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To the editor:

Mutations in succinate dehydrogenase B (*SDHB*) enhance neutrophil survival independent of HIF-1 α expression

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Neutrophils are unusual in their reliance on glycolysis to maintain their energy requirements¹ despite the presence of mitochondria and tricarboxylic acid (TCA) cycle intermediaries.² This metabolic adaptation is thought in part to underpin their survival and antimicrobial function in tissues that are typically hypoxic.³⁻⁵ Despite their unique metabolism, little is known about the importance of flux between metabolic pathways in determining neutrophil survival responses. Recent work has demonstrated the importance of the hypoxia-inducible factor (HIF)/prolyl hydroxylase domain (PHD)-containing enzyme oxygen-sensing pathway in this regard, identifying both HIF-1 α and PHD3 as critical regulators of neutrophil survival in hypoxia,^{6,7} with the extended survival of neutrophils in hypoxia being dependent on HIF-1 α expression. In parallel, an expanding body of work has addressed the role of HIF-1 α in coordinating macrophage functional responses to proinflammatory mediators.⁸⁻¹¹ This work led to the observation that, in macrophages, lipopolysaccharide (LPS) causes an intracellular increase in succinate levels, resulting in HIF-1 α stabilization and enhanced interleukin-1 β signaling.¹¹ Subsequently, the metabolic rewiring of antimicrobial (M1) and tissue repair (M2) macrophages has been elucidated, with important consequences of TCA cycle activity and integrity for regulation of nitric oxide and N-glycosylation signaling, respectively.¹² Whether TCA cycle activity and succinate accumulation regulates HIF-1 α and hypoxic survival in neutrophils is unknown.

Patients with rare germ line mutations in genes encoding the TCA cycle enzyme succinate dehydrogenase (SDH) allow us to directly question the role of the TCA cycle and mitochondrial respiratory chain in neutrophil survival responses. SDH oxidizes succinate to fumarate in the TCA cycle and is a ubiquinone oxidoreductase, also functioning in complex II of the respiratory chain.¹³ SDH comprises four subunits (A-D), with inherited mutations of each of the subunits linked to the development of pheochromocytoma (PHEO) and paraganglioma (PGL) after somatic

inactivation of the wild-type allele and loss of heterozygosity.¹⁴⁻¹⁶ We questioned whether heterozygous germ line mutations in *SDHB* (*SDHBx*) would reduce SDH activity in the peripheral blood neutrophils of these patients, leading to accumulation of intracellular succinate, HIF-1 α stabilization, and a pseudohypoxic survival phenotype, given the importance of the B subunit for SDH catalytic function and its high prevalence within PHEO/PGL patient populations.^{13,17,18}

To determine whether succinate is implicated in regulating neutrophil survival responses, we isolated peripheral blood neutrophils from patients with heterozygous germ line *SDHBx* mutations in whom an increase in intracellular succinate would be predicted. In total, 20 individuals with frameshift, splice, missense, or nonsense mutations were studied (supplemental Table 1, available on the *Blood* Web site). Although all but 1 patient displayed plasma succinate levels within the normal range, a significantly higher plasma succinate level was observed in patients with *SDHBx* (Figure 1A). To confirm the consequence of *SDHB* mutations on intracellular succinate and to measure other TCA cycle and glycolytic intermediaries, peripheral blood neutrophils were isolated from 3 individuals with *SDHBx* and 3 healthy controls, and relative metabolite abundance was determined by gas chromatography–mass spectrometry (Figure 1B). Succinate was significantly more abundant in neutrophils isolated from patients with *SDHBx* than from controls. This finding was paralleled by increases in lactic acid and citric acid, but no changes in other TCA cycle intermediaries (α -ketoglutaric acid, fumaric acid, or malic acid) were observed. Thus, neutrophils heterozygous for mutant *SDHB* gene expression display the predicted elevation in intracellular succinate, but with no decrease in downstream TCA cycle intermediaries. Citric acid levels were increased, which may reflect an increase in biosynthetic requirements outside the TCA cycle. In keeping with the increased succinate in *SDHBx* neutrophils, a detectable increase in protein succinylation was also observed (Figure 1C).

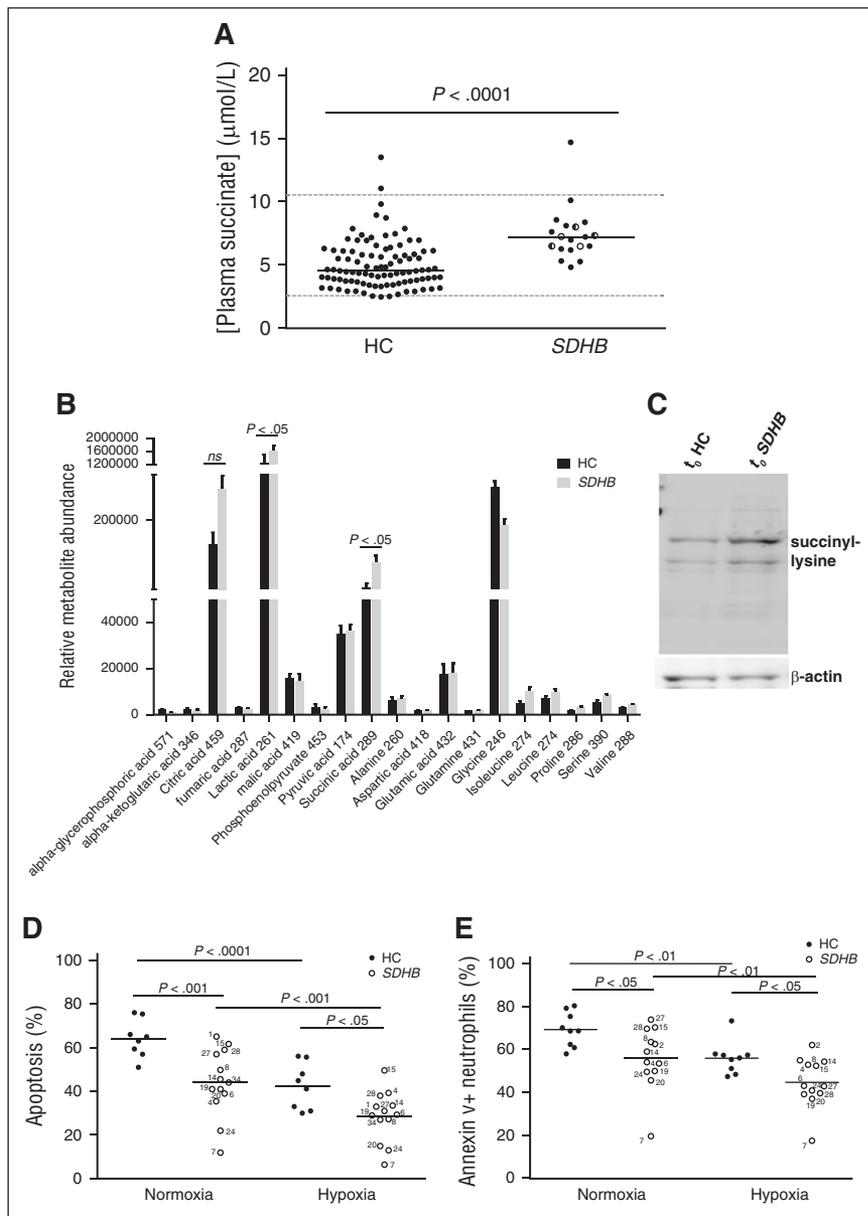
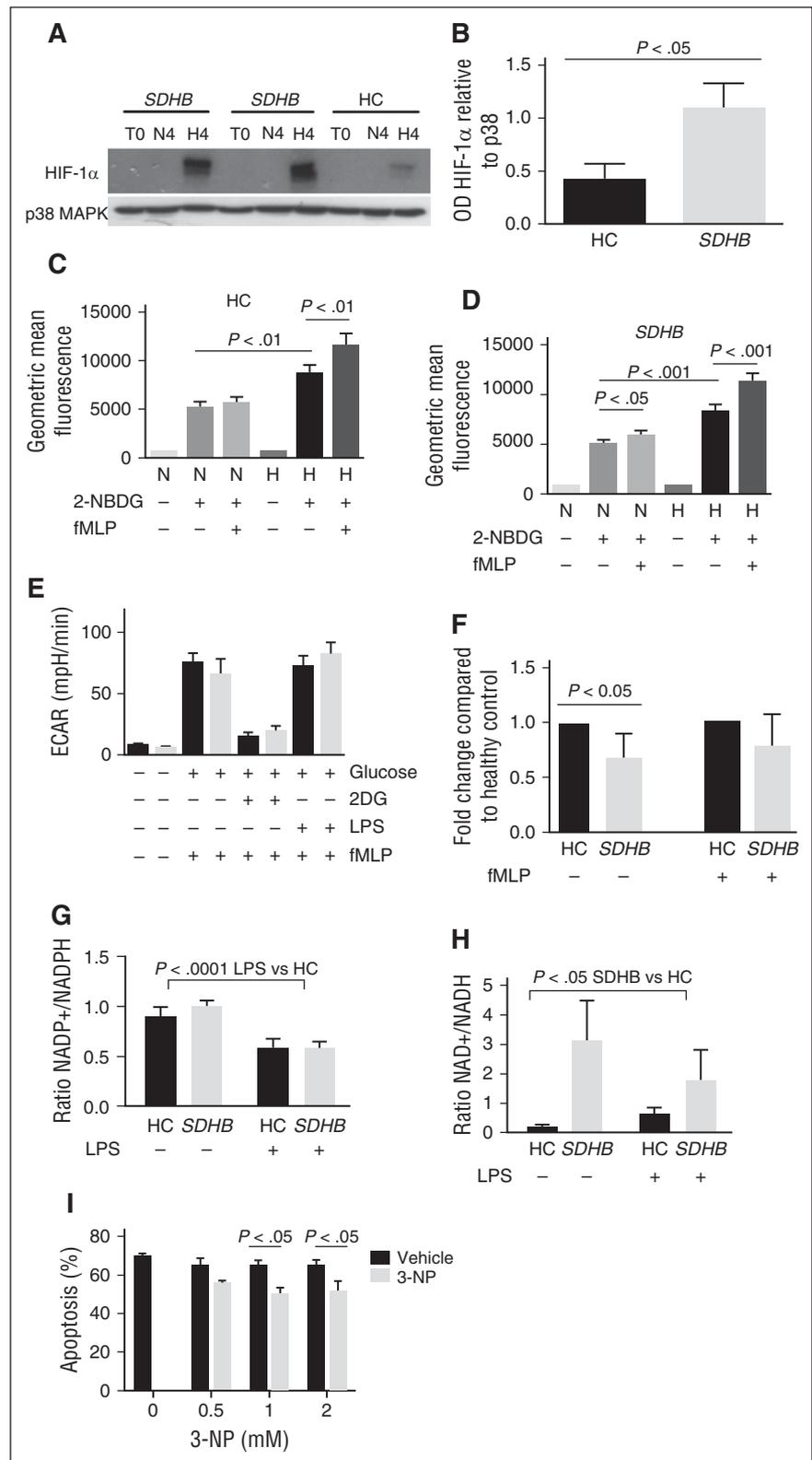


Figure 1. Heterozygous *SDHB* neutrophils display a specific metabolic signature and enhanced survival. (A) Plasma succinate concentrations were determined for 97 healthy controls (HC) and 19 patients with germ line mutations in *SDHB*. Filled, half-filled, and open circles represent patients without, with previous, and with current tumors, respectively. Solid lines represent median values, and dashed lines represent upper and lower limits of reference. (B-E) Human peripheral blood neutrophils from healthy controls (HC) and *SDHBx* patients were studied in parallel. (B) Relative metabolite abundance. Freshly isolated neutrophils were lysed in methanol, the liquid phase was subjected to gas chromatography–mass spectrometry, and relative quantification of 20 key metabolic intermediaries was performed. Data represent mean \pm SEM, $n = 3$. (C) Succinylation. Freshly isolated neutrophil lysates were separated by SDS-PAGE, and membranes were probed for succinylated protein expression relative to β -actin control; blot is representative of $n = 3$. (D-E) Apoptosis. Neutrophils were cultured for 20 hours in normoxia or hypoxia, and apoptosis was determined by morphology (D) and flow cytometry (Annexin V) (E). Solid bars represent mean; P values were determined by Mann-Whitney U test (A), unpaired Student t test (B), or 2-way ANOVA (D-E). ANOVA, analysis of variance; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SEM, standard error of the mean.

The consequence of *SDHB* heterozygosity for constitutive rates of neutrophil apoptosis and hypoxic survival responses was determined. *SDHBx* neutrophils displayed both reduced constitutive apoptosis and enhanced survival in hypoxia, as assessed both by cellular morphology (Figure 1D) and Annexin-V positivity (Figure 1E). Given the previous report of succinate-mediated HIF-1 α stabilization in bone marrow–derived macrophages,¹¹ and the importance of HIF-1 α for hypoxic neutrophil survival,⁶ we asked whether the reduced apoptosis in *SDHBx* neutrophils was secondary to increased HIF-1 α activity. HIF-1 α protein in *SDHBx* neutrophils was elevated in hypoxia (Figure 2A-B) but undetectable in normoxic cells, in which reduced rates of apoptosis were observed. Thus, the phenotype of enhanced neutrophil survival in the setting of *SDHB* heterozygosity occurs independently of HIF-1 α protein expression. In keeping with unaltered HIF-1 α activity in normoxic *SDHBx* neutrophils, we saw no alterations either in glucose uptake (Figure 2C-D), in which a hypoxic uplift is observed in healthy control cells, or in extracellular acidification rates, an

indirect measure of glycolytic activity (Figure 2E). Interestingly, we observed that neutrophils isolated from individuals with *SDHB* mutations displayed significantly reduced levels of oxidant stress (Figure 2F), a phenotype associated with enhanced neutrophil survival in chronic granulomatous disease patients.¹⁹ However, no differences in nicotinamide adenine dinucleotide phosphate (NADP)/reduced NADP (NADPH) redox ratios were detected between patients and controls, suggesting the *SDHB* phenotype to be independent of altered NADPH oxidase 2 activity (Figure 2G). This finding led us to question whether SDH was regulating apoptosis through its role as a mitochondrial ubiquinone oxidoreductase. *SDHBx* neutrophils demonstrated an increased ratio of oxidized to reduced nicotinamide adenine dinucleotide (NAD) (Figure 2H), and treatment of healthy human neutrophils with the irreversible SDH inhibitor 3-nitropropionic acid reduced constitutive neutrophil apoptosis (Figure 2I), thus implicating impaired mitochondrial complex II and compensatory changes in the electron transport chain in the enhanced survival of *SDHB*-mutant neutrophils.

Figure 2. *SDHB*x neutrophil survival is independent of HIF-1 α expression and linked to uncoupling of the mitochondrial electron transport chain. Human peripheral blood neutrophils from healthy controls (HC) and *SDHB* patients were studied in parallel. (A-B) HIF-1 α protein expression. Freshly isolated neutrophils and neutrophils aged for 4 hours in normoxia (N4) or hypoxia (H4) were lysed and separated by SDS-PAGE, the membranes were probed for HIF-1 α and p38 MAPK expression, and densitometry on hypoxic samples was performed. Representative blot shown (A), with mean densitometry \pm SEM, n = 6 (B). (C-D) Glucose uptake in healthy controls (C) and *SDHB* patients (D). Neutrophils were preincubated in glucose-free phosphate-buffered saline in normoxia (N) or hypoxia (H) for 1 hour before stimulation with 100 nM *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) in the presence of 200 μ M 2-(*N*[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)-2-deoxyglucose (2-NBDG) for 20 minutes. Uptake was determined by flow cytometry (FL1 geometric mean fluorescence). Data represent mean \pm SEM, n = 5. (E) Glycolytic capacity in healthy controls (filled) and *SDHB* patients (open). Neutrophils were cultured with or without glucose, 2-deoxy-D-glucose (2DG; 10 mM), or LPS (1 mg/mL) for 2 hours before stimulation with 100 nM fMLP, and peak extracellular acidification rates were determined by Seahorse. Data represent mean \pm SEM, n = 4. (F) Intracellular reactive oxygen species. 2',7'-Dichlorofluorescein fluorescence was quantified after 45-minute neutrophil culture in the presence or absence of fMLP (100 nM), and fold change in patient neutrophil fluorescence was calculated relative to healthy controls. (G-H) Electron transport. Ratios of oxidized to reduced NADP (G) and NAD (H) were measured by fluorometric enzyme cycling assay in freshly isolated and aged neutrophils (6 hours). (I) Apoptosis. Neutrophils were cultured for 20 hours in the presence or absence of 3-nitropropionic acid (3-NP; 0-2 mM), and apoptosis was determined by morphological appearance; data represent mean \pm SEM, n = 4. *P* values were determined by unpaired (B), paired (C, D, and I), or 1-sample (F) Student *t* tests, or by 2-way ANOVA (G-H). ECAR, extracellular acidification rate; OD, optical density.



These studies use a valuable patient group with a specific mutation in *SDHB* as an experimental system in which to delineate the role of the TCA cycle and mitochondrial respiratory chain in neutrophil survival responses. It provides the first description of elevated intracellular succinate levels in neutrophils isolated from

patients with heterozygous mutations in *SDHB* and the first evidence of a dysfunctional TCA cycle in resting-state peripheral blood neutrophils. In marked contrast to the role of succinate in facilitating HIF-1 α -dependent inflammatory responses in LPS-stimulated macrophages, we dissociate enhanced neutrophil survival from HIF-1 α

stabilization in the context of germ line mutations in SDH, linking it instead to a phenotype of impaired mitochondrial complex II function and reduced oxidative stress. Taken together, this work identifies key metabolic differences between neutrophils and macrophages and raises further important questions as to the metabolic control of neutrophil function and survival. Future work dissecting the consequences of *SDHB* mutations for neutrophil host-pathogen responses and inflammation resolution will be key in this regard.

*R.J. and K.E.M. contributed equally to this study.

†J.N.-P. and S.R.W. contributed equally to this study.

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