expression

ELISA: Unless otherwise stated, cells were treated with stimulus for 24h and the supernatants centrifuged at 400 *g* for 10 min to eliminate cell debris. Neat supernatants were quantified for cell-secreted NGFβ using an NGFβ ELISA

Development Kit (Peprotech) and ABTS Liquid Substrate (Sigma-Aldrich) following the manufacturers' protocol. Secreted levels of BDNF, NT3 and NT4 were measured using human BDNF Quantikine ELISA Kit and NT-3 or NT-4 DuoSet Kits (R&D Systems), according to the manufacturer's instructions.

Western blots: THP-1 cells were infected with *S. aureus* (MOI: 0.1:1) for 12h in medium supplemented with 0.5% FCS, supernatants collected and protein precipitated using trichloroacetic acid (TCA) at 10% (v/v) for 5 min at -20˚C, followed by 30 min at 4˚C. Samples were then centrifuged at 16,000 *g* for 15 min at 4˚C, the pellets washed with 1 ml ice-cold acetone, and air-dried at RT before resuspending in Laemmli sample buffer (Biorad) containing 350 mM DTT. Paraffin-embedded sections of control or *S.aureus*-infected wild-type or *Plg*-/- mouse liver were from experiments described elsewhere (*42*). Protein extraction was performed as described before (*43*). Briefly, tissue sections were deparaffinised by incubation at RT in Histo-Clear for 20 min, and then rehydrated with a graded series of ethanol followed by distilled water. Tissue samples were subsequently subjected to high-temperature extraction at 100˚C for 20 min in 20 mM Tris-HCl, pH 8.8, 2% SDS, 200 mM DTT (extraction buffer), followed by 2h at 80˚C. Extracts were clarified by centrifuging at 12,000 *g* for 15 min at 4˚C and quantified using the QuickStart Bradford Dye Reagent (Biorad).

In vitro cleavage of pro-NGF: Recominant human Pro-NGF (BioVision Inc.; 2 μg / well) was incubated with SH1000 *S.aureus* at 10^5 /ml in 1 ml of DMEM supplemented with 0.5% FCS for the indicated time periods at 37°C, before centrifuging at 4000 *g* for 10 min to pellet bacteria. Protein was precipitated from the resulting supernatant using 10% (v/v) TCA, as described above. Pro-NGF cleavage was analysed by Western blotting.

Immunofluorescence

Cells grown in 24-well plates on round glass coverslips were pre-stimulated where indicated with NGFβ for 2h at 37˚C. For phospho-TrkA or intracellular NGFβ staining, cells were infected with RFP-expressing *S. aureus* at MOI 5:1 for 1h at 37˚C and then, for NGFβ, incubated in media containing BD GolgiStop and GolgiPlug Protein Transport Inhibitors for a further 14h at 37˚C. After washing with ice-cold PBS, cells were fixed with 4% paraformaldehyde in PBS for 15 min and then blocked and permeabilised using DMEM containing 10% fetal calf serum, 10 mM glycine, 10 mM HEPES (pH 7.4) with 0.1% Triton X-100 for 10 min, or permeabilised in -20˚C methanol for 5 min. Cells were imaged using a Zeiss LSM710 confocal microscope.

Zebrafish experiments

Morpholino knock-down of trkA: trkA morpholino oligonucleotide $(MO)^{atg}$ (5'-TCGAATGAATCCAGCGGAATCTGGC-3') was designed based on *trkA* gene sequence. A standard control morpholino (Genetools) was used as a negative control. One cell stage eggs were injected with 1nl of morpholino at 0.5 mM.

Quantification of inflammatory response in zebrafish larvae: Zebrafish embryos were injected at 1 cell stage with either standard control morpholino (1 nl of 0.5 mM),

TrkA morpholino (1 nl of 0.5 mM), or 62.5 pg TrkA mRNA coinjected with TrkA morpholino (1 nl of 0.5 mM). Total RNA was extracted from 72 hpf WT larvae, the TrkA gene cloned using forward primer 5'- ATG GCT GAC CAT AGG GTG GC-3' and reverse primer 5'- CTA CTC CAG GAT GTC CAG GT-3', and mRNA synthesised using the mMessage mMachine kit (Ambion) according to the manufacturers instructions. TrkA construct was linearised with Kpn1 and transcribed using T7 polymerase. Tail transection was performed on anaesthetised 72 hpf *Tg(mpx:EGFP)i114* (*44*). Larvae and neutrophils expressing GFP were counted at the time point indicated following injury.

Transgenic line generation: The pME TrkA was generated by BP recombination (Life Technologies) after PCR amplification of the TrkA coding sequence using the primers: TrkA attB1 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGGCTGACCATAGGGTG-3' & TrkA attB2 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTCCAGGATGTCCAGGTA-3'. The expression vector was generated using the Tol2 Kit gateway system (*45*) by recombining pME TrkA with pDestTol2CG2, p3E-PolyA and the mpeg1 promoter entry clone (*46*). The resulting expression vector was used to generate *Tg(mpeg1:TrkA)sh396* as described (*47*).

Immunohistochemistry for TrkA: 72 hpf (hours post fertilization) zebrafish larvae were fixed overnight in 4% paraformaldehyde, with several washes in PBS plus 0.1% Triton-X-100 (PBT), followed by substantial washing in 0.05 M Tris-HCl, 0.2% Triton-X-100 and 0.1% BSA (TTB). Acetone cracking (1:2 with ethanol) was performed for 10 min at -20°C. Brief washes in TTB were followed by endogenous peroxidase blocking, 5 min in 3% hydrogen peroxide. Larvae were rinsed and blocked for 1h at room temperature in 10% sheep serum, 1% DMSO, 0.2% TritonX-100 and 0.05 M Tris-HCl. Rabbit anti-TrkA polyclonal antibody (Santa Cruz Biotechnology SC-118) was diluted 1:100 and incubated overnight at 4°C. Larvae were washed and incubated for 2h at room temperature in secondary goat anti-rabbit IgG-HRP at 1:100 (Southern Biotech, 4050-05). Larvae were DAB stained and viewed under a dissecting microscope.

Microinjections of S. aureus into zebrafish embryos: Zebrafish embryos at 30 hpf were microinjected into the circulation with bacteria as previously described (*48*). Briefly, anaesthetised embryos embedded in 3% w/v methylcellulose were individually injected using microcapillary pipettes filled with the bacterial suspension of known concentration. Following infection, embryos were observed frequently up to 120 hpf for evidence of mortality and numbers recorded at each time point.

Determination of in vivo bacterial burden: In order to recover bacteria from infected embryos, groups of zebrafish embryos were transferred individually (with 100 μl of E3) to microfuge tubes and mechanically homogenized using a micropestle (Eppendorf). The homogenates were serially diluted and plated out on BHI agar to determine *S. aureus* numbers.

Drosophila S2 cell experiments

Actively proliferating S2 cells were seeded at 1 x 10^6 cells/well onto 6-well plates in 1.5 ml of Schneider's *Drosophila* medium (S2 medium, Lonza) supplemented with 10% FCS. The cells were treated with 1 μM of ecdysone for 24h and subsequently treated with *S. aureus* peptidoglycan or conditioned medium or infected with SH1000 or *SaeR*-mutant strains at an MOI of 0.1:1 for 1h at 25˚C. Infected cells were pelleted, washed twice with PBS and reseeded in 1.5 ml/well of fresh S2 medium with 10% FCS containing 10 μg/ml lysostaphin for 30 min at 25˚C. After washing with PBS, the cells were incubated in S2 medium with 10% FCS at 25˚C for 9, 18 or 24h. Total RNA was extracted using an RNA purification plus kit (Norgen Biotek). Total RNA (500 ng) was used for first strand cDNA synthesis using the Maxima first strand cDNA synthesis kit (Thermo Scientific). The resulting cDNA was used as a template for SYBR green (Bioline)-based quantitative real-time PCR (qPCR) to determine *Spaetzle* (*spz1)* expression using the following primer pair: SpzC1_F 5'-GACGACACCTGGCAGTTAATT-3' & SpzC1_R, 5'-GGATTATAGCTCTGCGGAAAG-3'. Triplicate measurements for each time point were performed and values are expressed as means ± standard error. *Spz* mRNA values were normalised to the ribosomal protein-49 (RP49) as an internal control.

