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Original Paper

Inhibition of the lymphocyte metabolic switch by the oxidative burst of human neutrophils

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Abstract

Activation of the phagocytic NADPH oxidase-2 (NOX-2) in neutrophils is a critical process in the innate immune system and is associated with elevated local concentrations of superoxide, hydrogen peroxide (H_2O_2) and hypochlorous acid. Under pathological conditions, NOX-2 activity has been implicated in the development of autoimmunity, indicating a role in modulating lymphocyte effector function. Notably, T-cell clonal expansion and subsequent cytokine production requires a metabolic switch from mitochondrial respiration to aerobic glycolysis. Previous studies demonstrate that H_2O_2 generated from activated neutrophils suppresses lymphocyte activation but the mechanism is unknown. We hypothesized that activated neutrophils would prevent the metabolic switch and suppress the effector functions of T-cells through a H_2O_2-dependent mechanism. To test this, we developed a model co-culture system using freshly isolated neutrophils and lymphocytes from healthy human donors. Extracellular flux analysis was used to assess mitochondrial and glycolytic activity and FACS analysis to assess immune function. The neutrophil oxidative burst significantly inhibited the induction of lymphocyte aerobic glycolysis, caused inhibition of oxidative phosphorylation and suppressed lymphocyte activation through a H_2O_2-dependent mechanism. Hydrogen peroxide and a redox cycling agent, DMNQ, were used to confirm the impact of H_2O_2 on lymphocyte bioenergetics. In summary, we have shown that the lymphocyte metabolic switch from mitochondrial respiration to glycolysis is prevented by the oxidative burst of neutrophils. This direct inhibition of the metabolic switch is then a likely mechanism underlying the neutrophil-dependent suppression of T-cell effector function.

Key words: glycolysis, hydrogen peroxide, oxidative phosphorylation, T-cells.

INTRODUCTION

Leucocyte and platelet metabolism have been linked to disease states with bioenergetic biomarkers being used to assess progression of human disease [1–4]. In addition, recent data indicate that monocytes and lymphocytes modulate both glycolysis and oxidative phosphorylation during activation of both the innate and the adaptive immune systems [5–7]. The metabolism of circulating neutrophils, monocytes, lymphocytes and platelets are distinctive and these differences can be used to evaluate the relationship between bioenergetics and function [2,8]. Under basal conditions, neutrophils possess minimal, if any, mitochondrial function and meet their energy requirements through glycolysis [2,8,9]. In contrast, lymphocytes isolated from peripheral blood, primarily utilize oxidative phosphorylation for energy production [2]. However, lymphocyte metabolic pathways are highly adaptive. Notably, the transition of lymphocytes from a naïve to an activated state requires a metabolic switch from oxidative phosphorylation to aerobic glycolysis [10,11]. Similarly, this change is also observed in many highly proliferative cells and is thought to provide the necessary energy and molecular building blocks for DNA, protein and lipid biosynthesis, as well as cell signalling [11]. Disruption of the metabolic switch in T lymphocytes has been shown to inhibit cellular clonal expansion and cytokine production [12,13]. Furthermore, in contrast with regulatory and memory T-cells, glycolysis is required to support T-cell

Abbreviations: Ab, antibody; CD, cluster of differentiation; CFSE, carboxyfluorescein succinimidyl ester; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; 2DG, 2-deoxy-glucose; ECAR, extracellular acidification rate; FCCP, trifluorocarbonylcyanide phenylhydrazone; HK, Hexokinase; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; NCF1, neutrophil cytosolic factor 1; NET, neutrophil extracellular trap; NOX-2, NADPH oxidase-2; OCR, oxygen consumption rate; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute medium; SLE, systemic lupus erythematosus; Th, T-helper cells; XF-DMEM, extracellular flux assay medium.

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effector function including cytokine production and proliferation [10,11,13,14].

At sites of inflammation, cluster of differentiation [CD4; T helper (Th)1 and Th2] and CD8 (cytotoxic) effector T-cells are recruited to combat viral or bacterial infection. Neutrophils are key components of innate immunity and use reactive oxygen species (ROS) to eliminate pathogens. This is a co-ordinated process which also involves extrusion of neutrophil extracellular traps (NETs) which aid in the clearance of pathogens by direct histone, chromatin and anti-microbial protein interaction [15–17]. T-cells recognize peptides presented by major histocompatibility complex (MHC) class 1 and 2 molecules from the host cells as part of the T-cell surveillance programme. T-cells actively recruit macrophages and neutrophils to the site of inflammation as well as facilitate B-cell antibody (Ab) production. In the absence of T regulatory cells or their associated cytokines [transforming growth factor (TGF)-β, interleukin (IL)-4 and IL-10], T effector cells can promote chronic inflammation and even autoimmunity. Whereas ROS derived from the neutrophil oxidative burst have long been considered a promoter of inflammation and disease progression, new evidence suggests that these ROS have an immunoregulatory role [18–21]. The production of superoxide from NADPH oxidase (NOX-2) generates high micromolar concentrations of superoxide and hydrogen peroxide (H_2O_2) which then raises the interesting possibility that NOX-2-dependent ROS formation is an essential regulatory modulator of T-cell function. On the other hand, this appears paradoxical since exposure of a broad range of eukaryotic cells to H_2O_2, including those in the vasculature and the cardiomyocyte, results in damage to key metabolic pathways including oxidative phosphorylation and glycolysis [22–26]. Importantly, this has not been examined in lymphocytes which are frequently in close proximity to neutrophils at sites of inflammation [19].

Neutrophil–lymphocyte interactions have been investigated in the context of cytokine production, antigen presentation and oxidant generation and some studies suggest that levels of the oxidative burst which are too low fail to regulate the T-cell effector function and can thereby contribute to the development of autoimmune disease [19–21, 27]. In fact, many auto-antibodies associated with systemic lupus erythematosus (SLE) are targeted towards neutrophilic debris and associated dsDNA [15]. In support of this concept, decreasing the neutrophil oxidative burst has effectively inhibited NETosis but has failed to improve SLE outcomes in animal models [28]. Furthermore, some patients with chronic granulomatous disease (CGD), who have a defective oxidative burst caused by mutations in the NCF1 (neutrophil cytosolic factor 1) gene which encodes the p47 protein in the NADPH oxidase enzyme, were shown to be more susceptible to autoimmune conditions and have an elevated inflammatory state [29,30]. T-cell driven pathologies such as rheumatoid arthritis and encephalomyelitis were enhanced in mouse models with NCF1 mutations [18]. The same group suppressed arthritis severity by addition of compounds that restored the neutrophil oxidative burst [31]. Finally, the suppressive effects of some neutrophil subsets have been shown to contribute to the pathogenesis of HIV, particularly in the setting of advanced AIDS [27,32]. These studies and others suggest a direct immunoregulatory role of neutrophil-derived ROS in lymphocytes; however, a mechanism has yet to be determined [33–35]. Taken together these data led us to hypothesize that ROS from the neutrophil oxidative burst modulates lymphocyte effector function through its inhibitory effects on the metabolic switch from oxidative phosphorylation to glycolysis. This immunoregulatory effect of neutrophil-derived ROS may then be essential to the prevention of chronic inflammation and autoimmunity, especially in the presence of neutrophil necrotic debris and NETs. In the present study, we utilize extracellular flux technology in a novel co-culture system to investigate, for the first time, the metabolic adaptations and perturbations in lymphocytes cultured with neutrophils.

MATERIALS AND METHODS

Isolation and plating of human lymphocytes and neutrophils

All protocols and procedures for the collection, isolation, analysis and storage of blood or its components have been reviewed and approved by the Institutional Review Board at the University of Alabama at Birmingham. Neutrophils and lymphocytes were isolated from whole blood of healthy individuals as previously described [36]. Plating of the cells following cell counting and suspension was performed in extracellular flux assay medium (XF-DMEM). Lymphocytes were plated at 15×10^4 cells/well in the Seahorse 96-well microplate coated with Cell-Tak (Fisher). Neutrophils were added in co-culture at the cell densities specified. As there is some significant variation in the degree of the oxidative burst and mitochondrial function between donors, representative data from a single donor is reported for each experiment presented in the study. Key findings were verified on a minimum of three donors with 3–6 technical replicates per experiment. Overall, cells were obtained from 10 healthy individual donors.

Neutrophil and lymphocyte immune function assessment

Neutrophils were plated at 7.5×10^4 cells per well and PMA injected in the extracellular flux analyser and the oxidative burst measured as described below. Three hours after PMA treatment, 100 μl of XF-DMEM medium was replaced with medium containing 2 μM SytoxGreen (Life technologies). NETosis in neutrophils was monitored by SytoxGreen fluorescence after 10 min at 37°C using 480/530 nm (excitation/emission) on a microplate reader (PerkinElmer).

