Modulation of Oxidative Burst with Exposure to Cytokines in Neutrophil Cell Activation

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Introduction

- Neutrophils are essential for killing microorganisms and have a significant role in the regulation of inflammatory responses. Microenvironment, receptor signaling and metabolism all play key roles during neutrophil activation.
- Stimulated neutrophils activate membrane-associated NADPH oxidase (NOX2) resulting in a powerful oxidative burst during which a large amount of oxygen is consumed generating ROS. Generation of ROS is critical for effective microbial immunity but also play a significant role modulating inflammatory responses.
- In this study, neutrophil activation in the presence of cytokines known to be expressed within microenvironments in normal and disease states was evaluated.

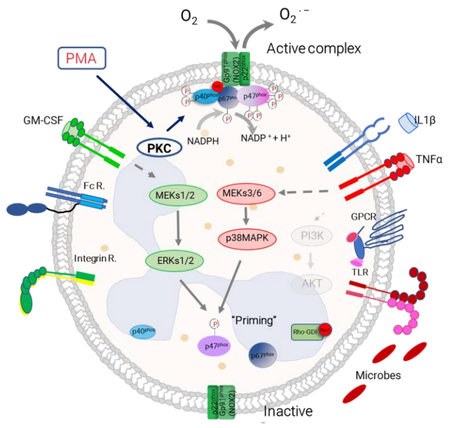

Figure 1. Neutrophils express several groups of receptors that can induce the activation of the NADPH oxidase (NOX2) complex. Ligand binding to cytokine receptors, TLR, and GPCRs can also transform neutrophils to a "primed" state where they are more susceptible to NOX2 activation by secondary stimulus. GM-CSF and TNFα prime NOX2 by MEK signaling pathways leading to phosphoryla-tion of Ser345 residue of p47^{phox}.

Experimental Approach

Method:

The Agilent Seahorse XF Neutrophil Activation Assay provides a direct, non-invasive, real- time detection and quantification of neutrophil activation by measuring oxygen consumption rate (OCR). This assay also provides a simultaneous measurement of proton efflux rate (PER) an indicator of glycolysis.

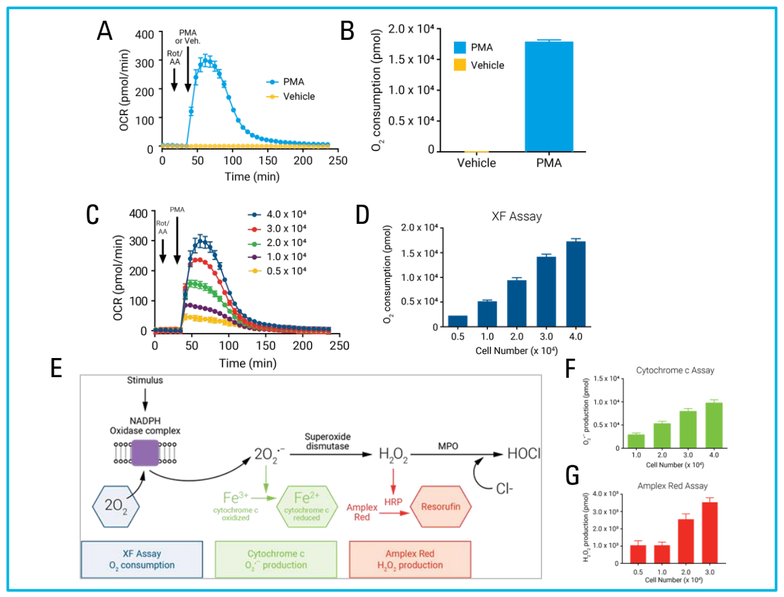

Figure 2. (A) XF Neutrophil Activation Assay kinetic trace of OCR, (C) with varying cell number. An initial injection of inhibitors rotenone and antimycin A (Rot/AA) to discard mitochondrial contribution to OCR, is followed by an injection of activator phorbol 12-myristate 13-acetate (PMA, 100 ng/ml) or vehicle control (black arrows). (B) and (D) O_2 consumption calculated from area under the curve (AUC) of XF kinetic trace. The cell titration demonstrates the sensitivity and linearity of method. (E) Scheme of oxidative burst noting assays used to measure neutrophil activation. Similar responses were obtained when compared with quantitation of superoxide production by cytochrome c assay (F) and quantitation of hydrogen peroxide production by Amplex Red assay (G).

