Metabolic regulation of immunological synergism between Toll-like receptor and Fc-receptor signalling

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ABSTRACT

Macrophages and dendritic cells (DCs), critical immune regulators, demonstrate synergistic cytokine production upon co-stimulation with Fc-receptor and Toll-like receptor (TLR) ligands. Since cytokine production upon TLR-triggering depends on glycolysis, this research investigated whether cytokine synergism also requires glycolysis. This was analysed in (co)-stimulated human macrophages, monocyte-derived DCs (MODCs) and retinoic acid DCs (RADCs). We showed that co-stimulation additionally enhances glycolysis compared to TLR-stimulation alone. However, synergism depended on glycolysis in RADCs only, whereas macrophages and MODCs showed synergistic gene expression, indicating translational and transcriptional regulation respectively. Elucidating these mechanisms could improve clinical manipulation of immune responses.

Keywords

Macrophages, dendritic cells, co-stimulation, glycolysis.

INTRODUCTION

Antigen presenting cells (APCs) like dendritic cells and macrophages play an important modulatory role in both innate and adaptive immune reactions. APCs are activated upon recognition and uptake of pathogens. Subsequently, cytokine production is induced, which plays a major role in orchestration of the immune response.[1] When the adaptive immune response has developed, pathogen-specific antibodies are produced that opsonize the pathogen.[2] Opsonized pathogens can be recognized by their molecular patterns and bound by the antibodies via pattern-recognizing receptors like Toll-like receptors (TLRs) and Fc-receptors (FcRs) respectively on APCs. While FcR-triggering on its own induces little cytokine production, co-stimulation of both FcR and TLRs synergistically enhances cytokine production.[1]

For the activation of dendritic cells and M1-type (pro-inflammatory) macrophages, a switch in cellular metabolism from oxidative phosphorylation to glycolysis is required.[3] In dendritic cells, TLR signaling promotes the function of hexokinase 2 (HKII) which catalyses the first step in glucose metabolism and is crucial for enhancement of glycolytic flux. In general, the glycolytic pathway supports *de novo* fatty acid synthesis in two ways. Glycolysis stimulates the pentose phosphate pathway (PPP) in which NADPH is generated and supplies carbons to the TCA cycle which generates citrate that is used in fatty acid synthesis. Fatty acids are then used to expand the endoplasmic reticulum and Golgi apparatus, stimulating cytokine production at translational level.[4,5]

Aforementioned findings derived from murine studies focused only on the metabolic regulation of TLR induced responses. In this research, we sought to determine what role metabolism, in particular glycolysis, plays in the synergistic effect in cytokine production upon co-triggering of TLRs and FcRs in human APCs. To this end, macrophages and dendritic cells were differentiated *in vitro* from human monocytes, isolated from peripheral blood, and stimulated with Pam and/or IgG or IgA. Cytokine production and cellular metabolic profiles with or without glycolytic inhibitors were analysed to determine the role of glycolysis in synergistic cytokine production.

METHODS

In vitro cell culture and differentiation

Mononuclear cells were isolated from human peripheral blood in a ficoll-amidotrizoaat density gradient. Monocytes were separated by MACS® Technology using CD14 MicroBeads (Miltenyi Biotec) and purity (>92%) was measured by flow cytometry. Monocytes were cultured at 37°C, 5% CO2 at a density of $1.75\text{-}2*10^6$ cells per well in 4 or 5 mL complete medium (RPMI-1640 containing 10% FCS, 100 U/mL Penicillin, 100 µg/mL Streptomycin and 2 mM Glutamate; ThermoFisher). M1 and M2 macrophages were differentiated in presence of GM-CSF (40 ng/mL) or M-CSF (20 ng/ml) respectively. For differentiation to monocyte-derived DCs (MODCs), GM-CSF (40 ng/mL) and IL-4 (0.86 ng/mL) were added. In addition to identical concentrations of GM-CSF and IL-4, retinoic acid (1 µM) was added to obtain retinoic acid DCs (RADCs).[6] Growth factors were added again at identical concentrations during medium refreshment after 2 or 3 days.

