

## Pharmacologic Augmentation of Hypoxia-Inducible Factor-1 $\alpha$ with Mimosine Boosts the Bactericidal Capacity of Phagocytes

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**Hypoxia-inducible factor (HIF)-1 $\alpha$  is activated on exposure to bacterial pathogens and regulates the innate immune functions of phagocytes. We show here that the HIF-1 $\alpha$  agonist mimosine can boost the capacity of human phagocytes and whole blood to kill the leading pathogen *Staphylococcus aureus* in a dose-dependent fashion and reduce the lesion size in a murine model of *S. aureus* skin infection. This provides the first proof of principle for a novel approach to the treatment of bacterial infection by pharmacologically augmenting the host phagocytic cell function.**

Phagocytic cells, such as neutrophils and macrophages, are a critical component of host innate immune defense against invading microorganisms. We have recently shown that the transcriptional regulator hypoxia-inducible factor (HIF)-1 $\alpha$  plays a significant role in supporting the inflammatory and bactericidal activities of neutrophils and macrophages in the murine model [1, 2]. In these studies, HIF-1 $\alpha$  was induced in macrophages and neutrophils by gram-positive and gram-negative pathogens independent of tissue oxygenation and functioned to stimulate the production of immune-defense molecules, including granule proteases, antimicrobial peptides, nitric oxide, and tumor ne-

crisis factor- $\alpha$ . This led to the postulate that HIF-1 $\alpha$  could serve as a master regulator of innate immunity [3] and, because pharmacological approaches for manipulating HIF-1 $\alpha$  levels have been considered in the context of angiogenesis and cancer biology research [4, 5], inspired commentary that drugs functioning to boost the transcription factor could support immune function under human infectious disease conditions [6]. Two caveats to this speculation are (1) that observations in the murine model can be translated to human phagocytic cells and (2) that augmentation of HIF-1 $\alpha$  can support phagocytic cell function in the context of bacterial infection in vivo. In the present study, we begin to address these questions in experiments involving the well-characterized HIF-1 $\alpha$  agonist mimosine and the common human bacterial pathogen *Staphylococcus aureus*.

**Methods.** For bactericidal assays, *S. aureus* (ATCC 33591) were grown in Todd-Hewitt broth (THB) to logarithmic phase (OD<sub>600</sub> of 0.4 or  $\sim 5 \times 10^7$  cfu/mL) and then pelleted, washed, and resuspended in PBS or RPMI 1640 tissue-culture medium to the desired concentration. Venous blood from healthy volunteers was used for whole blood and neutrophil isolation. Neutrophils were purified using the PolyMorphPrep Kit (Axis-Shield) in accordance with manufacturer's instructions. Human monocytic cell line U937 was propagated in RPMI 1640 plus 10% fetal calf serum, 1 mmol/L NaPyr, 10 mmol/L HEPES, and glucose. Whole blood or phagocytic cells were preincubated with mimosine (Sigma-Aldrich) (0–500  $\mu$ mol/L) for 2–4 h and then challenged with *S. aureus* (either  $10^5$  cfu in 100  $\mu$ L added to 300  $\mu$ L of whole blood or at an MOI of 1 bacterium/cell for isolated phagocytes). Aliquots were plated on THB agar after 30 (whole blood and neutrophils) or 60 (U937 monocytes) min for enumeration of surviving *S. aureus* colony-forming units. Experimental differences were analyzed by Student's unpaired *t* test (2-tailed).

A concentration of 1 mmol/L mimosine did not affect the growth characteristics of *S. aureus* in THB or RPMI 1640 medium (data not shown), and others have shown mimosine does not inhibit bacterial growth in cell-free human serum [7].