- Human neutrophils where isolated by from fresh whole blood using immunomagnetic depletion, with an erythrocyte depletion step.
- Neutrophils were seeded on Cell-Tak™ coated XF96 cell culture microplates/miniplates at a density of 4 x10^4 cells per well.

Results and Discussion

Mitochondrial respiration is not required for oxidative burst in neutrophils

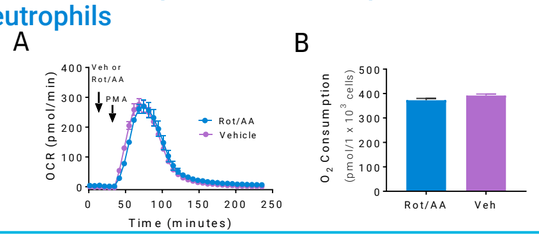

Figure 3. XF neutrophil activation assay with or without addition of Rot/AA prior to PMA stimulation. (A) XF kinetic trace of OCR. (B) O_2 consumption calculated from AUC.

Oxidative burst in neutrophils requires glucose

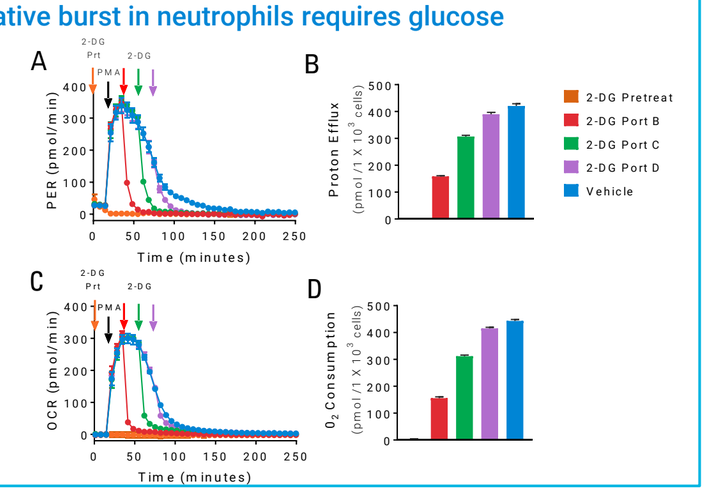

Figure 4. XF neutrophil activation assay with inhibition of glycolysis in real-time with injection of 2-DG using the programable injection ports of XF analyzer. Data include pretreated and vehicle control. XF kinetic trace of PER (A) and OCR (C). The total proton efflux (B) and total O_2 consumption (D) calculated from AUC.

Cytokine priming leads to enhanced neutrophil activation

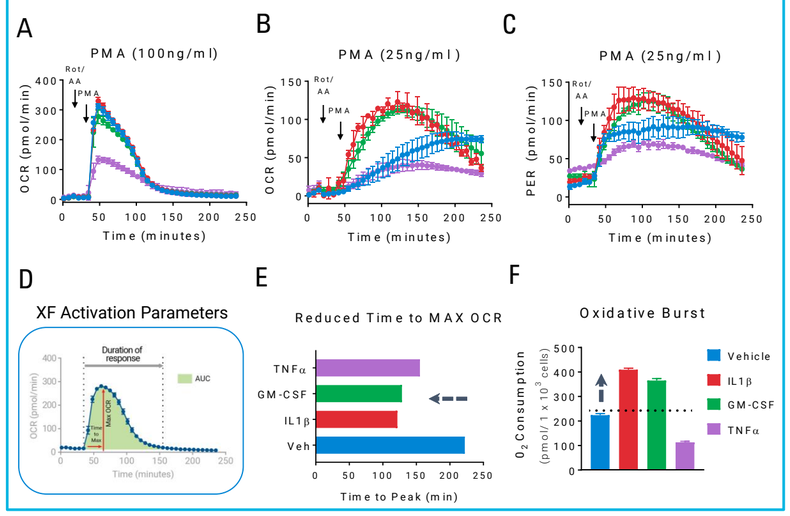

Figure 5. Neutrophils pretreated with IL1β (50ng/ml), GM-CSF (20ng/ml) or TNFα (100ng/ml) for 1h prior to XF neutrophil activation assay. (A) XF neutrophil activation with 100ng/ml PMA or (B) 25ng/ml PMA suboptimal dose. (C) XF kinetic trace of PER with 25ng/ml PMA (D) Scheme of the XF activation parameters. (E) Time to Max OCR. (F) O_2 consumption calculated from AUC.

