INTRODUCTION

Human eosinophils reside primarily in haematopoietic and mucosal tissues. Interest in eosinophil activity stems predominantly from their role in antiparasite immunity and allergic disease, but there is growing interest in their role in tissue homeostasis, especially adipose tissue. Eosinophil-mediated effector function involves degranulation, the release of antimicrobial cytotoxic molecules and respiratory burst, yet we know little about the immunometabolic processes that underpin these activities. Activation of other granulocyte populations such as neutrophils and mast cells enhances glycolysis to support biosynthetic intermediate production and rapid ATP generation. Eosinophil metabolism is assumed to be largely homologous to that of neutrophils, where, despite the presence of mitochondria, energy production stems primarily from glycolysis.
Nonmetabolic roles for mitochondria within eosinophils have been the focus of several investigations. Mitochondrial DNA can be released in a "catapult-like" fashion from eosinophils and contributes to antibacterial defence, although this remains controversial and has yet to be confirmed. Furthermore, the initiation of apoptosis has been reported as an alternative role to respiration for eosinophil mitochondria. As eosinophils produce large amounts of nicotinamide adenine dinucleotide phosphate oxidase (NOX2)-dependent extracellular reactive oxygen species (ROS) upon activation, it is commonly thought that oxygen consumption by eosinophils supports ROS production rather than oxidative phosphorylation (OXPHOS). Contrary to this, human eosinophils are sensitive to oligomycin (mitochondrial ATP synthase inhibitor), suggesting that in addition to glycolysis, mitochondria can indeed contribute, at least in part, to ATP production. As such, the role of the mitochondria in eosinophils remains unclear and requires investigation.

Glycolysis has been reported to be the main source of ATP in numerous cell types. This is especially true for immune cells such as T cells and mast cells, which undergo a glycolytic switch upon activation to support rapid ATP production. Little is known about the role of glycolysis in eosinophil-mediated immunity, but glycolysis-derived ATP is essential for the removal of schistosomula by human eosinophils, and cytokines such as IL-3, IL-5, granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNFα stimulate glucose uptake in these cells. The anti-apoptotic cytokines IL-3, IL-5 and GM-CSF, produced primarily by T-cell subsets, fibroblasts and epithelial cells, are critical for eosinophil activation and maturation. Differential effects of IL-3, IL-5 and GM-CSF have been identified, with IL-3 generally being a weaker inducer of eosinophil activation than either IL-5 or GM-CSF; IL-3 induces less glucose uptake, superoxide production and eosinophil-derived neurotoxin (EDN) release than IL-5 or GM-CSF. However, IL-3 can prolong ribosomal protein S6 signalling compared to IL-5 and GM-CSF, producing augmented levels of semaphorin-7A and heightened protein translation. Despite these differences in responses of eosinophils to IL-3, IL-5 and GM-CSF, the impact of these cytokines on human eosinophil metabolic adaptation that underpins these different functional outcomes has not been studied.

Here, for the first time, we demonstrate that human eosinophils are metabolically plastic cells, up-regulating both glycolytic and TCA cycle intermediates upon activation. We show that IL-3, IL-5 and GM-CSF all increase glycolysis, and importantly, that upon activation by these cytokines, eosinophils increase glutaminolysis and subsequent TCA cycling. This is significant as these cells were previously thought not to engage their mitochondria for metabolic purposes. In contrast to earlier studies, we report that the IL-5-induced metabolic switch initiates glycolysis and enhances mitochondrial respiration in a mechanism that is dependent on the STAT5/PI3K/Akt axis. Finally, the ability of eosinophils to compensate for inhibition of ROS production and the associated reduced levels of TCA cycle intermediates by increased aerobic glycolysis highlights their metabolic plasticity.