To specifically assess neutrophil extracellular killing, cytochalasin D (Sigma) was added to the bactericidal assay at a final concentration of 10  $\mu$ g/mL to inhibit phagocytic uptake [8, 9]. To degrade neutrophil extracellular traps (NETs), DNase was added at a final concentration of 100 U/mL (Worthington) [8]. NET production was visualized with 0.1 mmol/L Sytox orange stain (Molecular Probes), and the mixture was immediately visualized without fixation or washes by means of a Nikon TE200 inverted fluorescent microscope, with image capture by means

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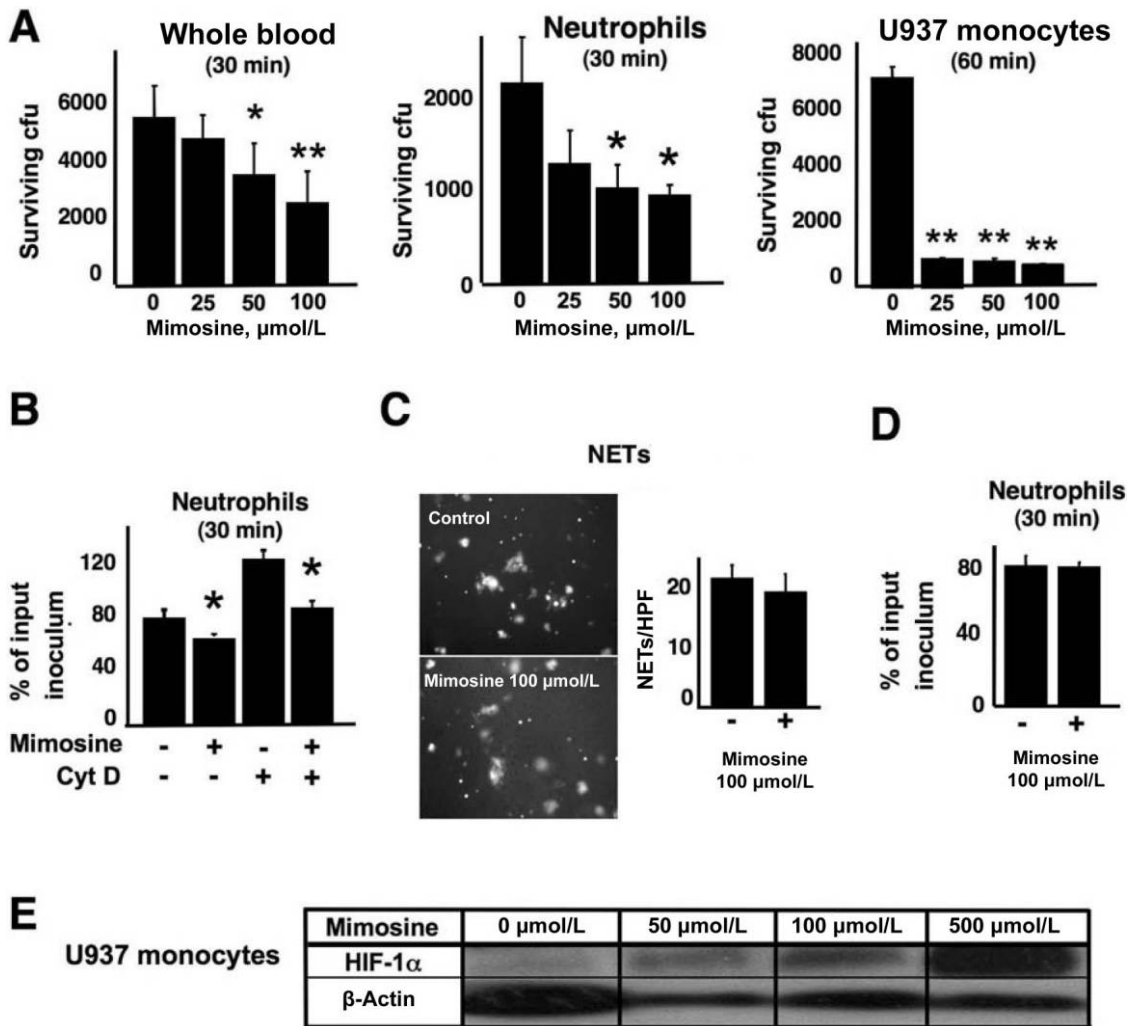
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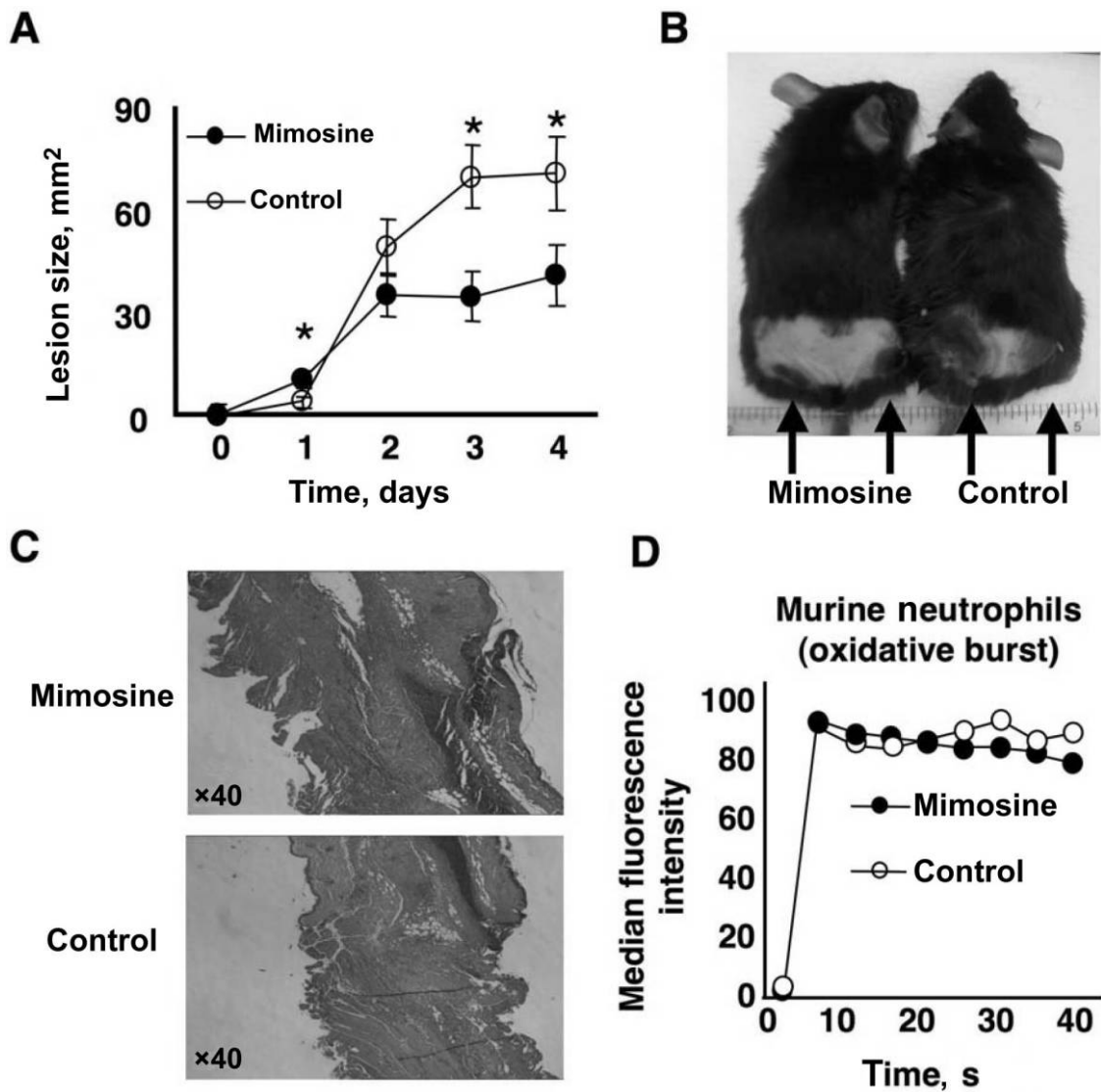


