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ACCELERATED PUBLICATION

A novel role for the transcription factor HIF-1α in the formation of mast cell extracellular traps

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MCs (mast cells) are critical components of the host innate immune defence against bacterial pathogens, providing a variety of intra- and extra-cellular antimicrobial functions. In the present study we show, for the first time, that the transcriptional regulator HIF-1α (hypoxia-inducible factor-1α) mediates the extracellular antimicrobial activity of human and murine MCs by increasing the formation of MCETs (MC extracellular traps).

Key words: AKB-4924, echinomycin, hypoxia-inducible factor-1α (HIF-1α), mast cell, mast cell extracellular trap, *Staphylococcus aureus*.

INTRODUCTION

MCs (mast cells) are one of the most multifunctional cells of the immune system, having been shown to participate in a wide variety of important biological functions, such as allergy, immune defence against parasites and tissue remodelling [1]. More recently, MCs have been appreciated as critical components of the host defence against bacterial pathogens, exerting a variety of intra- and extra-cellular antimicrobial functions. Not unlike professional phagocytic cells such as neutrophils and macrophages, MCs have been shown to eliminate bacteria through phagocytosis [2], release of antimicrobial peptides [3] and formation of MCETs (MC extracellular traps) [4]. However, the detailed signalling events, such as involvement of specific transcriptional regulators, mediating MC antimicrobial activity during an infection are still unknown.

HIF (hypoxia-inducible factor) plays a role in regulating the inflammatory and innate immune functions of neutrophils and macrophages [5,6]. HIF is an oxygen-sensitive heterodimeric helix–loop–helix transcription factor, composed of a constitutively expressed β-subunit and a tightly regulated α-subunit. HIF-1α-specific binding to HREs (hypoxia-resposive elements) regulates the transcription of target genes, including those encoding erythropoietin, glucose transporters, glycolytic enzymes, antimicrobial factors and the angiogenic factor VEGF (vascular endothelial growth factor) [7,8]. In the present study, we investigated the role of HIF-1α in the antimicrobial activities of MCs.

MATERIALS AND METHODS

MCs

In the present study the human MC line HMC-1 was used as a model cell line [9] and was cultured as described previously [4]. BMMCs (bone-marrow-derived MCs) from C57BL/6 WT (wild-type) and myeloid HIF-1α-deficient mice [5] were isolated and cultured as described previously [4]. All mouse experiments were performed following appropriate ethical approvals (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg, Germany or the University of California, San Diego Institutional Animal Care and Use Committee). To determine the purity and differentiation status of BMMCs, cells were stained with a PE (phycoerythrin)-labelled anti-mouse CD117 antibody (0.2 μg/10^6 cells) (Southern Biotechnology) and analysed by flow cytometry using a FACSCalibur™ flow cytometer (BD Biosciences). More than 95 % of the cells were confirmed to be positive for CD117.

Co-incubation of MCs and bacteria

MC antimicrobial activity against *Staphylococcus aureus* strain Newman was monitored *in vitro* at different time points of co-incubation. MCs were centrifuged for 10 min at 118 ***g***, washed once with sterile PBS and resuspended in infection medium {IMDM (Iscove's modified Dulbecco's medium) including 25 mM Hepes, L-glutamine and sodium bicarbonate, supplemented with 2 % nuclease-free heat-inactivated FBS (fetal bovine serum) [10], and, in the case of the BMMCs, 0.1 mM MEM (minimal essential medium)}. MCs were then seeded in 24-well plates (HMC-1, 250 μl per well) or 48-well plates (BMMCs, 125 μl per well) at a density of 4×10^6 cells/ml. Mid-exponential phase bacteria grown in Todd–Hewitt broth (125 rev./min) were harvested by centrifugation for 5 min at 2952 ***g*** and resuspended in infection medium. Bacteria were added in a volume of 50 μl to the seeded MCs at an MOI (multiplicity of infection) of 1 bacterium per cell and centrifuged at 250 ***g*** for 5 min. After co-incubation at 37 °C, 5 % CO_2 for different time points, the MCs were lysed with a final concentration of 0.025 % Triton X-100 and serial dilutions

Abbreviations used: BMMC, bone-marrow-derived mast cell; DAPI, 4′,6-diamidino-2-phenylindole; DPI, diphenylene iodonium; HIF, hypoxia-inducible factor; HRE, hypoxia-reponse element; IL, interleukin; MC, mast cell; MCET, MC extracellular trap; MOI, multiplicity of infection; PFA, paraformaldehyde; ROS, reactive oxygen species; WT, wild-type.

