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Role of Trained Innate Immunity Activation by Metabolic Reprogramming in Immune Response Regulation Against Emerging Viral Infections.

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Abstract:
Trained innate immunity (TII) is a memory-like adaptation of innate immune effectors that is accomplished through metabolic and epigenetic reprogramming of trained immune cells. In contrast to bacterial and fungal infections where the mechanisms of defense have been well studied, its contribution to antiviral defense is still poorly understood. This study focused on the mechanism of metabolic reprogramming that controls the activation of trained innate immunity and improves antiviral responses during emerging viral infections. Human macrophage derived monocytes and murine models were pre-stimulated with betulinic acid (b-glucan) or monophosphoryl lipid A (MPLA) inducing trained immunity followed by testing with vesicular stomatitis virus (VSV). Seahorse assay, LC-MS/MS, histone modifications (ChIP-qPCR), gene expression and cytokine production were analyzed. β-glucan and MPLA priming promoted hemodynamic glycolytic activation and accumulation of fumarate and succinate as well as stimulating histone marks (H3K4me3, H3K27ac). Trained macrophages had a significant increase in IL-6, TNF-a and IFN-v secretion and upregulation of antiviral genes (IFNB1, ISG15). Survival and virus load were shown to be increased in vivo in v-glucan-trained mice after infection. Metabolic reprograms of antiviral TITI through the connection between activation of glycolysis and epigenetic reprogramming and cytokine priming. These results present immunometabolism as a potential target of new generation antiviral drugs and adjuvants for vaccines.

Keywords: Keywords: Trained Innate Immune Response, Programmed Metabolism, Immunometabolism, Epigenetic Regulation, Antivirus, v-glucan, Cytokines.

دور تنشيط المناعة الفطرية المدربة عن طريق إعادة البرمجة الأيضية في تنظيم الاستجابة المناعية ضد العدوى الفيروسية الناشئة

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الملخص
المناعة الفطرية المُدرَّبة (TII) هي تكيفٌ شبيهٌ بالذاكرة لخلايا المناعة الفطرية الفعالة، ويتحقق ذلك من خلال إعادة برمجة أيضية وجينية للخلايا المناعية المُدرَّبة. وعلى عكس العدوى البكتيرية والفطرية، حيث دُرست آليات الدفاع جيدًا، فإن مساهمتها في الدفاع المضاد للفيروسات لا تزال غير مفهومة بشكلٍ كافٍ. ركزت هذه الدراسة على آلية إعادة البرمجة الأيضية التي تتحكم في تنشيط المناعة الفطرية المُدرَّبة، وتُحسّن الاستجابات المضادة للفيروسات أثناء العدوى الفيروسية الناشئة. تم تحفيز الخلايا الوحيدة المشتقة من البلاعم البشرية ونماذج الفئران مسبقًا بحمض البيتيولينيك (بيتا جلوكان) أو أحادي فوسفات الليبيد أ (MPLA) لتحفيز المناعة المُدرَّبة، ثم تم اختبارها باستخدام فيروس التهاب الفم الحويصلي (VSV). تم تحليل اختبار Seahorse، وLC-MS/MS، وتعديلات الهيستون (ChIP-qPCR)، والتعبير الجيني، وإنتاج السيتوكينات. عزز تحفيز بيتا جلوكان وMPLA تنشيط عملية تحلل الجلوكوز في الدورة الدموية وتراكم الفومارات والساكسينات، بالإضافة إلى تحفيز علامات الهيستون (H3K4me3، H3K27ac). أظهرت البلاعم المدربة زيادة

ملحوظة في إفراز IL-6 وTNF-α وIFN-γ، وزيادة في تنظيم الجينات المضادة للفيروسات (IFNB1، ISG15). كما تبين زيادة معدل البقاء على قيد الحياة والحمل الفيروسي في الجسم الحي لدى الفئران المدربة على بيتا جلوكان بعد الإصابة. إعادة برمجة التمثيل الغذائي لـ TITI المضاد للفيروسات من خلال العلاقة بين تنشيط تحلل الجلوكوز وإعادة البرمجة اللاجينية وتحفيز السيتوكينات. تقدم هذه النتائج التمثيل الغذائي المناعي كهدف محتمل لأدوية الجيل الجديد المضادة للفيروسات والمواد المساعدة للقاحات.
الكلمات المفتاحية: الاستجابة المناعية الفطرية المدربة، الأيض المبرمج، الأيض المناعي، التنظيم اللاجيني، مضاد للفيروسات، في-غلوكان، السيتوكينات.

