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# Technical Report

## Regulation of DC metabolism by nitric oxide in murine GM-CSF cultures

The regulation of immune cell differentiation and function by intracellular metabolism has recently attracted strong interest. In dendritic cells (DCs), the maturation process required for efficient priming of adaptive immunity has been linked to metabolic reprogramming. As DCs are very rare in tissues, researchers often turn to *in vitro*-generated BM-derived DC cultures to improve their yield. Accordingly, the study of DC metabolism has been propelled by the use of GM-CSF cultures [1]. In GM-CSF-derived DCs, TLR activation leads to an early (<1 hour) upregulation of glycolysis [2]. This glycolytic shift was postulated to fuel the synthesis of new fatty acids required to support the expansion of ER and Golgi membranes essential for producing a vast array of new proteins. However, conditional deletion of ACC1, the rate-limiting enzyme of fatty acid synthesis, had no effect on DC activation [3].

At later timepoints (>6h), stimulated GM-CSF-derived DCs favor aerobic glycolysis as their oxidative phosphorylation (OXPHOS) rates decrease [4, 5]. Increased glycolysis has been deemed essential for DC activation [5] and a consequence of the expression of inducible nitric oxide synthase (iNOS). iNOS produces nitric oxide (NO) which inhibits mitochondrial respiration [6]. Thus, NO was proposed to shift DC metabolism from OXPHOS to aerobic glycolysis. Yet, *bona fide* DCs do not express iNOS, thus suggesting that this effect is characteristic of GM-CSF cultures.

Indeed, until recently, DC differentiated from GM-CSF cultures were defined as MHC class II (MHCII)$^{+}$CD11c$^{+}$ and the wide range of MHCII expression in this subset was thought to reflect differences in basal activation. However, it has been shown that the MHCII$^{+}$CD11c$^{+}$ fraction within GM-CSF cultures comprises a MHCII$^{high}$CD11b$^{int}$ population resembling DCs (denominated GM-DCs) and a MHCII$^{low}$CD11b$^{high}$ population more closely related to macrophages (GM-Macs) [7]. Although both subsets respond to TLR stimuli, only the GM-Macs fraction upregulated iNOS gene expression upon LPS treatment [7]. This led us to hypothesize that these two subpopulations might also exhibit different metabolic signatures.

After 7 days of culture with GM-CSF (see Supporting Information for methods), we identified two populations within the CD11c$^{+}$ compartment based on the expression of MHCII and CD11b, as previously described [7]. In accordance with existing gene expression data [7], only the MHCII$^{low}$CD11b$^{high}$ population (GM-Macs) upregulated iNOS upon activation (Fig. 1A and B). We next analyzed the bioenergetics of these populations 24 hours after LPS stimulation. Interestingly, the maximal oxygen consumption rate (OCR) of the GM-DC subset slightly increased upon activation (Fig. 1C), indicating higher rates of mitochondrial respiration. Conversely, LPS treatment caused an almost complete loss of mitochondrial respiration in GM-Macs (Fig. 1C), which was rescued by the addition of the iNOS inhibitor SEITU, confirming the involvement of NO in this effect (Fig. 1D). Intriguingly, there was no change in glycolytic rates of GM-DCs after activation (Fig. 1E). Since previous work has shown that TLR activation induces glycolysis in bulk GM-CSF cultures, we speculated that the NO produced by the GM-Mac fraction may act on the DC fraction to inhibit their mitochondrial respiration. Therefore, DCs would engage in glycolysis to maintain their energy levels. To test this hypothesis, we cultured sorted GM-DCs and GM-Macs together in a 1:1 ratio and analyzed their glycolytic rate after overnight stimulation with LPS. The mixed culture displayed higher glycolytic capacity than the GM-DC fraction alone, which increased further with LPS treatment (Fig. 1E). To confirm that NO alone could promote a glycolytic metabolism in GM-DCs, we sorted and cultured them in the presence of a NO donor, DETA-NONOate (DETA). Interestingly, DETA alone increased the glycolytic capacity of GM-DCs (Fig. 1F). Furthermore, we confirmed that total loss of iNOS in GM-CSF cultures from Nos2$^{-/-}$ mice (Fig. 2A) rescued mitochondrial respiration upon LPS activation, albeit not completely (Fig. 2B). Most importantly, we observed that Nos2$^{-/-}$ GM-Macs were unable to inhibit the OCR in WT GM-DCs upon LPS activation, while WT GM-Macs drastically inhibited the OCR of Nos2$^{-/-}$ GM-DCs (Fig. 2C).