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of the culture suspension were plated on Todd–Hewitt agar. The surviving CFUs (colony-forming units) were enumerated and the percentage of surviving bacteria in comparison with a bacterial growth control (100 %), grown under the same conditions in the absence of MCs, was determined.

To analyse effector mechanisms underlying MC antimicrobial activity, MCs were treated with 10 μM DPI (diphenylene iodonium; Sigma) (in DMSO; 1 h prior to infection) to block NADPH-oxidase-dependent ROS (reactive oxygen species) synthesis. To differentiate an extracellular compared with an intracellular (phagocytotic) process, MCs were treated for 10 min with 10 μg/ml cytochalasin D (Sigma) (in DMSO) to block phagocytosis prior to infection. To degrade MCETs, cells were treated with 500 m-units/ml micrococcal nuclease (Sigma) starting 1 h prior to infection. The results were compared with MCs that were treated with respective amounts of vehicle controls.

Blocking and boosting of HIF-1α activity

To evaluate the impact of HIF-1α on the antimicrobial activity of MCs, cultures were pre-incubated with the HIF-1α antagonist echinomycin (Alexis Biochemicals), prepared in DMSO and added at 0.32 μM 4 h prior to infection, and/or the HIF-1α agonist AKB-4924 [11] (Aerpio Therapeutics), prepared in DMSO/acid (pH 4.3) and added at 10 μM 1 h prior to infection. The results were compared with MCs treated with the respective amount of vehicle controls.

Immunofluorescence microscopy

For visualization and analyses of HIF-1α accumulation and MCET formation, MCs were seeded on to poly-L-lysine-coated (Sigma) glass coverslides and treated as described above. MCETs were visualized without fixation using the Live/Dead Viability/Cytotoxicity kit (Invitrogen) for mammalian cells according to the manufacturer's protocol. For immunostaining of HIF-1α, cells were fixed with 4 % PFA (paraformaldehyde), washed with PBS, and blocked for 45 min with 2 % BSA, 2 % goat serum and 0.2 % Triton X-100 in PBS. Next, samples were incubated with a polyclonal rabbit anti-HIF-1α antibody (Novus Biologicals) diluted 1:100 in PBS containing 2 % BSA and 0.2 % Triton X-100 for 60 min at room temperature (21°C). A universal rabbit IgG (Dako) served as a negative control. After washing three times with PBS, samples were incubated with a secondary Alexa Fluor® 488-labelled goat anti-rabbit-IgG (1:500 in PBS containing 2 % BSA and 0.2 % Triton X-100) (Invitrogen) for 60 min at room temperature in the dark and mounted with ProlongGold with DAPI (4′,6-diamidino-2-phenylindole; Invitrogen).

The Live/Dead BacLight™ Bacterial Viability kit (Invitrogen) was used to determine the viability of *S. aureus* entrapped in the MCETs by fluorescence microscopy. After staining, according to the manufacturer's protocol, cells were washed three times with PBS, fixed with 1 % PFA for 5 min, washed, and mounted on to glass slides with ProlongGold with DAPI.

Mounted samples were examined using an inverted confocal laser-scanning two-photon Olympus Fluoview FV1000 microscope with Fluoview™ Spectral Scanning technology (Olympus). Confocal Z-stack images were obtained using a 60×/1.42 PlanApo objective. Alternatively, fluorescence images were recorded using a Zeiss Axiolab microscope (Zeiss 20×/0.5 Plan-Neofluor or 40×/0.65 Achroplan Zeiss objective) with an attached Sony Digital Photo Camera DKC-5000 at calibrated magnifications.

Statistical analysis

Data were analysed using GraphPad Prism 4.0 (GraphPad Software). Each experiment was performed in duplicate at least three times independently. Means and S.E.M. were calculated from all independent experiments and compared with their respective controls. The differences between groups were analysed using a paired Student's *t* test.

