Understanding the metabolic basis for T cell-mediated inflammation in the Central Nervous System

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Abstract

Relapsing remitting multiple sclerosis (RRMS) is a disease that affects hundreds of thousands of people in the US and millions worldwide. RRMS is most commonly diagnosed in the third decade of life, and as a disease with no cure and limited treatment options, is a lifetime sentence to progressively worsening debilitation. There is a critical need for novel therapeutic strategies to manage RRMS.

Current disease modifying therapies (DMT) for RRMS achieve therapeutic efficacy by suppressing the immune system, but suffer from the side effects of blanket immunosuppression. Indeed, while depleting the immune system provides important short-term benefits for RRMS patients, this approach is inherently limited due to the side effects of long-term immunosuppression. Efforts to develop more targeted ways of singling out specific lineages of cells that contribute significantly to pathology are under intense investigation. A very promising lineage of cells to target are pathologic subsets of $CD4^+$ T cells, agents of the adaptive immune system that play critical if not orchestrating roles in the RRMS disease process. A major focus of this thesis is identifying ways to defuse pathology driven by $CD4^+$ T cells with the capacity to produce the pro-inflammatory cytokine IL-17A, called Th17 cells.

In experimental autoimmune encephalomyelitis (EAE), a mouse model in which all currently approved DMT for RRMS have been validated, Th17 cells play myriad pathologic roles. Th17 contribute to central nervous system (CNS) demyelination by (i) inducing of glial cell death, (ii) organizing ectopic foci of

inflammation in meningeal tissues, (iii) disrupting the blood brain barrier, and (iv) recruiting peripheral immune cells such as neutrophils and monocyte-derived cells into the CNS. Genetic and pharmacologic strategies to destabilize the Th17 lineage ameliorate disease outcomes in EAE, and in many cases, prevent disease altogether. As a result, there is tremendous enthusiasm and value into discovering new ways to subvert Th17 development and functions. Inflammatory T cells require changes to their quiescent state metabolism to support the biosynthetic and bioenergetic requirements of their effector functions such as cytokine production. These metabolic adaptations often hinge on the potent induction of specific isoforms of metabolic machinery. Thus, revealing the metabolic basis for pathologic T cell functions in the context of EAE has the potential to identify novel targets through which this significant source of pathology may be defused. The overarching goal of this thesis is to identify and exploit metabolic peculiarities and vulnerabilities of T cells that drive disease in the EAE model, and especially the Th17 cell.

The major focus of the first half of the work presented herein is to gain a better understanding of how metabolism interfaces with inflammatory functions of T cells in the EAE model. Toward this end, I identify that T cells at the site of disease (spinal cord) in the EAE model are metabolically distinct compared to those in the periphery. In particular, spinal cord T cells exhibit a heightened glycolytic flux that correlates with elevated expression levels of specific isoforms of glycolytic machinery, including the M2 isoform of pyruvate kinase (PKM2). Interestingly, I find that glycolytic inhibition is relatively ineffective at regulating IL-

17A production in *ex vivo* cultures of EAE spinal cords, suggesting that sources of IL-17A may be refractory to this type of metabolic intervention. In the second half of my thesis work, I build upon these observations by using pharmacologic and genetic approaches to interrogate the metabolic and non-metabolic ways in which PKM2 can be manipulated to alter a Th17 response seemingly resilient to glycolytic inhibition.

The work presented in this thesis provide insight into the means by which T cell use metabolic machinery to facilitate their inflammatory responses in the demyelinating CNS. It is my hope that these studies have made even a small contribution to the development of improved therapeutic treatment strategies for RRMS and beyond.

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CHAPTER I: INTRODUCTION

I. MECHANISMS OF RELAPSING REMITTING MULTIPLE SCLEROSIS

Relapsing Remitting Multiple Sclerosis is a debilitating disease that affects hundreds of thousands of people in the US and millions worldwide[1,2]. RRMS is a chronic progressive disease of ever worsening debilitation with no cure. Disease onset generally occurs in the third decade of life, with pediatric cases making up a non-trivial proportion (~5%) of all RRMS diagnoses[3,4]. The goal of this section is to discuss the presentation of RRMS and the presumed mechanisms of disease particularly from the perspective of drug development efforts.

The clinical presentation of RRMS

Diagnosing RRMS

RRMS patients experience episodic flares (relapses) of sensorimotor and/or autonomic disturbances, followed by periods of near if not complete recovery (remissions)[3]. Manifestations of disease are presumed to be the result of neuro and glio-dysfunction in associated central nervous system (CNS) structures. The prototypical RRMS patient is a young (~30 years old) female (3:1 female to male predominance[5]) with symptoms reminiscent of RRMS and radiologic abnormalities in areas of the CNS associated with the observed dysfunction[3]. The McDonald Criteria[6], a set of guidelines for diagnosing RRMS, require a patient's symptoms be disseminated in time and space before a diagnosis of RRMS can be made. Both requirements are assisted by radiologic

studies – dissemination in time requires radiologic evidence of new disease activity in the brain or spinal cord. For T2-weighted MRI imaging, this means new lesions found 30 days after baseline scans made at initial presentation[6] (T2 hyperintensities do not distinguish between new and old lesions, thus necessitating longitudinal pre/post comparisons[7]). For gadolinium (Gd) enhanced imaging, the presence of enhancing and non-enhancing lesions at initial presentation is sufficient as Gd can only penetrate areas of blood brain barrier (BBB) compromise occurring in the past 3 weeks[6,8]. Dissemination in space requires involvement of at least 2 different CNS structures, primarily periventricular, juxtacortical, infratentorial, or spinal cord regions[6], regardless of whether lesion formation is symptomatic or silent.

The overt and insidious natures of disability in RRMS

By definition, a relapse is a transient (at least 24 hours duration[7], generally lasting from days to weeks[9]) bout of debilitation that is partially or totally reversible. Due to this feature of disease, RRMS patients accumulate disability relatively slowly. Eventually, however, many RRMS patients will convert to a second phase of disease characterized by rapid irreversible debilitation that accumulates independently from superimposed relapse-remission cycles. This phase of disease is called secondary progressive MS (SPMS). Whereas transient flares of disability associated with focal CNS radiologic abnormalities are the norm for RRMS, intractable loss of activities of daily living and profound brain atrophy are the norm for SPMS[10]. It can be difficult to discern the exact

timing of when a patient with RRMS transitions into SPMS, though recent guidelines suggest that this can be done with reasonable accuracy based on the use of standardized methods for scoring disability status in patients with a diagnosis of RRMS[11].

The Kurtzke Expanded Disability Status Scale (EDSS) offers an incisive representation of the spectrum of debilitation traversed by patients during their progression from RRMS to SPMS. Onset of SPMS generally occurs at EDSS=3, corresponding to moderate disability without impairment of activities of daily living[12,13]. Median time from SPMS onset to EDSS=6, a disease phase at which patients require constant unilateral ambulatory assistance (e.g. cane) ranges from 4-8 years[12,13]. The strikingly rapid accumulation of severe disability in SPMS reveals the importance of preventing or at least prolonging the latency to this phase of disease. Early retrospective population-based studies suggested a median duration of RRMS to SPMS of ~19 years[12,14], though a recent single-center prospective study shows the development and implementation of disease modifying therapies (DMT) has likely prolonged latency to SPMS[15]. While promising, within the 10 year time period of this prospective study, 59% of RRMS patients experienced significant worsening of EDSS as did nearly all patients with progressive MS variants, including SPMS. Thus, while current DMT are effective means of reducing the frequency of overt relapse-remission cycles during RRMS, they are clearly not as effective at preventing underlying disease processes that drive permanent disability in both RRMS and SPMS. This is a major unmet clinical need.

The importance of developing novel treatment strategies for RRMS

As a disease with early onset and no cure, a diagnosis of RRMS is a lifetime sentence to progressive disability accumulation that is often handed down to individuals early in life. While DMT exist for RRMS, complications that arise from their many limitations and flaws are frustrations that RRMS patients must endure for decades of treatment. DMT-related adverse events, high cost of medication (current DMT average over $60,000 per DMT per year in the U.S.[16]), and waning treatment efficacy are just some of many reasons that an estimated 40-80% of RRMS patients will discontinue their first line of DMT[17,18]. Due to these shortcomings, upgrading the current battery of RRMS DMT is an essential and much needed step toward improving management of patients with RRMS.

Immunomodulation as a therapy for RRMS

Analysis of the RRMS lesion suggests immune involvement in pathology

Efforts to rationally design next-generation DMT for RRMS have focused on identifying factors that contribute to the pathologic microenvironment of the RRMS lesion. On a cellular level, it is clear that the immune system plays a key role in disease, with myeloid cells (MC) such as macrophages being the predominant immune cell subsets found within the RRMS lesion[19-22]. Lesional MC burden correlates strongly with the extent of axonopathy[20], though how MC contribute to this pathologic neurodegenerative feature of the RRMS lesion is not

definitively understood. Macrophages, a MC subset, in the active RRMS lesion are frequently laden with engulfed myelin[22,23], indicating that phagocytosis is at least one of their major functions in the lesion, perhaps related to associated axonopathy. It is not clear if this is detrimental engulfment of healthy CNS tissues or the necessary clearance of debris from dead or dying neurons and glia. More broadly, whether parenchymal MC, an umbrella term for a diverse group of immune cells, serve purely deleterious roles in the RRMS lesion is controversial, especially considering that heterogeneity in their lineages may influence their downstream functions[24-26]. While there is a growing preponderance of evidence in mice to suggest MC of different lineages play distinct roles during immune-mediated CNS demyelination[27-32], due to technical limitations, there are no convincing data that this is the case in humans. Thus, while the presence of MC in the RRMS lesion is associated with disease, the actual role(s) they play in the overall disease process, and in particular, how these roles segregate between distinct MC lineages, remains unclear.