Bioinformatics

Identifying >10-membered cystine knot proteins: Based on the cystine knot pattern specification available in (*9*), the PROSITE (*49*) pattern Cx(35,45)Cx(5,18)Cx(10,15)Cx(25,32)CxC was produced to search for potential >10-membered cystine knot domains. Using EMBOSS fuzzpro (*50*) version 6.3.1, the pattern was searched in the human proteome (including isoforms) as downloaded from UniProt (*51*). Results were parsed using UNIX text processing tools and Perl scripts to produce a list of 491 human proteins (UniProt identifiers). This set was filtered to 166 proteins by annotation using the Gene Ontology (*52*) and InterPro (*53*) domain assignments to include membrane/extracellular and secreted proteins, and to exclude metalloproteins, IgGs, and intracellular proteins. A structural analysis was then performed where each of the sequences was searched using FUGUE (*54*) against a database of structural families called TOCCATA, which combines the structure classification resources SCOP (*55*) and CATH (*56*). For those query sequences with significant matches, the sequence was aligned with FUGUE to a representative selection of up to 5 members from the relevant family. The resulting alignment was annotated using the software JOY (*57*), which identifies cysteine bonds in the homologous structures. Any cysteines from the query sequences aligned to positions entirely consisting of cysteines were then marked and assessed for the likelihood of participation in disulphide bridges, to calculate the ratio of these cysteines inside or outside the pattern. Evidence of cysteine formations was also collected from annotations in UniProt. Multi-Factorial Analysis (MFA), as implemented in the FactoMineR package (*58,59*) for R, was used to compare the set of 166 proteins to *Drosophila* Spaetzle through the following attributes: recorded lengths between cysteines in the pattern, BLOSUM62 distance of the residue between C5 and C6 to the equivalent residue in *Spaetzle*, external and internal cysteines

predicted to form disulphide bridges (from the structural analysis), the disulphide bridges formed according to UniProt annotation, the presence of a signal peptide, evidence for homodimer formation (obtained from UniProt and protein-protein interactions recorded in the PSIQUIC Registry (*60*) databases), and the length of the protein. It was assumed that the presence of disulphide bonds on cysteines external to the pattern could be detrimental for the formation of the knot.

Phylogenetic tree: A multiple alignment was retrieved from UniProt using Clustal Omega (*61*) for the following protein identifiers: P01138, P05200, P01139, P21617, Q6YBR5, K7EZL3, P48607, Q8IRB0, P20783, P34130, P23560, Q9VZX1. Using the R package ape (*62*), a Drosophila NT1 short isoform rooted tree was produced using neighbour-joining. A hundred bootstrap iterations were used to assess branch stability.

HSAN4 and HSAN5 patient cohorts

Individuals were identified through referral to Regional Pain Genetics Clinic at Cambridge, UK and all had typical neurological features of Hereditary Sensory and Autonomic Neuropathy (see below). In all families, parents were both proven to be carriers and had a normal phenotype. The individuals with HSAN5 (with deleterious mutations in NGF) have previously been reported (16).

Family	Ethnicity	Consang-uineous	Predicted effect of gene mutation effect on Protein	Pain and temperature sensing	Cognitive abilities	Age of index case presentation and diagnosis
1	Arab	Yes	NGF Homozygous c.[680C>A]+[681_682delGG]; Loss of C-ter 15 amino acids	Both absent	Moderate learning difficulties	6m and 3y
2	Pakistani	Yes	TRKA Homozygous c.2288G>C; p.Cys763Ser	Both absent	moderate learning difficulties	1y and 8y
3	Pakistani	Yes	TRKA Homozygous c.228_229delGCinsTT p.Gln76fsHisGln*ter	Both absent	moderate/severe learning difficulties	1y and 5y
4	Caucasian	No	TRKA Heterozygous c.717+4A>T, p.mis-splicing exon 6; Heterozygous het c.1550G>A p.[Gly517Glu]	Both absent	moderate learning difficulties	6m and 16y

Statistical analysis

Analysis was performed using either SigmaPlot 12 or Prism version 5.0, GraphPad. Statistical significance was assumed at *p*-value below 0.05. Data presented as mean ± S.E.M. unless otherwise stated. The student's t-test was used to ascertain statistical significance unless otherwise stated. Survival experiments were evaluated using the Kaplan–Meier method. Comparisons between curves were made using the log rank test.

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Figure S1.