RESULTS AND DISCUSSION

HMC-1 cells have been reported to constitutively express HIF-1α [12], a finding we confirmed under our experimental conditions by immunofluorescence microscopy with HIF-1α-specific antibodies (Figure 1A). Infection of HMC-1 cells with *S. aureus* slightly increased HIF-1α accumulation, but this effect was not statistically significant ($P = 0.1366$, Student's *t* test). HMC-1 cells and BMMCs were treated with echinomycin, which blocks HIF-1α activation of target genes by binding to HRE sequences [13]. Echinomycin did not influence HIF-1α expression of HMC-1 cells (Figure 1A), a potential secondary effect reported in certain epithelial cell cultures under normoxia [14]. Pre-treatment of HMC-1 cells (Figure 1B) or BMMCs (Figure 1C) with echinomycin completely abolished their antimicrobial activity against *S. aureus* Newman. To further corroborate the involvement of HIF-1α, BMMCs were isolated from mice lacking HIF-1α in the myeloid cell lineage [5] and tested for their antimicrobial activity. As shown in Figure 1(D), HIF-1α-deficient BMMCs showed a significantly reduced antimicrobial effect against *S. aureus* compared with control BMMCs (Figure 1D).

Pharmacological augmentation of HIF has been shown to boost the bactericidal activity of macrophages and neutrophils [11,15]. In the present study, the new pharmacological agent AKB-4924 [11] was used to inhibit prolyl hydroxylases involved in the HIF-1α degradation pathway and to increase HIF-1α protein levels in MCs, as confirmed by immunofluorescence microscopy (Figure 1A) for HMC-1 cells and Western blot analysis (Supplementary Figure S1 at http://www.BiochemJ.org/bj/446/bj4460159add.htm) for HMC-1 cells and BMMCs. In an additional control experiment, the transcription of mRNA encoding known HIF-1α target genes in HMC-1 cells, namely IL (interleukin)-6 and IL-8 [12], was significantly induced upon AKB-4924 treatment (Supplementary Figure S2 at http://www.BiochemJ.org/bj/446/bj4460159add.htm). As shown in Figure 1(E), HMC1-cells pre-treated with 10 μM AKB-4924 for 1 h showed significantly increased antimicrobial activity against *S. aureus* compared with control cells treated with vehicle only. When echinomycin was used to block HIF-1α signalling, the AKB-4924-induced antimicrobial effect was abolished (Figure 1F). Corroborating these data, echinomycin diminished the AKB-492-mediated induction of mRNA encoding the HIF-1α target genes IL-6 and IL-8 in HMC-1 cells (Supplementary Figure S3 at http://www.BiochemJ.org/bj/446/bj4460159add.htm). Importantly, AKB-4924 also increased the antimicrobial activity of WT BMMCs, but not that of HIF-1α-deficient BMMCs, further validating the HIF-1α-specific effect (Figure 1G).

To differentiate whether the HIF-1α effect in boosting MC antimicrobial activity was operating through intracellular compared with extracellular mechanisms, AKB-4924-treated and control cells were treated with cytochalasin D to block phagocytosis. As shown in Figure 2(A), inhibition of phagocytosis did not alter the antimicrobial activity of HMC-1 cells, whether or not HIF-1α activity was boosted with AKB-4924. These results suggest that HIF-1α primarily mediates an extracellular bactericidal activity of the HMC-1 cells. Interestingly, when