Cell harvest and stimulation

Cells were harvested using Cellstripper™ (Corning) or TrypLE Select™ (ThermoFisher). For stimulation with complexed IgG or IgA, wells were coated by 1 hour incubation IgG or IgA (4 and 2 µg/mL respectively) in PBS at 37°C. IgA was used to stimulate RADCs, whereas M1 and M2 macrophages and MODCs were exposed to IgG. Pam3Cys (10 µg/mL) or an equal amount of complete medium was added, resulting in four conditions: non-stimulated, Pam3Cys-stimulated, IgG/IgA-stimulated or Pam3Cys- and IgG/IgA-stimulated cells. Specific

metabolic processes were addressed by treating cells with metabolic inhibitors as indicated: oligomycin (1 µM), 2-DG (1 or 10 mM), UK5099 (50 µM), oxamate (50 mM). For cytokine analysis, cells were stimulated in 96-wells plates with a density of 25,000 cells in 210 µL. After 24 hours of stimulation, supernatants were taken and stored at -20°C. For metabolic analysis, 10%FCS/RPMI was replaced by 10%FCS/XF medium (RPMI medium without phosphate or carbonate buffer). 30,000 cells per well were plated in XF^{e}96 cell culture microplates (Seahorse Bioscience). In order to analyse gene expression, 100,000 cells in 400 µL were stimulated for 3 hours. Cells were harvested, washed with PBS and lysed by Buffer RLT supplied with 1% β-mercaptoethanol. Cells were stored at -80°C until used for RNA isolation.

Metabolic analysis

To investigate cellular metabolism, the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were analysed with an XF-96 Extracellular Flux Analyser (Seahorse Bioscience) as described.[7] As lactate is produced during glycolytic function, ECAR can be used to measure glycolysis. Oxygen consumption is a measure of mitochondrial respiration. During the run, ECAR and OCR were measured in response to Pam3Cys (10 µg/mL), oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) and a combination of antimycin A and rotenone.

Analysis of produced cytokines

Interleukin-10 (IL-10) and tumour necrosis factor α (TNFα) concentrations in cell supernatants were measured by ELISA, using PeliKine IL-10 and TNFα ELISA kits (Sanquin) and TNFα ELISA Max^{TM} Deluxe kit (BioLegend). When using Sanquin kits, plates were coated overnight with coating antibodies (1:100 in 0.1 M Na_2CO_3 buffer, pH 9.6). To block aspecific binding, plates were incubated in PBS/1%BSA for one hour. Then, standard dilution series and samples were incubated for one hour, followed by one hour incubation with biotin-conjugated antibodies (1:100). Streptavidine-polyHRP (1:10,000) was incubated for 30 minutes. Substrate solution was incubated in the dark for approximately 15 minutes. Reaction was stopped by adding 1.8M H_2SO_4. Plates were read by transmission at 450 nm. When TNFα was measured by using BioLegend kits, manufacturer's protocol was followed. Except for adding stop solution, all steps were preceded by washing with washing buffer (0.005% Tween 20 (Sigma-Aldrich) in 1% PBS).

Gene expression analysis

For RNA isolation, RNeasy® Plus Mini Kit or RNeasy® Mini Kit (Qiagen) was used according to manufacturer's protocols, including treatment with DNAse in the latter. For cDNA synthesis, a mix was made containing 4 UI 5x First-strand buffer, 2 UI 0.1 M DTT, 1 UI RNAseOUT (40 U/ul), 1 UI random primers (250 ng/ul), 2 UI dNTPs (6.25 mM of dATP, dCTP, dGTP and dTTP each) and 1 UI mMLV. A control condition without mMLV was included. 11 µL of the mix was added to 9 µL RNA. The following program was used for cDNA synthesis: 10 min 25°C, 50 min 37°C, 15 min 70°C. Next, TNFα and IL-10 expression was measured by RT-PCR. Expression was normalized to housekeeping gene β 2-microglobulin. To 2 µL of 3x diluted cDNA, 8 µL of primer mix was added, containing 2 UI H2O, 5 UI 2x SYBR Green (Bio-Rad), 0.5 UI forward primer (1 µM) and 0.5 UI reverse primer (1 µM) (table 1). Expression values were calculated based on the ΔΔCt method.