There are many human studies that indicate T cells, agents of the adaptive immune system that drive inflammatory responses against specific antigens, are critical for disease in RRMS. Perhaps most convincing are genome-wide association studies that reveal the most significant genetic risk factors for development of RRMS are polymorphisms that predispose an individual to dysregulated T cell function, either at the level of antigen presentation or in cytokine reception[33-37]. Likewise, in preliminary Phase I/II studies[38,39], autologous bone marrow transplantation in patients with treatment-

refractory RRMS was shown to reduce disease activity in the CNS, and this was correlated with reconstitution of the presumably autoreactive T cell compartment[40,41]. Whereas T cells are scant in the healthy CNS, in individuals with RRMS, T cells accumulate in CNS perivascular spaces[42]. It is possible that this localization may be critical for the establishment of inflammatory foci via regional endothelial cell activation that subsequently permits invasion of other immune cell subsets (e.g. MC) with a more evident capacity to directly mediate CNS destruction. This hypothesized disease mechanism is made plausible by the routine trafficking of T cells throughout the perivascular spaces and cerebrospinal fluid (CSF) of healthy individuals[43-46], but raises the question as to why T cells become activated to precipitate downstream inflammation in RRMS, but not in healthy individuals. Perivascular cells with the capacity to activate inflammatory programs in T cells are potential mediators of this process, but the initiating factors and other specifics related to this pathologic interaction remain unclear.

Immune cell CNS burden and RRMS disease activity are correlated, but it has proven difficult, from human studies alone, to develop this observation into confirmed mechanisms of RRMS disease etiology and or pathogenesis. Nevertheless, it is clear that the RRMS lesion is characterized by the abnormal accumulation of immune cells and that preventing this accumulation impedes new lesion formation. Broad targeting of immune cells is an important and effective means of managing RRMS patients but comes with the dangers of blanket immunosuppression[47].

The successes and failures of immunomodulatory DMT reveal the known and unknown aspects of RRMS pathogenesis

There are currently 15 DMT approved for use in RRMS, all of which achieve therapeutic efficacy by successfully reducing the frequency of symptomatic relapse-remission cycles. The most effective of these DMT are alemtuzumab, natalizumab, and fingolimod[48], with the recently approved ocrelizumab likely to be added to this list[49]. The proposed mechanisms of action of these drugs are distinct, yet ultimately converge on a single biological outcome: suppression of the immune system. Alemtuzumab is a leukocyte-depleting agent often used in solid organ transplant to prevent transplant rejection[50]. Natalizumab prevents leukocyte infiltration of tissues, and as a result is also approved for use in diseases of non-CNS tissue-specific inflammation, such as Crohn's disease[51]. Fingolimod sequesters lymphocytes in peripheral immune organs (e.g. lymph nodes), preventing their trafficking into other tissues[52]. Ocrelizumab is a B cell-depleting monoclonal antibody[49,53], and is the only DMT approved for use in both RRMS and also progressive forms of MS[54,55]. The leading hypothesis as to why immunomodulation is therapeutic in RRMS is related to the histopathologic features of the characteristic active RRMS lesion: heavy immune cell burden that colocalizes with areas of demyelination[19,20,22]. Whereas microglia, the resident macrophage population of the CNS, are the only classically defined immune cells in the healthy CNS parenchyma, a wide array of immune cell subsets can be found packed into the RRMS lesion. This has led

many to believe that immune activity in the CNS makes important contributions to the MS lesion. In support of this hypothesis, immunosuppressive DMT prevent the formation of new CNS lesions, detected as Gd-enhanced foci on MRI[56-59]. Taken together, the success of immunomodulatory interventions to manage RRMS confirms the widely held belief that the immune system plays a critical role in RRMS pathogenesis.

What remains extremely controversial, however, is whether immune infiltration of the CNS is a causative driver of RRMS, or a response to ongoing neurodegenerative processes[10]. Resolving this nuanced debate may be critical for optimizing the rational design of future therapeutic advances for RRMS. Some hypothesize that RRMS is a disease of primary neurodegeneration with superimposed secondary immunopathology[60-62] and, if true, immunomodulatory treatments are inherently limited in their therapeutic potential. This belief is not without supporting evidence.

- The insidious progression of disability in the absence of symptomatic relapse does seem to question immune-mediated inflammation as a causal driver of neurodegeneration.
- Partial antibody-mediated depletion of a subset of T cells considered to be prominent contributors to demyelinating pathology, the $CD4^+$ T cell, yields no therapeutic benefit in RRMS[63].
- Death of oligodendrocytes (OLG), myelinating glia of the CNS, in the absence of peripheral immune cell burden is a common histological

observation in the early RRMS lesion[64], and may be the precipitating event that leads to the downstream axonal degeneration and neuronal loss[65] associated with disability accumulation.

- A recently developed mouse model of globally induced OLG death triggers an immune response in the CNS reminiscent of that observed in RRMS[66], suggesting neurodegeneration can precede and trigger secondary immune-mediated inflammatory responses in the CNS.

These observations have inspired debate as to whether neuroprotective rather than immunosuppressive DMT should be the focus of drug development efforts for RRMS[10,67]. Continued research into agents that provide relief for neurodegenerative processes is undoubtedly essential, particularly given that immunomodulation is therapeutically unproductive in managing disease in the progressive forms and stages of MS. For RRMS, however, prevention of new immune activity is sufficient to delay irreversible EDSS worsening and the onset of SPMS[15], indicating that the rate and severity of overall disability accumulation is at least influenced by immune activity in the CNS. Even if the exact order of events that lead to RRMS symptomatology are unknown, treating RRMS as a primary disease of aberrant immune activity in the CNS with secondary neurodegeneration has proven therapeutic benefit. In addition, the shortcomings of DMT may not necessarily be in what they do, but rather, in when and how they are deployed.

Shortcomings of immunomodulation in RRMS do not invalidate the approach

Often overlooked when considering the failure of current DMT to halt disease progression in RRMS are extrinsic factors that impact their efficacy. One such factor is latency to first DMT administration. A recent review of patients seen in MS clinics in Spain revealed the median latency between symptom onset and DMT treatment initiation in this patient population was a startling 2 years[68]. Given that hallmark features of neurodegeneration, like axonal transection and loss, occur early in the disease[69] and the solid evidence that intervening at this early time results in slower acquisition of disability and extended time to SPMS onset[13,70-72], it is understandable how a prolonged duration between first symptoms of disease and DMT initiation may play a major role in eventual outcomes decades later. This delay in early intervention is not without reason – premature administration of immunosuppressants in a patient presenting with general clinical features suggestive of RRMS (sensorimotor or autonomic disturbance with associated CNS radiologic abnormality) could be catastrophic if the suspected disease signs were instead, for example, of an infectious etiology. Thus, one of the main reasons that DMT fail to block disease progression in RRMS may actually have little to do with their mechanisms of action, and more to do with when they are first administered. In this vein, RRMS treatment guidelines recommend first generation DMT (interferons or glatiramer acetate), agents with relatively inferior therapeutic efficacies[48,73,74], as initial therapies of choice for RRMS patients[75,76]. Despite being more potent, second generation DMT (e.g. natalizumab, alemtuzumab, fingolimod) carry significant adverse effect profiles,

and the risk of treatment-related sequelae increases with extended administration. In the case of natalizumab, sustained treatment beyond 2 years is not generally recommended due to the compounding likelihood of developing an incurable and lethal opportunistic infection of the CNS called progressive multifocal leukoencephalopathy. That the most efficacious DMT for RRMS are those that cannot be tolerated for extended durations is a stark limitation to their therapeutic potential, and one that is likely related to their design as global, rather than targeted, immunotherapies.

Another potential reason for disease progression despite DMT treatment is that many DMT are designed to prevent the formation of new inflammatory foci in the CNS, but not necessarily to target ongoing inflammation *in situ*. To illustrate this point, as an induction immunosuppressant for organ transplant, alemtuzumab drives robust lymphoablation in the peripheral blood, but cannot reach lymphocytes present in tissues, even at sites of ongoing disease[77]. There is no strong human evidence that natalizumab, alemtuzumab, fingoliomod, or other approved second generation DMT[78] with potential immunomodulatory activity – teriflunomide (pyrimidine synthesis inhibitor that blocks lymphocyte proliferation), dimethyl fumarate (promoter of antioxidant responses in macrophages), or mitoxantrone (antineoplastic, type II topoisomerase inhibitor that blocks cell proliferation) – actually achieve therapeutic efficacy by targeting processes in the CNS. Thus, the benefits of DMT in RRMS, assuming efficacy is achieved via immunomodulation, may be predominantly driven by their effects on

the peripheral immune system, with their subpar efficacies at the site of disease explaining their reduced capacity to block neurodegenerative outcomes.