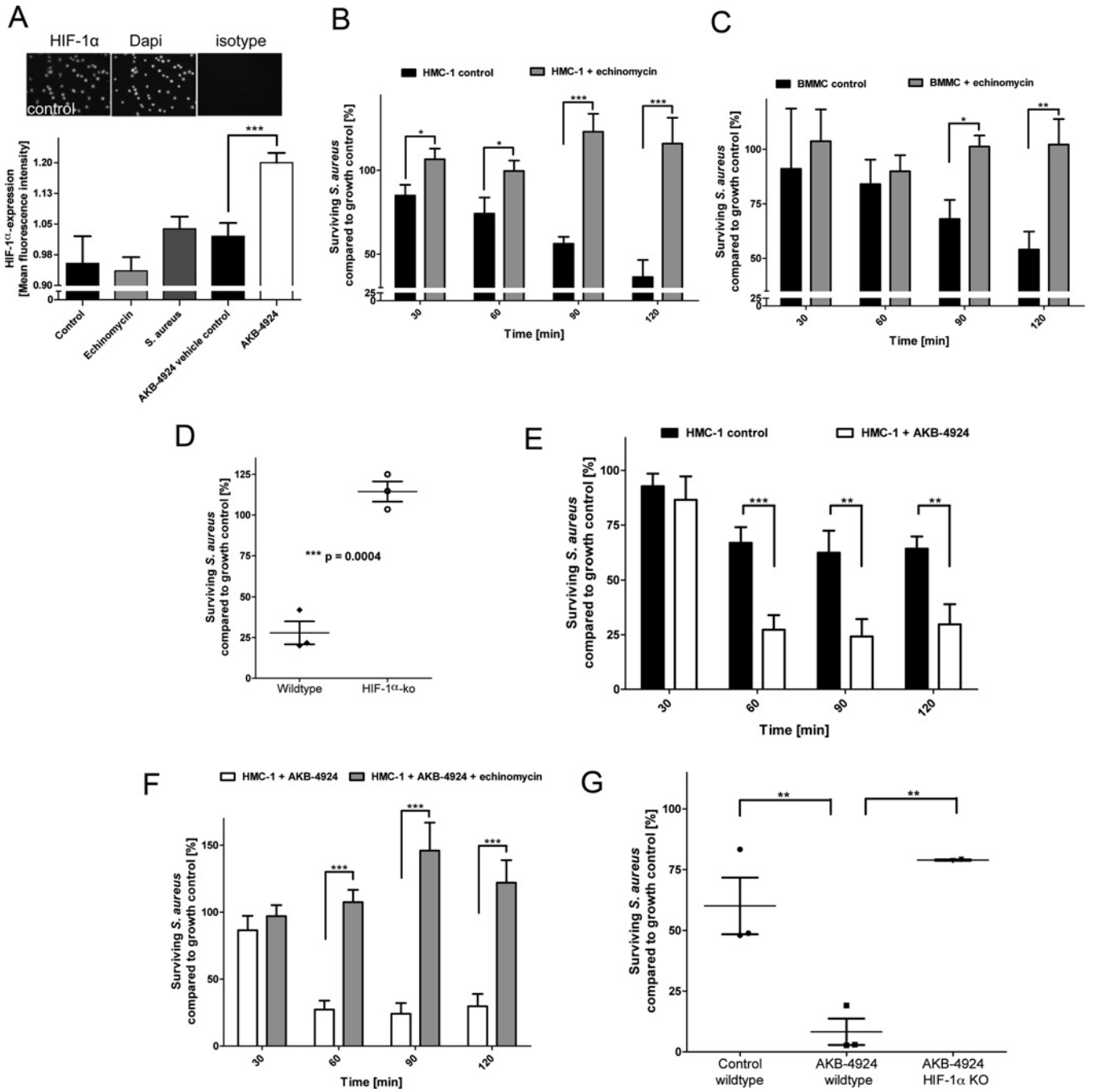

Figure 1 Role of HIF-1α in the antimicrobial activity of MCs

(**A**) Relative HIF-1α expression in HMC-1 cells. MCs were seeded on poly-L-lysine-coated glass coverslides, stimulated with respective reagents or bacteria, fixed with 4 % PFA and immunostained. HIF-1α expression was visualized and referred to total cell amount (DAPI) and respective isotype background staining. Mean fluorescence intensities were measured at equal exposure times and quantified using ImageJ (NIH). (**B**) The HIF-1α antagonist echinomycin blocks the antimicrobial activity of HMC-1 cells against *S. aureus*. (**C**) The HIF-1α antagonist echinomycin blocks the antimicrobial activity of BMMCs against *S. aureus*. (**D**) Loss of HIF-1α results in significantly reduced antimicrobial activity of BMMCs against *S. aureus*. Each data point shows the mean value from one independent experiment, using murine BMMCs from different individuals. (**E**) The HIF-1α agonist AKB-4924 boosts the antimicrobial activity of HMC-1 against *S. aureus*. HMC-1 cells were treated with 10 μM AKB-4924 or respective amounts of vehicle control 1 h prior to infection to increase HIF-1α activity. (**F**) Echinomycin abolishes the AKB-4924-induced antimicrobial activity of HMC-1 against *S. aureus*. HMC-1 cells were treated with 0.32 μM echinomycin 4 h prior to infection to block HIF-1α signalling, and 10 μM AKB-4924 or respective amounts of vehicle control was added 1 h prior to infection to increase HIF-1α activity. (**G**) AKB-4924 boosts the antimicrobial activity of BMMCs derived from WT, but not from myeloid HIF-1α-deficient mice. BMMCs were treated with 10 μM AKB-4924 or respective amounts of vehicle control 1 h prior to infection with *S. aureus* to increase HIF-1α activity. Each data point shows the mean value from one independent experiment, using murine BMMCs from different individuals. All data are presented as percentages of surviving bacteria in comparison with a bacterial growth control (100 %). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.005$, as measured by Student's paired *t* test. ko/KO, knockout.