Table 1 Primer sequences used to determine β 2-microglubulin, TNFα and IL-10 expression

Gene	Forward and reverse primer
B 2-microglobulin	TGCCGTGTGAACCATGTGA
	CCAAATGCGGCATCTTCAA
TNFα	TCTTCTCGAACCCCGAGTGA
	CCT CTGATGGCACCACCAG
IL-10	ACCTGCCTAACATGCTTCGAG
	CCAGCTGATCCTTCATTTGAAAG

Flow cytometry

Monocyte purity (>92%) was measured by using anti-CD14 (MΦP9). Cell differentiation was ascertained by the following markers: anti-CD1A (BL6 or HI149), anti-CD14 (MΦP9), anti-CD103 (Ber-ACT8), anti-CD163 (GHI/61) and anti-CD206 (15-2). Fc receptor binding was prevented by adding FcR-Block (eBioscience). Aqua-staining was used to distinguish life cells from dead cells. Cells were washed in FACS buffer. For staining, cells were incubated at 4°C for 30 minutes in the dark. After two washes in FACS buffer, cell staining was measured with FACSCanto Flow Cytometer (BD Bioscience).

Statistical analysis

Data were analysed with GraphPad Prism version 6 (GraphPad Software). Multiple t-tests were used according to the Holm-Sidak method to determine statistical significance (α=5%) of differences between TLR- and TLR/FcR-stimulation. One-way ANOVA including Tukey's multiple comparisons was used to analyse differences between ECAR- and OCR-values in stimulatory conditions.

RESULTS

Cell differentiation was achieved

Cell differentiation was shown to be achieved by measuring cell-type specific markers in flow cytometry. Moreover, M1 macrophages produced mainly TNFα, whereas M2 macrophages showed higher IL-10 levels, confirming their pro- and anti-inflammatory character.

Co-stimulation enhances glycolysis additionally

Stimulation of all cell types with TLR-ligand Pam3Cys or FcR-stimulus Ig showed an enhanced ECAR, indicating an increased glycolytic flux (figure 1). Interestingly, co-stimulation of M1 and M2 macrophages and RADCs enhanced glycolysis more than stimulation of FcR alone. Moreover, maximal glycolytic capacity of cells was increased upon (co)-stimulation. OCR was not significantly changed upon stimulation. Since metabolic effects were especially clear in M1 macrophages and RADCs, further experiments focused on these cells.

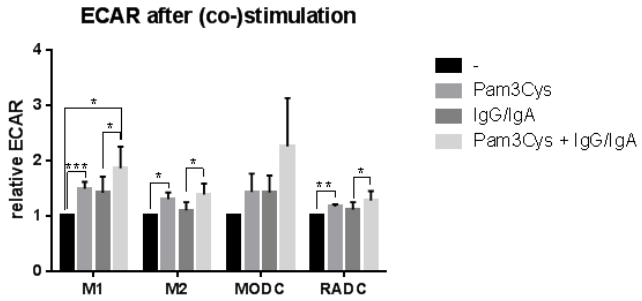

Figure 1 ECAR is enhanced by TLR- or FcR-stimulation, whereas co-stimulation seems to induce an additional increase. (Relative to unstimulated condition; M1: n=6; M2, MODC: n=4, RADC: n=5; error bars depict S.D.)

Synergistic cytokine production depends on glycolysis in RADCs

Consistent with literature, synergistic cytokine production was demonstrated upon co-stimulation of TLRs and FcRs in macrophages, MODCs and RADCs.[1] Although co-stimulation showed enhanced glycolysis compared to TLR or FcR-stimulation alone, blocking glycolysis with 2-deoxy-D-glucose (2-DG) did not eliminate synergistic TNFα production in macrophages and MODCs (figure 2A, M1 macrophages shown as example). In contrast, synergism upon co-stimulation in RADCs was dependent on glycolysis, since glycolytic inhibition abolished synergism (figure 2B). Effects on IL-10 production were less clear. For RADCs, this was possibly due to low IL-10 production which hindered accurate measurement.

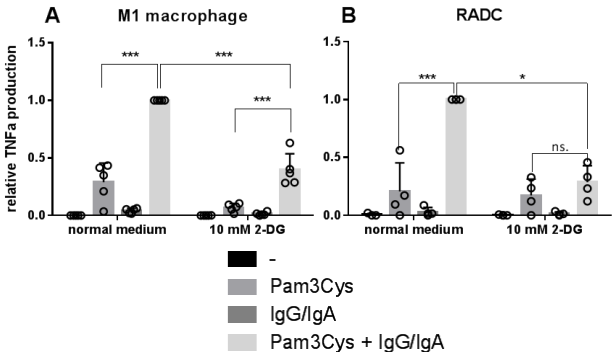

Figure 2 Inhibition of glycolysis with 2-DG abolishes synergistic TNFα production upon co-stimulation in RADCs (B) but not in M1 macrophages (A). (Relative to co-stimulation, normal medium; M1: n=5; RADCs: n=4; error bars depict S.D.)