Cytokine-directed therapies for RRMS

Treating RRMS with monoclonal antibodies against cytokines

Drug developers striving for improved tolerability of next-generation immunomodulating DMT have focused efforts on more targeted approaches, with one of the most popular being the neutralization of individual effector proteins produced by inflammatory immune cells. Targeting soluble inflammatory proteins, or cytokines, has been a tremendously effective means of managing autoimmune diseases. Monoclonal antibodies (mAb) are the agent of choice for this purpose, and when infused intravenously or subcutaneously can disseminate and bind their cognate cytokines, thereby neutralizing them and preventing their downstream functions. In rheumatoid arthritis (RA), mAb or decoy receptor-mediated blockade of tumor necrosis factor alpha (TNF-α), a cytokine produced by many cells after inflammatory stimulus, is profoundly therapeutic[79,80]. Likewise, the robust therapeutic efficacy of mAb against interleukin 17A (IL-17A), a cytokine produced by inflammatory T cell subsets commonly found in the skin, has revolutionized the treatment of psoriasis[81,82]. Attempts at directed cytokine neutralization in RRMS have produced very interesting, and sometimes surprising, results.

Blockade of TNF-α was initially hypothesized as a promising means to limit inflammation in RRMS[83,84], but this theory was quickly debunked when anti-TNF-α mAb infusion increased the rate of Gd-enhancing lesion formation and relapse in multiple studies of MS patients with diverse clinical presentations[85,86]. Likewise, while anti-TNF-α mAb are incredibly effective for a diverse array of peripheral inflammatory diseases (arthritidies, spondyloarthropathies, Crohn's disease), their use in these settings of preexisting autoimmunity also elevates risk of developing spontaneous demyelinating diseases reminiscent of RRMS[87,88]. In some but not all cases, neurologic disease abates with cessation of anti-TNF-α treatment[88] suggesting a true link between blockade of TNF-α and induction of demyelination. While there is no known mechanism explaining this correlation, it is possible that blocking a cytokine with predominantly pro-inflammatory functions would precipitate reactivation of latent pathogens, thus triggering an auto-reactive immune system to target tissues where these pathogens exhibit tropism, such as the CNS. Related studies in mouse models of RRMS suggest that loss of TNF-α signaling contributes to disease susceptibility via modifications to the microbiome content[89]. Interestingly, CNS demyelination was recently reported in an RA patient treated with mAb blockade of another classic inflammation-associated cytokine, IL-6[90]. These findings suggest that cytokines may play important roles in maintaining the immune-privileged/specialized nature of the CNS. Indeed, it has been shown that T cell-derived IFN-γ[91] and IL-4[92] are important for the development and maintenance of

neurotypic behavior in rodents. Taken together, not all cytokines may be exclusively detrimental in either the healthy or demyelinating CNS.

Given that the RRMS disease process is classically referred to as being T cell-mediated[3], a major goal of cytokine-directed mAb therapies has been to target effector proteins produced by inflammatory T cells. This approach has produced many surprises. Targeting interleukins 12 and 23, cytokines important for the generation of inflammatory T cell subsets, failed to prevent relapse in RRMS[93]. In addition, unanticipated results from numerous pre-clinical murine studies[94-96] derailed further efforts to treat RRMS by targeting interferon-gamma (IFN-γ), a classic T cell-derived inflammation-associated cytokine known to be elevated preceding relapse and to induce relapse when administered intravenously[84,97]. Not all efforts have been overt failures. mAb blockade of interleukin-17A (IL-17A) and granulocyte macrophage colony stimulating factor (GM-CSF) are showing favorable results in early clinical trials[82,98], though it is premature to conclude efficacy or otherwise at this time. Daclizumab, a mAb against the alpha subunit of the high affinity interleukin 2 receptor (IL-2Rα), reduces relapse rate and CNS lesion formation and is the only cytokine-directed mAb currently approved for RRMS. The pivotal Phase III DECIDE trial revealed daclizumab as being superior to the first line DMT interferon beta-1a (Avonex), in reducing relapse rate in RRMS patients, though like many other immunomodulating treatments, daclizumab failed to reduce overall disability progression in this study[74]. IL-2 is a cytokine produced predominantly by T cells that aids in their activation and expansion[99]. When T cells become activated, they

upregulate IL-2Rα to allow formation of the high affinity IL-2R(αβγ)[100]. Transduction of IL-2-mediated activating signals facilitates further propagation of T cell effector functions and impingement on this process was the hypothesized mechanism of action of daclizumab. Thus, it came as a surprise when mechanistic studies revealed daclizumab treatment did not directly affect T cell functions in RRMS patients, but rather expanded an anti-inflammatory immune cell subset called CD56(bright) natural killer cells[101,102]. Targeting cytokines to treat RRMS clearly has not been as straightforward as in other peripheral inflammatory conditions.

Animal models reveal other cytokines that may play a role in RRMS

Nearly all of the information discussed up until this point has been gleaned from human studies. However, many mechanistic insights into the RRMS disease process have come from animal models. One of the most common, experimental autoimmune encephalomyelitis (EAE), involves immunizing animals (usually rodents) against components of their own CNS myelin, thus triggering a targeted immune response against mainly spinal cord tissues that presents phenotypically as an ascending paralysis[103]. The predominant spinal cord pathology observed in EAE is in stark contrast to RRMS where inflammation is prevalent in both the brain and also the spinal cord. This is one of a few notable differences between EAE and RRMS (reviewed in [104]). Despite this, many of the DMT for RRMS were initially developed and validated using EAE as a therapeutic proving ground[51,105-109]. Thus, enough of the RRMS disease process

is recapitulated in EAE to make it an extremely informative and translatable model system, especially when striving to understand immunologic drivers of disease that can be targeted for therapeutic benefit in RRMS.

As in RRMS, immune cell infiltration, demyelination, and axonal transection/loss are common features of the EAE CNS[110]. The severity of the EAE paralytic syndrome strongly correlates with CNS immune cell burden[28] and peripheral inflammatory immune cells that subsequently migrate into the CNS are critical for driving demyelination[28,32,111]. Of particular importance to the EAE disease process are immune cells, a situation reminiscent of RRMS.

There is a strong evidence to support the claim that T cells orchestrate immune-mediated pathology in EAE:

- The immunization paradigm is designed to produce inflammatory myelin-reactive T cells, or in other words, T cells that will engage and propagate inflammatory responses upon encountering myelin[103]
- Genetic manipulations that produce mice carrying T cells with inherent reactivity against CNS myelin lead to spontaneous paralytic disease reminiscent of EAE[112-115].
- Transfer of inflammatory myelin reactive T cells into a recipient animal is sufficient to produce EAE in the recipient[116-119].

T cells contribute to disease in the EAE model by organizing and enhancing myeloid cell (MC)-mediated inflammation in the CNS. MC are agents of the

innate immune system known to potently drive pathology in EAE disease pathogenesis:

- Genetic manipulations that impair MC entry into the CNS delay EAE disease induction[28,120-122]
- EAE can be markedly attenuated when MC are unable to respond to or receive T cell-derived signals, like GM-CSF, that induce inflammatory signatures during their maturation[32,123].
- MC of the EAE spinal cord, particularly those that infiltrate from the periphery bear a robust pro-inflammatory transcriptional signature[27,29,30]

Many inflammation-associated cytokines, including GM-CSF[32,111,123-127], Interleukin 1 beta (IL-1β)[126,128], and IL-23[129-131] are involved in EAE to the extent that individually targeting them genetically or pharmacologically attenuates disease severity. That being said, the importance of certain cytokines to the overall EAE disease process varies significantly depending on the strain of mice under study. GM-CSF signaling is an absolute requirement for EAE to develop in C57BL/6 mice[32,119], but is largely dispensable in C3HeBJ/FeJ mice[132]. Similarly, the pathogenicity of IL-17A and its closely related family member IL-17F in the EAE model also varies from group to group and may be strain-dependent[133-135]. This calls into question the utility of targeting individual cytokines to treat RRMS, especially when it is unclear if these cytokines are executing their detrimental functions in the periphery (an area sensitive to mAb targeting) or in the CNS (an

area much less sensitive to mAb penetration[136]). Perhaps a better strategy would be to use small molecules to target a cellular process upregulated in and essential to the pathogenic signature of inflammatory immune cells. Indeed, an inflammatory program related to GM-CSF, IL-1β, IL-23, and IL-17A biology is that of the Th17 cell, and efforts to destabilize this T cell lineage have proven to be profoundly efficacious in the EAE model.

The contribution of Th17 cells to pathology in EAE

Induction of glial death and BBB dysfunction

Defined by their capacity to produce the cytokine IL-17A, Th17 cells are believed to play myriad pathogenic roles in RRMS. The mechanisms by which Th17 cells achieve their pathologic functions in EAE are largely attributed to the cytokines they produce. Perhaps the most direct example of this comes from studies of IL-17 signaling in $NG2^+$ glia (e.g. oligodendrocyte precursor cells or OPCs) during EAE (**Figure 1**). Deletion of Act1, a cytoplasmic adaptor for the IL-17 receptor complex, specifically in $NG2^+$ glia (but not neurons, astrocytes, or endothelial cells) robustly attenuates the severity of EAE driven by Th17, but not by Th1 cells[137]. In this study, IL-17 signaling through Act1 was shown to prevent the differentiation of $NG2^+$ glia into mature oligodendrocytes (OLG), and instead drive expression of pro-inflammatory mediators known for their pathologic roles in EAE (e.g. GM-CSF, IL-6), cleavage of caspase 3, and cell death. A follow up report from this same group revealed that the mechanism by which IL-17 inhibits

NG2$^+$ differentiation into OLG is by activating the NOTCH1 pathway, resulting in OPCs adopting the previously described hyperinflammatory phenotype and preventing their differentiation[138].