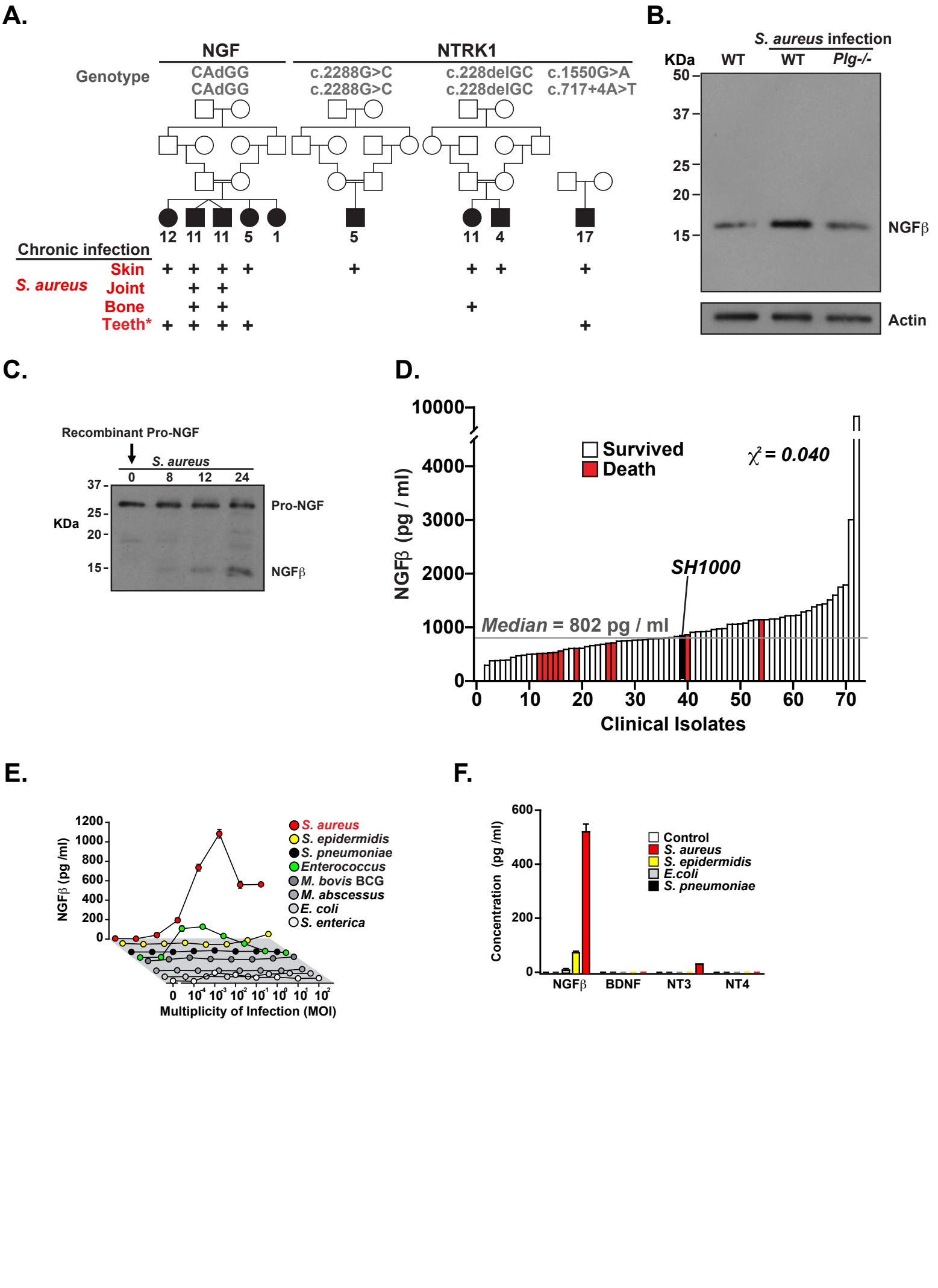

A.
NGF
NTRK1
Genotype
CAdGG
CAdGG
c.2288G>C
c.2288G>C
c.228delGC
c.228delGC
c.1550G>A
c.717+4A>T
12 11 11 5 1
5
11 4
17
Chronic infection
S. aureus
Skin
Joint
Bone
Teeth*
B.
S. aureus infection
KDa
WT
WT
Plg-/-
50
37
25
20
15
NGFβ
Actin
C.
Recombinant Pro-NGF
S. aureus
0 8 12 24
37
25
20
15
KDa
Pro-NGF
NGFβ
D.
10000
4000
3000
2000
1000
0
NGFβ (pg / ml)
Survived
Death
χ² = 0.040
SH1000
Median = 802 pg / ml
0 10 20 30 40 50 60 70
Clinical Isolates
E.
1200
1000
800
600
400
200
0
NGFβ (pg /ml)
S. aureus
S. epidermidis
S. pneumoniae
Enterococcus
M. bovis BCG
M. abscessus
E. coli
S. enterica
0 10⁻⁴ 10⁻³ 10⁻² 10⁻¹ 10⁰ 10¹ 10²
Multiplicity of Infection (MOI)
F.
600
400
200
0
Concentration (pg /ml)
Control
S. aureus
S. epidermidis
E.coli
S. pneumoniae
NGFβ
BDNF
NT3
NT4

Fig. S1.

***S. aureus*-specific immune role for NGFβ. A**. Individuals with hereditary neuropathies caused by deleterious mutations in genes encoding either NGFβ (NGF) or its high affinity receptor TRKA (NTRK1) develop recurrent infections with *S. aureus* of the skin (pustules, ulceration), joints (septic arthritis), bones (osteomyelitis) and teeth and gums (* as part of polymicrobial abscesses). **B.** Generation of NGFβ during in vivo infection may be plasminogen-dependent. Levels of mature NGFβ (detected by western blot in excised liver tissue) are increased during *S. aureus* infection of wild type but not plasminogen deficient (*Plg-/-*) animals. No Pro-NGF was detected in these experiments. The finding that *Plg-/-* produce less NGFβ is associated with, and may explain, the observed increased mortality during low dose in vivo *S. aureus* challenge (*42*) **C.** Cleavage of recombinant Pro-NGF in vitro by *S. aureus.* Incubation of recombinant Pro-NGF (2 μg / time point) with *S. aureus* (SH1000) in mammalian cell culture media (DMEM, 0.5% FCS) at 37^{o}C for the times indicated (hours) revealed cleavage of Pro-NGF to a band at 13-14 kDa consistent with mature NGF. Supernatants were centrifuged (to remove bacteria) and TCA precipitated before processing for western blot. **D.** NGFβ secretion by clinical isolates of *S. aureus.* NGFβ release by differentiated THP-1 cells in response to infection with clinical samples of *S. aureus*. Isolates triggering lower than median levels of NGFβ were associated with increased patient all-cause mortality (*red*) on Chi-squared testing. NGFβ secretion by the laboratory strain SH1000 in the same assay is shown for reference (*black*). **E.** Species specificity of NGFβ response. NGFβ release at 24h from differentiated THP-1 cells in response to infection with a panel of live bacteria over a range of multiplicities of infection (MOI): *Salmonella enterica, E. coli*, *Mycobacterium abscessus, Mycobacterium bovis* BCG, *Enterococcus faecalis, Streptococcus pneumoniae* (TiGR4), a clinical strain of *Staphylococcus epidermidis*, or *S. aureus* (SH1000). **F.** NGFβ is the major neurotrophin upregulated in response to *S. aureus*. Secretion of NGFβ, but not BDNF, NT3 or NT4, is significantly elevated after 24h in response to infection with live *S. aureus*, but not other types of bacteria. * 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 S2.