Lymphocyte survivability, cytokine production and clonal expansion were monitored by FACs analysis after an initial stimulation with 100 ng/ml PMA for the neutrophil oxidative burst on non-Cell-Tak coated Seahorse Extracellular flux microplates. Lymphocytes were washed from the plate by gentle pipetting, centrifuged at 300 ***g*** for 10 min and resuspended in 500 μl of R10 media, with PMA (50 ng/ml) and ionomycin (100 ng/ml) at 37°C and 5% humidified CO_2 for 4 days. Golgistop and GolgiPlug (10 μg/ml; BD Biosciences) were added during the last 12 h. Cells were harvested and washed once with PBS before being labelled with fluorescent LIVE/DEAD fixable dead cell

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dye (Molecular Probes, Invitrogen). Fluorochrome conjugated monoclonal antibodies antiCD-3 Alexa 780 (Clone: UCHT1), CD8 V500 (Clone: RPA-T8) and CD4 Qdot 655 (Clone: S3.5; BD Biosciences) were used for surface staining. Following fixation and permeabilization with Cytofix and Cytoperm (BD Biosciences), cells were washed and stained with intracellular markers interferon (IFN)-γ Alexa Fluor 700 (Clone: B27) and IL-2 PE (Clone: MQ1-17H12; BD Biosciences). Following staining, cells were washed and fixed in 2% paraformaldehyde (Sigma–Aldrich) and analysed on an LSRII flow cytometer within 24 h (BD Biosciences).

At least 100000 CD3+ events were acquired from each lymphocyte sample. Data analysis was performed using FlowJo version 9.7.6 software (Tree Star). CD3+ lymphocytes were gated based on forward and side scatter properties after the exclusion of doublets. Gates were set relative to positive controls and negative controls.

Lymphocyte clonal expansion was assessed by labelling with 1.25 μm CFSE (carboxyfluorescein succinimidyl ester; Molecular Probes) for 4 min at room temperature. After washing in PBS, cells were resuspended in 1 ml of complete RPMI (Roswell Park Memorial Institute medium) with 10% Ab serum. PMA, a protein kinase C activator and the calcium ionophore, ionomycin, were added at indicated concentrations and the lymphocytes were incubated for 4 days at 37°C and 5% CO_2. On the fifth day, the cells were centrifuged at 300 ***g*** for 10 min and resuspended in 1 ml of RPMI with 10% Ab serum with GolgiPlug and GolgiStop and with PMA (50 ng/ml) and ionomycin (100 ng/ml). They were incubated an additional 6 h at 37°C then kept overnight at 4°C. Surface and intracellular staining followed by flow cytometric analysis was performed as above.

Assessment of bioenergetic function and the oxidative burst

The induction of aerobic glycolysis in lymphocytes and the oxidative burst in neutrophils was accomplished by the addition of 100 ng/ml PMA or as otherwise indicated. PMA was loaded into port A at a 10× concentration and was injected after three basal oxygen consumption and extracellular acidification rate readings (OCR/ECAR). OCR and ECAR measurements were taken every 8 min until the oxidative burst response of neutrophils ceased, approximately 160 min, and then the bioenergetic profile of lymphocytes was obtained. The bioenergetic profile consists of basal OCR/ECAR measurements followed by the injection of 1 μg/ml oligomycin, 0.6 μM FCCP (trifluorocarbonylcyanide phenylhydrazone) and 10 μM antimycin A as previously described [36,37]. Briefly, oligomycin inhibits mitochondrial ATP synthase and the resulting drop in OCR and rise in ECAR are attributed to ATP-linked OCR and the compensation of glycolysis for the loss of mitochondrial ATP production. The protonophore, FCCP, uncouples the mitochondrial proton gradient and oxygen consumption from ATP synthase and drives maximal OCR. Antimycin A inhibits complex III of the electron transport chain and suppresses all mitochondrial oxygen consumption and the remaining OCR is considered non-mitochondrial [38].

In the co-culture experiment, cells were centrifuged on to the Cell-Tak-coated plate at 200 ***g***, the plate rotated and then centrifuged at 300 ***g*** as previously described [36]. The volume of the well was brought up to 180 μl with XF-DMEM and the plate incubated for 10–30 min in a non-CO_2 incubator. The Seahorse cartridge was loaded at 10× the final concentration of 100 ng/ml PMA in port A and 1 μg/ml oligomycin, 0.6 μM FCCP and 10 μM antimycin A in ports B, C and D respectively. The effect of catalase (Sigma–Aldrich) on lymphocyte bioenergetics was assessed by incubation at 1000 units/ml for 30 min prior to inducing the oxidative burst of neutrophils.

Glycolytic function of lymphocytes in co-culture was determined by the glucose stress test [38]. Glucose is essential for the oxidative burst of neutrophils and for that reason the lymphocytes and neutrophils were treated with PMA outside of the extracellular flux analyser for a period of 160 min before the medium was exchanged with XF-DMEM containing no glucose or pyruvate. The media exchange consisted of two washes of replacement medium to effectively lower glucose to <1% of its original concentration. The cells were allowed 20 min to equilibrate before being placed on the XF analyser. Three basal measurements were obtained before the injection of 5 mM glucose, 1 μg/ml oligomycin and 100 mM 2-deoxy-glucose (2DG). Glucose stimulated ECAR was termed glycolysis, whereas oligomycin-sensitive ECAR represented the glycolytic reserve. 2DG, the competitive inhibitor of hexokinase (HK), inhibited all glycolytic activity and the remaining ECAR was termed non-glycolytic.

Lymphocytes were exposed to bolus hydrogen peroxide and the intracellular redox cycling agent, 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) to determine the bioenergetic consequences of hydrogen peroxide in a neutrophil-free system [37]. Lymphocytes were isolated and plated as previously described [36] and 50, 100 and 500 μM bolus hydrogen peroxide was added to the cells for 30 min at 37°C prior to the first assay measurement. A 1, 3 and 5 μM DMNQ dose response was obtained under the same conditions prior to PMA injection and bioenergetics assessment.

Statistics

All OCR/ECAR traces and analyses were analysed for each representative donor with 3–6 replicate wells. The data are presented as mean ± S.E.M. A Student's *t* test was used to determine statistical significance ($P \leqslant 0.05$) using the standard Excel statistics package.

RESULTS

Isolation and characterization of lymphocytes and neutrophils

To assess the ability of isolated neutrophils to undergo an oxidative burst and NETosis, CD15+ neutrophils were isolated from whole blood of healthy volunteers as previously described [36]. The conditions required to elicit PMA-dependent oxidative burst, measured as the amount of oxygen consumed over 100 min and NET formation, measured as Sytoxgreen fluorescence, in neutrophils were first established (Figures 1A and 1B).

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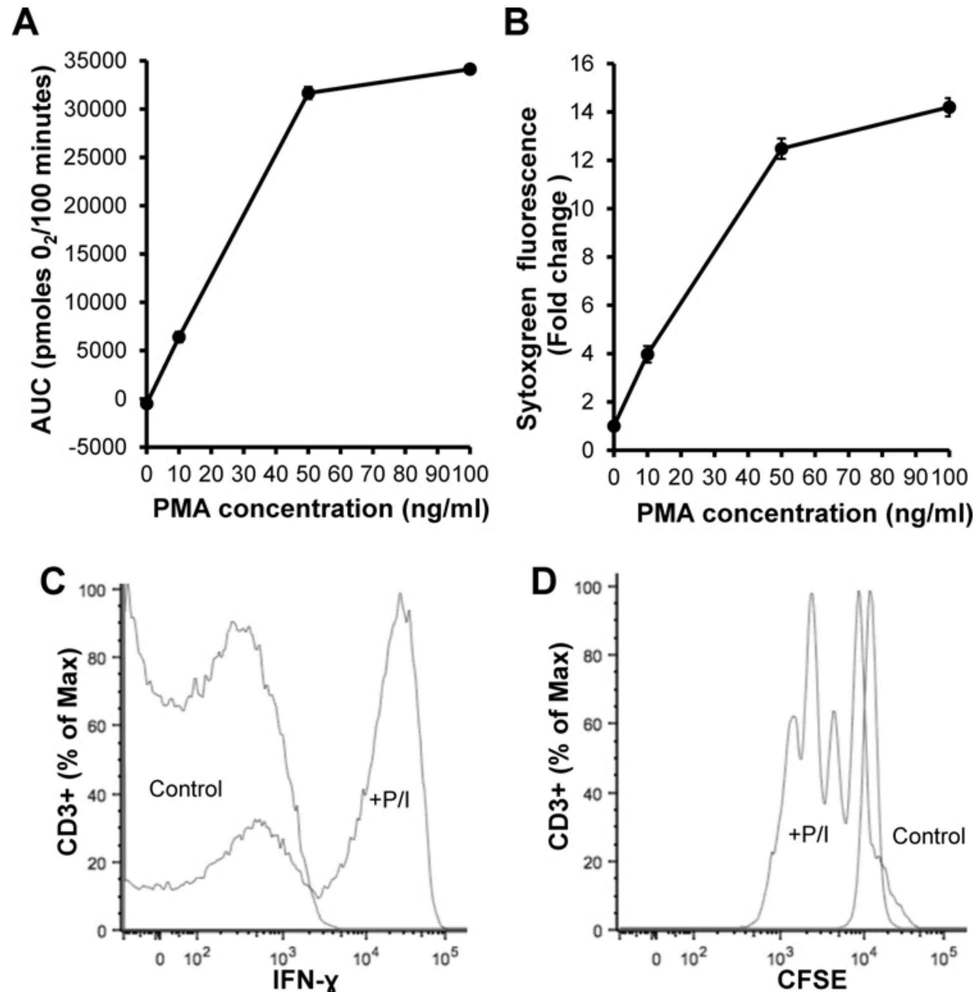

Figure 1 Stimulated neutrophil and lymphocyte immune function

Healthy donor CD15+ neutrophils were isolated from total polymorphonuclear cells and lymphocytes by CD14+ monocyte, CD61+ platelet and CD235+ red blood cell (RBC) depletion. (**A**) Neutrophils were plated on an extracellular Flux microplate and stimulated with 0, 10, 50 and 100 ng/ml PMA for 100 min and the total OCR measured as area under the curve (AUC). (**B**) Three hours after stimulation, cells were incubated with a final concentration of 1 μM Sytoxgreen and fluorescence measured after 10 min. (**C**) Lymphocytes stimulated with 50 ng/ml PMA and 100 ng/ml ionomycin (P/I) for 4 days are positive for IFN-γ expression and (**D**) undergo clonal expansion as seen by reduction of CFSE stain in subsequent daughter cells by FACS analysis. Values expressed as mean $\pm$ S.E.M. with technical replicates of 3–6.