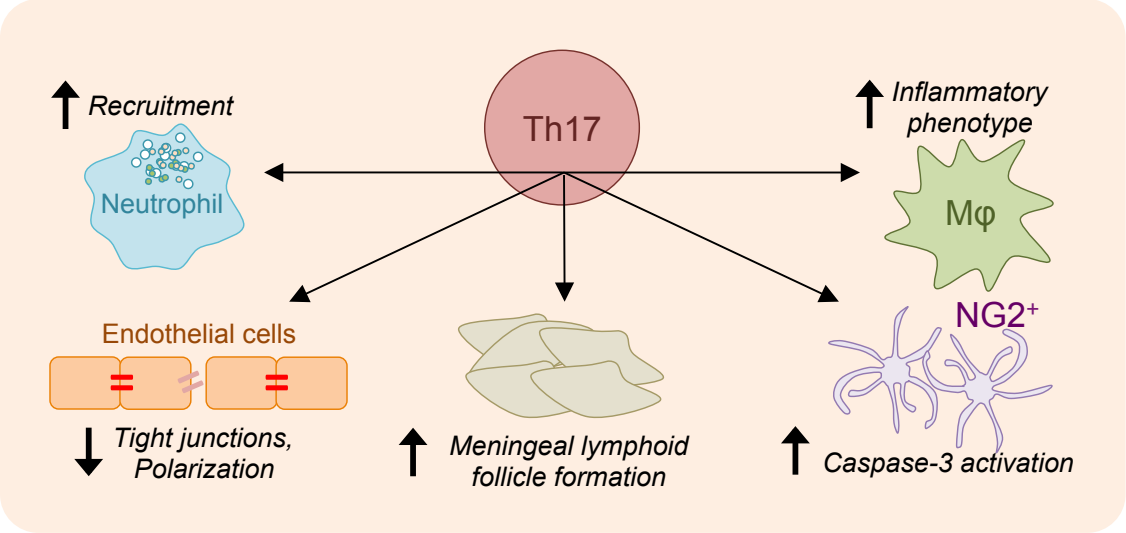


Figure 1: *Pathologic functions of Th17 cells in EAE that will be discussed in this section. Mφ = Macrophage*

In contrast to the dispensability of Act1 in endothelial cells for the development of EAE, IL-17 does appear to exert disease-relevant effects on the blood brain barrier. CXCL12 is a chemokine normally localized to the basolateral (abluminal) aspect of blood vessels in the healthy CNS where it is posited to prevent leukocytes from exiting the perivascular space and penetrating the underlying parenchyma[139-142]. However, in individuals with MS, blood vessels in lesioned areas exhibit lost CXCL12 polarization, the severity of which directly correlates with disease activity[141]. IL-17 is a major signal driving this pathologic structural change to the CNS vasculature, though the precise mechanism for this observation is unknown[140]. In addition, IL-17 and IL-22 produced by human-derived Th17 cells can disrupt tight junctions in *in vitro* blood brain barrier models

and enhance leukocyte recruitment into the diseased CNS presumably by binding to endothelial IL-17R and IL-22R characteristic of CNS vessels in "heavily infiltrated" lesions[143].

Orchestration of pathological meningeal ectopic lymphoid follicles

Another aspect of EAE-associated pathology controlled by IL-17 and IL-22 is the formation of meningeal ectopic lymphoid follicles (eLF) **(Figure 1**). Under homeostatic conditions, lymphoid follicle formation is commonly restricted to secondary lymphoid tissues (e.g. lymph node), where a coordinated response organizes T cells and B cells in a way that facilitates the maturation of an immune response against a particular antigen. eLF are characteristic hallmarks of ongoing inflammation at sites of inflammatory pathology (e.g. the synovium of the RA joint[144,145]). Th17 cells were recently shown to have the capacity to organize follicular-appearing lymphoid structures in the meninges during EAE, and this function seems to be related to their production of IL-17A and IL-22[146] and their surface expression of podoplanin[147]. Relatedly, Th17 cell expression of ligands for the lymphotoxin beta receptor (LTβR), a cell surface protein known for its role in the development of lymphoid follicles, is critical for the propagation of a meningeal Th17 response during EAE (though dispensable for follicle formation itself)[146]. Allelic variation at the LTβR locus was recently identified as risk-conferring in RRMS[35], further suggesting a role for it in disease. eLF are present in the meninges of secondary progressive MS patients and are a probable source of ongoing inflammation that, while not necessarily manifesting as the

symptomatic relapse-remission cycles earlier in disease, may contribute to the rapid progression of disability during this advanced phase[12,13]. Likewise, neurodegenerative changes are commonly observed in cortical tissue adjacent to meningeal follicles, and individuals with progressive MS who have these hallmarks of inflammation in their meninges die a staggering 15 years sooner than those with none[148,149]. It is intriguing to note that the signals that drive Th17 development are very similar to those that drive the differentiation of a distinct T cell lineage called follicular helper (TFH) cells that is more commonly associated with lymphoid follicle development[150]. As of yet, it is unclear how well current DMT are able to target inflammation in meningeal lymphoid follicles, though the apparent efficacy of ocrelizumab for progressive MS at least suggests that targeting key components of the follicle could hold therapeutic promise.

GM-CSF as a driver of EAE pathology

As exemplified by the effects of IL-22, Th17 cells achieve their pathologic functions by more than just production of IL-17 family members. One of the major pathologic activities of the Th17 lineage is their production of GM-CSF[124]. When performed in the C57BL/6 mice, EAE is completely prevented when T cells are unable to produce GM-CSF[124,151]. There are two major known drivers of GM-CSF production by T cells: IL-2 signaling through STAT5[127] and IL-1β signaling through MyD88[126]. A genetic polymorphism in the high affinity subunit of the IL-2 receptor (IL-2Rα/CD25) was among the first to be identified as a risk variant for RRMS with numerous follow-up studies validating this original finding[33-35]. Further

studies revealed that, while IL-2Rα itself does not have signaling capabilities, this polymorphism leads to heightened IL-2-mediated phosphorylation of STAT5 and correlates with elevated T cell production of GM-CSF[127]. Likewise, deletion of STAT5 in T cells precludes their ability to produce GM-CSF and induce EAE[152]. While there are no validated RRMS risk alleles involving inflammasome machinery, IL-1β, its receptor, or MyD88, IL-1β clearly plays an essential role in the induction of GM-CSF competent Th17 cells. Addition of IL-1β during classic Th17 differentiation with IL-6 and TGFβ potently induces GM-CSF production[151]. On the other hand, T cells lacking IL-1R1 or the downstream signaling protein MyD88 are deficient in their ability to produce GM-CSF and likewise do not drive disease in EAE[126]. Taken together, in the C57BL/6 EAE model, T cells are the major source of the GM-CSF that is required for disease. Deletion of the GM-CSF receptor on peripheral myeloid cells defined by expression of the chemokine receptor CCR2 completely prevents EAE development, indicating that these populations are the major recipients of the T cell-derived encephalitogenic signal. Interestingly, GM-CSF receptor deficient myeloid cells can infiltrate the CNS, but fail to upregulate phagocytic machinery as well as various inflammatory mediators, including IL-1β. Thus, there appears to be some feedback loop by which T cells and myeloid cells use IL-1β and GM-CSF to communicate and amplify pathologic responses required for the development of EAE. In summary, GM-CSF is a signal produced by Th17 cells that is critical for EAE due to its effects on peripheral myeloid cell populations (**Figure 1**), the specifics of which require further investigation.

Recruitment of neutrophils to the CNS

Th17 cells contribute to the EAE disease process by directly and indirectly recruiting neutrophils into the CNS. Adoptive transfer EAE studies demonstrate that Th17, but not Th1 cells, induces ELR+ chemokines CXCL1 and CXCL2 in spinal cords[153]. These chemokines are potent neutrophil chemoattractants (**Figure 1**) capable of being produced by many different cell types in response to a variety of IL-17 family members (reviewed in ref [154]). Confirming that the Th17-mediated recruitment of neutrophils to the CNS is pathogenic in EAE, blocking antibodies against the CXCL1/2 receptor CXCR2 as well as neutrophil-depleting (α-Ly6G) antibodies are both therapeutic in EAE, particularly in atypical variants of disease featuring prominent inflammation in the brain and brainstem[153,155,156]. These studies demonstrate the pathological significance of Th17 cells and their recruitment of neutrophils into the CNS during EAE. Importantly, human Th17 (but not Th1) cells can also directly recruit human neutrophils through their production of CXCL8 (IL-8 – human ortholog of CXCL1)[157].

Despite the many pathologic activities ascribed to them, Th17 cells exist for a reason. In particular, they are often credited with performing essential tasks like providing effective host defense to fungal and other extracellular pathogens, or maintaining barrier integrity at mucosal surfaces. Indeed, individuals with genetic mutations at the STAT3 locus have deficient Th17 cell responses and are highly susceptible to fungal and extracellular bacterial infections[158]. Importantly, Th17 cells fulfill this function alongside other IL-17A-producing cell subsets,

which, in addition to serving protective roles in host-pathogen interactions, also contribute to pathogenesis in EAE. Thus, in disease contexts relevant to RRMS, the amplification or inappropriate targeting of an IL-17A-skewed immune response is one that contributes significantly to disease[159]. Given the many different aspects of Th17 biology that contribute to their pathogenic mechanisms in RRMS, efforts to destabilize multiple aspects of their function could provide significant therapeutic relief in RRMS.