2 MATERIALS AND METHODS

2.1 Human eosinophil isolation

Human peripheral blood was collected from both male (n = 9) and female donors (n = 25) aged between 18 and 70 years into heparinized
Vacuettes™ (Greiner Bio-one). We recruited both atopic and nonatopic donors with eosinophils comprising between 1% and 8% of total circulating leucocytes. Specific donor demographics can be found in Table S1. All samples were collected with informed written consent, and ethical approval was obtained from Wales Research Ethics Committee 6 (13/WA/0190). Eosinophils were isolated by negative selection using immunomagnetic microbeads (autoMACS; Miltenyi Biotec). Detailed Materials and Methods can be found in the Appendix S1.

3 | RESULTS

3.1 | Eosinophils increase glycolysis in response to cytokines

In human eosinophils, IL-3, IL-5 and GM-CSF are the predominant cytokines associated with their activation.25 Therefore, the effect of IL-3, IL-5 and GM-CSF on eosinophil metabolism was investigated.

First, we investigated the mode of glucose transport in eosinophils. Gene expression levels of the main glucose transporters (GLUT1-4; SLC2A1-4) were determined using qPCR. While there was donor variability, GLUT1 (SLC2A1) and GLUT3 (SLC2A3) were expressed by all donors (Figure 1A). GLUT4 (SLC2A4) expression was not detected, and only some donors expressed detectable GLUT2 (SLC2A2; 3/7) (Figure 1A). Uptake of a fluorescent glucose analogue 2-N-glucosamine, and only some donors expressed detectable (SLC2A2; Miltenyi Biotec). Detailed Materials and Methods can be found in the Appendix S1.

3.2 | Cytokine-stimulated eosinophils consume oxygen for ROS production

It has been reported that eosinophils do not require their mitochondria for ATP production via OXPHOS.10,30 However, mitochondria are diverse organelles with multiple metabolic roles. We confirmed the presence of mitochondria using transmission electron microscopy (Figure 2A). Mitochondrial function was assessed by measuring oxygen consumption rate (OCR) in the presence of IL-3, IL-5 or GM-CSF (Figure 2B). Baseline OCR was increased in IL-5- and GM-CSF-treated cells postglucose starvation compared to the control and IL-3-treated cells (Figure 2C). We noted a decrease in OCR upon oligomycin treatment under all conditions, and this is indicative of oxygen consumption for ATP generation, which is in agreement with a previous study.5 Increasing the concentration of IL-3 delivered to the cells caused an increase in both ECAR and OCR (50 and 100 ng/mL; Figure 2D and E) suggestive of a differential kinetic response of eosinophils to IL-3 vs IL-5/GM-CSF.

In our experiments, eosinophils clearly consume oxygen, especially in response to treatment with IL-5 or GM-CSF. However, oxygen consumption can occur independently of OXPHOS for processes such as respiratory burst, involving the generation of ROS and subsequently hydrogen peroxide via NOX enzymes.13,14 To determine whether any of the cytokine-induced oxygen consumption was due to mitochondrial ROS production, we utilized the mitochondrial superoxide indicator MitoSOX and selected a time point to coincide with the extracellular flux assay (15 minutes). Regardless of the cytokine used for stimulation, mitochondria did not contribute to oxygen consumption via ROS production at the time point measured; rotenone was used as a positive control (Figure 2F and G).

To further investigate increased oxygen consumption upon stimulation, we investigated whether IL-3, IL-5 and GM-CSF induce total ROS production. We determined total oxidative stress levels using the fluorescent probe CellROX and flow cytometry, and phorbol 12-myristate 13-acetate (PMA) was used as a positive control. All three cytokines induced ROS production in comparison with the CellROX control with GM-CSF being the most potent (Figure 2H and I). Using the inhibitor diphenyleneiodonium (DPI), these responses were shown to be NOX-dependent, although this was only significant for GM-CSF and PMA-induced ROS production. These data raise the question of what happens to OCR when ROS production is inhibited, and we have addressed this in relation to IL-5 later in the manuscript, see Figure 5.
3.3 | Cytokine-stimulated eosinophils synthesize TCA cycle intermediates

In addition to the generation of ATP and ROS, mitochondria can act as biosynthetic hubs, synthesizing TCA cycle intermediates and nonessential amino acids; however, this has not been previously demonstrated in eosinophils. To test this, eosinophils were activated with IL-3, IL-5 or GM-CSF (10 ng/mL) for 4 hours. Upon activation, eosinophils incorporated $^{13}$C$_6$-glucose into TCA cycle intermediates, such as citrate, succinate, malate and fumarate (Figure 3A-E). The metabolite pools analysed were mostly composed of the unlabelled (m + 0) or m + 2 mass isotopologue indicating lack of sustained TCA cycling.