The concentration of PMA required to elicit the maximal oxygen consumption was 50–100 ng/ml and this corresponded well with the extent of NETosis (Figure 1B) as reported in the literature [39]. PMA was selected for these studies because of its consistency in stimulation of NOX-2 and because it is one of the few agents which can activate both neutrophils and lymphocytes. Negatively selected lymphocytes were activated to assess cytokine production and clonal expansion, key characteristics of activated lymphocytes. Lymphocytes cultured for 4 days with 50 ng/ml PMA and 100 ng/ml ionomycin showed an increase in IFN-γ production as compared with the medium control (Figure 1C). Lymphocyte proliferation was measured using the cell permeable stain, CFSE and demonstrated nearly 100% proliferation of lymphocytes as seen by loss of CFSE (Figure 1D).

Lymphocyte and neutrophil oxidative and glycolytic metabolism

The lymphocyte 'metabolic switch' from oxidative phosphorylation to aerobic glycolysis was determined in cell culture using extracellular flux analysis [40]. In Figure 2(A, C), the OCR and ECAR of lymphocytes are shown with and without the addition of PMA (100 ng/ml). Within 8 min of addition of PMA, both OCR and ECAR are stimulated. These effects are sustained for the subsequent 160 min after which a mitochondrial stress test was performed. Oligomycin, which inhibits the mitochondrial ATP synthase, resulted in the expected decrease in OCR which was stimulated on the addition of the uncoupler, FCCP. The addition of antimycin A inhibits all mitochondrial respiration and shows a slightly elevated non-mitochondrial respiration in PMA-treated lymphocytes. The addition of oligomycin

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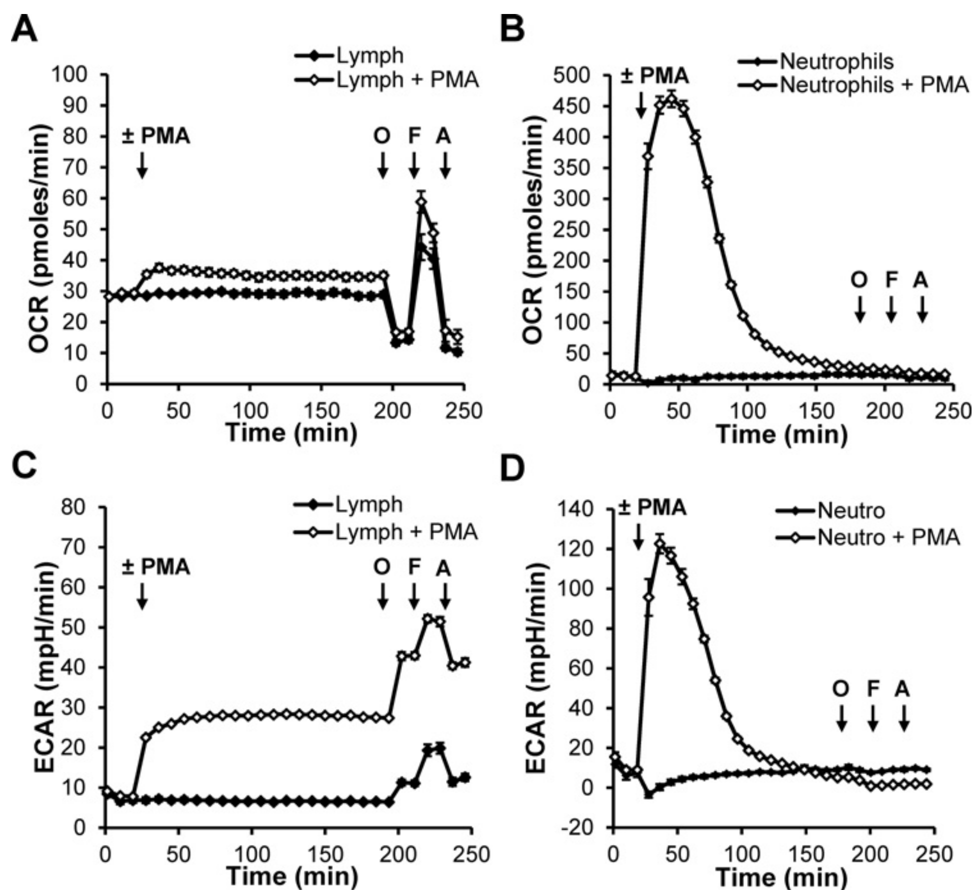

Figure 2 The mitochondrial and glycolytic function of lymphocytes and the oxidative burst of neutrophils with PMA stimulation

(**A**) Lymphocytes (15×10^4 cells/well) and (**B**) neutrophils (2.5×10^4 cells/well) isolated from freshly collected human blood were plated and stimulated with $\pm$ 100 ng/ml PMA and their OCR followed for 160 min before 1.0 μg/ml oligomycin (O), 0.6 μM FCCP (F) and 10 μM antimycin A (A) were injected for mitochondrial function profiling. (**C**) Lymphocytes and (**D**) neutrophil glycolytic function was monitored by ECAR concurrently with the OCR measurements. Values expressed as mean $\pm$ SEM with technical replicates of 3–6.

stimulates ECAR with or without PMA. Interestingly, the overall glycolytic capacity is greater after addition of PMA, consistent with increased glycolytic flux. The modest stimulation of ECAR on addition of FCCP and its inhibition by antimycin A occurs to the same extent with or without PMA and most probably represents proton production from the tricarboxylic acid (TCA) cycle. These data indicate that the lymphocytes are metabolically active and demonstrate the anticipated switch to aerobic glycolysis on addition of PMA.

Similarly, neutrophil OCR and ECAR were measured over the same time course (Figures 2B and 2D). As we have reported previously, neutrophils have minimal mitochondrial function and utilize a small amount of glycolysis under the basal state and show no significant response to the addition of mitochondrial inhibitors [2,8]. PMA induces a rapid increase in OCR which is due to the activation of NOX2 and the production of superoxide which is maximal at approximately 30 min after injection of PMA and progressively decreases over the subsequent 100 min [8]. Incubating the neutrophils or lymphocytes in glucose free medium or with the competitive inhibitor of HK, 2DG, resulted in complete suppression of the oxidative burst and glucose oxidation assessed by the ECAR measurement (result not shown).

As reported in the literature, low dose PMA and ionomycin had a synergistic effect on inducing lymphocyte activation [5,41]. However, ionomycin led to a transient elevation in ECAR and OCR in lymphocytes and resulted in loss of maximal mitochondrial function (Supplementary Figure S1). Additionally, ionomycin completely suppressed the oxidative burst of neutrophils (result not shown). Although ionomycin and PMA are often used together to activate lymphocytes, these data demonstrate that the calcium ionophore suppresses the metabolic processes in both cell types. Therefore, in the present study, PMA alone was used to stimulate lymphocyte and neutrophil activation unless otherwise specified.