Targeting Th17 cells to treat RRMS

Ways to destabilize the acquisition of Th17 characteristics

Due to the many pathologic functions of Th17 cells in EAE, there is a great deal of excitement surrounding efforts to curtail their functions in RRMS. Classic Th17 lineage specifying factors include hypoxia-inducible factor 1 alpha (Hif-1α), signal transduction and activator of transcription 3 (STAT3), and RAR-related orphan nuclear receptor gamma t (RORγT). Genetic deletion of each of these factors in T cells (i) blocks their differentiation into IL-17A-competent cells *in vitro* and *in vivo* and (ii) eliminates their ability to induce EAE in both active immunization and also passive transfer models. Due to these observations, the development of pharmacologic agents to disrupt the Th17 lineage is believed to hold tremendous therapeutic potential for the treatment of RRMS.

As with other T cell lineages, Th17 cells arise when TCR ligation and co-stimulation (signals 1 and 2) are coupled with the right set of extrinsic cues to

promote the engagement of a transcriptional program[160,161] that ultimately facilitates the induction of the namesake cytokine IL-17A. There are many extrinsic cues or cytokines that, in various combinations, can confer IL-17A competency, and all result in the upregulation of the "master regulator" of the Th17 lineage, RORγT. Two major signals involved in the upregulation of RORγT are STAT3 and Hif-1α (a STAT3-dependent target during Th17 differentiation[162]). T cells deficient in STAT3 are unable to differentiate into Th17 cells *in vitro*[163] or *in vivo*[164], and this deficiency renders them completely unable to drive EAE[164]. Activators of STAT3 include IL-6, IL-21, and IL-23 all of which are known drivers of RORγT induction, subsequent Th17 lineage acquisition, and EAE susceptibility[129,130,165-170]. One of the major lineage-committing activities of STAT3 is the early induction of Hif-1α, a transcription factor that is also needed for Th17 differentiation and development of EAE[162,171]. Importantly, attempts to create a Th17 cell in the absence of STAT3 or Hif-1α actually results in the induction of an anti-inflammatory population of regulatory T cells (Tregs) defined by their expression of the transcription factor forkheadbox P3 (FoxP3)[162,171,172]. Likewise, deletion of gp130, a subunit of the STAT3-activating IL-6R, on T cells results in a massive accumulation of Tregs during immune challenges *in vivo* that would otherwise induce Th17 cells[166].

These data indicate an overlap between the signals that lead to Th17 versus Treg specification, such that manipulations affecting one could lead to the reciprocal generation of the other. For Th17 and Tregs, this shared signal is mediated by the cytokine transforming growth factor β1 (TGFβ1). STAT3

activating signals alone are not sufficient to induce Th17 differentiation, and must be coupled with TGFβ1 or a similar signal (e.g. activin) in order to achieve this outcome[173,174]. *In vivo* evidence for the role of TGFβ1 in Th17-mediated inflammation in the context of EAE is substantial. Mice genetically engineered to overexpress TGFβ1 develop a much more severe EAE disease course than WT controls, and this is related to their massive accumulation of IL-17A producing T cells during peripheral priming and in the CNS at the peak of disease[174]. On the other hand, forced expression of a dominant negative RII subunit of the TGFβ receptor (TGFβRII) on T cells[175] or deletion of the TGFβRII receptor subunit on T cells[176] both result in near complete protection from EAE due to failure of Th17 cells to develop and/or expand after immunization. Relatedly, deletion of TGFβ1 from T cells likewise protects mice from EAE, suggesting auto/paracrine mechanisms by which TGFβ1 controls Th17 cells[177]. Despite the significant body of evidence implicating TGFβ1 as a major driver of Th17 lineage specification, the exact mechanisms by which TGFβ1 signaling regulates Th17 differentiation have been difficult to elucidate. Per the aforementioned studies, it is agreed that TGFβ1 signaling through the TGFβRII is absolutely required for Th17 differentiation *in vivo* during inflammation and in most *in vitro* contexts. Yet, the homeostatic *in vivo* development of Th17s in the intestine do not appear disturbed by T cell-specific deletion of TGFβRII[178]. Ligation of TGFβRII by TGFβ1 results in phosphorylation of the receptor-activated Smad proteins 2 and 3 (Smad2/3) that are proposed to serve as transducers of TGFβ1-mediated signals resulting in IL-17A production, yet there is conflicting evidence regarding the

specific contributions, if any, Smad2 and 3 make in this context[179-182]. It is interesting that Smad4, the "common Smad," so often studied for its role in co-activating Smad2 and Smad3 gene signatures, is completely dispensable for Th17 differentiation *in vitro* and *in vivo*[183]. In fact, a recent report reveals that Smad4$^{-/-}$ T cells, unlike WT T cells, have the unique capacity to efficiently develop into Th17 cells in the presence of IL-6 alone[176]. This exciting study sheds light onto the mechanisms by which TGFβ1 controls Th17 differentiation, ultimately identifying Smad2/3 as being required to switch Smad4 from serving as a tonic inhibitor of RORγT transcription to an activator.

It should be noted that the vast majority of what is known regarding the differentiation requirements of Th17 cells has been discovered using murine T cells. As of this writing, there is still great controversy surrounding the appropriate cues required for the differentiation of human Th17 cells[184]. Nevertheless, the classic mechanism to induce IL-17A competency in an undifferentiated T cell is through TCR/co-stimulation and some combination of a TGFβ1 and STAT3-activating signal. In addition, other factors, like IL-1β, are gaining increasing attention for their ability to at least potentiate[185] and even substitute for[178] these classic signals to promote Th17 lineage specification. In the case of IL-1β, perhaps its most significant impact on Th17 development with respect to EAE, as previously discussed, is the facilitation of GM-CSF competency[126]. Indeed, there are agreat many extrinsic cues that are known to result in Th17 differentiation, and, despite their name, Th17 cells execute a great many functions beyond IL-17A production. A better understanding of the complex integration of signals that

can drive Th17 specification is important not only because Th17s contribute to disease in many inflammatory settings, but also successfully destabilizing Th17 differentiation can often lead to the reciprocal induction of anti-inflammatory Tregs. Excitingly, cutting edge computational approaches that interrogate the dynamic transcriptional changes undertaken by differentiating Th17 cells to infer or predict the identities of key regulators of these processes are now being used to successfully identify numerous previously unappreciated regulators of Th17 lineage acquisition[160,161,186]. In addition to Th17 cells, many other cells are able to produce IL-17A, and their ability to do so in the absence of conventional signaling factors like STAT3 suggests there may also be alternative means by which Th17 cells can be generated (and exploited)[187]. That these studies are successfully identifying new points of entry through which drug developers can thwart a T cell lineage known to contribute so significantly to pathology in diseases like RRMS is extremely exciting.

Summary

RRMS is a disease characterized by interruptions in and eventual fracturing of neuronal networks associated with CNS inflammation. DMT used in RRMS effectively reduce the frequency of symptomatic flares and the most potent are those that result in peripheral lymphoablation. While DMT for RRMS reduce the frequency of new inflammatory foci developing in the CNS, they are less effective at preventing underlying neurodegenerative processes that likely contribute to irreversible disability accumulation. Failure to adequately address

this crucial aspect of disease is a significant shortcoming shared by all current RRMS DMT. Despite this and other flaws, available DMT reduce the rate of irreversible EDSS worsening and prolong latency to SPMS onset. Arguments against immunomodulation in RRMS should be considered alongside evidence that extrinsic factors, including delays between symptom onset and therapeutic intervention, as well as treatment initiation with inferior DMT, likely contribute to the insufficiencies of current DMT. Continuing to upgrade the arsenal of immunomodulating DMT used to treat RRMS has tremendous therapeutic potential and is deserving of intense focus.

Immune cell infiltration of the CNS is associated with lesion formation and relapse in RRMS. While it remains unclear if these cells serve purely pathologic roles in the demyelinating CNS, many believe they are likely crucial for relapse as therapies that ablate or sequester them to the periphery are effective means of managing RRMS. Monoclonal antibodies (mAb) that neutralize inflammation-associated cytokines have been at the forefront of drug development efforts. Clinical trials targeting the Th17 response have largely focused on mAb-mediated neutralization of IL-17A and GM-CSF. Promising initial results from these trials support the development of Th17-directed therapeutics for RRMS. However, if anything has been learned from past attempts to target cytokines with mAb to treat RRMS, it is that cytokines often play a more nuanced role in disease than originally predicted. Thus, it may be more beneficial to target specific lineages of cells rather than individual effector cytokines they produce. The critical role that metabolism plays in Th17 differentiation and function may

represent one such exploitable vulnerability by which Th17-mediated inflammation can be selectively defused.

II. GLUCOSE METABOLISM AND INFLAMMATION

While cytokines are useful to define a lineage of cells (e.g. IL-17A and Th17 cells), lessons gleaned from attempts to target them in RRMS suggest that it may be more beneficial to disrupt inflammatory programs rather than individual mediators thereof. One feature shared by inflammatory immune cells are the metabolic adaptations they must undergo in order to exit quiescence and acquire the capability to mount an immune response, pathologic or otherwise. A tremendous body of work in the cancer literature has helped to inform immunologists of the dynamic nature in which cells regulate their consumption of substrates such as glucose to meet the needs of functions like proliferation. The goal of this section is to discuss immune cells (particularly T cells) and the metabolic adaptations they undertake (particularly with respect to glyclolysis) during inflammatory responses, and how these adaptations may be leveraged and exploited as vulnerabilities for therapeutic gain in the treatment of RRMS.