Next, we determined whether TCA cycle intermediates are used as precursors for the synthesis of nonessential amino acids. Glutamate abundance was increased upon eosinophil stimulation with IL-3, IL-5 or GM-CSF (Figure 3G) and was largely present as the m + 2 mass isotopologue (Figure 3H). While the eosinophils demonstrated production of glutamine and aspartate at baseline, cytokine stimulation had no further effect on the production of these. However, in comparison with the untreated control,
cytokine-stimulated eosinophils had a reduced pool of $^{12}$C unlabelled amino acids, indicating consumption of these amino acids (Figure S2A-D).

Fully functional canonical TCA cycling requires two metabolite inputs: acetyl-CoA derived primarily from glucose and $\alpha$-ketoglutarate derived from glutamine (Figure S3A). Having established that eosinophils incorporate $^{13}$C-glucose into TCA cycle intermediates, we next wanted to determine whether cytokine-activated eosinophils engage glutaminolysis. To address this, eosinophils were activated with IL-3, IL-5 or GM-CSF in the presence of $^{13}$C-glutamine for 4 hours. Incorporation of $^{13}$C into TCA intermediates was increased in IL-3, IL-5 or GM-CSF treated eosinophils compared to the untreated controls (Figure S3B).

### 3.4 The STAT5/PI3K/Akt axis governs the immediate metabolic response to IL-5

The development and clinical implementation of IL-5 targeting therapies in the treatment of asthma$^{31}$ prompted us to consider the early signalling mechanisms that govern increased ECAR and OCR in response to IL-5 treatment. STAT5 is activated upon IL-3, IL-5 or GM-CSF ligation$^{32,33}$ and in certain circumstances can be activated by ROS production via the common $\beta$ chain.$^{34}$ We initially confirmed STAT5 phosphorylation in eosinophils treated with IL-3, IL-5 and GM-CSF. All cytokines induced STAT5 phosphorylation, but this only reached significance above baseline for IL-5 and GM-CSF (Figure 4A). Next, we wanted to determine whether inhibition of STAT5 affected the immediate ECAR...
**FIGURE 3** IL-3, IL-5 or GM-CSF treatment induces the production of TCA cycle intermediates. A, Schematic of uniformly labelled \(^{13}\)C\(_6\)-glucose incorporation into TCA cycle intermediates. Eosinophils were activated with IL-3, IL-5 or GM-CSF (10 ng/mL) for 4 h. Relative abundance of \(^{12}\)C and \(^{13}\)C (B) citrate, (C) succinate, (D) malate and (E) fumarate. F, Mass isotopologue distribution (MID) of m + 2 citrate, succinate and malate. G, Relative abundance of \(^{12}\)C and \(^{13}\)C glutamate including the (H) MID distribution. All data are from 3-6 independent experiments with each data point representing an individual donor. Data are expressed as mean ± SEM. Statistical analysis was performed using a two-way ANOVA; * \(P \leq .05\), ** \(P \leq .01\), *** \(P \leq .001\)