Introduction

The innate immune system is the first line of defense against intruding pathogens and traditionally termed as an immediate but nonspecific defense. However, evidence has been accumulating that innate immune cells can learn to have a memory-like phenotype called trained innate immunity (TII) [1,2]. This creates a condition in which monocytes, macrophages, and natural killer (NK) cells, when stimulated a second time, have enhanced responsiveness through epigenetic and metabolic reprogramming which is nonetheless not antigen specific [1]. In contrast to adaptive immunity, which is based on clonal expansion and antigen specificity, trained immunity is based on constitutive chromatin remodeling and transcriptional reprogramming with Digital increase of cytokine secretion and pathogen elimination [3,4]. Metabolic reprogramming has come to light as being a key determinant of innate host immune cell activation and function to date. Upon stimulation, innate immune cells are metabolically redirected from oxidative phosphorylation (OXPHOS) to that of aerobic glycolysis, fatty acid oxidation, and glutaminolysis, to meet their energetic and biosynthetic needs [4,5]. Not only these metabolic transitions maintain effector functions they also provide intermediates such as fumarate and succinate as cofactors for epigenetic modifying enzymes and thereby stabilize the trained phenotype [5,6]. For example, exposure to v-glucan results in fumarate accumulation and activation of glycolytic that leads to histone H3K4 methylation and transcription of IL-6 and TNFa by trained monocytes [6,2]. Despite significant progress, the metabolic basis of ongoing immunity in viral infections following training has to be incompletely defined. Whereas most studies were done for bacterial or fungal stimuli, the molecular and metabolic determinants of virus-mediated training remain largely untraced [7,3]. Hosts respond to viruses by limiting viral spread through immunometabolic adaptations that aim at limiting viral replication through hijacking of host cellular metabolism (e.g., glycolysis, lipid metabolism) [8,9]. It has been recently highlighted that metabolic reprogramming in infected macrophages/dendritic cells not only impacts on viral replication kinetics, but also determines cytokine polarization and antiviral gene expression [10,1]. In this context, the present study examines the relevance of metabolic reprogramming in the activation and regulation of trained innate immunity at an early stage of emerging viral infections. Specifically, it focuses on the roles of the glycolytic and mitochondrial pathways in cytokine profiles modulation, antiviral gene expression and the maintenance of the trained immune response. It can be hoped that the elucidation of these mechanisms will inform the rational design of metabolically targeted immunotherapies and next generation antiviral vaccines [7,2].

Materials and methods

Study design

This experimental study was set up to assess the role of metabolic reprogramming in the induction and regulation of trained innate immunity during viral infection. Human monocyte-derived macrophages (MDMs) and peripheral blood mononuclear cells (PBMCs) were used for *in vitro* model while a murine model of viral challenge was used for *in vivo* validation of the result. There were three general phases to the study: Induction of trained immunity by metabolic stimuli. Viral Challenge and secondary stimulation. Assessment of immunometabolic and transcriptional response. All procedures performed involving human blood samples and animals were in accordance with the institutional ethics committee approval and were performed in accordance with international biosafety and animal welfare guidelines (ARRIVE 2.0 standards).

Cell isolation and culture

Whole blood samples were obtained from healthy adult volunteers (20-35 years) by written informed consent. PBMCs were obtained by a density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Sweden). Monocytes were isolated by CD14+ magnetic bead separation (Miltenyi Biotec, Germany), and differentiated to become macrophages by seven days of culture in the medium of RPMI with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 50 ng/ml M-CSF (PeproTech, USA).

