Metabolic regulation of RA macrophages is distinct from RA fibroblasts and blockade of glycolysis alleviates inflammatory phenotype in both cell types

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Abstract

Recent studies have shown the significance of metabolic reprogramming in immune and stromal cell function. Yet, the metabolic reconfiguration of RA macrophages (MΦs) is incompletely understood during active disease and in crosstalk with other cell types in experimental arthritis. This study elucidates a distinct regulation of glycolysis and oxidative phosphorylation in RA MΦs compared to fibroblast (FLS), although PPP is similarly reconfigured in both cell types. 2-DG treatment showed a more robust impact on impairing the RA M1 MΦ-mediated inflammatory phenotype than IACS-010759 (IACS, complexli), by reversing ERK, AKT and STAT1 signaling, IRF8/3 transcription, and CCL2 or CCL5 secretion. This broader inhibitory effect of 2-DG therapy on RA M1 MΦs was linked to dysregulation of glycolysis (GLUT1, PFKFB3, LDHA, lactate) and oxidative PPP (NADP conversion to NADPH), while both compounds were ineffective on oxidative phosphorylation. Distinctly, in RA FLS, 2-DG and IACS therapies constrained LPS/IFN-γ-induced AKT & JNK signaling, IRF5/7, and fibrokine expression. Disruption of RA FLS metabolic rewiring by 2-DG or IACS therapy was accompanied by a reduction of glycolysis (HIF1α, PFKFB3) and suppression of citrate or succinate buildup. We found that 2-DG therapy mitigated CIA pathology by intercepting joint F480+iNOS*MΦ, Vimentin*fibroblast & CD3*T cell trafficking along with downregulation of IRFs and glycolytic intermediates. Surprisingly, IACS treatment was inconsequential on CIA swelling, cell infiltration, M1 & Th1/Th17 cytokines, and joint glycolytic mediators. Collectively, our results indicate that blockade of glycolysis is more effective than inhibition of complex I in CIA, in part due to its effectiveness on the MΦ inflammatory phenotype.

Keywords

RA macrophages; RA FLS; glycolysis; mitochondrial oxidative phosphorylation; CIA

INTRODUCTION

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease in which the cross-regulation between joint macrophages (MΦs) and fibroblast-like synoviocytes (FLS) plays a critical role in the inflammatory response and pannus formation [1]. Hypoxia, inflammatory cytokines and stimulation with pathogen-associated or damage-associated molecular patterns (PAMPs or DAMPs) trigger joint hyperproliferation and as a result, the higher energy demand necessitates cellular metabolic reprogramming [2]. Newly recruited naïve cells polarized into joint M1 MΦs, shift their metabolic activity from oxidative phosphorylation to glycolysis and pentose phosphate pathway (PPP) to support ATP production, cell proliferation and survival [3,4]. While the metabolic profile of RA FLS is linked to glycolysis, PPP and amino acid metabolism [5–8]. Earlier studies have shown that T cells in RA have a defective mitochondrial DNA reparation and shunt glucose towards the PPP [9–11]. RA synovium is a multi-cellular tissue in which the MΦs, FLS and T cells [12] are influenced by their milieu and the ability to differentially utilize metabolites. The metabolites produced from one cell type may have a pivotal regulatory impact on the function of other cells. Thus justifying the evaluation of metabolic pathways in an individual setting as well as in the arthritic joints.
Interestingly, biologics including TNFi reduce the expression of GLUT1, PKM2 and GAPDH in the RA synovial tissue (ST) biopsies of responders compared to non-responders [5]. Moreover, Tofacitinib therapy restrains glycolytic enzymes in RA explants [13]. Extending these findings, disease-modifying anti-rheumatic drugs (DMARDs) such as Methotrexate suppresses HK2 and SLC2A5 expression levels in RA FLS [14].

Recent studies have examined the implication of targeting the glycolytic factors in RA FLS and experimental models. These investigators have shown that PFK15, a small molecule inhibitor of PFKFB3 impairs RA FLS migration, MAPK and NF-κB signaling and lactate production as well as ameliorating collagen-induced arthritis (CIA) joint inflammation [15]. Moreover, KxB/N arthritis activity and bone erosion were attenuated in HK2Col1 mice [16]. In contrast, administration of glycolytic intermediate, fructose 1, 6-biphosphate (FBP), alleviated zymosan-induced arthritis by intercepting neutrophil migration and production of inflammatory cytokines [17]. Our groups have shown that switching the joint metabolic regulation to mitochondrial oxidative phosphorylation via AMPK activation mitigates K/BxN-induced arthritis in part by dysregulating NF-κB signaling [18]. Earlier investigations have highlighted the significance of metabolic reconfiguration in RA pathogenesis. However, the majority of these studies are focused on the significance of hypermetabolic activity in RA FLS, and its contribution to RA MΦ pathology remains undefined.