**FIGURE 4** The STAT5/PI3K/Akt axis is responsible for the metabolic switch in IL-5-treated eosinophils. A, Representative immunoblot of eosinophils treated for 15 minutes with IL-3, IL-5 or GM-CSF (10 ng/mL) for pSTAT5\(^{694}\) and \(\beta\)-actin. Corresponding densitometry analysis of pSTAT5 normalized to \(\beta\)-actin. B, ECAR and (C) OCR before and following addition of a STAT5 inhibitor (STAT5i; N-[(4-oxo-4H-chromen-3-yl)methylene]nicotinohydrazide; 100 µmol/L), IL-5 (10 ng/mL) and 2-DG (100 mmol/L), including "precytokine" activation and "postcytokine" activation pooled OCR and ECAR data. D, ECAR and (E) OCR before and following addition of a PI3K inhibitor (LY294002; 10 µmol/L) or Akt1/2 inhibitor (1,3-dihydro-1-[1-(4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl)phenyl)methyl]-4-piperidinyl]-2H-benzimidazol-2-one trifluoroacetate salt hydrate; 10 µmol/L), IL-5 (10 ng/mL) and 2-DG (100 mmol/L). Data are expressed as mean ± SEM of 5 (A), 2-3 (B-C) and 4 (D-E) independent experiments with each data point representing an individual donor. Statistical analysis was performed using a Friedman test with Dunn's multiple comparisons (A) or a two-way ANOVA with Sidak's multiple comparison test (B-E); * \(P \leq .05\), ** \(P \leq .01\), *** \(P \leq .001\)
FIGURE 5 DPI inhibits oxidative metabolism in IL-5-stimulated eosinophils. A, ECAR (mpH/min) and (B) OCR (pmoles/min) of eosinophils treated with IL-5 (10 ng/mL) ± DPI (100 nmol/L), glucose (5.5 mmol/L), oligomycin (1 µmol/L) and 2-DG (100 mmol/L). Eosinophils were activated with IL-5 (10 ng/mL) ± DPI (100 nmol/L) for 4 h in the presence of 13C-glucose. C, Relative abundance of 12C and 13C and (D) mass isotopologue distribution (MID) of glycolytic intermediates pyruvate and lactate. E, Relative abundance of 12C and 13C and (F) MID of TCA cycle intermediates citrate, succinate, fumarate and malate. G, Relative abundance of 12C and 13C and (H) MID of amino acids glutamate and aspartate. Data are expressed as mean ± SEM of 4 (A-B) and 3 (C-H) independent experiments with each data point representing an individual donor. Statistical analysis was performed using an unpaired t test (B) or a two-way ANOVA (C-H); *P ≤ .05, **P ≤ .01, ***P ≤ .001
and OCR responses of eosinophils treated with IL-5. Pretreatment with the STAT5 inhibitor N’-((4-oxo-4H-chromen-3-yl)methylene)nicotinohydrazide (STAT5i) completely abrogated the ECAR and OCR response in IL-5-stimulated eosinophils (Figure 4B and C). Calculations of “precytokine” and “postcytokine” data can be found at Figure S4.

In addition to cytokine-mediated STAT5 activation, both IL-5 and ROS can activate the PI3K/Akt axis; therefore, we next investigated the role of PI3K/Akt in human eosinophil metabolism. Treatment with either the PI3K inhibitor LY294002 or the Akt1/2 inhibitor abrogated IL-5-stimulated induction of ECAR (Figure 4D). The same trend was observed for OCR whereby the PI3K inhibitor reduced the immediate induction of OCR in eosinophils treated with IL-5 (Figure 4E). However, treatment with the Akt1/2 inhibitor did not reduce IL-5-induced OCR (Figure 4E), suggesting other downstream PI3K pathways may be involved. These data show that one of the key immediate effects of IL-5 on eosinophils is up-regulation of glycolysis, and this is dependent on the STAT5/PI3K/Akt axis.