Glycolysis in inflammatory T cells

Biological processes supported by glucose in quiescent versus activated T cells

All cells require external nutrients to ensure survival[188]. Even quiescent T cells, not known for engaging in proliferative or cytokine producing behaviors, require growth factor signaling to stimulate uptake of external nutrients for the active maintenance of cellular homeostasis[189]. Glucose is a major substrate consumed by T cells and it can be used to generate ATP as well as biosynthetic

precursors for anabolic processes in glycolysis. A full breakdown of the following discussion on glycolytic and mitochondrial metabolism in T cells can be found in a number of excellent reviews[190-193]. Upon uptake through a glucose transporter, the hexokinase enzymes catalyze the ATP-dependent phosphorylation of glucose to glucose 6-phosphate, thereby committing it to an intracellular fate.

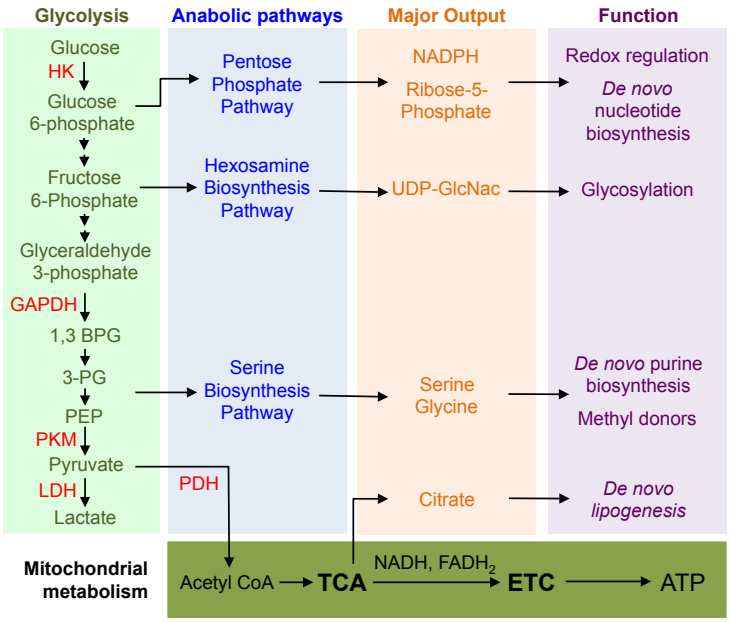


Figure 2: *Major metabolic biological functions supported by glucose metabolism. Enzymes are listed in red. HK = hexokinase, GAPDH = glyceraldehyde 3-phosphate dehydrogenase, PKM = pyruvate kinase muscle type, LDH = lactate dehydrogenase, PDH = pyruvate dehydrogenase. TCA = tricarboxylic acid cycle; ETC = electron transport chain*

Through a series of cytoplasmic reactions called glycolysis, the initial step of which is mediated by hexokinase, glucose is converted into two molecules of pyruvate (**Figure 2**). At steady state, resting T cells preferentially transport pyruvate into the mitochondria where it is converted to acetyl CoA by the enzyme pyruvate dehydrogenase. Acetyl CoA can then be used to generate $FADH_2$ and NADH through the mitochondrial tricarboxylic acid (TCA) cycle. These electron

carriers contribute electrons to the electron transport chain (ETC), and in an O_2-dependent process, ultimately lead to the generation of up to 36 molecules of ATP. The utilization of glucose-derived pyruvate to generate ATP through the TCA and ETC in the mitochondria is termed oxidative phosphorylation (OXPHOS). There are numerous mechanisms by which the rate of OXPHOS is controlled including substrate availability. For example, in situations of low ADP availability, ETC activity slows via a process called respiratory control[194]. Likewise, when O_2 availability is low, cells preferentially shunt glucose-derived pyruvate to lactate in a process called anaerobic glycolysis[195]. In this context, pyruvate to lactate conversion is an essential means by which NAD^+ consumed during and required for glycolysis can be regenerated. In a process termed, "aerobic glycolysis," highly proliferative cells, such as cancer cells, increase their conversion of pyruvate to lactate in the setting of high O_2 concentrations[196,197]. Despite the many different rationalizations for why cancer cells engage this metabolic adaptation, termed the "Warburg effect", there is actually very little understood regarding its benefits[198]. The mechanisms favoring aerobic glycolysis likely involve transcriptional induction[171,199] of metabolic machinery, like LDH[200], that support lactate production and repression of those that favor pyruvate oxidation in the mitochondria, such as pyruvate dehydrogenase kinase (PDK)[201]. It is important to note that activated T cells increase their rates of OXPHOS in addition to engaging aerobic glycolysis, though the latter process, at least *in vitro*, is potentiated to a greater degree[199].

Elevated glycolytic flux is an important way to increase the availability of biosynthetic precursors needed for building daughter cells (**Figure 2**). For example, glucose 6-phosphate generated in the first step of glycolysis can be used for *de novo* nucleotide biosynthesis via the pentose phosphate pathway (PPP). Another key component of the PPP is the production of NADPH, an important cofactor for *de novo* lipogenesis and the maintenance of the integral redox-regulating molecule glutathione. Fructose 6-phosphate is an important precursor for UDP-GlcNAc generation through the hexosamine biosynthetic pathway. UDP-GlcNAc is essential for proper glycosylation of numerous proteins involved in T cell development and functions, and disrupting flux through this offshoot of the glycolytic pathway has profound effects on T cell biology[202-205]. Likewise, conversion of 1,3 bisphosphoglycerate to 3-phosphoglycerate (3-PG) provides an important substrate for *de novo* serine biosynthesis. In fact, highly proliferative cancer cells are believed to express a lower activity isoform of the pyruvate kinase enzyme (to be discussed in detail later) in order to promote shunting of 3-PG to serine biosynthesis[206]. Taken together, T cells finely tune their metabolic state to meet the various biosynthetic and bioenergetic demands of their effector responses, and interventions that disturb metabolism can drastically alter these downstream functions.

Mechanisms for and consequences of elevated glycolysis during T cell activation

A major underlying implication of many immunometabolic studies is that stimuli that drive inflammatory responses must, in some way, be able to alter the

metabolic state of the responding cell. The PI3K-AKT-mTOR pathway appears to be one of the main mechanisms by which this occurs. In $CD4^+$ T cells, the engagement of the cell surface co-stimulatory protein CD28 (in association with T cell receptor signaling) activates the PI3K-AKT signaling cascade[207,208]. Immediately downstream of PI3K activation, events occur to facilitate the potentiation of glycolytic flux. PI3K-AKT activation, occurring minutes after CD3/CD28 stimulation[208], is known to support the translocation of glucose transporter 1 (GLUT1), the major glucose transporter in T cells, from the cytoplasm to the cell membrane within hours of CD3/CD28 stimulation[209]. The mechanisms behind this re-localization are not well characterized, but may involve posttranslational modifications that promote GLUT1 trafficking to, stabilization in, and activity at the cell membrane[207,210,211]. Supporting an intersecting role for PI3K-AKT and GLUT1 in T cell metabolism, transducing T cells to overexpress a constitutively active version of AKT or GLUT1 increases glucose uptake, cell size, proliferation, and activation marker expression[207]. PI3K-AKT activation also affects the activity of other glycolytic machinery. Phosphorylated AKT stabilizes the localization of hexokinase-II (HK-II), an isoform of the hexokinase enzyme, to the outer mitochondrial membrane (OMM). At this location, HK-II is believed to have increased access to mitochondria-derived ATP, which it can then use to phosphorylate glucose to G6P, the initiating reaction in glycolysis[212-214]. Activation of PI3K can also directly alter the glycolytic capacity of a cell via AKT-independent mechanisms. PI3K is known to initiate cytoskeletal-remodeling events via Rac. A recent report[215] shows that, in

the basal state, the majority of cellular aldolase a, the glycolytic enzyme that catalyzes fructose 1,6 bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate in glycolysis, is sequestered in F-actin and thereby impeded in its ability to fulfill its enzymatic functions. One consequence of PI3K-driven Rac-mediated cytoskeletal remodeling is the release of aldolase a from F-actin. aldolase a liberation is required for maximal glycolytic flux and is blocked by inhibitors of PI3K and Rac but not AKT or mTOR. Localization to cytoskeletal filaments does not seem to be a peculiarity specific to aldolase a, but rather, is a common trait shared with other glycolytic machinery[216]. Finally, there is a great body of work implicating mTOR as an essential player in T cell homeostasis. Genetic and pharmacologic approaches to disrupt mTOR activity produce profound effects on T cell functions *in vivo*[217,218], and in many cases, these defects are attributed to functional metabolic deficiencies. For example, mTOR complex 1 (mTORC1 – defined by association with the adaptor protein Raptor) governs cholesterol and lipid biosynthesis to regulate Treg function *in vivo*[219]. Likewise, in effector T cell lineages, mTORC1 activity is important for induction of a glycolytic program that allows for exit from quiescence and acquisition of Th1/17[218] and Th2 functionality[220]. Rapamycin is a compound that inhibits mTOR activity (particularly mTORC1) and is commonly used for its immunosuppressive effects in settings like renal transplantation[221,222]. Rapamycin is known to block activation-induced increases in T cell glycolytic activity, and in so doing, promote the differentiation of induced-Tregs during *in vitro*-skewing paradigms that would otherwise generate Th17 cells[171]. Likewise, two publications in 2016[223,224]

showed that during division, asymmetric inheritance of mTORC1 activity confers one of two daughter T cells with a heightened glycolytic profile secondary to the potentiating effects of mTORC1 on c-myc, a master regulator of metabolic adaptation in T cells. Functionally, cells that inherit more mTORC1 activity fail to persist long term and contribute to responses characteristic of memory T cells. In line with the view that mTOR activation critically mediates the T cell metabolic state, T cells deficient in the mTOR-inhibiting protein Tsc1 have a disrupted quiescent state metabolism that renders them hypertrophic and, interestingly, more prone to death upon stimulation[225]. While PI3K-AKT signaling is by no means the only activator of mTOR, collectively, studies of the PI3K-AKT-mTOR signaling axis have provided insight into how extrinsic cues promoting T cell cytokine production and proliferation, such as TCR/CD28 ligation can converge on and engage metabolic pathways to support these biosynthetic and bioenergetic expenditures.