In this study, we sought to exhibit whether inhibition of glycolysis or complex1 would influence different facets of RA MΦs, FLS, and CIA mechanism of action. We found that 2-DG in contrast to IACS (IACS-010759, complexli) therapy, diminishes the inflammatory phenotype of RA M1 MΦs by reversing ERK, AKT, and STAT1 signaling along with IRF8/3 transcription and CCL2 and CCL5 secretion. In comparison to IACS, 2-DG manifested a wider suppressive effect on RA M1 MΦ markers that was accompanied by transcriptional downregulation of GLUT1, PFKFB3, LDHA, NOS2 and lower lactate production. Distinct from these results, in RA FLS, treatment with 2-DG and IACS negated the LPS/IFNγ-induced AKT and JNK signaling, IRF5/7 transcription and IL-6, IL-8, CCL2 or CCL5 secretion. Consistently, 2-DG and IACS treatment dysregulated RA FLS metabolic rewiring by intercepting HIF1α and PFKFB3 transcriptional regulation and reducing citrate or succinate accumulation. Similarly, both therapies were capable of reversing Th1/Th17 cell differentiation. Although 2-DG therapy attenuated CIA disease activity by intercepting joint cell infiltration and inflammatory phenotype; surprisingly IACS administration was ineffective in this preclinical model. Collectively, a lack of response to complexli therapy in the CIA indicates that disruption of glycolysis can be more effective in RA patients.

RESULTS

2-DG more effectively impairs RA MΦ inflammatory phenotype and metabolic activity than IACS

To determine the role of glycolysis and oxidative phosphorylation on RA MΦ pathology, inhibitors of glycolysis and complex1 were examined in response to immunostimulation. We showed that 2-DG curtailed LPS/IFNγ-induced ERK, AKT and STAT1 signaling. Whereas LPS/IFNγ-activated NF-κB signaling was unaffected, baseline pJNK levels were reduced by 2-DG and IACS therapies (Fig. 1A, quantified in Suppl-B1). Despite, suppression...
of TNFα (60%) by IACS, only 2-DG therapy negated CCL2 and CCL5 (80–85%) production in LPS/IFNγ-activated RA MΦs (Figs. 1B–E). Interestingly, both IACS and 2-DG treatments reduced LPS/IFNγ-driven M1 transcription factors (TFs), IRF1 and IRF5 in RA CD14+CD86hiCD206lo MΦs (Fig. 1F, Suppl-A1–A2). While, M1 or M2 TFs, IRF8 (50% reduction) and IRF3 (2.5x increase) were uniquely modulated by 2-DG therapy (Fig. 1G). However, LPS/IFNγ-induced IRF4/9 transcription was unaltered by 2-DG or IACS therapy (Fig. 1H).

LPS/IFNγ-induced ECAR levels were restrained by 2-DG treatment in MΦs (Fig. 2A). RA MΦ glycolytic reprogramming was reversed by 2-DG through reduction of GLUT1, PFKFB3, and LDH expression (50–70%) and lactate secretion (65%; Figs. 2B, 2D). In contrast, neither 2-DG nor IACS had any influence on PKM2, RAPTOR, PDK1 transcription in RA M1 MΦs (Fig. 2C).

We analyzed the impact of these treatment strategies on PPP function. We found that 2-DG and IACS rescued NADP depletion mediated by LPS/IFNγ stimulation in RA MΦs (Fig. 2E). Yet, only 2-DG therapy intercepted LPS/IFNγ-induced conversion of NADP to NADPH (40%) and NOS2 expression (99%)(Figs. 2E–G). Still, none of the compounds affected CARKL expression in RA MΦs (Fig. 2H).

Next, the implication of these therapeutic approaches was characterized on oxidative phosphorylation. Surprisingly, 2-DG and IACS therapies were inconsequential on LPS/IFNγ-modulated OCR or expression levels of citrate, succinate, PPARγ and AMPK in RA MΦs (Figs. 2I–M). On the other hand, in healthy naive MΦs, treatment with 2-DG or IACS potentiates AMPK phosphorylation (Fig. 2M, quantified in Suppl-B2). Taken together, while 2-DG therapy counteracts RA M1 MΦ inflammatory response, glycolysis and PPP, it is ineffective on oxidative phosphorylation. In contrast, IACS treatment is mostly ineffective in deregulating RA M1 MΦ-enhanced inflammatory phenotype, glycolysis and PPP.

In RA FLS, 2-DG and IACS treatments disrupt inflammatory response, while hypermetabolic activity is partially influenced by IACS

Next, the significance of restricting glycolysis or activating mitochondrial oxidative phosphorylation was assessed on RA FLS pathology. We found that LPS/IFNγ-induced JNK and AKT activation were suppressed by 2-DG and IACS, while ERK and STAT3 signaling were exclusively diminished by 2-DG (Fig. 3A, quantified in Suppl-C1 and timepoint was selected based on Suppl-C4 findings). Concurrently, IκB degradation or STAT1 activation triggered by LPS/IFNγ were unaffected by both therapies (Fig. 3A, quantified in Suppl-C1). Despite IACS’s ability to suppress specific LPS/IFNγ-activated signaling pathways, it was uninvolved in RA FLS migration, contrary to 2-DG therapy (Figs 3B–C). Conversely, both strategies disrupted the production of IL-6, IL-8 and CCL2 (60–90%) or baseline CCL5 as well as the transcription of IRF5 and IRF7 (40–90%) in LPS/IFNγ-stimulated RA FLS (Figs. 3D–H). Although LPS/IFNγ-induced IRF1/4 levels were reduced by 2-DG (35–65%), IACS was ineffective in this process (Fig. 3I). Additionally, the amplified levels of IRF3/9 by LPS/IFNγ stimulation were unaffected by all compounds (Fig. 3J).
We noted that 2-DG but not IACS therapy negated transcriptional regulation of glycolytic intermediates, GLUT1, HK2, PKM2, LDHA, and secretion of lactate in LPS/IFNγ-activated RA FLS (Figs. 4A, 4C). In contrast, IACS similar to 2-DG participated in intercepting glycolysis by limiting LPS/IFNγ-modulated HIF1α and PFKFB3 in RA FLS (Fig. 4B). The LPS/IFNγ-mediated conversion of NADP to NADPH, induction of NOS2 or repression of CARK1 levels was only intercepted by 2-DG (Fig. 4D–G). We found that the LPS/IFNγ-induced citrate or baseline production of succinate from RA FLS was reversed by both inhibitors (50–80%) (Fig. 4H–I). However, only 2-DG was capable of increasing PPARγ expression levels (4x) (Fig. 4J). Intriguingly, induction of pAMPK signaling was observed by 2-DG and IACS treatment in unstimulated RA FLS (Fig. 4K, quantified in Suppl-C2). These results indicate that IACS inhibits the LPS/IFNγ-modulated RA FLS inflammatory response and oxidative metabolite buildup while displaying a modest impact on reversing glycolysis. Nonetheless, the advantage of 2-DG over IACS is its ability to obstruct RA FLS migration and its wider influence on glycolysis, PPP and PPARγ-dependent oxidative phosphorylation.