3.5 | ROS inhibition reduces TCA cycling of IL-5-stimulated eosinophils

To determine whether the observed cytokine-stimulated metabolic changes in eosinophils were promoted by ROS production, we next determined whether NOX had a role in increased ECAR and OCR with a focus on IL-5 as before. Bioenergetic analyses were used to show that DPI had no effect on IL-5-stimulated glycolysis (Figure 5A) but significantly reduced peak OCR (Figure 5B). SITA using 13C-glucose showed increased incorporation of 13C into pyruvate and lactate (indicated as an increased m + 3 mass isotopologue) in the presence of DPI (Figure 5C and D). This was accompanied by a reduction in the relative abundance of all TCA cycle intermediates (Figure 5E), represented by a decreased abundance of the m + 2 mass isotopologue (Figure 5F). DPI treatment negatively impacted on the synthesis of amino acids glutamate and aspartate, from 13C-glucose, by reducing 13C incorporation and the m + 2 mass isotopologue (Figure 5G and H). Collectively, these data demonstrate that NOX-mediated ROS may have a critical role in driving mitochondrial metabolism.

4 | DISCUSSION

The study of eosinophil metabolism has been challenging, but recent years have seen the introduction of novel, refined technologies that allow metabolic analyses on low cell numbers with more sensitive readouts. This has been driven by the burgeon of the field of immunometabolism and the increasingly recognized role of cellular metabolism in immune cell fate and function. Cellular metabolism through energy production (ATP) and biosynthetic intermediate generation orchestrates numerous effector roles such as cytokine production, migration, and proliferation and can have a profound impact on various human pathologies. Aside from their well-recognized energetic and biosynthetic roles, individual metabolites can have alternative roles. For example, TCA cycle metabolites, succinate and fumarate act as inflammatory signalling molecules. In LPS-stimulated macrophages, succinate stabilizes hypoxia-inducible factor-1α to promote increased glycolysis and IL-1β production. Therefore, elucidating the cellular metabolic response of eosinophils not only improves our basic understanding of eosinophil function, especially how it might apply to tissue homeostasis, but also has implications for revealing immunopathogenetic and therapeutic strategies in eosinophilic disorders.

The rapid engagement of aerobic glycolysis by eosinophils in response to cytokines demonstrated here was accompanied by accumulation of both intra- and extracellular lactate. Lactate creates an acidic environment in which eosinophils are known to thrive, such as in the lung. Furthermore, excess lactate retains T cells in pro-inflammatory environments, curtailing their migration. If the same occurred for eosinophils, this would provide a mechanism to retain viable eosinophils in an acidic inflammatory tissue environment. This increased glycolytic rate that supports the accumulation of lactate is presumably due to either GLUT1- or GLUT3-mediated glucose uptake as these transporters were expressed by all donors or through kinetic effects on the direct phosphorylation of glycolytic enzymes.

A key feature of the work presented here is clarity surrounding the role of mitochondria in eosinophil metabolism. It is well established that eosinophils utilize their mitochondria for apoptotic purposes; however, definitive metabolic contributions have remained elusive. Here, we confirmed that cytokine-stimulated eosinophils were sensitive to oligomycin treatment through a decrease in OCR. This indicates that mitochondria in eosinophils have an important role in mediating metabolic responses to cytokines which are in agreement with a previous study.

While the conversion of glucose to lactate seems to be the predominant mitochondrial pathway in response to cytokine stimulation, we used stable isotope tracing to show that eosinophils use both glucose and glutamine to generate TCA cycle intermediates and support OXPHOS upon activation. To our knowledge, we are the first to provide evidence that carbons from glucose and glutamine are incorporated into TCA metabolites upon cytokine stimulation in eosinophils. Collectively, we reveal a novel role for human eosinophil mitochondria that extends beyond apoptosis and antibacterial defence. We demonstrate that eosinophils can utilize their mitochondria for TCA cycling contributions to OXPHOS and biosynthesis of amino acids. In support of a role for mitochondrial metabolism in eosinophils as we described here, a recent study indicated that peripheral blood eosinophils have increased oxidative parameters in comparison with neutrophils. However, this interpretation was based solely on decreased oxygen consumption upon exposure to oligomycin and did not definitively characterize the metabolic fuels consumed by eosinophils.

The effects of IL-3, IL-5 and GM-CSF on eosinophil metabolism were broadly similar. To better understand the signalling processes that govern cytokine-mediated changes to eosinophil cellular metabolism, we chose to focus on a single cytokine. IL-5 was chosen as it is a therapeutic target for treating eosinophilic asthma via monoclonal antibodies to IL-5 itself or IL-5Ra. IL-5 ligation in human eosinophils has been shown to activate the JAK/STAT pathway, specifically STAT5. With use of a specific STAT5 inhibitor, we determined that increases in
both ECAR and OCR upon IL-5 stimulation were dependent on STAT5 signalling. Because activated eosinophils increased their glucose utilization substantially, our attention turned to the PI3K/Akt axis as it is known to control glycolysis in other immune cell types such as T cells and macrophages.\textsuperscript{42,43} PI3K and Akt inhibitors had a profound effect on the IL-5-mediated metabolic switch, especially glycolysis, showing that the IL-5-induced metabolic switch in human eosinophils is mediated by the STAT5/PI3K/Akt signalling axis. The IL-5-induced OCR was abrogated with PI3K inhibition but not Akt. This suggests that there are alternative downstream PI3K pathways contributing to increased oxygen consumption, such as the PI3K/Rac pathway.\textsuperscript{44} Respiratory burst in eosinophils has been closely linked previously with the Rac pathway, thus offering a plausible explanation for our observations.\textsuperscript{13} Elucidating roles of multiple Akt-independent downstream PI3K targets and their contributions to eosinophil metabolism warrants further investigation.

Finally, we considered the link between ROS production and metabolic pathway activity in eosinophils again focussing on the effects of IL-5. Treatment of eosinophils with IL-5 has been shown to induce ROS production,\textsuperscript{23} and here, we show that IL-5 increases oxygen consumption. As NOX-dependent respiratory burst is a fundamental effector function of eosinophils,\textsuperscript{14} we sought to investigate the role of ROS in eosinophil metabolism. Inhibiting NOX-dependent ROS production reduced the abundance of TCA cycle intermediates while increasing the accumulation of glucose-derived lactate suggesting that ROS may be a driver of eosinophil mitochondria metabolism in particular. This highlights that different bioactive molecules in the immediate microenvironment of eosinophils shape their metabolic plasticity.

Our study outlines the metabolic requirements of mitochondria in cytokine-activated eosinophils. We also show that ROS may enable metabolic plasticity. Taken together, this provides further insight into the mechanistic control of eosinophil function. It is likely that terminally differentiated cells such as eosinophils do not require extensive energy production and biosynthesis to support homeostasis or activation. Instead, multiple cytokines and important mediators such as eosinophil-derived neurotoxins and peroxidases are contained within preformed granules. However, cytokine-mediated activation clearly up-regulates cytoplasmic and mitochondrial metabolic pathways. This raises further questions about the links between eosinophil function and metabolism including the bioenergetic demands of piecemeal degranulation and the effects of mitochondrial DNA release on the metabolic status of eosinophils. Eosinophils are a characteristic feature of type 2 immune responses linked to immunopathology in asthma and other inflammatory disorders but also to tissue defence and repair processes in helminthic parasite infection\textsuperscript{45} and in other settings including metabolic homeostasis in adipose tissue.\textsuperscript{6} Greater understanding of the regulation of eosinophil recruitment, retention and survival would provide mechanistic insight and offer new metabolically targeted therapeutic approaches for respiratory and other eosinophilic diseases. Cell-specific delivery systems of pharmacological agents via for example Siglec-8 could be one route\textsuperscript{46} or more general approaches to limiting lactate in the tissue microenvironment during pathology might have broad effects on multiple cells types,\textsuperscript{39} including eosinophils.

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CONFLICT OF INTEREST
The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS
NJ, EEV, LCF, JGC and LMS performed the experiments. PSH, PL and CAT provided intellectual discussion. NJ, EEV, PSH, PL and CAT designed the experiments. NJ, EEV, PL and CAT wrote the manuscript. All authors critically revised and approved the manuscript.

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