**Figure 1.** Enhancement of the killing activity of human phagocytes by the hypoxia-inducible factor (HIF)-1 $\alpha$  agonist mimosine. *A*, Killing of *Staphylococcus aureus* by human whole blood, purified human neutrophils, or cultured U937 human monocytes after pretreatment with varying concentrations of mimosine. *B*, Influence of mimosine (100  $\mu$ mol/L) on the killing of *S. aureus* by neutrophils, even with the inhibition of phagocytotic uptake (plus cytochalasin D [cyt D]). *C*, No influence of mimosine on the production of neutrophil extracellular trap (NET) by human neutrophils (original magnification,  $\times 100$ ). *D*, Elimination of the enhancement of neutrophil bactericidal activity by mimosine after degradation of neutrophil NETs by exogenous DNase. *E*, Western blot showing HIF-1 $\alpha$  induction in U937 monocytes on treatment with mimosine. Experiments were performed in triplicate and were repeated 3 times with similar results; shown are representative experiments with mean  $\pm$  SE values. \* $P < .05$  and \*\* $P < .001$ .

of a CCD (charge-couple-device) camera [9]. For quantification, NETs were enumerated by counting 3 transects of 5 independent wells. Experimental differences were analyzed by Student's unpaired *t* test (2-tailed).

Western blot assays were performed with U937 monocytes, which were incubated with mimosine (0–500  $\mu$ mol/L) for 4 h at 37°C. Total protein was extracted with RIPA buffer, and concentrations were determined using the BioRad assay (BioRad Laboratories). All samples were separated by 4%–12% Tris-tricine gel electrophoresis (Invitrogen), transferred to nitrocellulose, and detected with 1:1000 primary rabbit anti-HIF-1 $\alpha$  (Novus Biologicals), secondary peroxidase-conjugated goat anti-rabbit antibody (Dako), and the enhanced chemiluminescent (ECL) system (Amersham Biosciences).

C57B1/6 mice (10–12 weeks old; Charles River Laboratory) were used for an established model of *S. aureus* skin infection [8]. Briefly, logarithmic-phase *S. aureus* were resuspended in PBS and mixed 1:1 with sterile Cytodex microcarrier beads (Sigma-Aldrich), and inocula of  $5 \times 10^7$  cfu were injected into both shaved flanks of each mouse either in 100  $\mu$ L (final volume) of PBS (control) or in 1 mmol/L mimosine in PBS. Mice were treated again at 6 and 24 h with 50  $\mu$ L of PBS (control) or 1 mmol/L mimosine in PBS intralesionally. Lesion size was measured daily for 4 days. On day 4, the mice were killed, and skin was removed for histological analysis. Injection of 1 mmol/L mimosine alone according to the above treatment schedule did not produce detectable skin lesions (data not shown). Experimental differences were analyzed by Student's unpaired *t* test (2-tailed).



**Figure 2.** Reduction in the severity of *Staphylococcus aureus* skin infection in vivo by the hypoxia-inducible factor (HIF)-1 $\alpha$  agonist mimosine. *A*, Graph depicting lesion size generated by *S. aureus* subcutaneous infection with or without treatment with 3 doses of mimosine during the first 24 h. Shown are mean  $\pm$  SE values. \* $P < .05$ . *B*, Image showing representative gross appearance of skin lesions in mimosine-treated mice vs. control mice. *C*, Histopathologic staining (hematoxylin-eosin; original magnification,  $\times 40$ ) showing similar neutrophil influx at the infection site of mimosine-treated mice and control mice (day 4). *D*, Oxidative-burst capacity. Induction of HIF-1 $\alpha$  by mimosine does not promote the capacity of murine neutrophils for oxidative burst, as assessed by flow cytometry.

For the oxidative-burst assay, neutrophils were isolated from the peritoneal cavity of 12-week-old C57B1/6 mice (Charles River Laboratory) 4 h after injection of 3% thiogluconate with or without 1 mmol/L mimosine and then resuspended in PBS plus 5 mmol/L glucose without Ca<sup>2+</sup> or Mg<sup>2+</sup>. Oxidative burst was initiated by adding PBS plus 5 mmol/L glucose containing Ca<sup>2+</sup> and Mg<sup>2+</sup> and was measured using the FcOxy BURST Green Assay (Invitrogen) in accordance with the manufacturer's instructions.

**Results and discussion.** HIF-1 $\alpha$  contributes to the bactericidal activity of murine phagocytes, and mice lacking the transcription factor in their myeloid lineage fail to restrict systemic

spread of bacterial skin infection [2]. To determine whether pharmacologic induction of HIF-1 $\alpha$  could increase the bactericidal capacity of human phagocytes, we pretreated human whole blood, freshly isolated neutrophils, or cultured U937 monocytic cells with 25, 50, or 100  $\mu$ mol/L (final concentration) of the established HIF-1 $\alpha$  agonist mimosine and then measured killing activity against the leading human pathogen *S. aureus*. A dose-dependent enhancement of bactericidal activity was observed—the highest dose of mimosine (100  $\mu$ mol/L) increased *S. aureus* killing by whole blood or neutrophils >2-fold and by U937 monocytic cells by 4-fold (figure 1A). When cytochalasin D was used to inhibit phagocytosis, enhancement of killing by mimo-

sine was still observed, suggesting that the bactericidal effectors induced by mimosine can function extracellularly (figure 1B). This effect of mimosine did not reflect an increase in the production of NETs (figure 1C) but was eliminated by treatment with DNase, which degrades NETs (figure 1D). Western blot analysis of HIF-1 $\alpha$  levels in the U937 monocytes confirmed a dose-dependent induction in response to the increasing concentrations of mimosine (figure 1D). These results indicate that stabilization of HIF-1 $\alpha$  could represent a pharmacologic target to enhance the bacterial killing activity of human phagocytes.

We hypothesized that pharmacologic enhancement of the phagocytic activity of myeloid cells could in and of itself serve to limit the spread of bacterial infection in vivo. *S. aureus* is the most important agent of human skin and soft-tissue infections, and we and others have studied the virulence mechanisms of this pathogen in a murine subcutaneous-challenge model [10, 11]. To provide the first proof of principle for HIF-1 $\alpha$ -targeted immune enhancement of therapy for bacterial infection, we challenged mice subcutaneously with *S. aureus* and treated the lesion site with 3 doses (at 0, 6, and 24 h) of mimosine or PBS control. The progression of infection was followed for 4 days. Those mice treated with mimosine had necrotic lesions that were on average 50% smaller than those in the untreated mice ( $P < .05$ ) (figure 2A and 2B). These results show that a pharmacologic agent with no intrinsic antibiotic activity can mitigate the progression of a bacterial infection through its effects on host innate immune function.

Corroborating observations made in mice with genetic deletion of overexpression of HIF-1 $\alpha$  [2], no qualitative difference were observed in the numbers of infiltrating neutrophils at the subcutaneous infection site after treatment with mimosine (figure 2C), nor were differences in respiratory-burst activity observed in neutrophils isolated from mice treated intraperitoneally with mimosine (figure 2D). Rather, HIF-1 $\alpha$  is known to induce production of neutrophil antimicrobial peptides and granule proteases [2], the key effectors of killing within NETs [8, 12]. Given that HIF-1 $\alpha$  does not appear to be required for efficient phagocytic uptake of bacteria [1]; the increased bactericidal activity attributable to HIF-1 $\alpha$  agonism may occur extracellularly.

The activation state of neutrophils and macrophages must be tightly regulated to provide a rapid microbicidal response to invading pathogens while avoiding undesired inflammatory dam-

age to host cells and tissues. By placing certain key microbicidal functions under the control of HIF-1 $\alpha$ , phagocytes are maintained in a baseline “off state” as they circulate in the oxygen-rich bloodstream and then are activated on recruitment and migration to the hypoxic conditions present at sites of tissue infection [2]. HIF-1 $\alpha$  may further promote prolonged survival of the phagocytes in the hypoxic microenvironment [13].

In human medicine, challenges to the effective control of bacterial infection arise from factors such as antibiotic resistance, lowered host immunity, and compromised epithelial barrier integrity. In such cases, targeted manipulation of the activation state of phagocytes toward increased bactericidal capacity could prove to be of therapeutic utility.

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