Th1 and Th17 differentiation is disrupted by 2DG and IACS therapies

Th1/Th17 cell differentiation is expanded in RA patients and recent studies exhibit that these cells have a distinct metabolic signature that is accompanied by redirecting glucose into PPP [9,10]. We showed that the LPS-induced polarization of Th1 and Th17 cells is impaired by IACS (Fig. 5A–C). Interestingly 2-DG and IACS markedly diminish Th1 cell polarization by LPS, while IL-12-driven Th1 cell development is only impaired by 2-DG therapy (Figs. 5D–E). In contrast, Th17 cell polarization instigated by LPS or IL-6+TGFβ was reduced by 2-DG and IACS (Figs. 5F, 5J). We found that suppression of Th17 cell differentiation fostered by 2-DG or IACS treatment was due to the reduction of IL-6 and IL-1β but not TGFβ (Figs. 5G–I). While IL-12-induced IFNγ production was not impacted by IACS, IFNγ secretion triggered by LPS (indirectly through myeloid cells) was suppressed by IACS treatment. This observation suggests that perhaps LPS operates through a different mechanism in enhancing Th1-mediated IFNγ than IL-12. Overall, our data indicate that except for IL-12-mediated Th1 differentiation, inhibition of glycolysis or complex1 impairs Th1 and Th17 cell development.

2-DG therapy attenuates CIA pathogenesis by abrogating MΦ, fibroblast and T cell migration and joint inflammatory phenotype

To understand the importance of hyperglycolysis on RA pathology, CIA mice were treated with 2-DG. 2-DG therapy mitigated CIA joint swelling starting on day 31 until day 52 when animals were sacrificed (Fig. 6A). CIA joint inflammation, lining thickness and bone erosion were resolved by 2-DG therapy in part via diminishing infiltration of joint F480+ iNOS+MΦs, Vimentin+fibroblasts and CD3+ T cells (Figs 6B–D, higher magnification in Suppl-D1). In line with the lower frequency of joint F480+ iNOS+MΦs (Suppl-D2–D3), expression of CIA M1 markers and TFs, including NOS2 and IRF1/5/7/8/9 (50–80%), were downregulated by 2-DG therapy (Figs 6E–F). Further, 2-DG increased CIA blood glucose levels, as a result of intracellular glucose suppression which was facilitated by GLUT, PFKFB3, ENO1, HIF1α, cMYC (50–90%) transcriptional downregulation (Figs. 6I–J). Interestingly, transcription levels of oxidative factors, RICTOR, CARK1 and PPARγ were
also restrained in the 2-DG group relative to control (Fig. 6J). Supporting the histological findings, expression of M1 monokines, TNFα, IL-1β, CCL2, CCL5, IL-6, IL-12 (40–90%), and inflammatory lymphokines, IFNγ and IL-17 (40–90%) were ablated by 2-DG therapy in CIA ankles (Figs. 6G–H). Notably, 2-DG amelioration of CIA was also accompanied by suppression of RANK, RANKL, CTSK and TRAP expression levels (80–90%) and bone erosion (Figs. 6K, 6C). In short, the 2-DG treatment effectively mitigated CIA pathology by primarily intercepting the glycolytic phenotype in MΦs in addition to impairing joint effector T cell differentiation and fibroblasts-linked inflammatory phenotype.

**IACS therapy was ineffective in CIA**

To elucidate the significance of complex I function in CIA, IACS, a highly selective inhibitor [19–21] was used. Surprisingly, we found that IACS did not influence CIA disease activity, joint inflammation, lining thickness, or bone erosion (Figs. 7A–C). In agreement with these findings, the number of joint F480+iNOS+MΦs, F480+Arginase+MΦs, Vimentin+fibroblasts, or CD3+T cells were comparable in IACS and placebo groups (Figs. 7B, 7D, Suppl-D). Interestingly, although transcriptional levels of joint TNF, CCL2, CCL5, IL-6 were similar in both strategies, the concentration of IL-1β, IL-12 and NOS2 were elevated in the IACS relative to the placebo group (Figs. 7E, 7G). Nevertheless, despite potentiated joint IL-1β and IL-12 levels, Th1/Th17 cell polarization was unchanged in CIA mice treated with IACS compared to control (Fig. 7H). Moreover, while there were no differences noted in M1 TFs (IRF1/5/7/8/9), the M2 TFs, IRF3, were reduced by 50% in the IACS group (Fig. 7F). In the CIA, the blood glucose levels were transiently elevated by IACS at 30 and 40 mins post-glucose injection, whereas its effect plateaued at 60 mins (Fig. 7I). Remarkably, IACS therapy was responsible for transcriptional downregulation of both glycolytic (PDK1, RAPTOR; 60–70%) and oxidative (RICTOR, PPARγ; 50–60%) regulators in CIA ankle joints (Fig. 7J). Collaborating histologic evaluation, IACS treatment was inconsequential on joint RANK, RANKL, CTSK and TRAP expression levels (Fig. 7C, 7K). In conclusion, our data indicate that the lack of IACS effectiveness in the CIA may be due to its inability to reverse the inflammatory or glycolytic phenotype in MΦs.