Induction of trained immunity

To induce trained immunity, cells were pre-stimulated with b-glucan (5ug/mL, InvivoGen) or monophosphoryl lipid A (MPLA, 1ug/mL, Sigma-Aldrich) for 24 hours. After washing and resting for five days in fresh medium, cells were re-challenged with viral mimics (polyinosinic - polycytidylic acid [poly I:C], 10ug/mL) or live-

attenuated vesicular stomatitis virus (VSV, MOI 0.1) for 24 hours. Untreated cells were used as controls. This two-step stimulation model models the induction and recall of trained immunity [1,2].

Animal model
C57BL/6 Mice (6-8 weeks, n = 24) Mice were divided into four different groups (control, trained - beta-glucan, infected - VSV, trained + infected). 7 days before VSV challenge (1 x 106 PFU, intranasal), beta-glucan was given (1 mg/kg, intraperitoneal). Cytokine and metabolite analysis in blood and spleen samples was taken at 24, 72 and 120 hrs. after infection.

Metabolic assays
Extracellular flux analysis of seahorse
The metabolic profile of trained macrophages was performed by a seahorse XF96 Analyzer (Agilent Technologies, USA). Mitochondrial respiration and glycolytic flux were determined as OCR and ECAR, respectively. Data was normalized to cell number and total protein concentration.

Metabolomics
Global metabolomics was done by liquid chromatography-mass spectrometry (LC-MS/MS, Thermo Fisher Scientific). Metabolites in glycolysis and tricarboxylic acid (TCA) cycle as well as fatty acid oxidation were quantified. Data analysis was performed by the MetaboAnalyst 5.0 platform.

Cytokines and gene expression analysis
Cytokine levels (IL-6, TNF-alpha, IL-1 beta, IFN-beta) were determined by commercial enzyme immunoassays (ELIAs) (R&D Systems, USA) following manufacturer's protocols and instructions.
Total RNA was extracted from treated cells by RNeasy Mini Kit (Qiagen, Germany) and cDNA synthesis was performed by high-capacity reverse transcription kit (Applied biosystems). Quantitative real time PCR (qPCR) was performed using SYBR Green Master Mix (Thermo Fisher) and a StepOnePlus apparatus. Gene expression of IL-6, TNFA, IFNB1, HK2 and PGC1a using the 2^-Delta-Delta Ct method normalised to GAPDH [7,11].

Epigenetic analysis
The histone modifications related to trained immunity were identified by chromatin immunoprecipitation (ChIP)-qPCR. Briefly, chromatin immunoprecipitation was performed with the antibodies against H3K4me3 and H3K27ac (Cell Signaling Technology). Enriched DNA regions corresponding to IL-6 and TNFA promoter were quantified through qPCR.

Flow cytometry
The expression of activation markers (CD80, CD86, HLA-DR) on the cell surface and of intracellular cytokines by flow cytometry (BD FACSAria III) was analyzed. Data were analyzed with the help of FlowJo software version 10.8.

Statistical analysis
All experiments were carried out in triplicate and all data was expressed as mean ± standard deviation (SD). Statistical analyses were performed by GraphPad Prism 10.0 (GraphPad Software, USA). One-way analysis of variance was used for intergroup comparison followed by Tukey's multiple comparison test. Differences were considered to be statistically significant at $p < 0.05$. Correlations between metabolic parameters (ECAR/OCR) with cytokines were done using Pearson correlation coefficient.

Results
Innate immune responsiveness was greatly improved in the 24-hour exposure of Human monocyte-derived macrophages (MDMs) to 24 hours of 1/2-2 -glucan or monophosphorylate lipid A (MPLA). When 1:C challenged with the viral mimic poly I:C was used, secretion of IL-6, TNF- 1:C and IL-1, were significantly increased in the 1:C-trained macrophages over the untrained controls ($p < 0.001$). The effects of MPLA training caused a moderate cytokine increase ($p < 0.05$) and proved that both metabolic and TLR4-based stimuli can form a trained immunity phenotype.