Aerobic glycolysis: controversial relationship with T cell inflammation

Evidence for glycolysis as a driver of inflammatory T cell functions

In a 2011 landmark study[171], glycolytic inhibition with the commonly used glycolysis inhibitor 2-deoxyglucose (2-DG) was shown to prevent the generation of Th17 cells, and instead induce Tregs. Using transfer models of EAE, Shi and colleagues demonstrated that glycolytic inhibition was sufficient to prevent the differentiation of a Th17-mediated encephalitogenic response. In my own studies,

using a similar model I showed that antagonism of GAPDH with an experimental compound 3-brompyruvic acid (3-BrPa) was sufficient to prevent the generation of a Th1-mediated encephalitogenic response, and was much more effective at blocking IFN-γ compared with IL-17A-mediated inflammatory processes in the EAE spinal cord[226]. Similarly, dichloroacetate (DCA), an inhibitor of the pyruvate dehydrogenase kinase enzymes that promote pyruvate to lactate conversion, blocks Th17 but not Th1 differentiation *in vitro* and *in vivo*[227]. Conversely, complete genetic ablation of GLUT1 on T cells prevents their ability to drive both Th1 and Th17-mediated inflammation *in vivo*[209]. These studies show that the differentiation of T cells that drive autoimmune inflammation can be subverted by inhibition along the glycolytic pathway, and further imply that intervention at various levels of the glycolytic cascade may differentially affect different subpopulations of T cells.

Another means by which glycolysis may control T cell functions is through the metabolic intermediates generated during glucose catabolism. For example, fructose 6-phosphate (F6P) produced during glycolysis is an essential molecule for UDP-GlcNAc formation via the hexosamine biosynthetic pathway (HBP). UDP-GlcNAc is a precursor substrate utilized for N-glycan branching, a post-translational modification that alters the localization and turnover of proteins important for T cell development and function[205]. High rates of aerobic glycolysis shunt F6P away from HBP, reduce UDP-GlcNAc availability and N-glycan branching, and promote Th17 differentiation. Conversely, inhibition of glycolysis with rapamycin or galactose reduces glycolytic flux, increases N-glycan

branching, and as a result, promotes FoxP3^{+} Treg generation by a mechanism dependent on the presence of N-glycan branching machinery[202]. GlcNAc supplementation *in vivo* is therapeutic in EAE and other models of inflammation, perhaps due to reciprocal dampening of inflammatory T cell responses and promotion of suppressive ones[203,204]. While the exact mechanisms underlying these observations are still relatively unclear, they illustrate a very intriguing possibility that glycolytic manipulations alter T cell functions primarily by altering the availability of glucose-derived substrates for N-glycan branching.

Tumor infiltrating lymphocytes (TIL) play a critical role in anti-tumor immunity, but their ability to perform this essential function can be hampered by metabolic constraints of the intratumoral environment and/or co-inhibitor receptor signaling[228,229]. Approaches to reinvigorate the depressed metabolic state of TIL are therefore under active investigation and hold tremendous therapeutic promise. Deficient glucose uptake is an important metabolic deficiency of TIL[228,230] that may contribute to the depletion of glycolytic intermediates. Phosphoenolpyruvate (PEP), one such byproduct of glycolysis that is depleted in TIL, was recently demonstrated as an important regulator of intracellular Ca^{2+}, and the depletion of PEP that occurs secondary to glucose restriction results in a failure to sustain Ca^{2+} signaling required for T cell activation and cytokine production[231]. T cells that overexpress phosphoenolpyruvate carboxykinase 1 (PCK1), an enzyme involved in the conversion of TCA cycle oxaloacetate to PEP, are better able to maintain PEP levels in low glucose conditions, maintain intracellular Ca^{2+} levels, and control tumor outgrowth *in vivo*[231]. Likewise, tumor

cells outcompete TIL for nutrients such as glucose, thereby imposing a metabolic restriction on TIL that impairs their ability to contribute to IFN-γ-associated anti-tumor immunity[228]. Thus, altered glycolytic flux in TIL contribute to their defective anti-tumoral response. It is important to note, however, that it is not clear that correction of glycolytic dysfunction would revert TIL insufficiency. In fact, evidence suggests that the prolonged exposure to the nutrient-restricted tumor microenvironment generates TIL that require forms of metabolic reinvigoration that are not strictly glycolysis-potentiating[230]. Nevertheless, it does seem clear that genetically engineering T cells to be able to withstand tumor-induced glucose starvation is sufficient to improve anti-tumoral immunity.

A recent study[200] using an IFN-γ reporter mouse strain (Yeti mice[232]) provides perhaps the best demonstration of the important role aerobic glycolysis plays in T cell-mediated inflammation *in vivo*. Yeti mice are an IFN-γ reporter strain genetically engineered to have an IRES-enhanced-YFP sequence inserted downstream of the *Ifng* locus and stabilized with a bovine growth hormone polyA tail. This highly stabilized form of the IFN-γ mRNA transcript results in hyperproduction of IFN-γ and spontaneous autoimmune syndromes when bred to homozygosity[233]. Of relevance to immunometabolism, prior reports[234,235] had established a tight relationship between aerobic glycolysis and IFN-γ production in $CD8^+$ T cells. In these studies, low flux through the glycolytic pathway was observed to allow GAPDH to bind to 3'-AU-rich elements in IFN-γ (and IL-2) mRNA transcripts, suppressing their translation. Thus, interventions that reduce glycolytic flux might be expected to reduce IFN-γ production by freeing up more

GAPDH to repress transcription. To assess the role aerobic glycolysis plays in supporting IFN-γ production in Yeti mice, Peng and colleagues deleted LDH-A, the LDH subunit that, in $CD4^+$ T cells, is expressed nearly exclusively, thereby blocking Yeti $CD4^+$ T cells from being able to undergo the defining step of aerobic glycolysis[200]. As anticipated, deletion of LDH-A in Yeti T cells blocked IFN-γ production and normalized their phenotype, but this occurred via an unexpected GAPDH-independent mechanism. Instead of increasing association of GAPDH with IFN-γ mRNA transcripts, LDH-A deletion resulted in rapid consumption of acetyl CoA through the TCA cycle, thus depleting a necessary substrate for activation-induced permissive histone acetylations required for opening of the *Ifng* locus during T cell activation. In summary, T cells require the ability to perform LDH-A-mediated aerobic glycolysis in order to undergo epigenetic modifications that facilitate their engagement of inflammatory programs that drive disease *in vivo* in Yeti mice.

While these reports strongly support the view of glucose as a contributor to pathologic T cell responses in disease contexts with prominent autoinflammatory components, there is a significant and growing body of evidence supporting a contrasting view.

Evidence for OXPHOS as the driver of inflammatory T cell functions

Seminal studies on Warburg metabolism in immune cells largely focused on aerobic glycolysis as a driver of inflammatory T cell responses[171,199,207,236,237]. However, many of the fundamental observations made in these works are

performed in artificial *in vitro* culture systems where media are optimized to maximize cell growth, proliferation, and cytokine production. A recent study[201] established the mechanism by which metabolic observations made *in vitro* might exaggerate the role that glucose plays in fueling the inflammatory responses of T cells. The authors found that supraphysiologic levels of glucose (~11mM in RPMI used to culture T cells and 25mM in IMDM used to culture Th17 cells versus 4mM *in vivo*) in the *in vitro* setting artificially drive expression of pyruvate dehydrogenase kinase 1 (Pdk1), the enzyme that inhibits oxidation of pyruvate to acetyl CoA, thus disproportionately favoring fermentation of pyruvate to lactate. In this study, Th17 cells generated *in vitro* and *in vivo* were found to be indistinguishable based on their ability to proliferate and produce inflammatory cytokines. Despite near identical effector functionality, the elevated Pdk1 in the *in vitro*-derived Th17 cells rendered them uniquely susceptible to glycolytic inhibition, whereas Th17s generated *in vivo* continued to produce cytokine in the presence of the glycolytic inhibitor 2-deoxyglucose (2-DG). While glycolytic inhibition appeared unable to affect *ex vivo* cytokine production by *in vivo* Th17 cells, inhibition of ATP-synthase with oligomycin did block IL-17A production. The authors conclude that *in vitro* Th17 cells rely on aerobic glycolysis to fuel IL-17 production whereas *in vivo* Th17 cells rely on OXPHOS. In my own studies[226], I found that the GAPDH inhibitor 3-BrPa was much better at suppressing IFN-γ compared with IL-17A production in spinal cord homogenates from mice with EAE, further supporting the notion that *in vivo*-generated Th17 cells can circumvent glycolytic inhibition to produce IL-17A.