**DISCUSSION**

The current study describes a distinct metabolic regulation for RA MΦs compared to RA FLS, which can guide the therapeutic strategies employed in patients. We exhibited that the strong inflammatory phenotype in RA M1 MΦs is accompanied by activation of glycolysis and PPP along with a blunted oxidative phosphorylation. Consequently, treatment of RA M1 MΦs with 2-DG but not IACS strongly reverses the inflammatory response, glycolysis and PPP; while both are ineffective on oxidative phosphorylation. In contrast, in RA FLS, treatment with 2-DG or IACS intercepts inflammatory phenotype and glycolysis in addition to rescuing the oxidative phosphorylation. Likewise, Th1 and Th17 cell differentiation was reversed by 2-DG or IACS therapy. Nevertheless, in RA preclinical model where MΦs control innate immunity, 2-DG therapy counteracted disease pathology; whereas the IACS treatment was ineffective (Fig. 8).
Previous studies demonstrate that RA patients display increased glycolysis together with joint lactate accumulation which correlates with the DAS28 score [22,23]. While in RA synovial fluids (SFs), levels of citrate are closely linked to DAS28 and WBC, suggesting that these patients suffer from hyper-glycolysis and dysregulation in oxidative phosphorylation [24,23]. To unravel the described metabolic abnormalities, we focused our studies on delineating the regulation of glycolysis, PPP and oxidative phosphorylation in RA MΦs, RA FLS and the CIA model.

We found that in RA MΦs, LPS/IFNγ-activated ERK, p38, AKT, STAT1, and NF-κB pathways, however, only ERK, AKT, and STAT1 activation were reversed by 2-DG therapy. Interestingly, baseline JNK activation in RA MΦs was repressed by both compounds. Despite IACS’s ability to abrogate electron transfer in complex1 [21], its inefficiency to intercept M1 MΦ signaling was reflected in its narrow impact on the M1 inflammatory phenotype. Distinct from others, we were unable to demonstrate that complex1i could impede CCL2 expression or rescue NF-κB, p38 and pERK signaling [25]. The inconsistency in our results may be due to the amplified inflammatory phenotype observed in response to TLR4 agonist in RA compared to NL MΦs. Nevertheless, IACS was able to suppress M1 TFs IRF1/5 transcription, whereas 2-DG impaired IRF1/5/8 induction and upregulated expression of the M2 TF, IRF3. This partial anti-inflammatory effect of IACS was also observed with the reduction of HIF1α which did not translate into lower ECAR or lactate production from RA MΦs. In contrast, 2-DG impaired RA MΦ metabolic reprogramming by diminishing expression of GLUT1, PFKFB3, HIF1α, LDHA, and secretion of lactate. Intriguingly, activation of HIF1α in M1 MΦs is shown to be connected to enzymes in the early (GLUT1) and later stages of glycolysis (LDHA) [26,27]. On the contrary, PKM2, RAPTOR and PDK1 transcription were neither impacted by LPS/IFNγ stimulation nor influenced by the 2 therapies. Although AKT/mTORC1, STAT1 and NF-κB signaling are paramount for cultivating glycolytic MΦs [28–30], our data suggest that restricting 2 of the 3 essential pathways by 2-DG therapy could effectively counteract the RA MΦ inflammatory response. Others have shown that HIF1α signaling is linked to AKT/mTORC1 and NF-κB activation and its glycolytic effect in MΦs is in part dependent on PKM2 and PDK1 [31,32]. Distinct from earlier reports, we showed that HIF1α is triggered in RA M1 MΦs and curtailed by 2-DG, however, its function is dissociated from PKM2 and PDK1 mechanism of action. Recent Accelerating Medicines Partnership (AMP) studies have identified 2 inflammatory RA ST myeloid subsets, one exhibits a distinct IL-1β signature (CCR2+IL-1β+S1008+CD14*), and the other displays an IFNγ-associated phenotype (STAT1+CXCL10+ISG+) and both may be metabolically active [12]. Others have shown that in M1 MΦs, SLC16A3 is responsible for the induction of glycolytic genes, HK2, GLUT1, PFKFB3, lactate, and expression of inflammatory monokines [33].