Together, our results support the existence of two cell populations with different metabolic profiles within the CD11c$^{+}$MHCII$^{+}$ compartment of GM-CSF cultures. More importantly, they expose a potential caveat of metabolic studies conducted using this model. In contrast to previous reports using bulk GM-CSF cultures [4], we observed that the GM-DC fraction increases their mitochondrial respiration after overnight stimulation with LPS without changing their glycolytic activity. Furthermore, our work shows that the GM-Mac fraction produces NO upon activation, which can diffuse and act on surrounding cells, including GM-DCs. By interacting with components of the electron transport chain, NO blocks mitochondrial respiration in GM-DCs, which engage in glycolysis to maintain energy levels. This could explain the results previously obtained for unsorted GM-CSF cultures [4] and cautions that accurate analysis of intrinsic DC or macrophage metabolism strictly necessitates sorting of the populations.

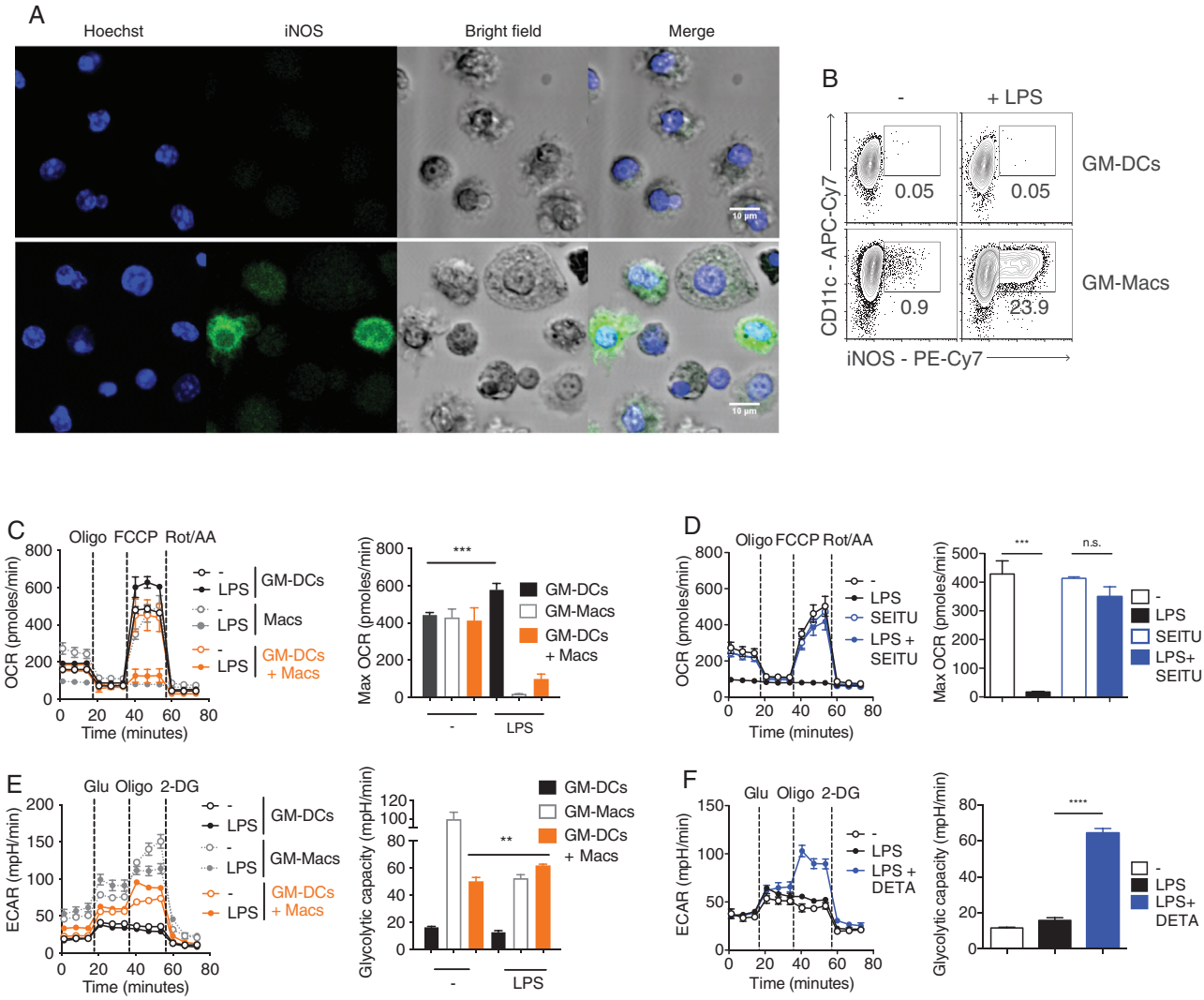


**Figure 1.** GM-Macs influence GM-DC metabolism upon activation. Sorted GM-DCs (upper panel) and GM-Macs (lower panel) were cultured with LPS for 24 hours and iNOS expression was determined by (a) confocal microscopy and (b) flow cytometry. (c) Oxygen consumption rates (OCR, left) and quantification of maximal OCR (Max OCR, right) from GM-DCs and GM-Macs or a 1:1 mixture of GM-DCs+Macs cultured with or without LPS for 24 hours, measured by extracellular flux analysis. (d) OCR (left) and