TNFα alone induced a small oxidative burst

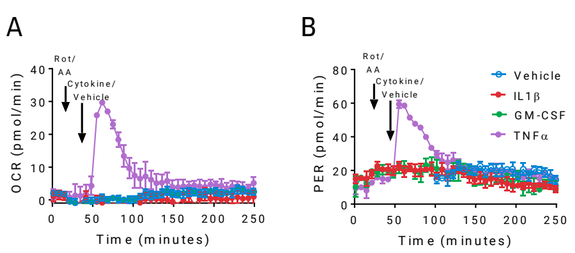

Figure 6. XF kinetic trace of OCR (A) and PER (B) with IL1β (50ng/ml), GM-CSF (20ng/ml) or TNFα (100ng/ml) acute injection.

Results and Discussion

Extended treatment with GM-CSF results in higher basal PER and enhancement of activation by PMA

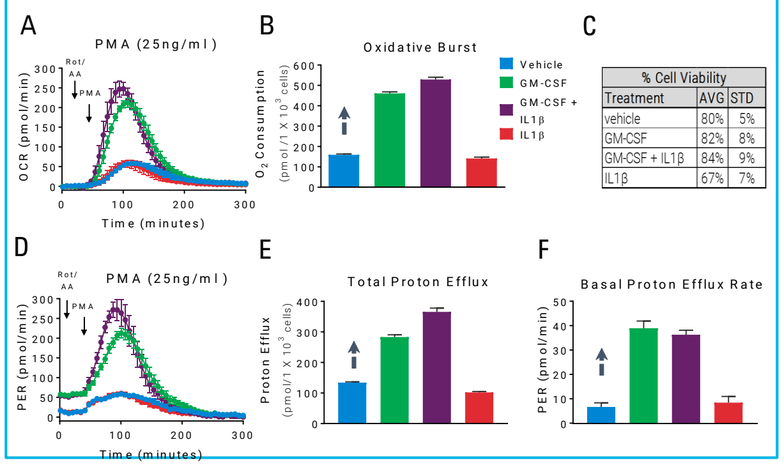

Figure 7. Neutrophils pretreated for 18h with IL1β (50ng/ml), GM-CSF (20ng/ml) or combination in RPMI media with 10% autologous serum. (A) XF kinetic trace of OCR. (B) O_2 consumption calculated from AUC. (C). Neutrophil Cell Viability after 18hr treatment. (D) XF kinetic trace PER. (E) Total Proton Efflux calculated from AUC. (F) Basal PER prior to PMA activation.

Conclusions

- The XF neutrophil activation assay is a specific, quantitative and kinetic assay performed on live cells in real time. The XF analyzer is used to quantify oxygen consumption rate (OCR) as a direct non-invasive measure of neutrophil activation. The simultaneous measurement of PER provides further insight into how neutrophils meet the energy demands upon activation.
- Pretreatment and serial administration of 2-DG demonstrates the requirement of glycolysis to meet energy and substrate demands of oxidative burst. Neutrophils stimulated in the presence or absence of mitochondrial inhibitors Rot/AA, indicates mitochondrial respiration is not required.
- TNFα alone induced a small oxidative burst within 1h of treatment, as observed in real time using the XF analyzer. This acute effect appeared to reduce the ability of neutrophils to respond to subsequent PMA activation within 1h.
- Treatment with GM-CSF and IL1 β alone had no acute effect, but primed neutrophils for enhanced response to PMA. GM-CSF pretreatment for 18h not only enhanced PMA induced oxidative burst, but increased basal PER indicative of an increased basal glycolysis, signifying the cells were metabolically poised for NOX2 activation.
- XF neutrophil activation assay provides a simple and direct way to examine the effect of modulators such as drug treatments and microenvironment on oxidative burst (ROS production) and metabolic effects thru parallel measure of glycolysis.

References

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