In delineating the oxidative PPP activity in RA M1 MΦs, we noted that only 2-DG but not IACS rescued the conversion of NADP to NADPH and its cofactor iNOS (NOS2) [34,35]. Interestingly both compounds were incapable of restoring OCR in RA M1 MΦs or affecting the oxidative markers, citrate, succinate, PPARγ or AMPK. These observations are due to the blockage of the TCA cycle at the level of isocitrate dehydrogenase (IDH) and succinate dehydrogenase (SDH) [36,37]. Recent studies have shown that the immuno-responsive gene (IRG1) and itaconic acid are responsible for the 1st and the 2nd TCA truncation [38,39].
Interestingly in RA FLS, LPS/IFNγ-induced signaling was differentially impacted by 2-DG and IACS. While JNK and AKT phosphorylation was suppressed by both compounds, activation of ERK and STAT3 was reversed by 2-DG. Despite the impact of IACS treatment on several signaling pathways in RA FLS, migration of these cells was only restricted by 2-DG therapy. Previous studies have shown that activation of PI3K, MAPK and NF-κB signaling plays a central role in RA FLS migration and invasiveness [40–43]. Corroborating these findings, 2-DG’s inactivation of AKT, ERK and JNK signaling was involved in RA FLS infiltration.

However distinct from RA MΦs, treatment with IACS and 2-DG impaired RA FLS inflammatory response and IRF5/7 transcription driven by LPS/IFNγ. Consistently, expansion of glycolysis via adenoviral HK2 expression, potentiates RA FLS migration and IL-8, IL-6 or MMP3 transcription compared to the control group [16]. Supporting these findings, the accumulation of joint lactate, glutamine and succinate advances RA FLS invasiveness [5,6]. Although GLUT1, HK2, PKM2, LDHA expression and lactate production triggered by LPS/IFNγ were only downregulated by 2-DG; IACS treatment subsided HIF1α and PFKFB3 transcription in RA FLS. Others have exhibited that HIF1α-potentiated GLUT1, HK2, and LDHA expression or induction of PFKFB3 and PKM2 amplify glycolysis in RA FLS [5,6,13]. Nevertheless, the expression levels of NADPH or CARKL in the oxidative or nonoxidative phases of PPP were not influenced by IACS, unlike 2-DG. Intriguingly, IACS like 2-DG therapy had a robust inhibitory effect on LPS/IFNγ-induced RA FLS citrate and succinate buildup. Hence, the lack of Krebs blockage in RA FLS may in part be accountable for the anti-inflammatory effect of IACS in these cells.

We demonstrate that inhibition of glycolysis or complex1 disrupts Th1 and Th17 cell polarization. Previous studies have illustrated that RA naïve CD4+ T cells have a specific metabolic reprogramming, that shifts glycolysis to PPP, leading to a biased Th1/Th17 cell differentiation [44,9,45]. Substantiating our findings, earlier studies revealed that AMPK inhibition expands Th1/Th17 cell development by enabling an unopposed mTORC1 signaling [46,47].

To determine how the interaction between arthritic joint leukocytes and fibroblasts is impacted by inhibition of glycolysis or complex1, CIA mice were treated with 2-DG or IACS. Treatment with 2-DG alleviated CIA by impairing joint monocytes, T cells and fibroblast infiltration in addition to restraining M1 MΦ, Th1 and Th17 cell polarization. Interestingly, levels of specific M1 markers and TFs such as NOS2, TNFα, IL-1β, CCL2, CCL5, IL-6, IL-12 and IRF1/5/7/8/9 were impaired in CIA joints by 2-DG therapy compared to the control group. Reversal of the inflammatory phenotype by 2-DG was accompanied by suppression of GLUT1, PFKFB3, ENO1, HIF1α, cMYC and diminished bone erosion. In contrast, IACS treatment despite having a transient and modest impact on glycolysis was ineffective on CIA joint cell migration, inflammation and bone destruction. Extending these observations, previous investigations report that 2-DG or BrPa therapy ameliorates K/BxN by impairing CD4+IL17+ cell development [48] or joint inflammatory phenotype [6]. Moreover, AMPK deficiency or the combination of metformin & 2-DG had a mild to insignificant impact on K/BxN-induced in wild-type mice [18] or those treated with 2-DG alone [48]. We postulate that the inefficiency of IACS to attenuate CIA disease...
activity is partly because of its inability to negate the M1 MΦ phenotype which controls the function of joint effector T cells and fibroblasts. In conclusion, constraining glycolysis may be more effective than activating oxidative phosphorylation as RA M1 MΦs have a broken TCA cycle.

**MATERIAL AND METHODS**

**Patient samples**

Peripheral blood (PB) was collected in accordance with our protocol approved by the University of Illinois at Chicago Institutional Ethics Review Board. RA patients participated in this study, after providing informed consent. RA patients were diagnosed according to the 1987 revised criteria of ACR[49]. Human PB mononuclear cells (PBMCs) were isolated by density gradient centrifugation (400g, 30 min) using Ficoll-Paque Premium (GE Healthcare). Monocytes were negatively selected using the EasySep human monocyte enrichment kit (19058; StemCell Technologies) according to the manufacturer’s instructions. Monocytes were cultured in 20% FBS/RPMI for 2–3 days to obtain *in vitro* differentiated naïve MΦs. RA FLS were isolated from digested (Dispase/Collagenase/DNase) fresh RA ST as previously described[50–52].

**Cell isolation and culture**

Macrophages were differentiated from monocytes for 3 days in the presence of 20% FBS/RPMI. RA FLS were cultured in 10% FBS/RPMI and used between passages 3 to 9, grown to ≥90% confluency. At the time of pretreatment, RA MΦs or FLS were switched from 20% or 10% to 0% FBS in the media o/n. Murine RAW 264.7 cells, were purchased from ATCC (TIB-71) and were cultured in 10% FBS/DMEM.