HMC-1 cells were treated with DPI to block the NADPH-dependent formation of ROS, baseline as well as AKB-4924-boosted antimicrobial activity was blocked (Figure 2B).

Previously, ROS-dependent formation of MCETs has been identified as a significant contributor to the antimicrobial activity of MCs [4]. MCETs are the result of a specialized cell death

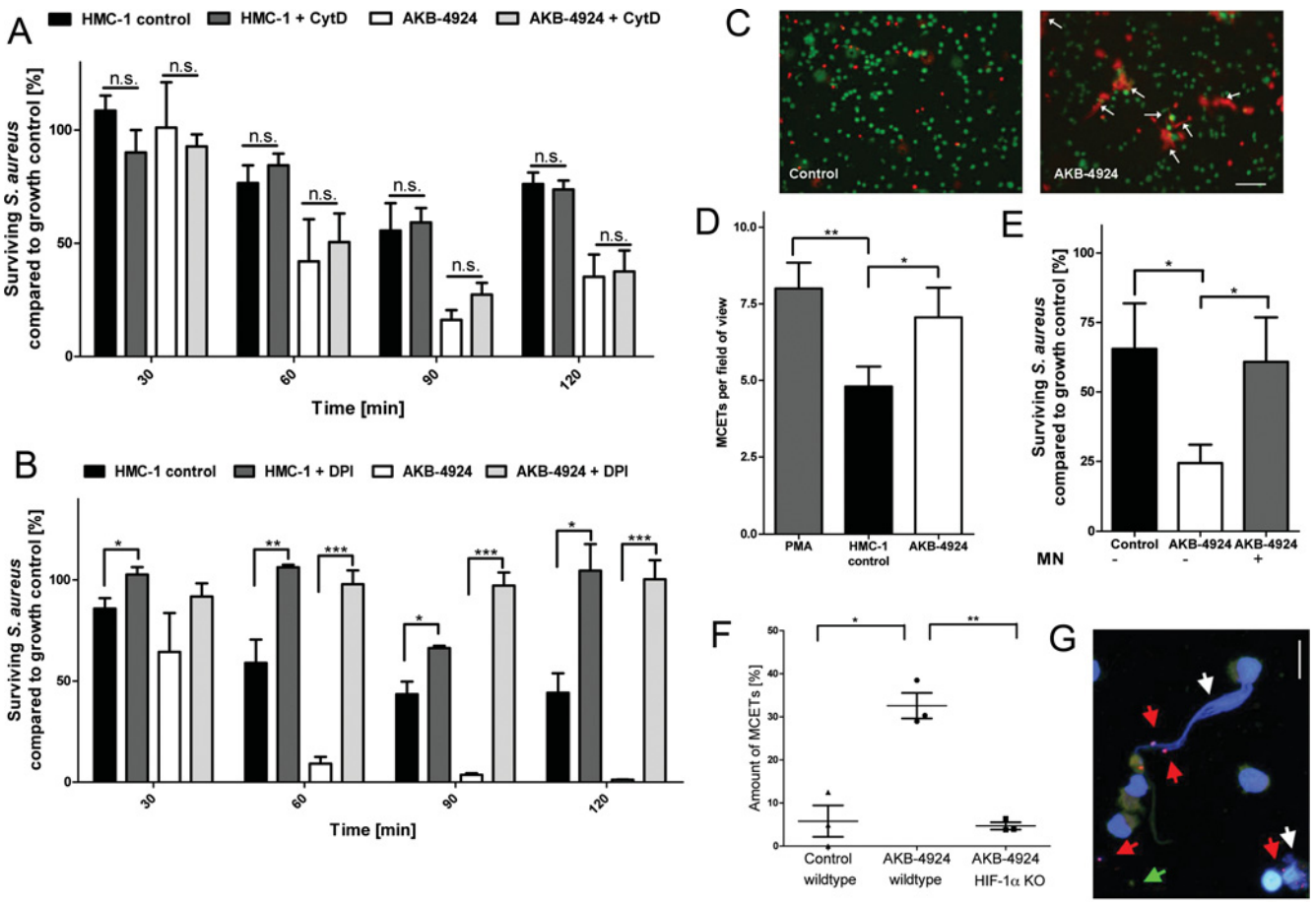

Figure 2 HIF-1α mediates the extracellular antimicrobial activity of MCs

(**A**) HIF-1α-mediated antimicrobial activity of HMC-1 cells against *S. aureus* via an extracellular mechanism. HMC-1 cells were treated with 10 μM AKB-4924 or a vehicle control to boost HIF-1α signalling (1 h prior to infection) and 10 μg/ml cytochalasin D to block phagocytosis (10 min prior to infection). (**B**) HIF-1α-mediated antimicrobial activity of HMC-1 cells against *S. aureus* is ROS-dependent. HMC-1 cells were treated with 10 μM DPI to block NADPH-oxidase-dependent formation of ROS simultaneously with 10 μM AKB-4924 to boost HIF-1α activity. (**C**) Boosting of HIF-1α with AKB-4924 results in the induction of MCETs. Representative fluorescent images of MCETs, stained with the Live/Dead Viability/Cytotoxicity kit for mammalian cells (staining cytoplasm of viable cells in green and DNA released by dead cells in red). The extracellular deposition of DNA is indicated by white arrows. Scale bar = 25 μm. (**D**) MCETs were counted per field of view with approximately 125 MCs after stimulation for 2.5 h with 10 μM AKB-4924 or vehicle control, or 1.5 h with 25 nM PMA as a positive control. (**E**) HMC-1 cells were treated with 10 μM AKB-4924 or a vehicle control to boost HIF-1α activity and 500 m-units/ml micrococcal nuclease (1 h prior to infection) to degrade MCETs. Then, HMC-1 cells were infected with *S. aureus* (MOI = 1) for 90 min and the antimicrobial activity was measured. (**F**) AKB-4924 boosts the formation of MCETs of BMMCs derived from WT, but not from myeloid HIF-1α-deficient, mice. Each data point shows the mean value from one independent experiment, using murine BMMCs from different individuals. (**G**) Representative high-resolution confocal fluorescent Z-stack images of MCETs boosted with AKB-4924 and infected with *S. aureus*. Analysis of viable (green, green arrows) compared with dead (red, red arrows) bacteria entrapped in MCETs (white arrows) as determined by the Live/Dead BacLight™ Bacterial Viability assay. Scale bar = 8 μm. All results in (**A**), (**B**) and (**D**–**F**) are presented as percentages of surviving bacteria in comparison with a bacterial growth control (100 %). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.005$, as measured by Student's paired *t* test. CytD, cytochalasin D; KO, knockout; MN, micrococcal nuclease; n.s., not significant.

process [16] in which extracellular fibres consisting of a DNA backbone and embedded antimicrobial peptides, histones and proteases locally entrap and kill bacterial pathogens such as *S. aureus* [4]. As directly visualized and quantified by fluorescence microscopy, significantly more MCETs were detected after treating HMC-1 cells and BMMCs with AKB-4924 to boost HIF-1α levels (Figures 2C, 2D and 2F). We used the Live/Dead Viability/Cytotoxicity assay, based on the DNA-intercalating dye ethidium homodimer-1, for visualization of extracellular DNA. This procedure stained both the MCETs and the nuclei of the MCET-releasing cells, confirming that these cells are dead. These data are in agreement with previously published data indicating that MCETs are released coincident with cell death [4]. Importantly, AKB-4924-induced MCET formation was absent from HIF-1α-deficient BMMCs (Figure 2F). Supporting the antimicrobial role of MCETs, treatment of HMC-1 cells with micrococcal nuclease to degrade MCETs resulted in a significant loss of MC antimicrobial activity, even in the case of boosting of HIF-1α with AKB-4924 (Figure 2E). Additionally, visualization of bacterial viability after boosting of HIF-1α with AKB-4924 revealed dead bacteria entrapped in MCETs (Figure 2G).

In summary, the results of the present study show, for the first time, that the transcription factor HIF-1α is a key regulator of the extracellular antimicrobial activity of MCs. An augmentation of HIF-1α activity resulted in a boosting of the antimicrobial activity of human and murine MCs by inducing the formation of MCETs. This new knowledge has significant implications for understanding the role of MCs in host defence against bacterial infections.