Max OCR (right) from GM-Macs stimulated with LPS in presence or absence of SEITU. (e) Extracellular acidification rates (ECAR, left) and calculated glycolytic capacity (right) from GM-DCs and GM-Macs or a 1:1 mixture of GM-DCs+Macs cultured for 24 hours with or without LPS. (f) ECAR (left) and calculated glycolytic capacity (right) from GM-DCs cultured for 24 hours with or without LPS and DETA. Representative results from 2 (a and b) or 3 (c–f) independent experiments are shown. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ by one-way ANOVA.

Thus, results obtained with bulk GM-CSF cultures cannot be extrapolated to conventional DCs. *Ex vivo* cDCs do not express iNOS or increase their glycolytic rate upon overnight LPS activation [4]. Moreover, Flt3L-*in vitro*-generated cDCs increase their mitochondrial respiration after TLR ligation [8], similarly to MHCII$^{high}$CD11b$^{int}$ cells (Fig. 1C). However, the signaling pathways triggering these metabolic changes or its functional relevance *in vivo* remain unknown.

The information available on the metabolism of human DCs is derived almost exclusively from DCs differentiated from blood monocytes with GM-CSF + IL-4 [9]. Detailed information regarding *bona fide* DCs and macrophage populations in human GM-CSF cultures or their iNOS expression is not reported and requires further investigation. Additional insight on the metabolic requirements of *bona fide* human DC subsets could be essential for developing new DC-based immunotherapies that target metabolic processes.

Our study also introduces the question of whether DC metabolism is differentially regulated under inflammatory conditions through NO production *in vivo* in a paracrine manner. NO may be a critical modulator of immune and non-immune cell metabolism during acute inflammation [10], as recently shown in a Leishmania infection model whereby NO acts as a signal to shut down inflammation [11]. However, we did not detect any defects in the activation of iNOS-deficient GM-CSF DCs treated with LPS (unpublished

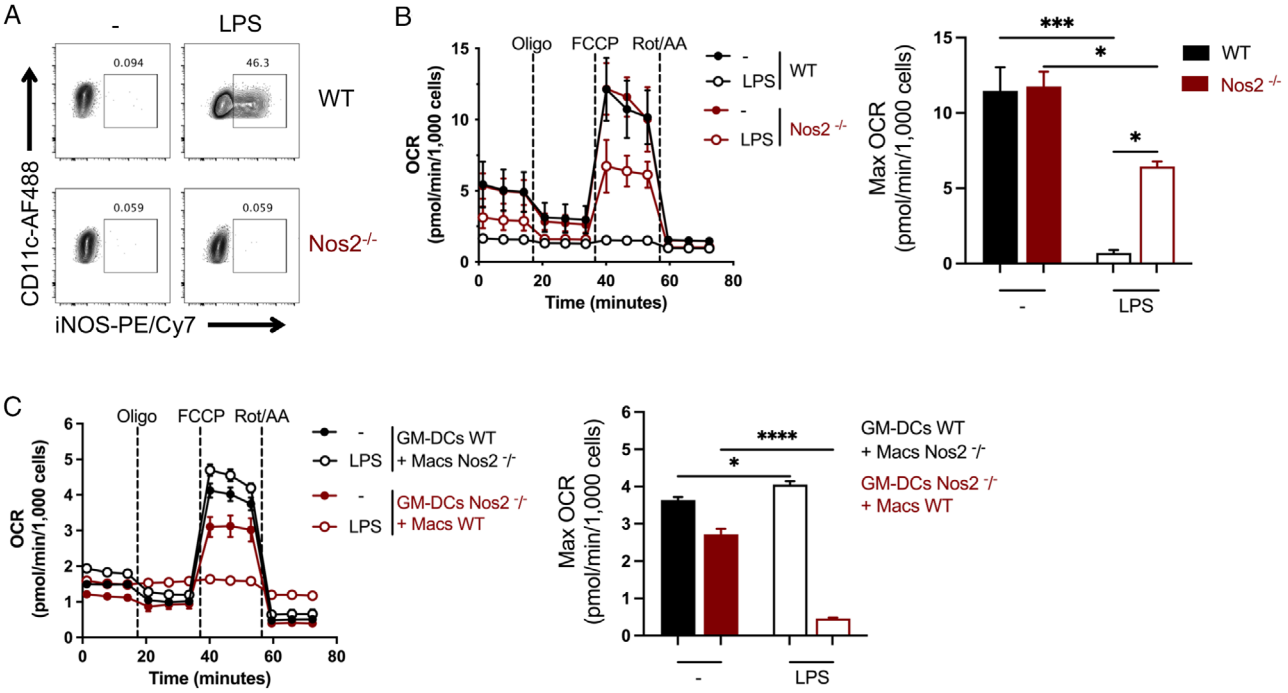


**Figure 2.** NO inhibits mitochondrial respiration of GM-CFS cell culture. Sorted GM-DCs, GM-Macs, or total cells of GM-CFS cultures from WT and Nos2$^{-/-}$ mice were activated for 24 hours with LPS and iNOS expression was determined by (a) flow cytometry. OCR (left) and Max OCR (right) from total (b) or a 1:1 mixture of GM-DCs+GM-Macs from WT and Nos2$^{-/-}$ (c) GM-CSF cultures stimulated with or without LPS for 24 h, measured by extracellular flux analysis. Representative results from three independent experiments are shown. *P < 0.05, ***P < 0.001, ****P < 0.0001 by one-way ANOVA.

observations), opening the question of whether the metabolic rewiring through NO and DC activation are indeed linked.

In conclusion, our findings caution against the use of mixed *in vitro* cultures to study DC metabolism and highlight the need to implement models that resemble DC populations found *in vivo*.

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The detailed *Materials and methods* for Technical comments are available online in the Supporting information