**Differentiation and induction experiments**

*In vitro* MΦs and RA FLS were pretreated in 0% FBS/RPMI o/n with DMSO, 2-DG (5mM) or IACS (100nM). Thereafter cells were treated with PBS or LPS/IFNγ (100ng/ml each). Either RNA was collected after 6h or conditioned media or cells were collected after 24h. All mRNA genes were normalized to GAPDH.

**ELISA analysis**

DuoSet ELISAs (R&D Systems) was used to quantify a variety of human and murine cytokines.

**Western blot analysis**

Cell lysates from RA MΦs or RA FLS were probed for pERK, p-p38, pJNK, pAKT, pSTAT1, pSTAT3, pAMPK, AMPK or degradation of IκB (Cell signaling) and the protein expression was normalized to actin levels (Santa Cruz Biotechnology). All antibodies were used at 1:1000 dilution except for actin that was used at 1:5000. The raw Western blots are shown in Supplementary Material.
Real-time RT-PCR

RNA isolated using TRIzol (Life Technologies) was reverse transcribed to cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems) for subsequent qRT-PCR analysis. We used Taqman primer/probe mixes predesigned by Integrated DNA Technologies and Taqman gene expression master mix (Applied Biosystems) to perform qRT-PCR. Data are presented as fold changes in RNA levels compared to control treatment, calculated following the $2^{-\Delta\Delta Ct}$ method.

Flow cytometry

Murine Th1 and Th17 frequency were quantified in mouse splenocytes cultured in 0.25µg/ml of CD3 Ab and CD28 Ab (Biolegend) and cells were untreated (PBS) or treated with IL-12 (10ng/ml; Th1+Ctl, Biolegend), TGFβ+IL-6 (1+20ng/ml respectively; Th17+Ctl, Biolegend) or LPS (100ng/ml) in presence or absence of IACS (100nM) for 4 days and supernatants were measured for cytokine secretion by ELISA. Thereafter, following APC-conjugated CD4 Ab (eBioscience) staining, cells were fixed and permeabilized and splenocytes were stained with FITC-conjugated IFNγ or PE-labeled IL-17 Ab (eBioscience). RA monocytes-differentiated into MΦs for 3 days were treated with PBS or LPS/IFNγ (100ng/ml each) for 24h before staining with FITC-labeled CD14 Ab (Biolegend), APC-conjugated CD86 (Biolegend) or PC7-labeled CD206 (Biolegend). Cells were gated based on the unstained and the gating strategy is provided in Supplementary F and G.

Metabolite quantification

RA MΦs or RA FLS were stimulated for 24h under starvation conditions (0% FBS). Metabolites including pyruvate, L-lactate, citrate and succinate concentrations were measured in the collected conditioned media using colorimetric assays (Sigma-Aldrich) according to the manufacturer’s instructions.

Seahorse cell energy phenotype test

The concentration of LPS/IFNγ and the dose of 2-DG or IACS were titrated in RAW cells using Seahorse assay. We tested the glycolytic capacity and oxygen consumption of control and (1000ng/ml each) LPS/IFNγ-stimulated RAW 264.7 cells (5×10^3 cells/well) in presence of 2-DG (10mM) or IACS (1000nM) using the Cell Energy Phenotype Test Kit (103325–100; Agilent Technologies) as per manufacturer’s instructions [53,54]. Cells were pre-conditioned with the inhibitors and stimuli in 0% FBS/DMEM for 24h before ECAR and OCR evaluation.

Scratch assay

Confluent RA FLS cell cultures were scratched reproducibly, while cultured in 5% FBS/RPMI. Subsequently, RA FLS were treated with LPS/IFNγ (100ng/ml) with DMSO, 2-DG (5mM) or IACS (100nM). For comparison, cells were either untreated or treated with bFGF (100ng/ml), functioning as a positive control. After 24h, RA FLS were fixed with 10% formalin (1h) and then stained with 0.05% crystal violet (1h). Microscopic images were produced to count the number of RA FLS that migrated to reclaim the cell-free scratch area.
Preclinical studies

All animal studies were approved by the UIC Animal Care and Use Committee. Eight-week-old DBA1 mice were immunized with collagen type II (Chondrex, Redmond, WA) on days 0 and 21 [55–58]. CIA mice were treated i.p. 3 times weekly with 2-DG (7.5mg/kg, Sigma), IACS-010759 (10mg/kg; ChemieTek) [19] or control starting on day 25 until day 51 [55,56,59,60,57,58]. Mice were sacrificed on day 52 post-onset and ankle circumference was measured with Caliper [59]. At the end of the experiment, ankles were dissected and homogenized for qRT-PCR (Trizol), ELISA (PBS+ protease inhibitor cocktail; Roche Diagnostics), or processed for immunohistochemistry (10% formalin).

Immunohistochemistry

Formalin-fixed mouse ankles were decalcified and paraffin-embedded. Slides were deparaffinized in xylene and antigen retrieval was achieved as previously described. Mouse ankle sections were stained for F480 (1:100, Genetex), iNOS (Santa Cruz, 1:200), Arginase 1 (Santa Cruz, 1:200), CD3 (1:100, Genetex) and Vimentin (Lab vision, 1:2500). Joint staining and H&E scoring for inflammation, synovial lining thickness and bone erosion were evaluated on a 0–5 scale [58].