AUTHOR CONTRIBUTION

Katja Branitzki-Heinemann and Cheryl Okumura performed the research, analysed and interpreted data, and wrote the paper. Lena Völlger performed the research, analysed and interpreted data, and provided a critical reading of the paper. Toshiaki Kawakami and Yuko Kawakami provided isolated and differentiated BMMCs, critically proofread the paper

before submission. Hassan Naim provided interpretation of data and critically proofread the paper before submission. Victor Nizet designed the research, interpreted data and wrote the paper. Maren von Köckritz-Blickwede designed and performed the research, analysed and interpreted data, and wrote the paper.

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A novel role for the transcription factor HIF-1α in the formation of mast cell extracellular traps

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MATERIALS AND METHODS

Western blot analysis of HIF-1α protein levels

HMC-1 cells or BMMCs were treated with 10 μM AKB-4924 or DMSO vehicle control for 2.5 h in FBS-free IMDM at 37 °C with 5 % CO_2. Then, the cells were centrifuged at 118 ***g***/4 °C for 7 min to remove the supernatant. The pellet derived from $(1–2)\times10^7$ cells (HMC-1 cells) or 5×10^6 cells (BMMCs) was resuspended in 100 μl of lysis buffer [25 mM Tris/HCl, 50 mM NaCl, 0.5 % sodium deoxycholate, 0.5 % Triton X-100 (pH 8.0) and proteinase inhibitors] and incubated with agitation for 15 min at 4 °C. After centrifugation at 13414 ***g*** for 15 min at 4 °C, the supernatant was harvested and the amount of protein was determined. Similar protein amounts of total cell extracts derived from control compared with AKB-4924-treated cells were denaturated in Laemmli buffer [150 mM Tris/HCl (pH 6.8), 6 % SDS, 30 % glycerine and 0.02 % Bromophenol Blue] for 5 min at 95 °C. Samples were separated by SDS/PAGE (10 % gels), and then proteins were transferred to PVDF membranes. The membranes were blocked for 45 min in TBS-TM [TBS-T (Tris-buffered saline + 0.1 % Tween 20) supplemented with 5 % non-fat dried skimmed milk] at room temperature. HIF-1α and β-actin protein were detected by incubating the membrane with respective primary antibodies against HIF-1α (rabbit anti-HIF-1α, Cayman Chemicals, 1:500 dilution) and β-actin (mouse anti-β-actin, Santa Cruz Biotechnology, 1:10000 dilution) diluted in TBS-TM overnight at 4 °C with shaking. After washing in TBS-T, membranes were incubated for 45 min at room temperature with the respective secondary antibodies: horseradish peroxidase-conjugated goat anti-rabbit IgG (Thermo Scientific, 1:10000 dilution) for HIF-1α and goat anti-rabbit IgG (Thermo Scientific, 1:10000 dilution) for β-actin, diluted in TBS-TM. After washing, protein was visualized using the SuperSignal West Femto Maximum Sensitivity Substrate reagents (Pierce).

Real-time PCR

RNA was isolated from untreated and treated HMC-1 cells using the RNA isolation kit from Qiagen according to the manufacturer's protocol. Different treatments of HMC-1 cells are indicated in the respective Figure legends. RNA was DNase-treated (Turbo DNase, Ambion), and 1 μg of total RNA was reverse-transcribed (iScript, Bio-Rad Laboratories). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories) with the following primers: IL-6 forward, 5′-GAGAAAGGAGACATCTAACAAGAGT-3′; IL-6 reverse, 5′-GCGCAGAATGAGATGAGTTGT-3′; IL-8 forward, 5′-AGCTCTGTGTGAAGGTGCAG-3′; IL-8 reverse, 5′-AATTTCTGTGTTGGCGCAGT-3′; GAPDH (glyceraldehyde-3-phosphate dehydrogenase) forward, 5′-GAAGGTGAAGGTCGGAGTCAACG-3′; and GADPH reverse, 5′-TCCTGGAAGATGGTGATGGGAT-3′. Data were normalized to GAPDH, and relative expression was calculated compared with untreated cells.

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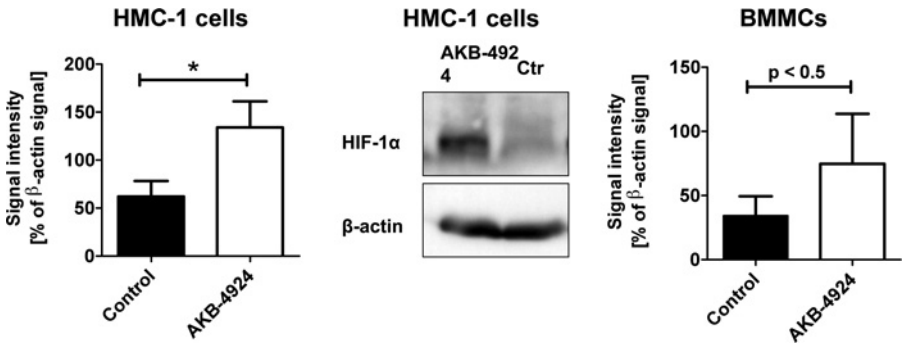

Figure S1 AKB-4924 leads to accumulation of HIF-1α protein

HMC-1 cells and BMMCs were treated for 2.5 h with 10 μM AKB-4924 or a DMSO vehicle control. Similar protein amounts of total cell extracts (2.5 μg for BMMCs and 12 μg for HMC-1 cells) were separated by SDS/PAGE (10% gel). The relative amount of HIF-1α protein (95 kDa) was analysed by Western blotting using β-actin (42 kDa) to normalize the data. $^*P < 0.05$, measured by paired Student's t test, for $n = 4$ (HMC-1 cells) or $n = 3$ (BMMCs) independent experiments.

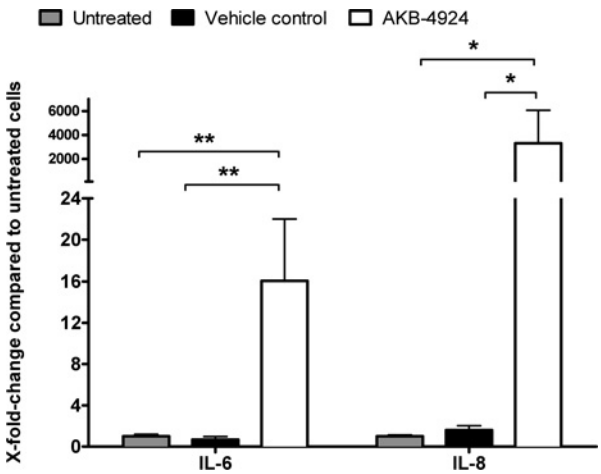

Figure S2 AKB-4924 induces transcript expression of HIF-1α-target genes

HMC-1 cells were treated for 24 h with 10 μM AKB-4924 or a DMSO vehicle control and relative transcript expression was analysed by real-time PCR using GAPDH as a housekeeping gene to normalize the data. Data are indicated as the fold change compared with untreated cells. $^*P < 0.05$ and $^{**}P < 0.01$, measured by paired Student's t test, for $n = 3$ independent experiments.

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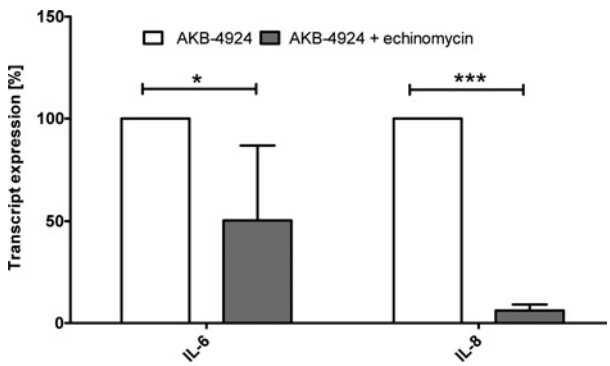

Figure S3 The HIF-1α antagonist echinomycin reduces the AKB-4924-induced transcript expression of HIF-1α target genes

HMC-1 cells were incubated in the presence or absence of 0.32 μM echinomycin for 4 h, followed by treatment with 10 μM AKB-4924 for 2.5 h. The relative expression of mRNA transcripts was analysed by real-time PCR, using GAPDH as a housekeeping gene to normalize the data. Data are indicated as the percentage transcript expression compared with samples treated with AKB-4924 only (without echinomycin). $^*P < 0.05$ and $^{***}P < 0.001$, measured by paired Student's t test, for $n = 2$ independent experiments, each run in duplicate.