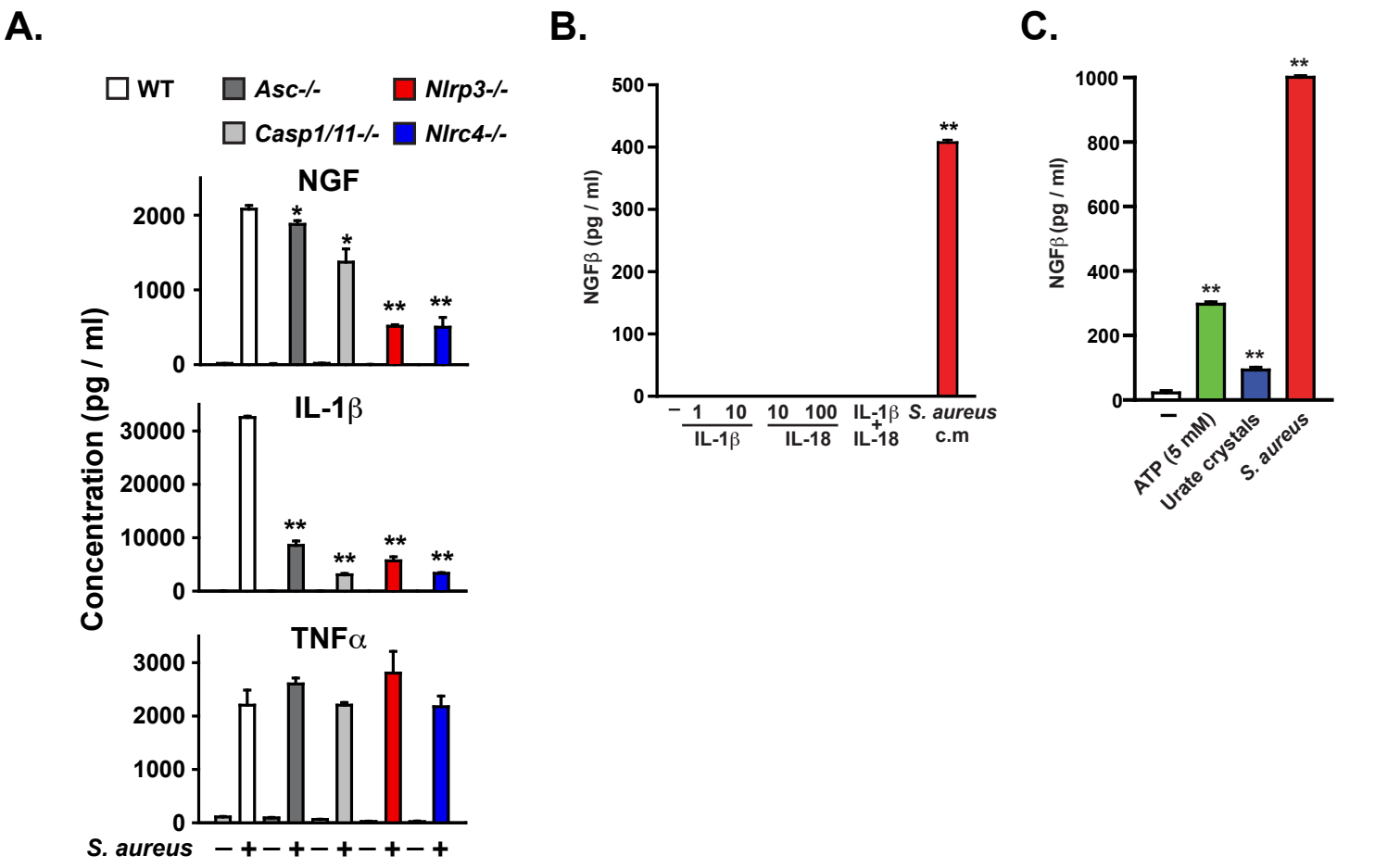


Fig. S2

Release of NGFβ from macrophages is dependent on activation of inflammasome components. ***A***. NGFβ release after 24h of infection with S. aureus was significantly impaired in bone marrow-derived macrophages (BMDM) from Nod-like receptor (NLR)P3, NLRC4 and, to a lesser extent, Caspase-1 & 11 (Casp1/11) and Apoptosis-associated speck-like protein containing caspase recruitment domain (ASC) knock-out mice, compared to wild-type (WT). As expected IL-1β, but not TNFα, secretion was also significantly reduced in the absence of inflammasome components. Unusually the effect on NGFβ secretion was considerably greater in cells deficient in NLRP3 and NLRC4 compared to those lacking ASC or Caspase 1/11, suggesting differential regulation of NGFβ by inflammasome components (through an as yet unidentified mechanism). **B.** Addition of IL-1β, IL-18 or both (at highest concentration) to primary human macrophages were unable to trigger NGFβ release suggesting NLR dependence is not via generation of these cytokines. Concentrations in pg/ml. **C.** NLRP3 activation alone may be sufficient to trigger NGFβ release in human macrophages. Primary human macrophages are triggered to produce NGFβ by treatment with NLRP3 stimulators ATP (5 mM) and monosodium urate crystals (MSU, 200 μg/ml). * 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 S3.

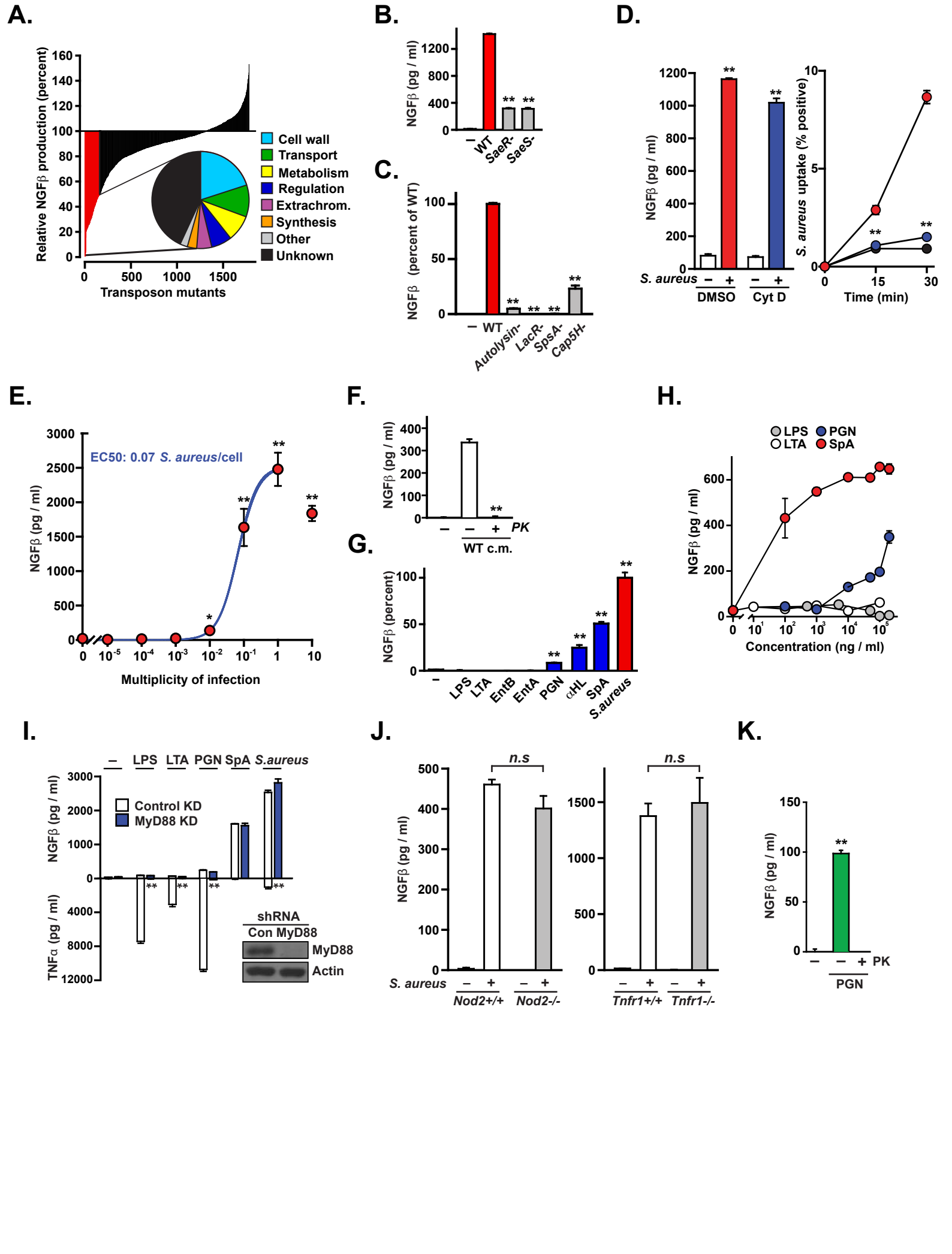

A.
Relative NGFβ production (percent)
Transposon mutants
Cell wall
Transport
Metabolism
Regulation
Extrachrom.
Synthesis
Other
Unknown
B.
NGFβ (pg / ml)
WT
SaeR-
SaeS-
C.
NGFβ (percent of WT)
WT
Autolysin-
LacR-
SpsA-
Cap5H-
D.
NGFβ (pg / ml)
S. aureus
DMSO
Cyt D
S. aureus uptake (% positive)
Time (min)
E.
EC50: 0.07 S. aureus/cell
NGFβ (pg / ml)
Multiplicity of infection
F.
NGFβ (pg / ml)
PK
WT c.m.
G.
NGFβ (percent)
LPS
LTA
EntB
EntA
PGN
αHL
SpA
S.aureus
H.
LPS
PGN
LTA
SpA
NGFβ (pg / ml)
Concentration (ng / ml)
I.
LPS
LTA
PGN
SpA
S.aureus
Control KD
MyD88 KD
NGFβ (pg / ml)
TNFα (pg / ml)
shRNA
Con MyD88
MyD88
Actin
J.
n.s
n.s
NGFβ (pg / ml)
S. aureus
Nod2+/+
Nod2-/-
Tnfr1+/+
Tnfr1-/-
K.
NGFβ (pg / ml)
PK
PGN

Fig. S3

Components of *S. aureus* trigger NGFβ release. A. Transposon library screening identifies bacterial components triggering NGFβ release. The Nebraska library of *S. aureus* transposon mutants were assessed for their ability to stimulate NGFβ release from differentiated THP-1 cells 24h after infection. Functional annotations (class assignment) of mutants triggering less than 50% of controls revealed putative effects of genes involved in cell wall synthesis, macromolecular transport, metabolism, cellular regulation and other processes (See Supplementary Material and Methods for details). **B,C.** Selected hits from functional screening of the Nebraska Transposon library were independently verified by phage transduction into a different strain background (SH1000) or by using validated (independently generated) knockout strains. As an example, mutants lacking (B) the 2 component system *saeR/saeS* or (C) autolysin, lactose phosphotransferase system repressor (*LacR*), signal peptidase IA (*spsA*) or capsular polysaccharide biosynthesis protein (*Cap*) *5H*, all had reduced capacity to stimulate NGFβ secretion (expressed as pg/ml in (B) and as a percent of wild-type response in (C); *red*). **D.** Internalisation of *S. aureus* is not required to trigger NGFβ release. *S. aureus*-triggered release of NGFβ by primary human macrophages is unaffected by pre-treatment (1h) with cytochalasin D (Cyt D; 10 μM; *blue*) while phagocytosis of RFP-labelled *S. aureus* (quantified by flow cytometry) was reduced to levels seen with cells kept at 4°C (*black circles*). **E.** NGFβ secretion by primary human macrophages in response to a range of multiplicities of infection (MOI) with *S. aureus* (SH1000). Cells were infected for 1h, washed and lysostaphin added to prevent ongoing extracellular bacterial growth. Data for MOI up to 1 fitted with four parameter logistic curve suggesting half maximal NGFβ release (EC50) at 0.07 *S. aureus* per cell. **F,G.** NGFβ release from primary human macrophages can be stimulated (F) by conditioned media (c.m.) from wild type *S. aureus* in a proteinase K-sensitive manner and (G) by a number of *S. aureus*-derived exoproducts (protein A (SpA; 10 μg/ml), peptidoglycan (PGN; 10 μg/ml) and α-hemolysin (α-HL; 1 μg/ml)) but not by lipopolysaccharide (LPS; 100 ng/ml), lipoteichoic acid (LTA; 5 μg/ml), enterotoxin A (EntA; 100 ng/ml) or enterotoxin B (EntB; 10 μg/ml). **H.** Dose-response curves for NGFβ production (measured at 24h) from differentiated THP-1 cells 24h after addition of LPS (*grey*) and *S. aureus*-derived LTA (*white*), peptidoglycan (PGN; *blue*) and Protein A (SpA; *red*). **I,J.** A number of purified *S. aureus*-derived exoproducts (protein A, peptidoglycan and α-hemolysin) were able to stimulate NGFβ release, which did not require MyD88 signaling or the known receptors for protein A (TNFR1; *Ref. 63*) and the muramyl dipeptide (MDP) fragment of peptidoglycan (NOD2; *Ref. 64*) **I.** The effect of LPS, LTA, PGN and *S. aureus* on NGFβ and TNFα release (measured at 24h) was assessed from differentiated THP-1 cells transduced with control (*white*) or MyD88 (*blue*) shRNA lentiviral particles (validated by western blot analysis; i*nset*). **J.** NGFβ release in response to *S. aureus* infection by *Tnfr1-/-* or *Nod2-/-* macrophages (*grey*) is similar to that from control animals (*white*). **K.** Peptidoglycan-mediated NGFβ release from macrophages is triggered by PGN-associated proteins, since the ability to stimulate NGFβ secretion is abolished by pre-treatment of PGN with Proteinase K. * 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 S4.

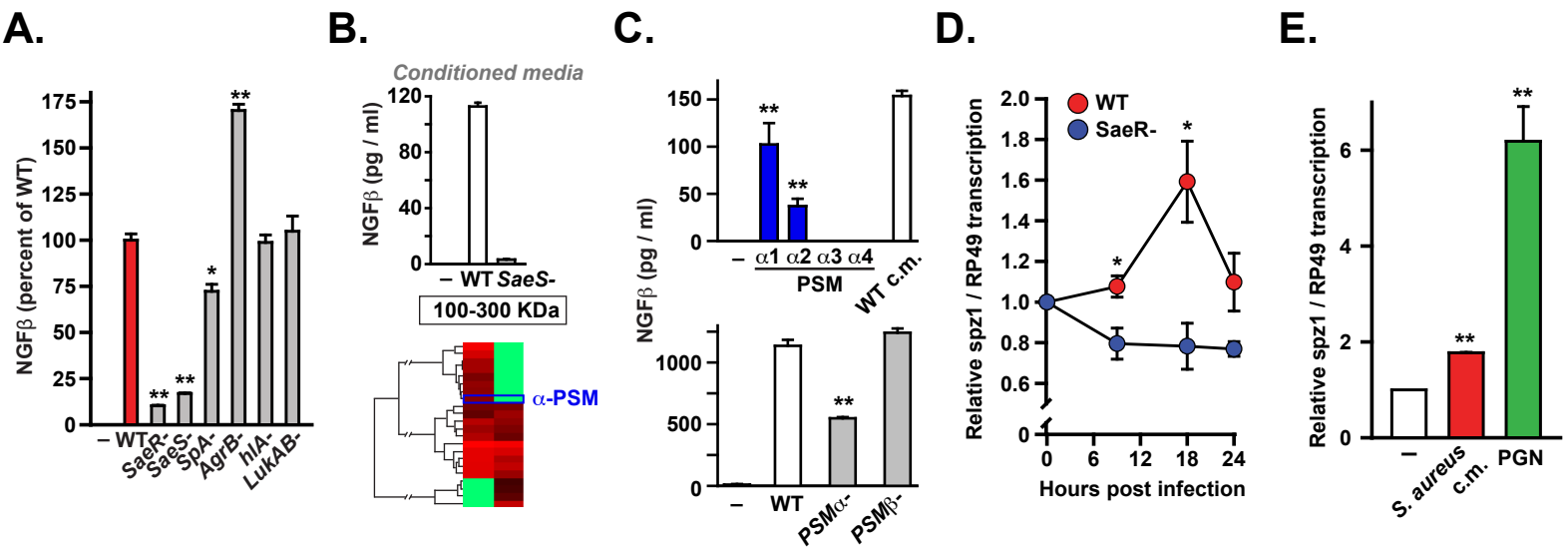


Fig. S4
Identification of novel *S. aureus* components mediating NGFβ release from macrophages and comparisons with Spaetzle release from *Drosophila* S2 cells. A. NGFβ secretion by *S. aureus* mutants for individual exotoxins. Although stimulation of NGFβ secretion was reduced by *saeR-/saeS*- and protein A (*spA*-) mutants, NGFβ production by mutants for accessory gene regulator B (*agrB*-) or individual exotoxins (alpha hemolysin, *hla*- and leukotoxin AB, *lukAB*-) mutation was unaffected or higher than controls. **B.** Comparative mass spectroscopy of conditioned media (100-300 kDa fraction) from wild type and *saeS*- mutant *S. aureus* reveals a number of potential mediators of NGFβ release including alpha phenol soluble modulins (α-PSMs). **C.** Recombinant α-PSMs (1-4; blue) trigger NGFβ release from primary human macrophages compared to conditioned media (c.m.) from wild type *S. aureus* while *PSMα*- but not *PSMβ*- mutant bacteria trigger reduced amounts of NGFβ release from infected macrophages. **D,E.** Transcription of *Drosophila* Spaetzle (*spz*) by S2 cells is induced by (D) infection with live wild type S. aureus (*red*) but not *saeR*- mutant bacteria infection and (E) conditioned media from wild type *S. aureus* (c.m.; *red*) and peptidoglycan (PGN; *green*). Spz mRNA values were normalised to ribosomal protein-49 (RP49) expression. * 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 S5.