A preceding study supports the idea that glucose limitations *in vivo* force T cells to adopt alternative metabolic programs to fuel their inflammatory functions. When adapting to lower concentrations of glucose, an AMPK-dependent mechanism programs T cells to increase their expression of glutamine transporters and rely on glutamine-based OXPHOS for ATP production[235]. This study revealed that deletion of AMPKα1 in T cells prevents their ability to respond to inflammatory stimuli *in vivo*, and posits that this deficit is due to the impaired ability of these cells to dynamically respond to the different metabolic constraints of their changing environments. Studies on T cell metabolism in graft versus host disease (GVHD) also demonstrate metabolic processes other than aerobic glycolysis, such as fatty acid oxidation (FAO) and OXPHOS, are major metabolic drivers of inflammatory T cell responses *in vivo*[238,239], though this interpretation changes dramatically based on the population of T cells used as reference for baseline metabolism[240]. In addition to glucose, T cells also modulate their metabolism of glutamine, and this may be the primary source of material for OXPHOS and biosynthetic precursor formation in GVHD and other settings of chronic inflammation such as lupus[241,242]. Whereas *in vivo* administration of 2-DG does little to ameliorate GVHD (either due to efficacy or toxicity problems[240], addition of a glutaminase inhibitor potently suppresses disease[243]. It is hard to reconcile these reports with evidence that GLUT1 deletion on T cells prevents their ability to drive disease in GVHD and IBD models[209], as well as others showing efficacy of glycolytic inhibitors in GHVD[240] and EAE[227]. It is also difficult to rationalize why effector T cells would go through the trouble of increasing their

glycolytic flux if doing so were dispensable for downstream functionality. A unifying rationalization, supported by the observation that T cells generated by immunization differ metabolically from those generated during GVHD[238], is that distinct metabolic processes may support different aspects of the T cell effector response (e.g. induction versus maintenance). Suffice to say, the shortcomings of *in vitro* systems likely provide a skewed perspective on the relationship between aerobic glycolysis and T cell-mediated inflammation.

Another aspect of T cell metabolism that differs when comparing *in vitro* and *in vivo*-derived cells is the metabolic properties of an immunomodulatory subset of $CD4^+$ T cells called regulatory T cells (Treg). When generated *in vitro*, Tregs rely on fatty acid oxidation[236] and not aerobic glycolysis[171] for maintenance of their suppressive phenotype. However, when assessed *ex vivo*, Tregs are highly glycolytic[244,245]. The relationship between glycolysis and suppressive function in Tregs is achieved, at least in part, at the level of α-enolase, the enzyme that catalyzes 2-phosphoglycerate to PEP in glycolysis. Two products can be produced from the α-enolase gene (*Eno1*) that differ in their contributions to metabolism and potentially inflammation[246,247]. *Eno1* encodes a single mRNA transcript with two distinct translational start sites that can thus generate two differing protein products. Translation of the full-length α-enolase gene results in the production of the canonical 48kDa α-enolase enzyme that participates in glycolysis; however, translation initiation at an alternative internal translational start site located 400 base pairs downstream results in a truncated 37kDa gene product that is known as Myc promoter binding protein 1 (MBP-1)[246,247]. This

internal translational start site is not present in β or γ-enolase, alternative isoforms of enolase that are largely expressed in muscle tissue[248]. MBP-1 is a known repressor of c-myc[246,247,249,250], a pro-proliferative transcription factor that is an essential driver of tumorigenesis and the master regulator of metabolic adaptation in T cells[199]. By an unknown mechanism, glycolytic inhibition of Tregs increases expression of α-enolase isoforms, including MBP-1[244], and this MBP-1 localizes to the nucleus where it blocks transcription of a specific spliced isoform of FoxP3 that is known to potently suppress inflammatory immune responses, particularly those mediated by RORγT[251]. Thus, blocking glycolysis impairs Treg function via a mechanism involving MBP-1 mediated repression of FoxP3. Impairments in peripheral blood Treg glycolysis is a shared feature in patients with multiple sclerosis and type I diabetes[244] and is also observed in patients with glycogen storage disease type 1b[252], a disease that predisposes individuals to develop autoimmune conditions. Perhaps relatedly, T cells isolated from rheumatoid arthritis (RA) patients actually show impaired rather than augmented glycolysis[253,254]. This defect is driven by reduced expression of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), an enzyme that catalyzes conversion of fructose 6-phosphate (F6-P) to fructose 2,6 bisphosphate (F2,6-BP). F2,6-BP is a potent allosteric activator of 6-phosphofructokinase-1 (PFK1), the enzyme that catalyzes the rate-limiting conversion of F6-P to fructose 1,6-bisphosphate during glycolysis. PFKFB3 deficiency makes T cells in RA prone to apoptosis and impairs generation of Tregs.

Collectively, these reports suggest that the relationship between glycolytic metabolism and inflammation is likely nuanced and perhaps context- or site-specific. For example, deficient T cell expression of PFKFB3 occurs in RA but not lupus patients[253]. Moreover, in mouse models of GVHD, PFKFB3 transcript is actually elevated in T cells isolated from inflamed organs, and pharmacologic inhibition of PFKFB3 ameliorates disease outcomes[240]. While impaired glycolysis in peripherally isolated T cells is reported in numerous autoimmune settings, including multiple sclerosis, due to understandable limitations, there remain no studies on the metabolic profiles of pathologic T cells isolated from the inflamed CNS. The metabolism of these cells engaged in active inflammation in the CNS is likely to be very different from those that are circulating. In addition, while studies in RA, for example, may inform the understanding of immune cell metabolism in inflammatory disease, it may not be appropriate to extrapolate these observations to the setting of MS where T cells exhibit profoundly different metabolically related behaviors (to be discussed)[42,145,255]. Further supporting the notion that disease processes in RA and MS diverge by more than just the site of disease, the standard of care DMT for RA induces MS-like syndromes and worsens disease in pre-existing MS[85-88]. Thus, just as different cytokines are known to be associated with different diseases (e.g. IL-17A and psoriasis, TNF-α and RA), compounding evidence suggests that different metabolic pathways may contribute to the functionality of different flavors of pathologic T cell responses. Taken together, targeting T cell metabolism to modulate their effector function is likely to be much more complex than originally hypothesized. Further evidence

for this comes from studies on nutritional interventions and their impact on inflammatory syndromes.

Nutritional interventions and Multiple Sclerosis

Exploring the relationship between metabolism and inflammation has opened an intriguing line of inquiry into how dietary modifications might impact disease outcomes in RRMS. However, there is currently no strong evidence that diet-based interventions are effective for this indication (reviewed in detail in [256]). Isolated reports of nutrients such as polyunsaturated fatty acids (e.g. Omega-3/6 fatty acids) reducing disability accumulation and mortality associated with MS have failed validation in a randomized double-blind placebo-controlled trial[257]. Likewise, after exciting studies in the EAE model[258,259] identified dietary sodium intake as a potential modulator of autoimmune inflammation of the CNS, follow-up studies in humans failed to consistently confirm a relationship between urine sodium concentrations and MS disease activity[260]. Even dimethyl fumarate (DMF), an agent initially believed to prevent inflammation through hypothetical effects on TCA cycle activity, is now believed to achieve therapeutic efficacy by primarily non-metabolic mechanisms[261]. In fact, a Cochrane Systemic Review of the literature in 2012 concluded there was no conclusive evidence that dietary modifications impact disease in RRMS[262].

A 2016 study[263] demonstrating that caloric restriction or a ketogenic diet is therapeutic in the EAE model reinvigorated this line of investigation. Ketone bodies, and particularly, beta-hydroxybutyrate (BHB) produced during such diets

are known to exert anti-inflammatory effects on the immune system[264] and may be playing a role in the observed prevention and treatment of EAE. The major novel finding of this study was that dietary restriction could modify severity of ongoing EAE. Furthermore, in a randomized controlled trial, ketogenic or fasting mimicking diets were associated with elevated plasma BHB concentration and therapeutic outcomes as measured by EDSS accumulation and relapse rate. However, due to the limited number of participants in this study, its unblinded nature, and its failure to incorporate unbiased measurements of disease activity (e.g. new lesion formation on MRI), it is difficult to draw conclusions as to the actual efficacy of these particular dietary modifications in RRMS disease outcomes.

Just as there is no clear evidence that diet affects disease activity in MS, there is also very little evidence suggesting a relationship between hyper-nutritional states (e.g. metabolic syndrome) and outcomes in MS. Metformin, an anti-hyperglycemic agent commonly prescribed for blood sugar control in diabetes, has been shown to have therapeutic benefits in the EAE model[265]. There are many possible mechanisms by which metformin might affect the EAE disease process, including the modulation of T cell metabolism via AMPK activation[241,243]. A recent study published in JAMA Neurology[266] reports reduced T2 lesion load in MS patients with comorbid metabolic syndrome treated with either oral Metformin or pioglitazone, an anti-hyperglycemic with a distinct mechanism of action from metformin. While this study supports the notion that the systemic nutritional state may impact disease activity in MS, it too is difficult

to interpret given the small number of study participants (10-20 per treatment arm), the lack of blinding, and the lack of a placebo-controlled study design. