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Mutations in Succinate Dehydrogenase B (*SDHB*) enhance neutrophil survival independent of HIF-1alpha expression.

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Neutrophils are unusual in their reliance on glycolysis to maintain their energy requirements[1] despite the presence of mitochondria and TCA cycle intermediaries[2]. This metabolic adaptation is thought in part to underpin their survival and anti-microbial 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 HIF/PHD oxygen-sensing pathway in this regard, identifying both HIF-1α and PHD3 as critical regulators of neutrophil survival in hypoxia[6,7], with the extended survival of neutrophils in hypoxia being dependent upon HIF-1α expression. In parallel, an expanding body of work has addressed the role of HIF-1α in coordinating macrophage functional responses to pro-inflammatory mediators[8-11]. This work led to the observation that, in macrophages, LPS causes an intracellular increase in succinate levels resulting in HIF-1α stabilization and enhanced IL-1β signaling[11]. Subsequently, the metabolic re-wiring of anti-microbial (M1) and tissue repair (M2) macrophages has been elucidated, with important consequences of TCA cycle activity and integrity for regulation of NO and N-glycosylation signaling respectively[12]. Whether TCA cycle activity and succinate accumulation regulates HIF-1α activity and hypoxic survival in neutrophils is unknown.

Patients with rare germline 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 oxidises 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 PHAEO and PGL following somatic inactivation of the wild type allele, and loss of heterozygosity[14-16]. We questioned whether heterozygous germline mutations in *SDHB (SDHBx)* would reduce SDH activity in the peripheral blood neutrophils of these patients, leading to accumulation of intracellular succinate, HIF-1α stabilization and a pseudo-hypoxic survival phenotype given the importance of the B subunit for SDH catalytic function and its high prevalence within PHAEO/PGL patient populations[13,17,18].

SDHB mutations enhance neutrophil survival

To determine whether succinate is implicated in regulating neutrophil survival responses we isolated peripheral blood neutrophils from patients with heterozygous germline *SDHBx* mutations in whom an increase in intracellular succinate would be predicted. In total, 20 individuals were studied with a combination of frame-shift, splice and non-sense mutations (Supplementary Table 1). Although all but one 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 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 determined by gas chromatography-mass spectrometry (Figure 1b). Succinate was significantly more abundant in neutrophils isolated from patients with *SDHBx* than controls. This 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). Thus neutrophils hetezygous 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 out-with the TCA cycle. In keeping with the increased succinate in *SDHBx* neutrophils, a detectable increase in protein succinylation was also observed (Figure 1c).

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-1α stabilization in BMDMs[11], and the importance of HIF-1α for hypoxic neutrophil survival[6], we asked whether the reduced apoptosis in *SDHBx* neutrophils was secondary to increased HIF-1α activity. HIF-1α 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-1α protein expression. In keeping with unaltered HIF-1α 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 CGD patients[19]. However, no differences in NADP/NADPH redox ratios were detected between patients and controls suggesting the SDHB phenotype to be independent of altered NOX2 activity (Figure 2g). This 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 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.

These studies utilize 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 intra-cellular 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-1α–dependent inflammatory responses in LPS-stimulated macrophages, we dissociate enhanced neutrophil survival from HIF-1α stabilization in the context of germline mutations in SDH, linking it instead to a phenotype of impaired mitochondrial complex II function and reduced oxidative stress. Taken together, his 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.

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Authorship

Contribution: R.J, K.M., J.A.W., D.S., A.H., A.A.R.T., R.S.D., T.P., A.L., and P.S. performed the research; E.D., and J.N-P., identified and recruited patients; R.J., K.M., J.A.W., B.G., B.K. interpreted the data; and P.C., J.N-P., M.K.B.W. and S.R.W. designed the research and wrote the manuscript.

Conflict of Interest

The authors declare no competing financial interests.

The online version of this article contains a data and methods supplement.

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Figure Legends

Figure 1. Heterozygous *SDHB* neutrophils display a specific metabolic signature and enhanced survival.

(A) Plasma succinate concentrations were determined for 97 controls and 19 patients with germline mutations in *SDHB*. Filled, half-filled and open circles represent patients without, with previous, and with current tumours respectively. Solid lines represent median values and dashed lines upper and lower limits of reference. (B-E) Human peripheral blood neutrophils from healthy controls (HC, filled) and *SDHBx* patients (open) were studied in parallel. (B) Relative metabolite abundance. Freshly isolated neutrophils were lysed in methanol and the liquid phase subjected to gas chromatography-mass spectrometry, and relative quantification of 20 key metabolic intermediaries performed. Data represents mean +/- SEM, n=3. (C) Succinylation. Freshly isolated neutrophil lysates were separated by SDS-PAGE and membranes probed for succinylated protein expression relative to β-actin control, blot representative of n=3. (D,E) Apoptosis. Neutrophils were cultured for 20 hours in normoxia or hypoxia and apoptosis 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 t-test (B) or 2-way ANOVA (D,E).

Figure 2. *SDHBx* neutrophil survival is independent of HIF-1α expression and linked to uncoupling of the mitochondrial electron transport chain.

Human peripheral blood neutrophils from healthy controls (HC, filled) and SDHB patients (open) were studied in parallel. (A,B) HIF-1α protein expression. Freshly isolated neutrophils and neutrophils aged for 4 hours in normoxia (N) or hypoxia (H) were lysed, separated by SDS-PAGE, membranes probed for HIF-1α and p38 MAPK expression and densitometry on hypoxic samples performed. Representative blot shown (A) with mean densitometry +/- SEM, n=6 (B). (C,D) Glucose uptake. Neutrophils were pre-incubated in glucose-free PBS in normoxia (N) or hypoxia (H) for 1 hour prior to stimulation with 100nM fMLP in the presence of 200μM 2-NBDG for 20 mins. Uptake was determined by flow cytometry (FL1 geometric mean fluorescence). Data represents mean +/- SEM, n=5. (E) Glycolytic capacity. Neutrophils were cultured +/- glucose, 2DG (10mM) or LPS (1mg/ml) for 2 hours prior to

stimulation with 100nM fMLP and peak extracellular acidification rates determined by Seahorse. Data represents mean +/- SEM, n=4. (F) Intra-cellular ROS. DCF fluorescence was quantified following 45 mins neutrophil culture in the presence or absence of fMLP (100 nM), and fold change in patient neutrophil fluorescence calculated relative to healthy controls. (G,H) Electron transport. Ratios of oxidized to reduced NADP (G) and NAD (H) were measured by fluorimetric 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 3NP (0-2 mM) and apoptosis determined by morphological appearance, data represents mean +/- SEM, n=4. *P* values were determined by unpaired (B), paired (C,D,I) or one-sample (F) t-tests or 2-way ANOVA (G,H).

Figures

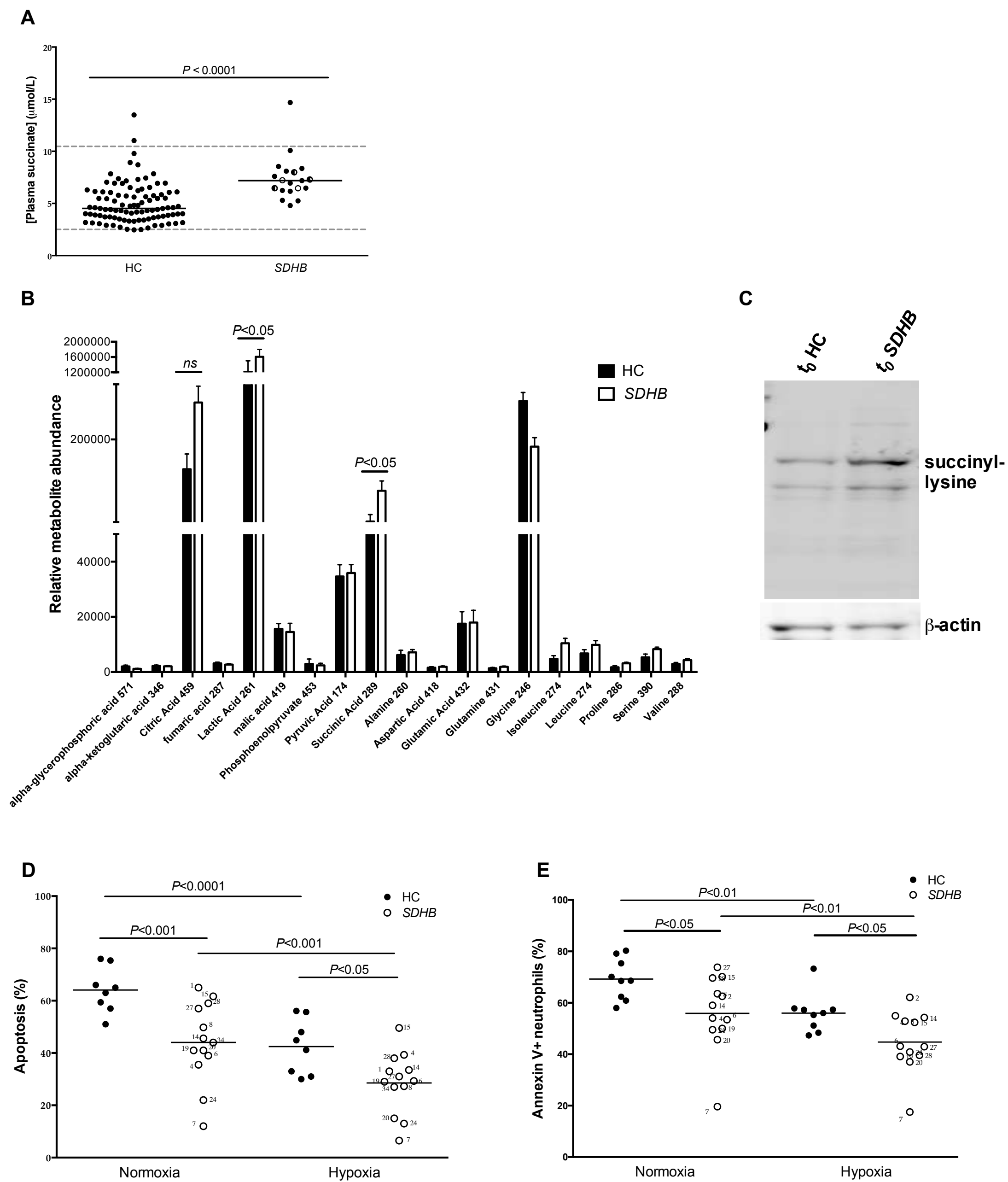

Figure 1

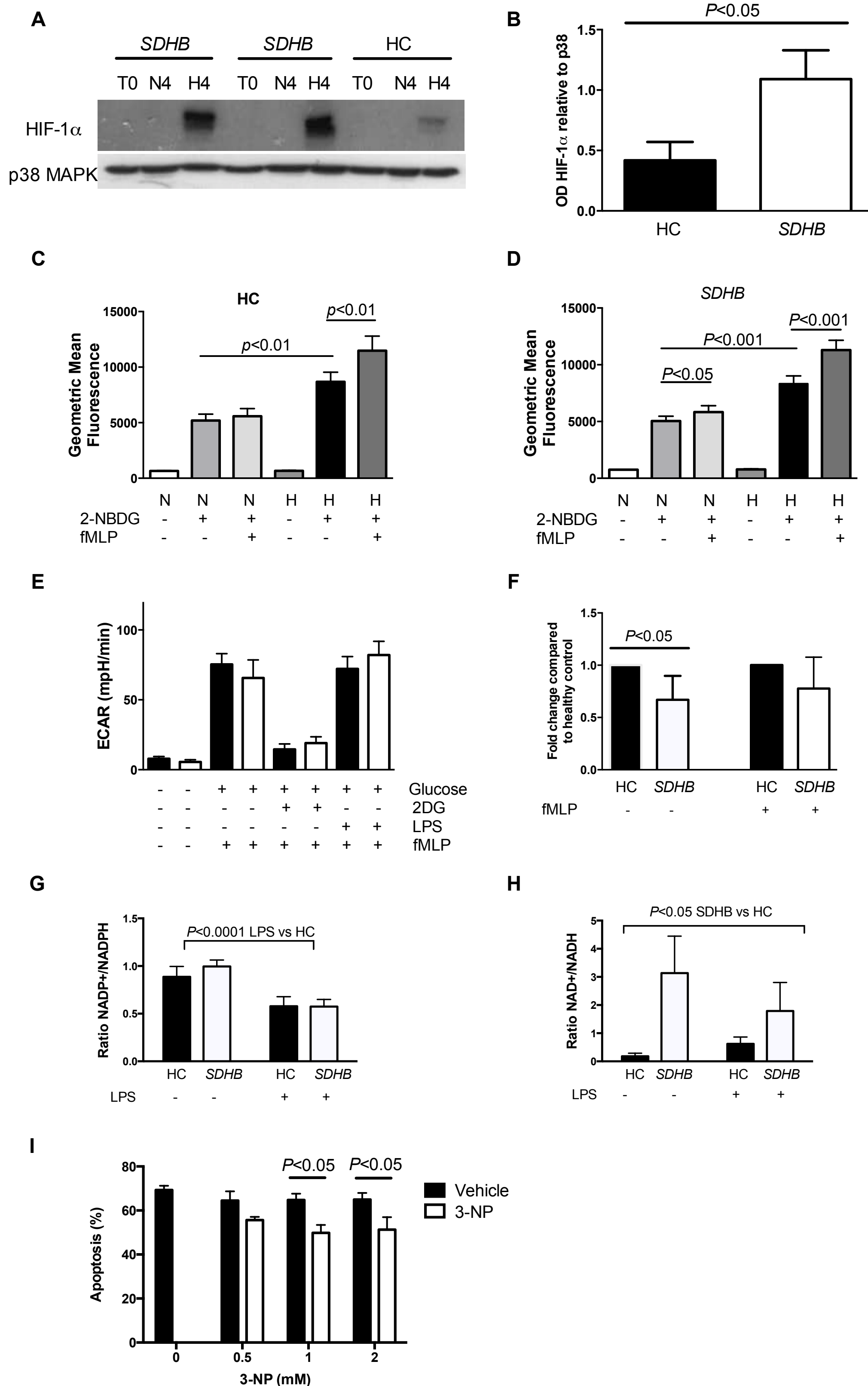

Figure 2