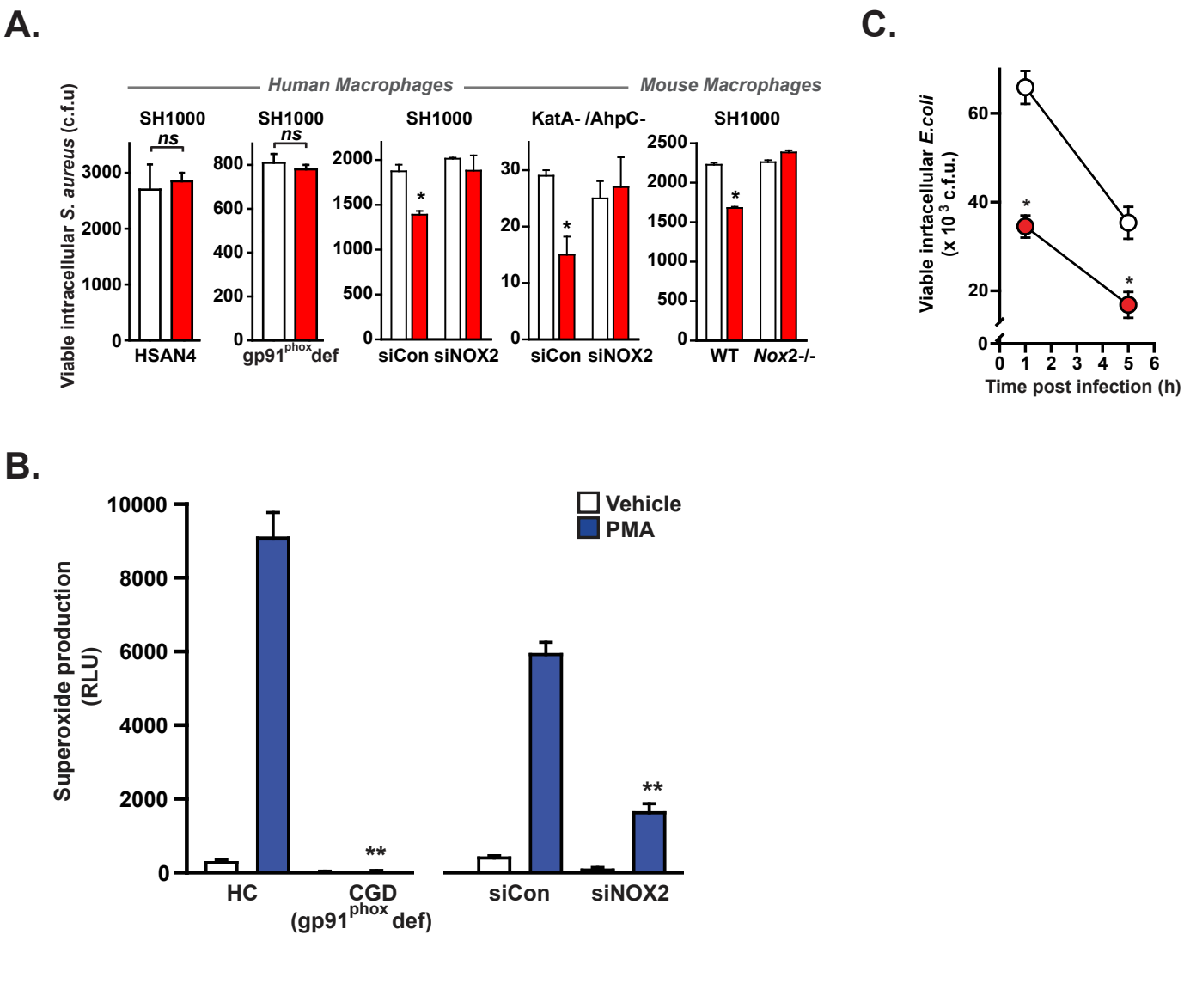
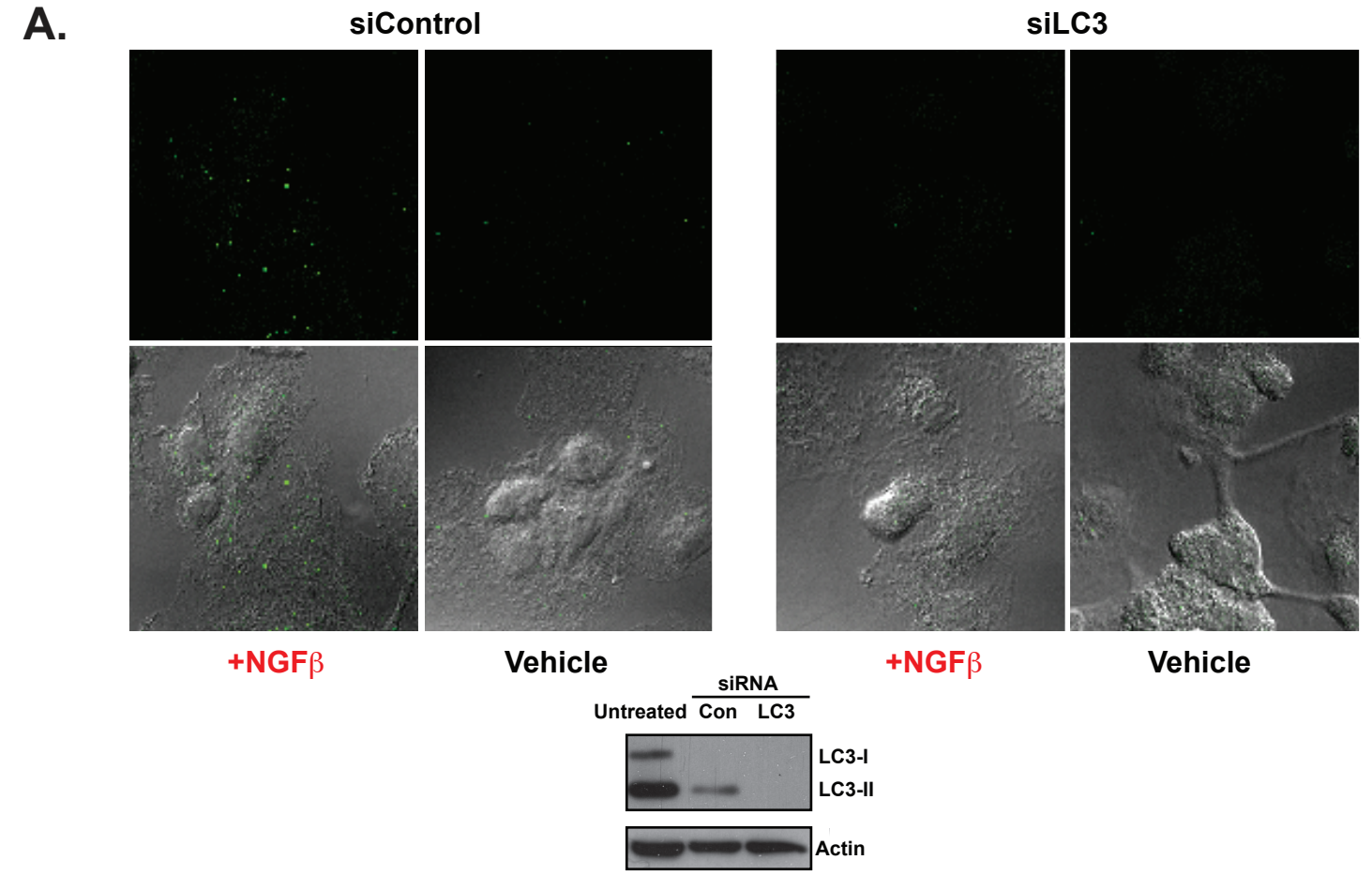
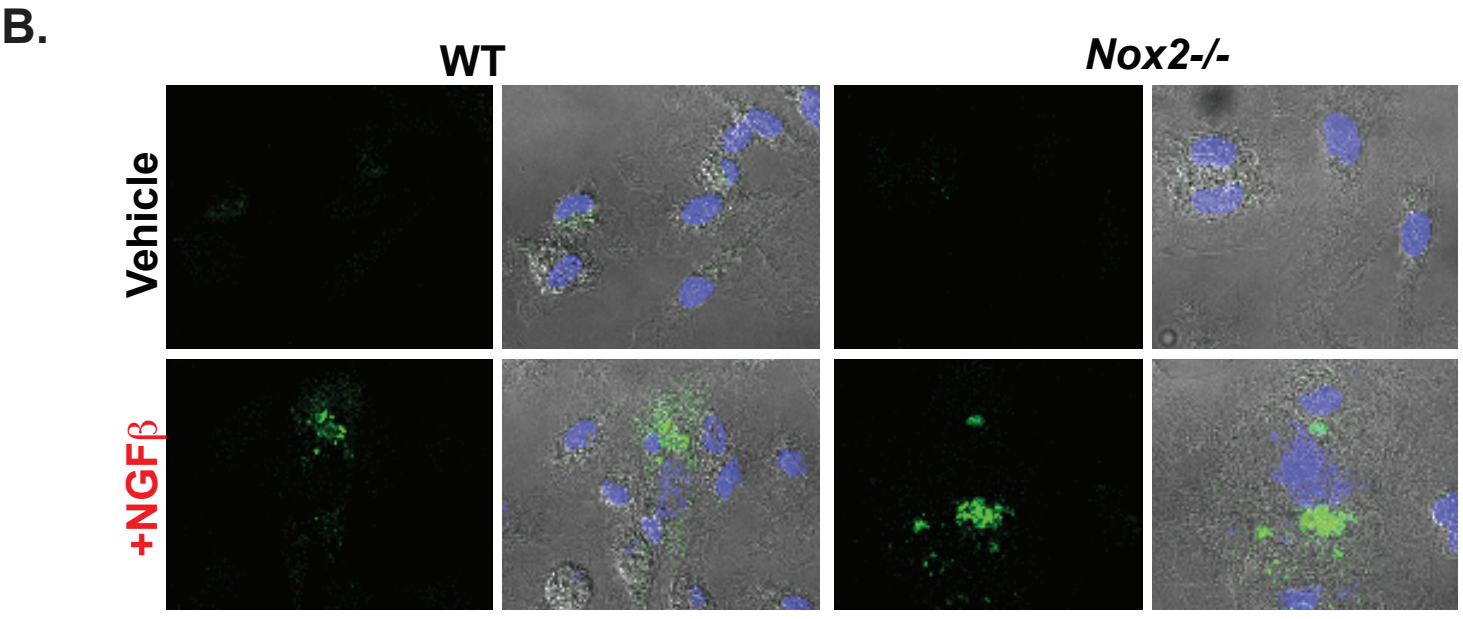


Fig. S5

TrkA-stimulated intracellular killing is superoxide-dependent and not restricted to *S. aureus.* A. The ability of TRKA activation (*red*) to enhance intracellular killing of *S. aureus* was lost in macrophages from patients with TRKA mutations (c.15550G>A/c.717+4A>T) or Chronic Granulomatous Disease (CGD; gp91phox deficiency), following NOX2 siRNA knockdown in human macrophages from healthy individuals, and in *Nox2-/-* mouse macrophages while being enhanced when cells were infected with catalase / alkyl hydroperoxide reductase deficient (*KatA-/AhpC-*) *S. aureus*. **B.** Superoxide generation (in response to PMA; 1 μM) is lost in macrophages from a Chronic Granulomatous Disease (CGD) patient with gp91 deficiency and reduced in macrophages from healthy volunteers following siRNA knockdown of NOX2. **C.** TRKA activation (using GA at 500 nM; *red*) enhances intracellular killing of other superoxide-susceptible bacteria such as *E. coli* (MOI 10:1) in differentiated THP-1 cells. * 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 S6.


A.
siControl
siLC3
+NGFβ
Vehicle
+NGFβ
Vehicle
siRNA
Untreated
Con
LC3
LC3-I
LC3-II
Actin


B.
WT
Nox2-/-
Vehicle
+NGFβ

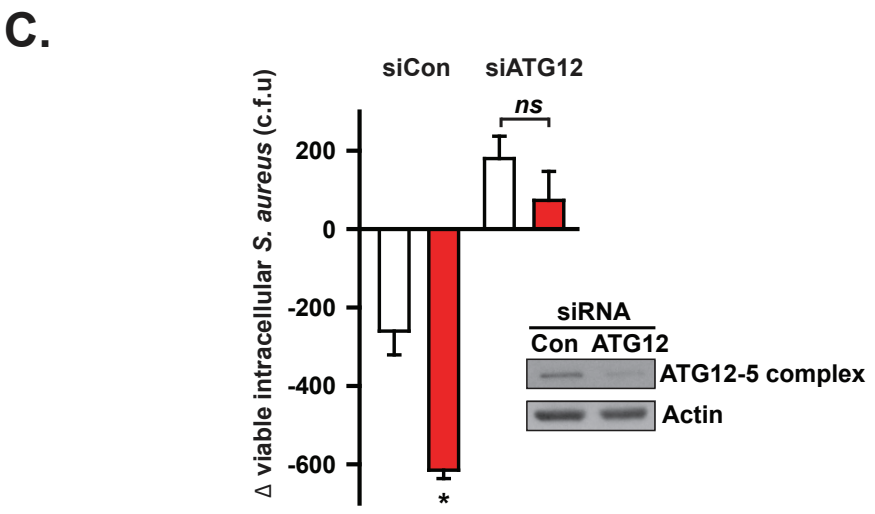

C.
siCon
siATG12
ns
200
0
-200
-400
-600
Δ viable intracellular S. aureus (c.f.u)
*
siRNA
Con
ATG12
ATG12-5 complex
Actin

Fig. S6

Effects of NGFβ on autophagy. A. Addition of NGFβ to human macrophages increased the number of autophagosomes (identified as LC3-positive vesicles) in cells transfected with control but not LC3 siRNA. *Inset:* Knockdown of LC3 was confirmed by western blot. **B.** NGFβ-induced autophagosome production was not dependent upon superoxide generation, since the numbers of LC3-positive vesicles were similar between macrophages derived from wild-type and *Nox2*-deficient mice. **C.** Blocking autophagy, through siRNA knockdown of ATG12 (confirmed by western blot detection of ATG12-ATG5 complex), attenuated the effects of TRKA activation (*red*) on intracellular killing of *S. aureus.* * 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 S7.

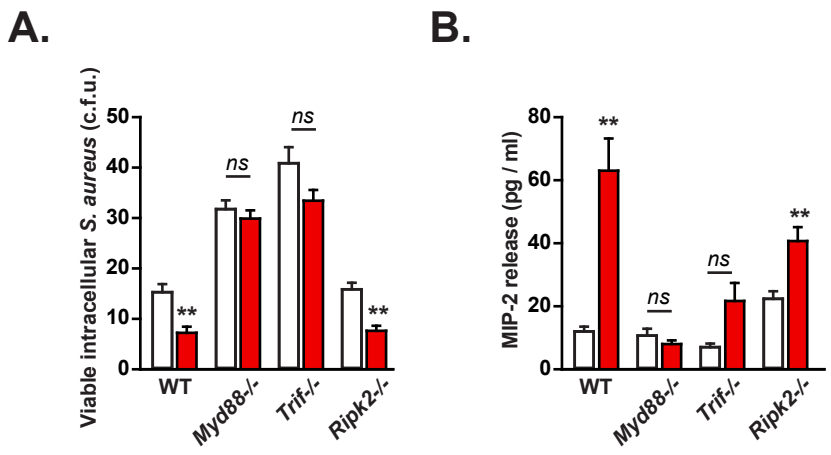


Fig. S7

Role of TLR signaling in TRKA effector responses. TRKA-dependent effector responses are depend on intact TLR signalling. **A,B.** TRKA-enhanced (A) intracellular killing of intracellular *S. aureus* and (B) cytokine production in uninfected cells were abrogated in *Myd88-/-* and *Trif-/-* but not *Ripk2-/-* macrophages. Cytokine experiments were carried out on uninfected macrophages treated with NGFβ (100 ng/ml; 3.7 μM). * 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 S8.