Table 1: The trends of secretion of cytokines after secondary viral challenge

Treatment Group	IL-6 (pg/mL)	TNF-α (pg/mL)	IL-1β (pg/mL)	IFN-β (pg/mL)	p vs Control
Control (untrained + VSV)	420 ± 38	305 ± 26	120 ± 18	210 ± 22	–
MPLA-trained + VSV	610 ± 42	475 ± 34	195 ± 21	275 ± 30	< 0.05
β-Glucan-trained + VSV	985 ± 56	735 ± 48	320 ± 27	460 ± 36	< 0.001

These results confirm that β-glucan induces a stronger cytokine amplification than MPLA, establishing a clear hierarchy in training efficacy.
Upon vesicular stomatitis virus (VSV) infection, poor IFNB1 (3.8-fold) and ISG15 (2.9-fold) upregulation were demonstrated in the group of macrophages trained with beta-glucan as compared to the untrained, infected controls ($p < 0.01$). MPLA-trained cells also showed increased transcriptional activation but this was not as high so this was consistent with a role for metabolic priming in antiviral defense. The induction of MxA and OAS1 in the trained PBMCs indicated the enhanced interferon-mediated protection.

Table 2: Differential gene expression profile in the trained and control macrophages

Gene	Function	MPLA-trained	β-Glucan-trained	p value
IL-6	**Inflammatory cytokine**	2.4 ± 0.3	4.7 ± 0.5	< 0.01
TNFA	**Inflammatory cytokine**	2.1 ± 0.4	3.9 ± 0.6	< 0.01
IFNB1	**Antiviral response**	2.8 ± 0.5	3.8 ± 0.4	< 0.05
ISG15	**Interferon-stimulated gene**	2.2 ± 0.3	2.9 ± 0.3	< 0.05
HK2	**Glycolysis enzyme**	1.8 ± 0.2	3.1 ± 0.4	< 0.05

The significant enhancement in both inflammatory and antiviral gene transcripts was suggestive of a long-lasting transcriptional reprogramming reported for trained immunity.
Seahorse extracellular flux analysis indicated that extracellular acidification rate (ECAR), and moderate decrease in oxygen consumption rate (OCR) resulting in a low OCR/ECAR ratio in the presence of trained macrophage (165% and moderate hampers the glycolytic metabolic shift) was observed in the presence of beta glucose. The same basic pattern was observed with MPLA trained cells, they were less blind than control cells, but still not on par with the HE trained cells.

Table 3: The metabolic flux parameters

Parameter	Control	MPLA-trained	β-Glucan-trained	p vs Control
OCR (pmoles O_2/min)	265 ± 25	210 ± 18	185 ± 20	< 0.05
ECAR (mpH/min)	24 ± 3	46 ± 4	64 ± 5	< 0.001
OCR/ECAR ratio	11.0 ± 1.2	4.6 ± 0.5	2.9 ± 0.4	–
ATP production (µmol/10^6 cells)	1.00 ± 0.08	0.82 ± 0.06	0.73 ± 0.05	< 0.05

These results show that trained macrophages favor glycolysis mode of energy production, intermittently even under resting conditions - that was they have a metabolic memory.
LC-MS/MS profiling revealed higher accumulation of fumarate, succinate and citrate, TCA intermediates that are known to impact epigenetic enzymes. also, cell trained under conditions of B. cereus compromise of (g-)membranes, the Beta-glcurnan trained cells showed the highest increase of fumarate (2.6-fold, $p< 0.001$) confirming the existence of metabolic- and transcriptional reprogramming link.

Table 4: showed fold changes of the major metabolites

Metabolite	Pathway	MPLA-trained	β-Glucan-trained	p
Fumarate	**TCA cycle**	1.8 ± 0.2	2.6 ± 0.3	< 0.001
Succinate	**TCA cycle**	1.5 ± 0.2	2.2 ± 0.2	< 0.01
Citrate	**TCA cycle**	1.4 ± 0.1	2.0 ± 0.2	< 0.05
Lactate	**Glycolysis**	1.9 ± 0.3	2.8 ± 0.4	< 0.01

The combined metabolic evidence supports glycolytic enhancement and TCA remodeling as central mechanisms sustaining trained immunity.
Activating histone marks H3K4me3 and H3K27ac were significantly (H3K4me3($p<0.01$), (H3K27ac($p<0.01$)) enriched at IL-6 and TNFA promoter regions in the midst of the macrophages trained with beta-glucan, and a strong correlation was observed between histone markings and enhancements in cytokine mRNA expression ($r=0.82$, $p<0.001$, Dominguess-Andres, Neeta). MPLA promoted similar but weaker enrichment patterns giving rise to possible stimulus specific epigenetic signatures.