In addition to inhibition of general glycolysis, metabolic processes that glycolysis feeds into were investigated. Inhibition of mitochondrial pyruvate shuttling with UK5099 or inhibition of ATPase with oligomycin did not affect cytokine production. However, blocking of lactate-dehydrogenase (LDH) with oxamate strongly decreased overall cytokine production.

Synergism at mRNA level in macrophages and MODCs but not in RADCs

At transcriptional level, synergistic gene expression was shown upon co-stimulation compared to TLR-stimulation in M1 and M2 macrophages and MODCs (figure 3A). This effect was independent of glycolysis, since presence of 2-DG during stimulation did not alter gene expression. In contrast, RADCs did not show this synergism upon co-stimulation, indicating that cytokine production after co-stimulation is regulated at translational level (figure 3B).

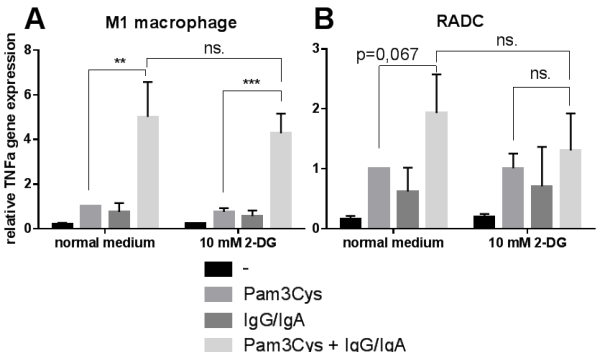

Figure 3 Synergistic TNFα gene expression is present upon co-stimulation in M1 macrophages (A), but not in RADCs (B). (Relative to Pam3Cys-stimulation; M1: n-4; RADC: n=3; error bars depict S.D.)

CONCLUSION

Co-stimulation of TLRs and FcRs on macrophages and dendritic cells induces synergistic cytokine production.[1] This research aimed to investigate whether cellular metabolism is involved in this phenomenon. As shown in murine cells in previous research[5], we demonstrated that glycolysis also increases upon Pam3Cys- and Ig-stimulation in human immune cells. Moreover, this study showed that glycolysis seemed to be additionally enhanced upon co-stimulation in all cell types. Literature already demonstrated that glycolysis supports cytokine production at translational level.[4,5] Since inhibition of glycolysis decreased overall cytokine production, this was confirmed in this study. However, synergistic cytokine production did not depend on enhanced glycolysis in macrophages and MODCs. Moreover, neither inhibition of mitochondrial pyruvate shuttling nor inhibition of LDH eliminated synergistic cytokine production upon co-stimulation. This indicates that synergism in these cells is not regulated at translational level. In contrast, since mRNA levels were synergistically enhanced upon co-stimulation, regulation takes place at transcriptional level.

Contrary to M1 and M2 macrophages and MODCs, synergistic cytokine production in RADCs was dependent on glycolysis. Blocking glycolysis with 2-DG abolished synergistic cytokine production upon co-stimulation. No synergism was present at mRNA level, pointing towards translational regulation. This corresponds with glycolytic support of cytokine production by promoting endoplasmic reticulum (ER) expansion, hence facilitating protein synthesis.[4,5] Glycolysis contributes to ER expansion by stimulating fatty acid synthesis via citrate production in the tricarboxylic acid (TCA) cycle and stimulation of the pentose phosphate pathway (PPP) which provides NADPH. However, inhibition of pyruvate shuttling, thereby blocking the TCA cycle, did not eliminate synergistic production. PPP-activation by

glycolysis might be sufficient to stimulate ER expansion. Direct inhibition of PPP could clarify the role of this pathway. Moreover, other molecules could supply carbon atoms for citrate production.

RA could affect differentiation and cellular function in various ways, since both genomic effects by nuclear receptor binding and nongenomic effects, such as stimulating kinase cascades, have been shown.[8] Previous studies presented several effects of RA on cytokine production that might be involved, e.g. suppression of NFκB-dependent gene expression, which includes TNFα.[9] These processes might be involved in forcing cytokine regulation to translational level in RADCs. To clarify increased translation, effects of RA on ribosomal activity could be studied. For instance, expression of ribosomal RNA 18S might be enhanced.