Lymphocyte metabolic function in the presence of activated neutrophils

To determine the effect of neutrophil activation on lymphocyte metabolism, a co-culture method was developed. Neutrophils were plated at 5000–75000 cells/well on the extracellular

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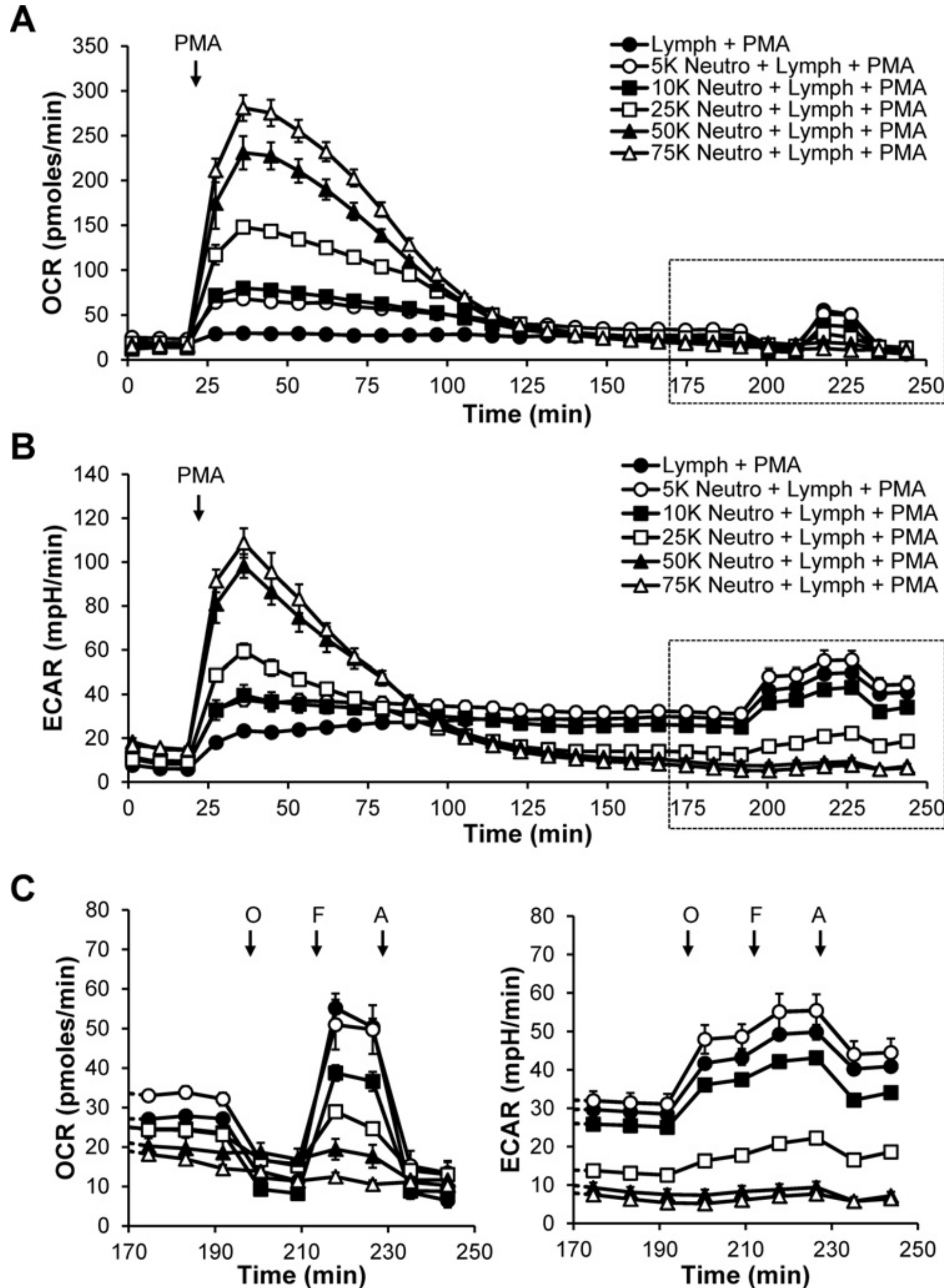

Figure 3 The neutrophil dose-dependent mitochondrial and glycolytic dysfunction of lymphocytes
Neutrophils were isolated and plated at 0, 5, 10, 25, 50 and 75 $\times 10^3$ (K) cells/well with 150 $\times 10^3$(K) lymphocytes. (**A**) OCR and (**B**) ECAR were concurrently monitored for three basal measurements prior to 100 ng/ml PMA injection in all co-culture groups and 160 min following injection. (**C**) Following the oxidative burst of the neutrophils, 1.0 μg/ml oligomycin (O), 0.6 μM FCCP (F) and 10 μM antimycin A (A) were injected for mitochondrial and glycolytic profiling. Values expressed as mean $\pm$ S.E.M. with technical replicates of 3–6.

flux microplate with 150000 lymphocytes. Neutrophils (25000) co-cultured with lymphocytes without PMA had no effect on basal lymphocyte ECAR or OCR (result not shown). After basal OCR/ECAR was established, PMA was injected and the cells monitored for 160 min. With the addition of PMA, lymphocyte OCR and ECAR were stimulated as observed with either cell type alone (Figures 3A and 3B). At 160 min after PMA injection, at which point oxygen consumption and extracellular acidification due to the oxidative burst was complete, the mitochondrial stress test was performed as shown in Figure 3(C). It is important to note that at the time of assessing lymphocyte mitochondrial function the neutrophils are not responsive to the mitochondrial

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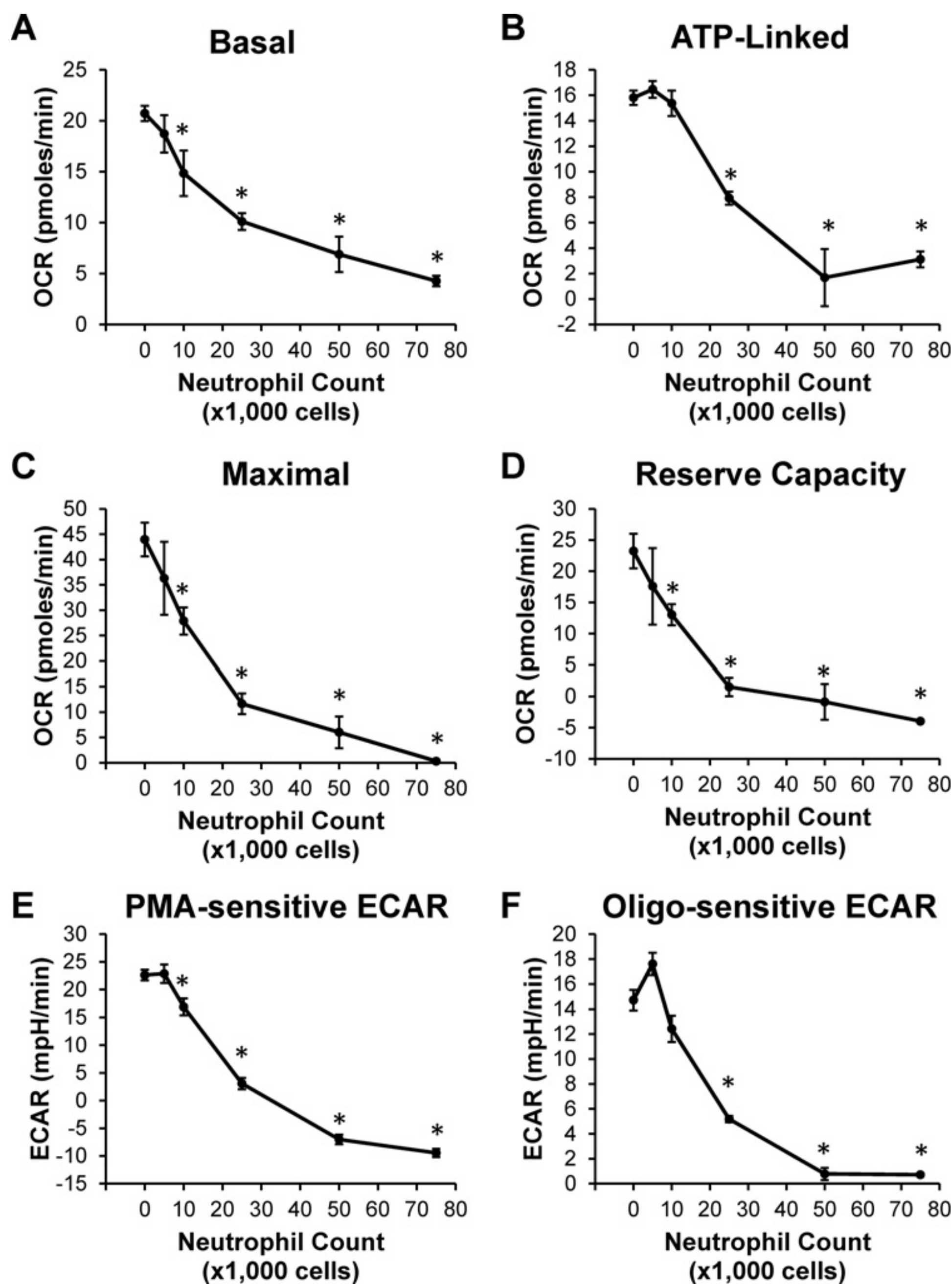

Figure 4 Lymphocyte mitochondrial and glycolytic profiling
Lymphocyte metabolic function was calculated using the OCR and ECAR measurements after oligomycin, FCCP and antimycin A injection. (**A**) Basal mitochondrial respiration (basal OCR–non-mitochondrial OCR), (**B**) ATP-linked respiration (oligo-sensitive OCR), (**C**) reserve capacity (maximal–basal OCR) and (**D**) maximal mitochondrial respiration (maximal OCR–non-mitochondrial OCR) were calculated and plotted by neutrophil count (per 1000 cells). Lymphocyte aerobic and oligomycin-stimulated glycolysis was measured using the ECAR values after (**E**) 100 ng/ml PMA injection (PMA-sensitive), (**F**) and 1.0 μg/ml oligomycin injection (oligo-sensitive) respectively and plotted by neutrophil count. Values expressed as mean $\pm$ S.E.M. with technical replicates of 3–6.*$P \leqslant 0.05$.

inhibitors and the oxidative burst is essentially complete. The detailed analysis of the lymphocyte OCR and ECAR parameters are shown in Figure 4. The activated neutrophils decreased basal, ATP linked, maximal and reserve capacity with a significant effect detected for 10000–25000 neutrophils (i.e. 1–2.5 neutrophils for every 15 lymphocytes). The PMA and oligomycin sensitive ECAR for the lymphocytes were sensitive to activated neutrophils over a similar range of cell densities (Figures 4E and 4F).