Table 5: lists the level of enrichment of histone marks

Histone Mark	Target Gene	Control	MPLA-trained	β-Glucan-trained	p
H3K4me3	**IL-6 promoter**	1.0 ± 0.1	2.3 ± 0.3	3.8 ± 0.4	< 0.01
H3K4me3	**TNFA promoter**	1.0 ± 0.1	2.0 ± 0.2	3.4 ± 0.3	< 0.01
H3K27ac	**IL6 promoter**	1.0 ± 0.1	1.8 ± 0.2	2.9 ± 0.3	< 0.05

This enrichment of marks suggests the functional connectivity of transcriptional activation of inflammatory genes by metabolic cues.

Pre-treatment of mice with v-glucan before the infection with VSV increased survival rate staying at 83.3% versus 41.6% for control mice ($p < 0.01$). Innate activation and the antiviral effector response were enhanced as indicated by a significant increase in serum IL-6 at 24 h post-infection, and a 58% reduction in viral load in the lungs.

Table 6: the results of *in vivo* efficacy

Group	Survival Rate (%)	Lung Viral Load (Log_{10} copies/mg)	Serum IL-6 (pg/mL)	p vs Control
Control (VSV only)	41.6 ± 4.2	6.1 ± 0.3	395 ± 45	–
β-Glucan pre-treated + VSV	83.3 ± 5.0	4.1 ± 0.2	760 ± 60	< 0.01

These findings verify that metabolic training increases systemic upregulation and defenses against viral challenge in vivo.

Cluster analysis of ease of chromatin accessibility response (ECAR) applications showed that the data were highly correlated between ECAR and IL-6 ($r = 0.86$, $p < 0.001$) as well as between fumarate and H3K4me3 ($r = 0.88$, $p < 0.001$), indicating that metabolic metabolites regulate chromatin accessibility and cytokine gene transcription directly.

Table 7: Relationships between Metabolic and Immunological Measures of Trained Macrophages

Variable Pair	r value	Significance
ECAR vs IL-6	0.86	$p < 0.001$
ECAR vs TNF-α	0.79	$p < 0.01$
Fumarate vs H3K4me3	0.88	$p < 0.001$

Discussion

The present observations give reasonably strong evidence of metabolic reprogramming being a key determinant of trained innate immunity (TII) and augment antiviral responses. Exposure of monocyte-derived macrophages to the LPS agree v-glucan or monophosphorylate lipid A (MPLA) significant upregulation of cytokines (IL-6, TNF-alpha, IL-1 beta) and antiviral genes upon viral challenge was observed, providing evidence that innate immune cells have the capacity to develop a memory-like functional phenotype via metabolic and epigenetic rewiring [12,9]. These results were in line with other evidence showing that v-glucan trained macrophages display persistent metabolic and chromatin modifications with a long lifespan which remains active long after the initial stimulus [4 ,10]. Mechanistically, the results show that Dectin-1-AKT-mTOR signaling was triggered by training stimuli and causes a metabolic switch towards aerobic glycolysis [5,3]. The studied increase in extracellular acidification rate (ECAR) and a reduction in OCR/ECAR ratio suggest long-term glycolytic phenotype, which was confirming previous studies, that glycolysis acts as both an energy supplier and transcriptional activity regulator [7,11]. This paradigm has been recently extended to include evidence that indicates that metabolic signals resulting from the production of glycolytic metabolites, such as lactate and pyruvate, synergize to strengthen Trained Immunity (TI) and cytokine production [12,13] it was observed additional accumulation of fumarate, succinate and citrate, metabolites from a biosynthesis pathway that prevent histone demethylation to boost H3K4me3 and H3K27ac histone marks at inflammatory gene promoters [6,8]. These results were in agreement with earlier studies in which fumarate accumulation induced histone methylation and the long-term inflammatory response [14,15]. Taken together, it supports the notion that immunometabolic intermediates act as epigenetic cofactors, which encode innate immune memory independent of antigen specificity [16,17]. Moreover, vesicular stomatitis virus (VSV) infection showed elevation in expression of IFNB1, ISG15 and OAS1 that corresponded to upgraded type I IFN (IFN-I) responses in trained macrophages and PBMCs [2,10]. Recently, IFN-I was proven to affect early antiviral signaling and limiting viral replication, therefore has well-established benefits in enhancing viral clearance [9,18]. The functional protection afforded by the trained immunity was confirmed in vivo, with the euthanized three hours after infection, the levels of PR9 in the serum were found to be increased by beta glucan, which was shared with other reports indicating beta glucan-induced cross-protection to infection triggered by influenza and herpesviruses [19,20]. In this study, the high correlation between glycolysis (ECAR) and cytokine ($r = 0.86$, $p <$

0.001) levels highlights the key position of the glycolytic metabolism to sustain trained cell's function [21,4]. Similarly, category A IGR (r = 0.88) underscores the close link between fumarate and H3K4me3 deposition indicating straight effects of metabolic intermediates on chromatin state and eventually gene changes [15,14]. This association of metabolism to epigenetics validates the emerging context of immunometabolic-epigenetic coupling in innate immune training (1). Recent studies have pointed to the fact that manipulating metabolic enzymes can modulate trained immunity. Targeting of glycolytic regulators such as hexokinase-2 and PFKFB3 change the output of cytokines and inflammatory acceleration [22,23]. Thus, pharmacological agents that can cause a mild activation of glycolysis could increase antiviral resistance but excessive activation of glycolysis could limit its ability to alleviate viral replication risking chronic inflammation or immunopathology [7,11]. Thus, the controlled activation of metabolic processes may result in a therapeutic trade-off between antiviral control and immunological tolerance [20,24]. The implications of these results were confirmed for vaccine design, because the addition of metabolic adjuvants was likely to accentuate vaccine induced immune recognition. Studies on BCG vaccination and trained immunity have shown that the metabolic training improves the protection against unrelated viral pathogens such as SARS-CoV-2 and respiratory syncytial virus [6,25,1]. The combination of metabolic modulators with antiviral vaccines was therefore a new approach to enhance broad-spectrum immune response [26,20]. Although these were infected outcomes, various other limitations need to be presented. Although the current study focused mostly on monocytes and macrophages, other innate populations, including natural killer (NK) cells and innate lymphoid cells (ILCs), have been shown to have memory-like properties [27,28]. Moreover, only one model of the virus (VSV) was investigated and future studies should explore whether similar mechanisms hold in clinically relevant viruses such as influenza, the Covid virus, and dengue [16,29]. Finally, by combining metabolomics and transcriptomics in single-cell multiomics, cell-type specific heterogeneity in trained immune responses might be revealed [20,21]. The results justify that metabolic-epigenetic coupling was at the basis of the maintenance and functioning of trained innate immunity, suggesting new pathways for antiviral prophylaxis as well as metabolically targeted immunotherapy [12,7].

Conclusion

This study confirms the importance of metabolic reprogramming in driving trained innate immunity (TII) and immune protection against viruses. beta-glucan and MPLA induced long-lasting glycolytic activation and accumulation of fumarate and succinate and enrichment of activating histone marks, which resulted in increased cytokine and interferon responses. These findings provide direct correlation between metabolism, epigenetic restructuring and immune memory. Targeting immunometabolic pathways may show us some new antiviral and vaccine strategies on how to upregulate innate immune readiness. Trained immunity is a state of protection that can be metabolically encoded for use in future immunotherapeutic development.

Recommendation

Future research should expand the study of trained innate immunity to include clinically relevant viruses such as influenza and the current pandemic coronavirus (SAR-CoV-2) and other innate immune cells such as NK and IL weeks. Capitalizing on multi-omics approaches will elucidate the molecular basis of metabolic and epigenetic training. Evaluating small molecule modulators for essential metabolic enzymes may identify potential immunometabolic adjuvants while translational research in humans may help identify safe metabolic elevation for improving vaccine efficacy and antiviral protection.

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