Collaboration of receptors as presented in this study has been shown before. For instance, co-stimulation of Dectin-1 and TLR2 leads to synergistic cytokine production as well. Dennehy et al demonstrated that Syk, which also mediates FcR-signalling, plays an essential role in this cooperation in murine macrophages by sustaining IκB degradation, thereby elongating nuclear localization of NFκB.[10] Since synergism in macrophages and MODCs was based on enhanced transcription, it could be investigated whether this phenomenon plays a role in FcR/TLR2 collaboration. Another process that could be involved was demonstrated by Vogelpoel et al in unpublished data, showing converging stimulation of interferon regulatory factor 5 (IRF5). TLR-signaling induced IRF5-activation by phosphorylation, whereas Syk-activation stimulated nuclear translocation.

In conclusion, co-stimulation of TLR and FcR enhances glycolysis additionally, but this effect is only required for synergistic cytokine production in RADCs. Whereas macrophages and MODCs showed transcriptional regulation, RADCs showed regulation at translational level. Further research could focus on differences between signalling in cells with transcriptional versus translational regulation and the pathways involved in pro-inflammatory at one hand and anti-inflammatory cytokines at the other hand. This knowledge can be used to manipulate immune responses in clinical settings.[11] For instance, we demonstrated that metabolic changes are important in the shift of RADCs, a model of intestinal tolerogenic DCs[6], towards a pro-inflammatory profile, producing more TNFα. Targeting metabolism could be a therapeutic strategy to either stimulate or inhibit the pro-inflammatory response: an anti-bacterial immune response should be increased, whereas dampening of inflammation is needed in inflammatory bowel disease for example.

ROLE OF THE STUDENT

Elsa Kuijper, bachelor student Biomedical Sciences, worked under supervision of dr. Bart Everts at the department of Parasitology in the LUMC. The topic was proposed by B. Everts, who also designed experiments in consultation with the student. Experiments were performed and analysed by the student in cooperation with ing. Alwin van der Ham and B. Everts. The rapport was written by the student.

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REFERENCES

1. Vogelpoel LTC, Baeten DLP, Jong de EC, Dunnen den J. FcγRIIa cross-talk with TLRs, IL-1R, and IFNγR selectively modulates cytokine production in human myeloid cells. *Immunobiology 220*, (2015), 193-199.
2. Van Egmond M, Vidarsson G, Bakema JE. Cross-talk between pathogen recognizing Toll-like receptors and immunoglobulin Fc receptors in immunity. *Immunological Reviews 268*, (2015), 311-327.
3. Pearce EL, Pearce EJ. Metabolic pathways in immune cell activation and quiescence. *Immunity 38*, (2013), 633-643.
4. Pearce EJ, Everts B. Dendritic cell metabolism. *Nature Reviews 15*, (2015), 18-29.
5. Everts B, Amiel E, Ching-Cheng Huang S et al. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKε supports the anabolic demands of dendritic cell activation. *Nature Immunology 15*, 4 (2014), 323-332.
6. Bakdash G, Vogelpoel LTC, Capel van TMM, Kapsenberg ML, Jong de EC. Retinoic acid primes human dendritic cells to induce gut-homing, IL-10-producing regulatory T cells. *Mucosal Immunology 8*, 2 (2015), 265-278.
7. Pelgrom LR, Ham van der AJ, Everts E. Analysis of TLR-induced metabolic changes in dendritic cells using the Seahorse XFe96 Extracellular Flux Analyzer. In *Methods in Molecular Biology 1390*, Springer Science+Business Media New York (2016), 273-285.
8. Al Tanoury Z, Piskunov A, Rochette-Egly C. Vitamin A and retinoid signaling: genomic and nongenomic effects. *Journal of Lipid Research 54*, (2013), 1761-1775.
9. Zeng R. Generation and transcriptional programming of intestinal dendritic cells: essential role of retinoic acid. *Mucosal Immunology 9*, 1 (2016), 183-193.
10. Dennehy KM, Ferwerda G, Faro-Trindade I. Syk kinase is required for collaborative cytokine production induced through Dectin-1 and Toll-like receptors. *European Journal of Immunology 38*, (2008), 500-506.
11. Everts B, Pearce EJ. Metabolic control of dendritic cell activation and function: recent advances and clinical implications. *Frontiers in immunology 5*, (2014), 203.