Statistical analysis

For comparisons among multiple groups, one-way ANOVA followed by Tukey’s multiple comparison test was employed using GraphPad Prism 9 software. The data were also analyzed using two-tailed Student’s t-tests for paired or unpaired comparisons between two groups. Values of \( p < 0.05 \) were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. 2-DG therapy strongly reverses RA M1 MΦ differentiation.
A. RA MΦs were pretreated with DMSO (D), 2-DG (5mM) or IACS (100nM) o/n before treating the cells with PBS or LPS/IFNγ (100ng/ml each) for 30min (timepoint was selected based on the earlier findings shown in Suppl-C4). Lysates were probed for pERK, p-p38, pJNK, pAKT, pSTAT1, pSTAT3 or degradation of IkB (1:1000, Cell signaling) and normalized to actin (1:5000, Santa Cruz Biotechnology)(density quantified in Suppl-B1), n=4. RA MΦs pretreated with DMSO (D), 2-DG (5mM) or IACS (100nM) o/n were untreated (PBS) or stimulated with LPS/IFNγ (100ng/ml each) for 24h before measuring the protein levels of TNFα (B), CCL2 (C), CCL5 (D), IL-6 (E) by ELISA or treated for 6h before analyzing the transcription levels of IRF1/5 (F), IRF8/3 (G) and IRFs (H) by real-time RT-PCR, n=5–6. The data are shown as mean ± SEM, * represents p<0.05 and ** denotes p<0.01.
Figure 2. Treatment with 2-DG but not IACS negates glycolysis and PPP in RA M1 MΦs.

The effect of 2-DG (10mM) or IACS (1000nM) was examined on the glycolytic capacity (ECAR, A) and oxygen consumption (OCR, I) of control and (1000ng/ml each) LPS/IFNγ-stimulated RAW cells (5×10^3 cells/well) using the Cell Energy Phenotype Test Kit (103325–100; Agilent Technologies) as per manufacturer’s instructions, n=5–6. Cells were pre-conditioned with the inhibitors and stimuli in 0% FBS/DMEM for 24h before ECAR and OCR evaluation. RA MΦs pretreated o/n with DMSO, 2-DG (5mM) or IACS (100nM) were untreated (PBS) o/n; prior to being untreated (PBS) or stimulated with LPS/IFNγ (100ng/ml each) for 6h before determining the transcriptional regulation of GLUT1, PFKFB3, LDHA (B), PKM2, RAPTOR, PDK1 (C), NOS2 (G), CARKL (H), PPARγ (L), (HIF1α is presented in Suppl-A4), n=7. Supernatants were harvested from RA MΦs pretreated with DMSO, 2-DG (5mM) or IACS (100nM) o/n; prior to being untreated (PBS) or stimulated with LPS/IFNγ (100ng/ml each) for 24h before measuring L-Lactate (MAK329, D), NADP or NADPH (MAK038, E-F), Citrate (MAK333, J) and Succinate (MAK335, K) by a Sigma Kit via colorimetric assay, (pyruvate is quantified in Suppl-A3), n=6. RA MΦs were pretreated with DMSO, 2-DG (5mM) or IACS (100nM) o/n were either treated with PBS or LPS/IFNγ (100ng/ml each)(M, upper panel) for 30min. In a different experiment, normal MΦs were treated with DMSO, 2-DG (5mM) or IACS (100nM) o/n before Western blotting (M, lower panel). Lysates from different experiments were probed for AMPK, pAMPK (1:1000, Cell Signaling) or actin (1:5000) expression, (density quantified in Suppl-B2), n=3. The data are shown as mean ± SEM, * represents p<0.05 and ** denotes p<0.01.
Figure 3. 2-DG and IACS nullify RA FLS-induced inflammatory response.
A. RA FLS were pretreated with DMSO, 2-DG (5mM) or IACS (100nM) o/n before treating the cells with PBS or LPS/IFN-γ (100ng/ml each) for 30min (timepoint was selected based on the earlier findings shown in Suppl-C4). Lysates were probed for pERK, p-p38, pJNK, pAKT, pSTAT1, pSTAT3 or degradation of IkB (1:1000, Cell signaling) and normalized to actin (1:5000, Santa Cruz Biotechnology), (density quantified in Suppl-C1), n=4. Representative images (B) and quantification of the number of cells in the scratch area (C) are shown for RA FLS scratch assay where cells were untreated (PBS) or were treated with bFGF (+Ctl; 100ng/ml) or pretreated with DMSO, 2-DG (5mM) or IACS (100nM) o/n before being untreated (PBS) or stimulated with LPS/IFN-γ (100ng/ml each) for 24h, n=4.
RA FLS pretreated with DMSO, 2-DG (5mM) or IACS (100nM) o/n; were untreated (PBS) or stimulated with LPS/IFN-γ (100ng/ml each) for 24h or 6h before evaluating the protein levels of IL-6 (D), IL-8 (E), CCL2 (F) and CCL5 (G) by ELISA; or mRNA concentration of IRF5/7 (H), IRF1/4 (I) or all analyzed IRFs (J) by real-time RT-PCR, n=5–6. The data are shown as mean ± SEM, * represents p<0.05 and ** denotes p<0.01.
Figure 4. 2-DG therapy intercepts glycolysis, PPP and oxidative phosphorylation, while IACS partially rescues these components in RA FLS.

RA FLS pretreated with DMSO, 2-DG (5mM) or IACS (100nM) o/n were untreated (PBS) or stimulated with LPS/IFN-γ (100ng/ml each) for 6h before determining the transcriptional regulation of GLUT1, HK2, PKM2, LDHA (A) HIF1α, PFKFB3 (B), NOS2 (F), CARKL (G) and PPARγ (J), n=4. Supernatants were harvested from RA FLS pretreated with DMSO, 2-DG (5mM) or IACS (100nM) o/n; before being untreated (PBS) or stimulated with LPS/IFN-γ (100ng/ml each) for 24h for measuring L-Lactate (MAK329, C), NADP or NADPH (MAK038, D-E), Citrate (MAK333, H) and Succinate (MAK335, I) by a Sigma Kit via colorimetric assay, (pyruvate is quantified in Suppl-C3), n=6. RA FLS were pretreated with DMSO, 2-DG (5mM) or IACS (100nM) o/n with PBS or LPS/IFN-γ (100ng/ml each) (K, upper) treatment for 30min. Alternatively, RA FLS were only treated with DMSO, 2-DG (5mM) or IACS (100nM) o/n before lysates were harvested (K, lower). Lysates were probed for AMPK or pAMPK (1:1000, Cell Signaling) or actin (1:5000) expression, (density quantified in Suppl-C2), n=4. The data are shown as mean ± SEM, * represents p<0.05 and ** denotes p<0.01.
Figure 5. 2-DG and IACS therapies abrogate Th1 and Th17 cell polarization.
Frequency of Th1 (A, B) and Th17 (A, C) was quantified in mouse splenocytes cultured with 0.25µg/ml of CD3 Ab and CD28 Ab (Biolegend) and cells were untreated (PBS) or treated with IL-12 (10ng/ml; Th1+Ctl, Biolegend), TGFβ+IL-6 (1+20ng/ml respectively; Th17+Ctl, Biolegend) or LPS (100ng/ml) in presence or absence of IACS (100nM) for 4 days, n=6. Alternatively, splenocytes cultured with 0.25µg/ml of CD3 Ab and CD28 Ab (Biolegend) were untreated (PBS) or treated with LPS (100ng/ml), IL-12 (10ng/ml) or TGFβ+IL-6 (1+20ng/ml respectively) in the presence of DMSO, 2-DG (5mM) or IACS (100nM) for 4 days before measuring protein levels of IFNγ (D, E), IL-17 (F, J), IL-1β (G), IL-6 (H) and TGFβ (I), n=6. The data are shown as mean ± SEM, * represents p<0.05 and ** denotes p<0.01.
Figure 6. 2-DG therapy mitigated CIA joint inflammation, glycolytic intermediates, M1 MΦ and erosive markers.

A. Ankle circumference was measured in the CIA mice treated with placebo or 2-DG (7.5mg/kg BW) i.p. 3x/week from day 25–51, n=7 mice (14 ankles). B. Ankles harvested on day 52 were stained with H&E, F480, iNOS, Arginase, Vimentin and CD3, n=5. Inflammation, lining and erosion (C) as well as F480, iNOS, Arginase, Vimentin and CD3 staining (D) was scored on a 0–5 scale, (higher magnification and quantification in Suppl-D). Harvested ankles were homogenized and mRNA or protein levels of NOS2 (E), IRF1/5/7/8/9/3/4 (F), TNF, IL-1β, CCL2, CCL5, IL-6 and IL-12 (G), IFNγ and IL-17 (H) were quantified by real-time RT-PCR or ELISA, n=7. I. Blood glucose was measured from 0–60min by Accu-Chek in 18h fasting CIA mice treated with placebo or 2-DG, n=7 mice. Transcriptional regulation of glycolytic and oxidative factors (J) and osteoclastic mediators (K) was quantified in ankle joints of CIA treated with placebo or 2-DG and shown as heatmap plot and/or bar graph, n=7. The data are shown as mean ± SEM, * represents p<0.05 and ** denotes p<0.01.
Figure 7. IACS treatment was ineffective in CIA pathology.

Ankle circumference was measured in the CIA mice treated with placebo or IACS-010759 (10mg/kg; ChemieTek) i.p. 3x/week from d 25–51, n=7. 

B. Ankles harvested on day 52 were stained with H&E, F480, iNOS, Arginase, Vimentin and CD3, n=5. Inflammation, lining and erosion (C) as well as F480, iNOS, Arginase, Vimentin, CD3 staining (D) was scored on a 0–5 scale, (higher magnification and quantification in Suppl-D). Harvested ankles were homogenized and mRNA or protein levels of NOS2 (E), IRF1/5/7/8/9/3/4 (F), TNF, IL-1β, CCL2, CCL5, IL-6 and IL-12 (G), IFNγ and IL-17 (H) were quantified by real-time RT-PCR or ELISA, n=7. I. Blood glucose was measured from 060min by Accu-Chek in 18h fasting CIA mice treated with placebo or 2-DG, n=7 mice. Transcriptional regulation of glycolytic and oxidative factors (J) and osteoclastic mediators (K) was determined in ankle joints from CIA & CIA+IACS and were shown as heatmap plot or bar graph, n=7. The data are shown as mean ± SEM, * represents p<0.05 and ** denotes p<0.01.
Figure 8.
Illustrates the misbalance of glycolysis over oxidative phosphorylation in RA MΦs and FLS and the mechanism by which blockade of glycolysis or complex 1 modulates the function of each cell type as well as disease pathology in the CIA preclinical model.