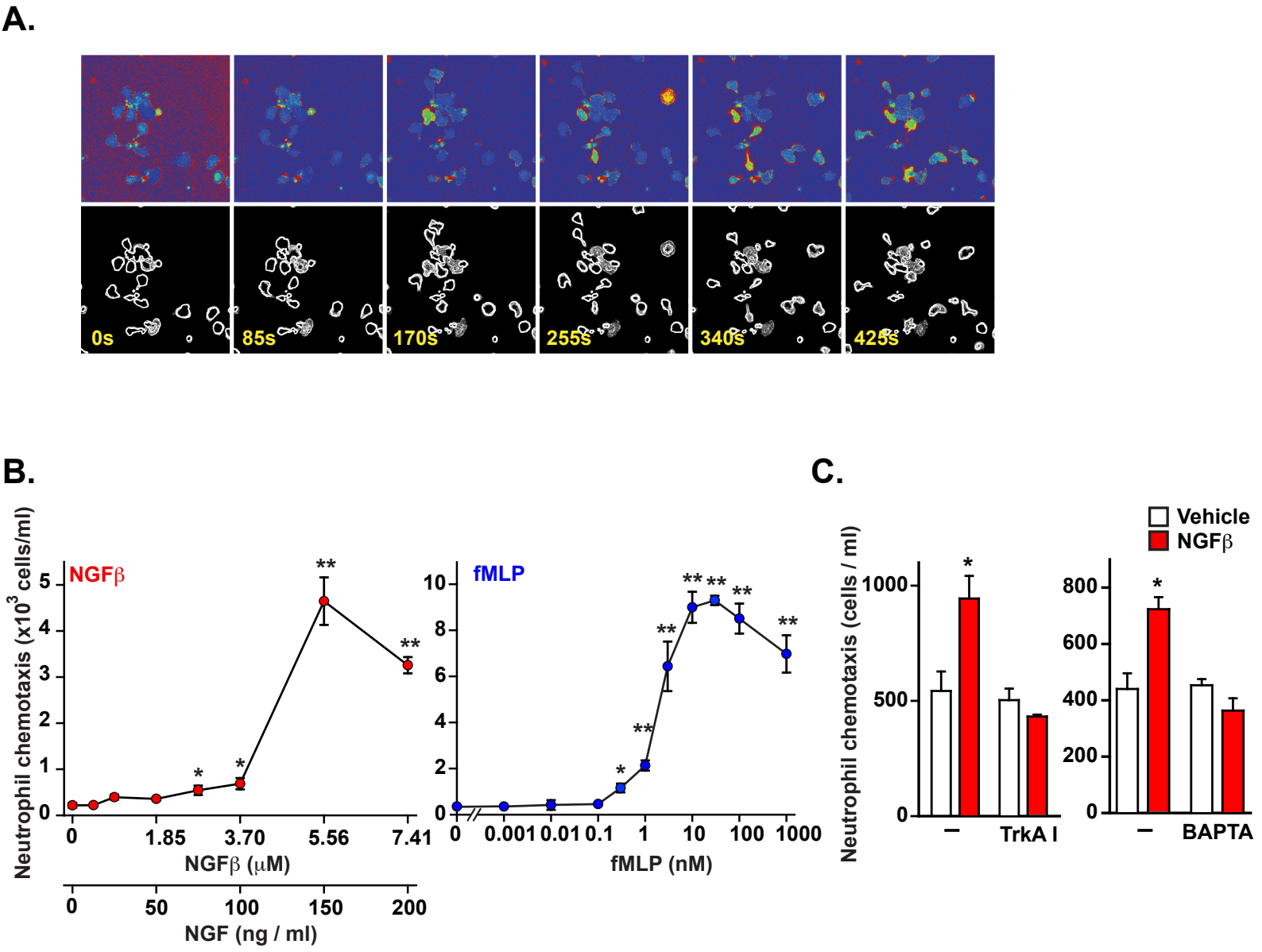


Fig. S8

Chemokinesis and chemotaxis of neutrophils in response to NGFβ. A. Pseudo-coloured images of Fluo3/FuraRed fluorescence ratios (monitoring intracellular calcium levels; *top*) and edge-enhanced images (highlighting movement; *bottom)* of primary human neutrophils in response to NGFβ stimulation (150 ng/ml). **B.** Chemotaxis of human neutrophils, assessed using transwell migration assays, in response to increasing concentrations of NGFβ or fMLP at concentrations shown. **C.** Chemotaxis in response to NGFβ (*red;* 150 ng/ml) was inhibited by pretreatment with the specific TRKA inhibitor (388626-12-8; 8 μM) or the calcium chelator BAPTA (20 μM). * 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 S9.

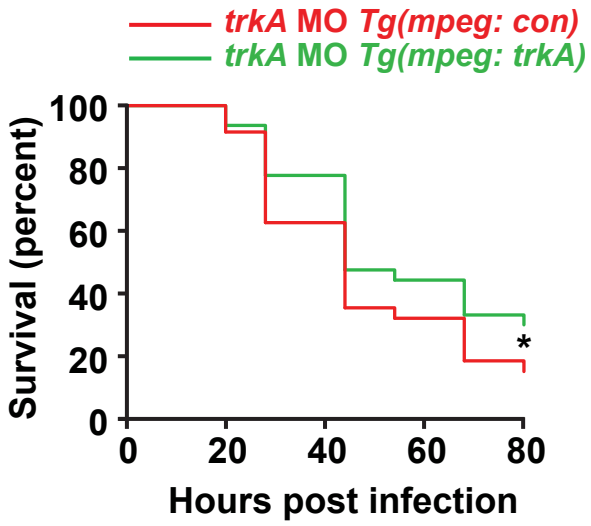


Fig. S9
Impact of conditional macrophage re-expression of trkA in trkA morphant zebrafish. Partial rescue of susceptibility to *S. aureus* infection caused by *trkA* knockdown in transgenic zebrafish expressing Morpholino-resistant *trkA* (trkA MO tg(mpeg1: trkA); *green*) but not control vector (trkA MO tg(mpeg1: con); *red*) in macrophages. * denotes $p \leq 0.05$; ** denotes $p \leq 0.005$. All experiments were carried out as 3 independent repeats using a total of 63 trkA transgenic and 59 vector control fish.

Movie S1

NGFβ-triggered calcium signaling and movement in neutrophils. Primary human neutrophils from healthy individuals, co-loaded with Fluo3-AM and FuraRed-AM, were imaged by live cell confocal microscopy (frame speed: 7.5s) following addition of NGFβ and shown as pseudo-coloured Fluo3/FuraRed ratios. Representative of 3 independent repeats.

Additional Data table S1 (separate file)

S1. **Nebraska transposon library.** NGFβ release from differentiated THP-1 cells was measured 24h after infection with individual mutants from the Nebraska transposon library.
S2. Signaling Protein array. Protein array was performed 15 min after NGF addition to primary human macrophages.