Activated neutrophils release a broad range of mediators on activation including proteases and ROS. To determine whether

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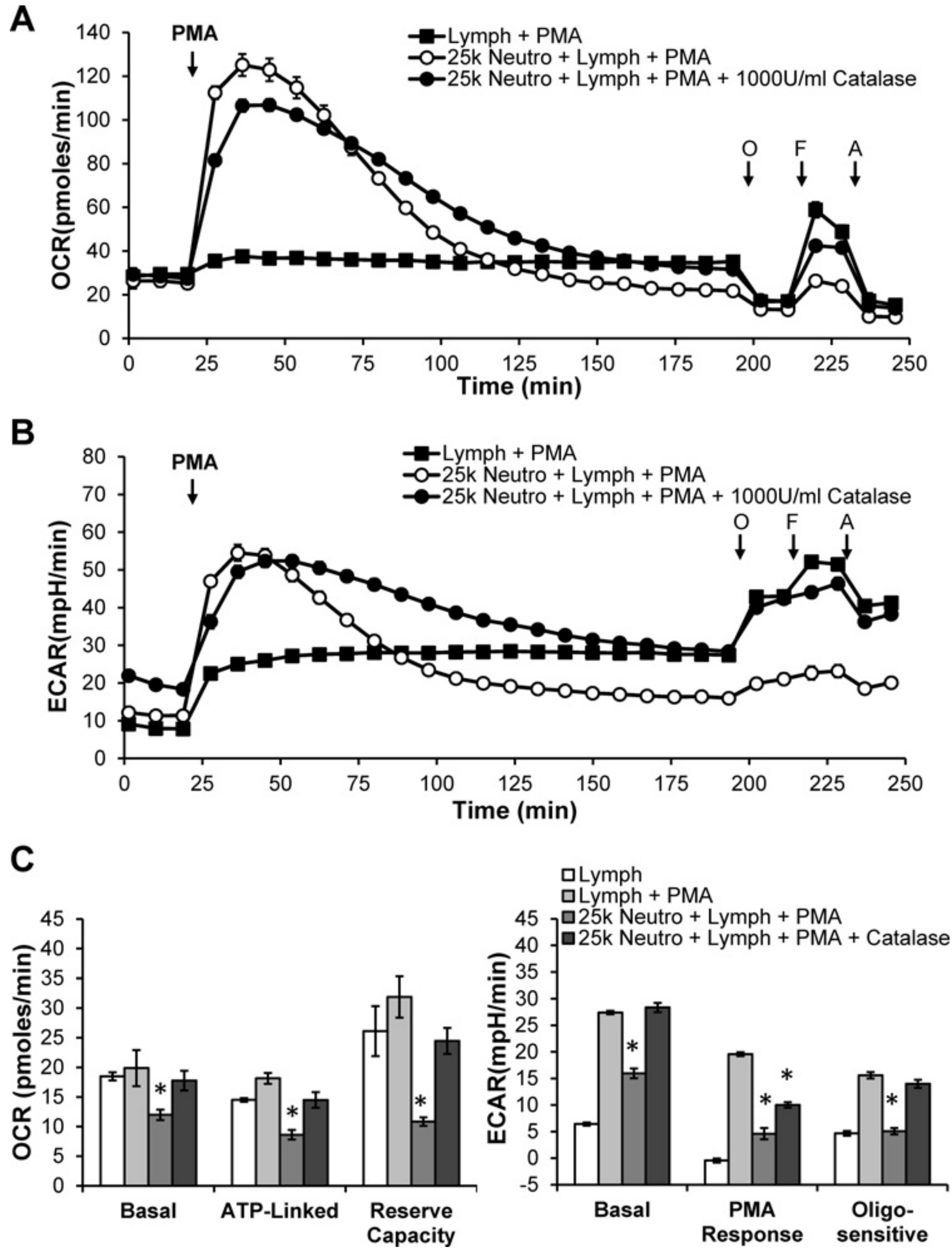

Figure 5 Lymphocyte metabolic dysfunction and prevention with catalase
Lymphocytes (15×10^4 cells/well) and neutrophils (2.5×10^4 cells/well) in co-culture were stimulated with PMA in the presence or absence of a 1000 units/ml catalase 30 min pre-treatment and (**A**) OCR and (**B**) ECAR followed until oligomycin, FCCP and antimycin A injection. (**C**) Basal mitochondrial, ATP-Linked, reserve capacity and basal, PMA-sensitive and oligomycin-sensitive ECAR were measured in lymphocytes ± PMA and in PMA stimulated lymphocyte and neutrophil ± catalase. Values expressed as mean ± S.E.M. with technical replicates of 3–6.*$P \leqslant 0.05$ compared with PMA stimulated lymphocytes.

H_2O_2 is mediating the effects on ECAR and OCR in the co-culture of neutrophils (25000) and lymphocytes, 1000 units/ml of catalase, which removes H_2O_2, was added 30 min prior to the start of the assay and was present for the duration of the oxidative burst. Figures 5(A) and 5(B) show the PMA-stimulated lymphocyte OCR and ECAR following the oxidative burst in the presence of neutrophils. Interestingly, the extent of the oxidative burst measured by OCR and ECAR was prolonged in the presence of catalase, suggesting the H_2O_2 is also damaging the oxidative burst components in the neutrophil. The decline in mitochondrial and glycolytic function following the oxidative burst as shown in Figure 4 was clearly evident and prevented by

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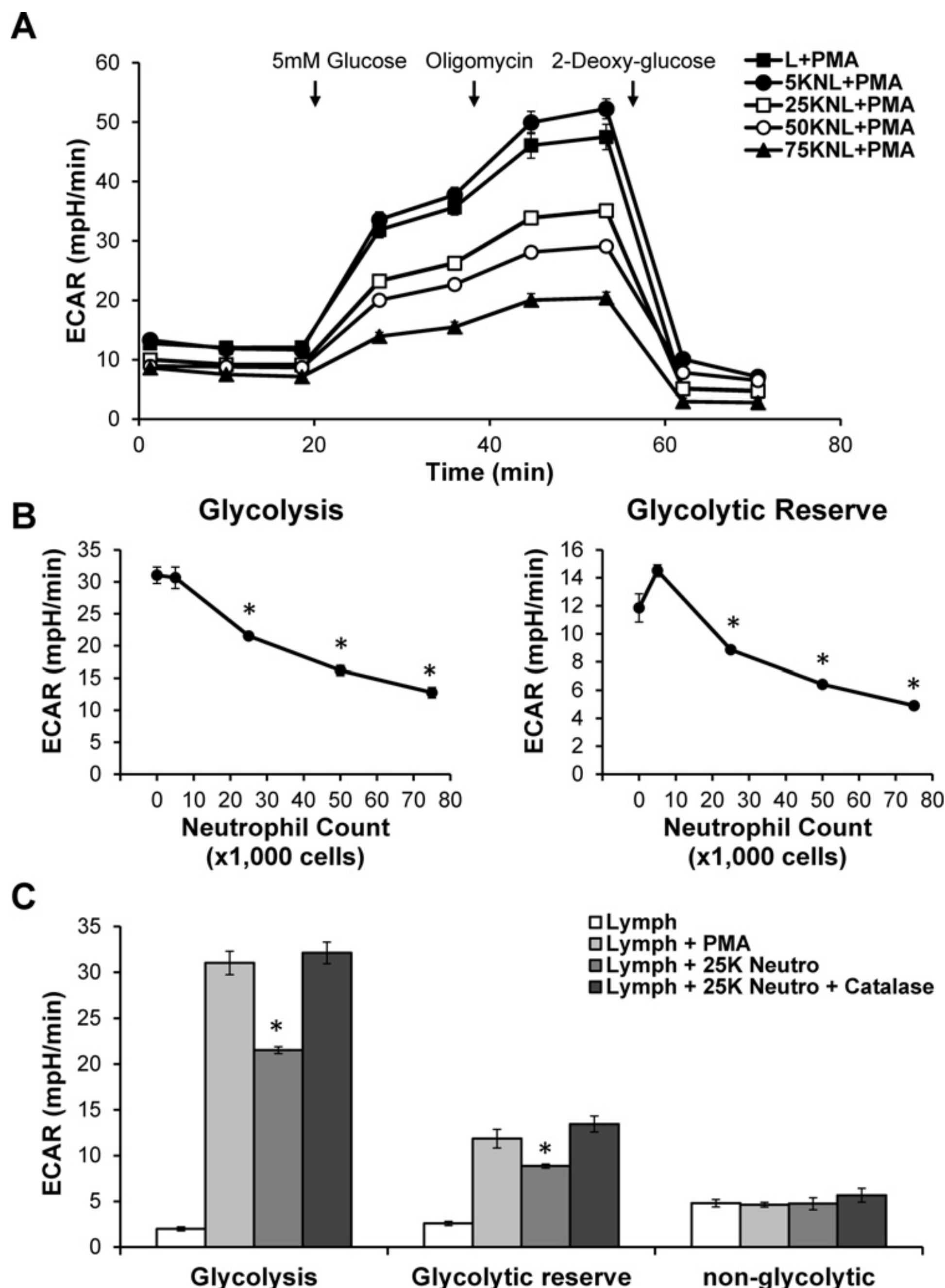

Figure 6 Lymphocyte glucose stress test in co-culture

Neutrophils were isolated and plated at 0, 5, 10, 25, 50 and 75×10^3 (K) cells/well with 150×10^3(K) lymphocytes and were stimulated with PMA and no-glucose media exchanged after 160 min. (**A**) ECAR was followed for three readings then 5 mM glucose, 1.0 μg/ml oligomycin and 20 mM 2-DG were injected. (**B**) Glycolysis (glucose ECAR–2-DG ECAR) and glycolytic reserve (oligomycin-sensitive ECAR) were measured and plotted by neutrophil count (per 1000 cells). (**C**) These and non-glycolytic ECAR were measured in lymphocytes $\pm$ PMA and in PMA stimulated lymphocytes and neutrophils $\pm$ 1000 units/ml catalase. Values expressed as mean $\pm$ S.E.M. with technical replicates of 3–6.*$P \leqslant 0.05$.

catalase (Figure 5C). In contrast, superoxide dismutase (SOD; 100 units/ml) administration failed to prevent the metabolic dysfunction in the lymphocytes (result not shown).

The extracellular acidification arises from any processes in the cell generating protons that are released and change the pH of the medium. To determine the contribution of glycolysis to these values the glycolytic stress test was performed in the neutrophil and lymphocyte co-cultures with PMA and the medium replaced for the glucose stress test at 160 min. In this assay, glucose is omitted from the medium and three basal measurements are obtained (Figure 6A). Next, 5 mM glucose is returned to the medium in the first injection and results in a rapid increase in ECAR in the control

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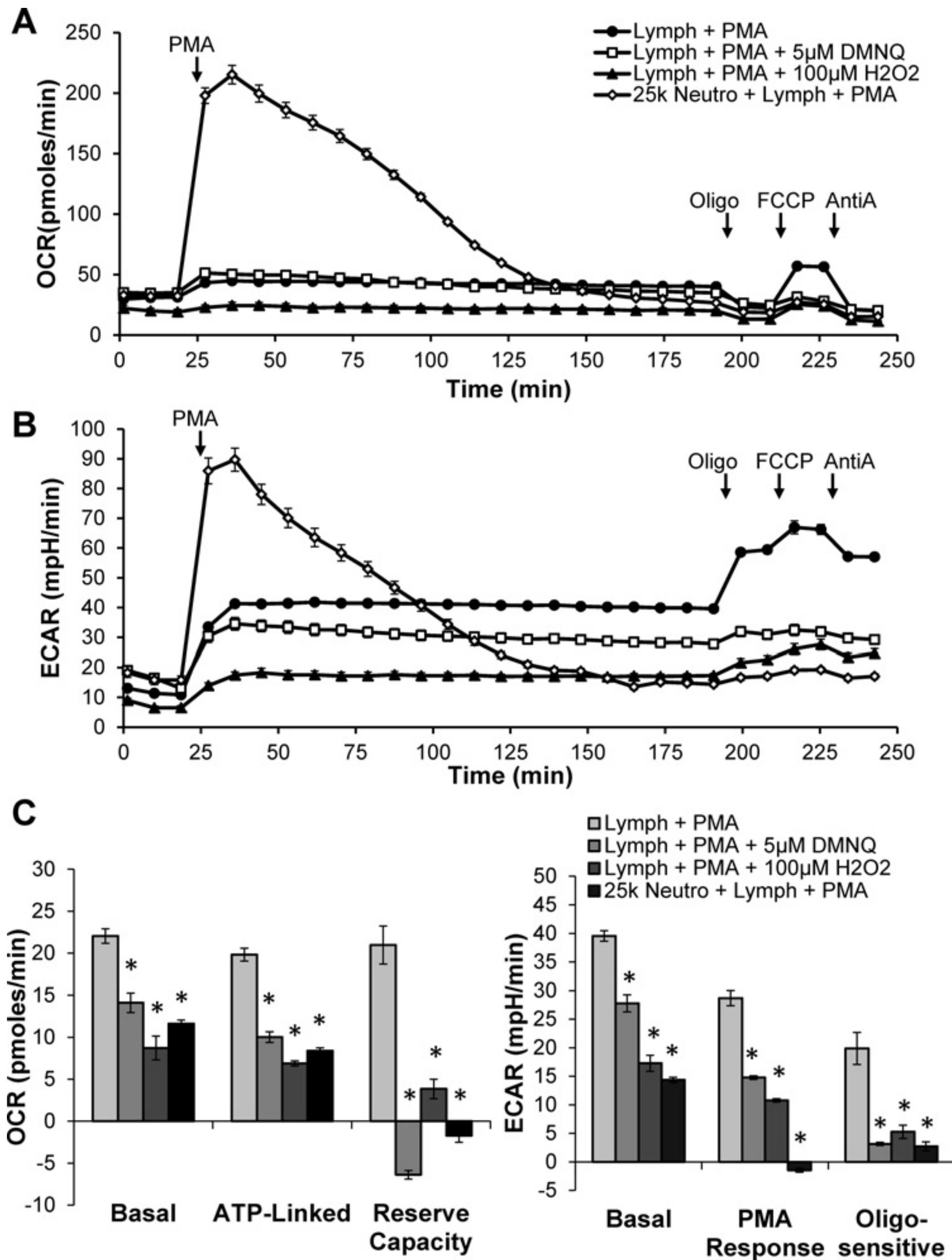

Figure 7 Metabolic regulation of activated lymphocyte by bolus hydrogen peroxide and DMNQ
Isolated lymphocyte (**A**) OCR and (**B**) ECAR were assessed after a 30 min pre-treatment of 100 μM H_2O_2, 5 μM DMNQ or 25000 neutrophils and the PMA sensitive changes measured for 160 min before the injection of oligomycin, FCCP and antimycin A. (**C**) The observed changes in post-PMA basal OCR/ECAR, ATP-linked OCR/oligo-sensitive ECAR, reserve capacity and PMA sensitive ECAR were quantified and compared with lymphocyte + PMA control. OCR/ECAR traces were obtained from a single healthy donor's lymphocytes and neutrophils and values expressed as mean $\pm$ S.E.M. with technical replicates of 3–6.*$P \leqslant 0.05$.

lymphocytes treated with PMA which is suppressed in a neutrophil dependent fashion. Addition of oligomycin further stimulates glycolysis as mitochondrial ATP production is inhibited and addition of the inhibitor of HKs 2-DG suppresses the ECAR to basal levels prior to the addition of glucose. Glycolysis was measured as the difference between ECAR after glucose injection and the non-glycolytic rate following 2-DG. Glycolytic reserve is the oligo-sensitive ECAR. Increasing neutrophil number significantly suppressed aerobic glycolysis and the glycolytic reserve in lymphocytes (Figures 6A and 6B). Notably, the neutrophil-dependent inhibition of glycolysis was prevented by catalase (Figure 6C) suggesting that this process is H_2O_2 mediated.

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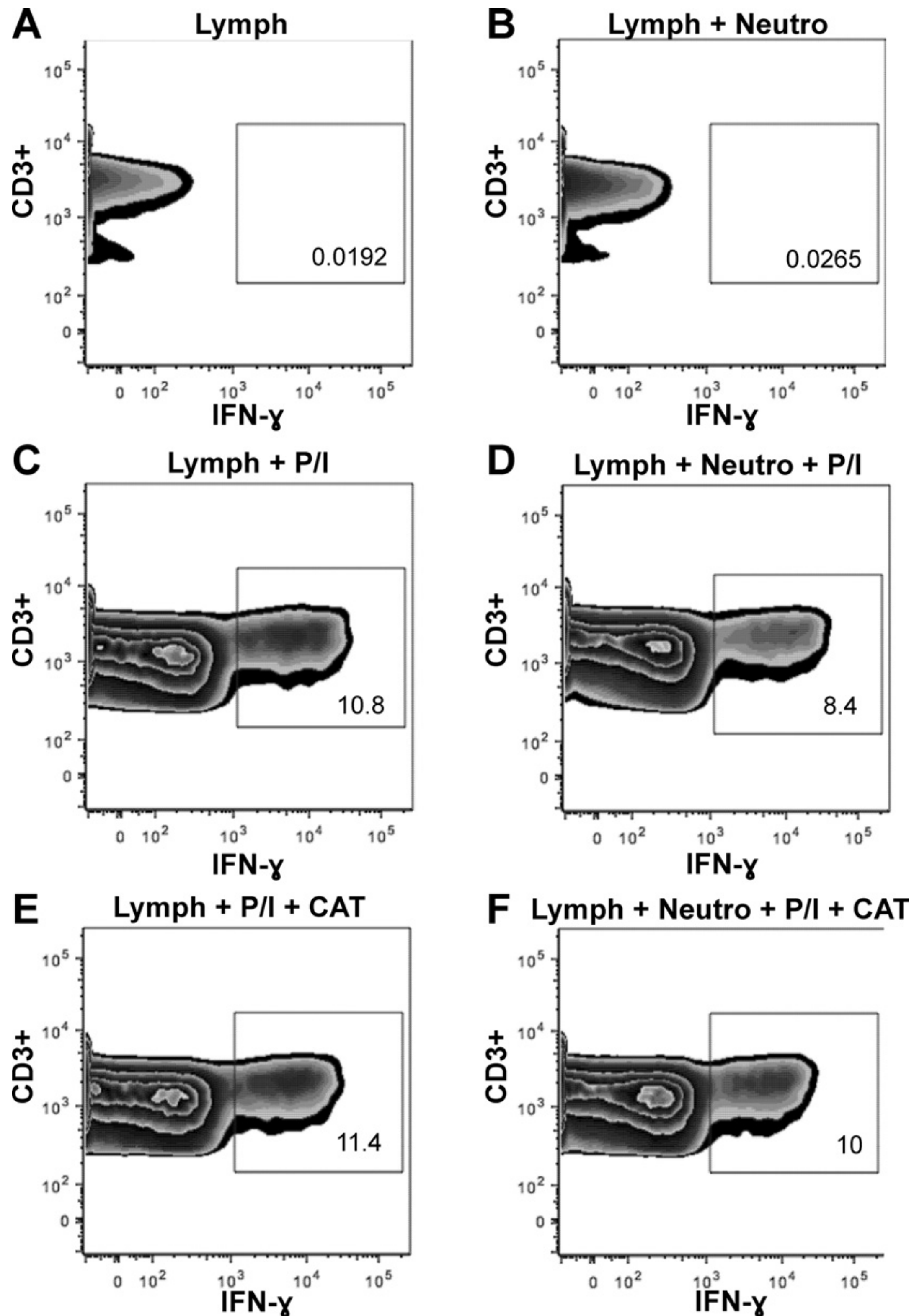

Figure 8 **Lymphocyte IFN-γ production in co-culture**
CD3+ lymphocytes, from a representative healthy donor, are gated for IFN-γ secretion after 4 days of incubation with (**A**) no stimulation: medium control, (**B**) co-culture with neutrophils or (**C**) stimulation with 50 ng/ml PMA and 100 ng/ml ionomycin (P/I). (**D**) Co-culture of PMA/ionomycin stimulated lymphocytes with neutrophils demonstrates reduced IFN-γ secretion. Catalase (1000 unit/ml) pre-treatment for 30 min of (**E**) lymphocytes alone and (**F**) lymphocyte and neutrophil co-culture, shows similar IFN-γ expression compared with the lymphocytes with PMA and ionomycin.

Lymphocyte metabolic function in a neutrophil free system with bolus hydrogen peroxide or DMNQ

To assess the metabolic regulation of H_2O_2 in the absence of neutrophils, lymphocytes were exposed to 50, 100 and 500 μM bolus doses 30 min prior to the first OCR/ECAR measurement. The ability of PMA to increase basal OCR and ECAR was suppressed in a dose-dependent manner with 100 μM H_2O_2 causing suppression of ATP-linked OCR, reserve capacity and oligomycin-sensitive ECAR to a similar degree as the same subject's neutrophils (25000) (Figure 7). Interestingly, the H_2O_2 produced by 25000 neutrophils (40 μM) was approximately twice as effective as the bolus reagent in inducing inhibition of lymphocyte bioenergetics. To address if this difference was due to proximity of H_2O_2 production, DMNQ, an intracellular generator of superoxide and hydrogen peroxide was exposed to lymphocytes 30 min prior to the assay. No significant increase in OCR was observed over the untreated control, indicating that, at the highest dose (5 μM), low levels of these oxidants were being generated [37]. However, this still resulted in significant suppression of basal, PMA-sensitive and oligomycin-sensitive changes in OCR and ECAR. This suggests that the modulation of lymphocyte metabolism by the neutrophil oxidative burst is particularly sensitive to local concentration of H_2O_2.

Perturbation of lymphocyte metabolism coincides with immune dysfunction

FACs analysis was used to elucidate the functional consequences of the neutrophil oxidative burst on lymphocyte cytokine production and proliferation after 4 days. Lymphocytes and neutrophils were plated in co-culture as described above on non-Cell-Tak coated microplates to maintain the same cell conditions and proximity as implemented during the bioenergetics assessments. After 160 min of PMA treatment lymphocytes were washed from the plate and centrifuged at 300 ***g*** for 10 min to remove neutrophil cellular debris and treated with PMA and ionomycin as described above. Following the 4 day incubation, 11% of CD3+ lymphocytes were positive for high IFN-γ production compared with control (Figure 8). Cytokine production was reduced to 8% when lymphocytes were co-cultured with 2.5×10^4 (25000) activated neutrophils (Figure 8D). Increasing neutrophil (50000–75000) number led to a greater and significant dose-dependent suppression of IFN-γ production (result not shown). Catalase markedly inhibited the neutrophil-dependent suppression of lymphocyte IFN-γ production and had a moderate stimulatory effect on IFN-γ production in activated lymphocytes (Figure 8F).

Clonal expansion was stimulated with PMA/ionomycin and measured by CFSE staining 4 days later. Figure 9 demonstrates a representative FACs profile showing significant lymphocyte proliferation in all groups compared with medium alone; however a neutrophil-dependent decline in lymphocyte proliferation was observed (Figures 9C and 9D). This effect was prevented by addition of 1000 units/ml catalase during the 160 min neutrophil oxidative burst at the start of the 4 day incubation (Figure 9F). Interestingly, the short duration of catalase exposure also stimulated lymphocyte proliferation, which we ascribe to scavenging of endogenously generated hydrogen peroxide (Figure 9F).

DISCUSSION

Neutrophils have a life span measured in hours to days before they migrate through the vasculature in response to local inflammation, undergo programmed cell death and NETosis or are cleared by the spleen or phagocytosis [42,43]. As expected, the neutrophil oxidative burst and NETosis occur rapidly after PMA stimulation under the conditions used for bioenergetic measurements (Figures 1A and 1B). These processes are associated with chronic inflammation and the generation of auto-antibodies recognizing NET DNA or other neutrophil intracellular components [15,44,45]. The role of neutrophils in chronic inflammation and autoimmunity appears to be highly dependent on their interaction with effector T-cells [19]. Monocytes are the typical means by which lymphocytes are activated and are also capable of generating an oxidative burst, though to a lesser extent than neutrophils. Upon MHC–T-cell receptor interaction and antigen recognition, naive lymphocytes activate, proliferate and secrete cytokines or undergo cytotoxic degranulation. PMA can stimulate lymphocyte activation in the absence of antigenic stimuli as evidenced by the increased IFN-γ production and cell proliferation (Figures 1C and 1D). Neutrophils and lymphocytes have been shown to co-exist at sites of inflammation as well as lymphoid organs resulting in decreased lymphocyte activation and proliferation [19,44]. This suggests that there are mechanisms through which T-cell activation is inhibited in the presence of activated neutrophils and possibly monocytes which may be important in preventing the development of autoimmune conditions or controlling inflammation.

Aerobic glycolysis is a means by which highly proliferating cells can generate ATP and the molecular building blocks for cell growth and DNA synthesis. High-dose PMA stimulated aerobic glycolysis in freshly isolated lymphocytes within 8 min and was stable for over 2 h as seen by the rapid rise in ECAR in Figures 2 and 3. This rapid induction is probably associated with the reported Glut1 (glucose transporter 1) trafficking to the cell surface through phosphoinositide 3-kinase (PI3K)/AKT and/or mitogen-activated protein kinase (MAPK)-dependent signalling with possible activation of glycolytic enzymes [e.g. HK, IPFK (inducible phosphofructokinase); 11,46–48]. The role of PMA in lymphocyte activation and the induction of aerobic glycolysis, however is not well understood. A significant increase in OCR is observed after PMA stimulation, which is consistent with the increased energetic demand associated with activation (Figure 2). PMA was used in place of other stimuli in part to demonstrate that many of these metabolic changes occur quickly and independently of nuclear transcription and to disentangle the confounding roles of cell surface receptor expression and cytokine signalling which would ultimately depend on the inflammatory environment.

Lymphocyte mitochondrial and glycolytic function showed a $>50\%$ decrease in activity when 25000 neutrophils were present, (a 1:6 ratio of neutrophils to lymphocytes) suggesting a relatively small number of activated neutrophils can affect the metabolism of lymphocytes and perhaps other cell types in close proximity (Figure 4). The oxidant, H_2O_2, is generated rapidly from the dismutation of superoxide during the neutrophil oxidative burst.

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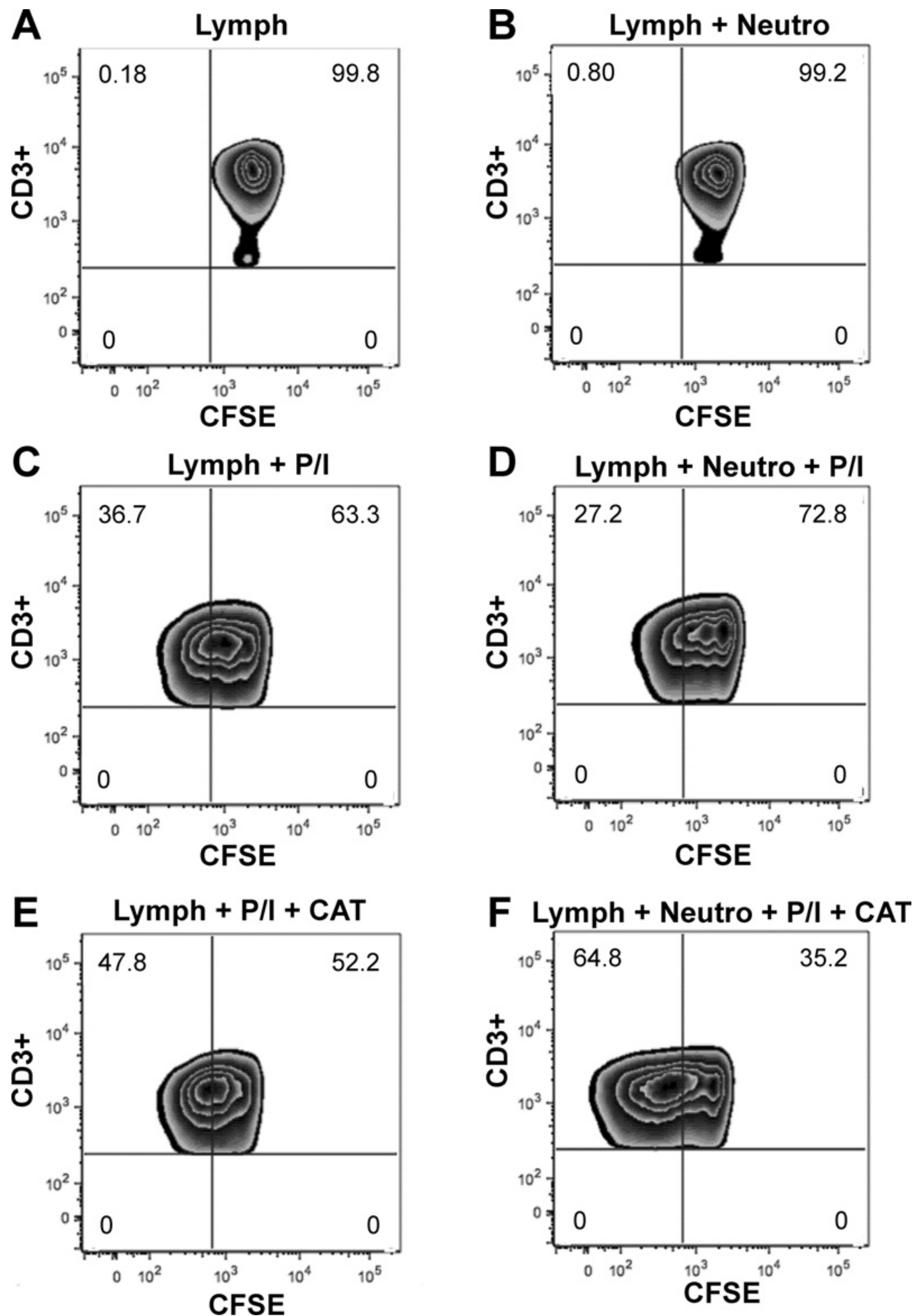

Figure 9 Lymphocyte clonal expansion in co-culture
CFSE analysis of lymphocyte proliferation from a representative healthy donor is demonstrated. Negative controls for (**A**) medium alone and (**B**) neutrophils in co-culture show minimal proliferating cells in the absence of stimulation. (**C**) The addition of 50 ng/ml PMA and 100 ng/ml ionomycin (P/I) results in approximately 37 % proliferation of lymphocytes. (**D**) This proliferation is decreased when exposed to the neutrophil oxidative burst. (**E**) Catalase (1000 units/ml) pre-treatment of lymphocytes alone (**F**) and in neutrophil co-culture, shows loss of neutrophil-dependent suppression of proliferation as well as increased proliferative capacity compared with the lymphocytes with PMA and ionomycin.

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Hydrogen peroxide is a non-radical species that can freely diffuse through membranes and inhibit both glycolytic and mitochondrial machinery [25,26]. Catalase prevented the neutrophil-mediated bioenergetic dysfunction and allowed lymphocytes to undergo the metabolic switch even in the presence of activated neutrophils (Figures 5 and 6). Bolus H_2O_2 effectively suppressed oxidative and glycolytic metabolism in a neutrophil-free system (Figure 7). Interestingly, assuming oxygen consumption resulted in a molar equivalent H_2O_2 production by the neutrophil oxidative burst, the total amount of H_2O_2 generated was less than half that provided by bolus H_2O_2 to achieve the same effect on lymphocyte bioenergetics. Similarly, the intracellular redox cycling agent, DMNQ, modulated lymphocyte metabolism to a similar degree as neutrophils in a dose-dependent manner (Figure 7). The possibility that neutrophils could facilitate a much greater degree of metabolic suppression due to the conversion of hydrogen peroxide to hypochlorous acid (HOCl) by myeloperoxidase was also considered, however the HOCl scavenger, taurine (5 mM), did not prevent inhibition of lymphocyte bioenergetics (result not shown).

Lymphocyte activation and modulation of the glycolysis is essential for effector cell function. Recently, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), a glycolytic enzyme, was shown to post-transcriptionally regulate IFN-γ expression by direct binding of mRNA when engagement of glycolysis was blocked [13]. The same group indicated that T-cell proliferation could precede with ATP obtained from oxidative phosphorylation or aerobic glycolysis, depending on the substrates available (e.g. galactose compared with glucose). The neutrophil-dependent ROS formation resulted in modulation of both mitochondrial function and glycolysis and was associated with changes in T-effector cell function and proliferation (Figures 8 and 9).

Clonal expansion and cytokine production reflect the ability of lymphocytes to respond to an activation stimulus and perform their immune-modulating effects locally and systemically. IFN-γ production is stimulated upon lymphocyte activation with PMA; however, 25000 neutrophils were able to suppress IFN-γ production by $>20\%$ (Figures 8C and 8D). Lymphocyte proliferation is suppressed after only a short exposure to neutrophil-derived H_2O_2 (Figures 9C and 9D). Interestingly, catalase treatment in the absence of neutrophils increases IFN-γ production and proliferation (Figures 8E, 8F, 9E and 9F). This is consistent with studies showing that a ROS generating system, possibly a NADPH oxidase, is present and functional in activated lymphocytes which would allow for auto-suppression of effector immune functions [31,49]. Indeed, the immediate increase in OCR observed with PMA in lymphocytes alone appears to be due to an increasing non-mitochondrial oxygen consumption, consistent with activation of an NADPH oxidase. Furthermore, catalase treatment reversed the inhibitory effects of the neutrophil oxidative burst on lymphocyte IFN-γ production and caused an even greater degree of proliferation, further supporting this hypothesis. To determine if endotoxin contamination could also be the cause, a Limulus amebocyte lysate (LAL) was performed and revealed a negligible amount of endotoxin (35 pg) exposure to the cells (result not shown). Furthermore, endotoxin is not a super-antigen and requires an antigen presenting cell for lymphocyte activation, which is absent from our co-culture system. Taken in the context of the metabolic switch and their suppressed cytokine production, these data indicate that H_2O_2 is regulating the immune response by preventing these cells from undergoing a metabolic switch to support these essential effector functions.

In conclusion, impaired metabolism and reduced activation potential in lymphocytes is mediated by H_2O_2 generated from the neutrophil oxidative burst. The relative proportions of neutrophils and lymphocytes at the site of inflammation are subject to duration, severity and type of infection [19]. Our study shows the coincident disruption of the metabolic switch and loss of activation, prompting future studies to investigate the specific metabolic targets. This mechanism may be in place to prevent the unrestrained activation of lymphocytes in the presence of neutrophil necrotic debris and thus any impaired oxidative burst capacity could enhance lymphocyte activation and so contribute to chronic inflammation and autoimmunity.

CLINICAL PERSPECTIVES

- The present study was undertaken to discern the means by which neutrophils suppress lymphocyte immune function, an interaction which is becoming increasingly appreciated in current literature.
- In the present study, we demonstrate that NADPH oxidase-derived hydrogen peroxide from activated neutrophils results in significant mitochondrial and glycolytic dysfunction in lymphocytes. Disruption of the lymphocytes metabolic switch from oxidative phosphorylation to a more glycolytic metabolism corresponds to decreased cytokine production and clonal expansion.
- This may be an important mechanism by which neutrophil-derived oxidative signalling regulates lymphocyte effector function, a process which may be essential to suppressing the inflammation associated with autoimmune disorders and chronic inflammation.

AUTHOR CONTRIBUTION

Philip Kramer, Lynn Prichard, Victor Darley-Usmar performed experimental design, analysis of data and interpretation of results and writing and revising the manuscript. Philip Kramer conducted Seahorse experiments. Lynn Prichard and Philip Kramer conducted flow cytometry experiments. Turner Overton and Sonya Heath interpreted results and contributed to the writing and revising of the manuscript. Balu Chacko and Saranya Ravi contributed to the design and performance of the experiments and manuscript revision.

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DISCLOSURE

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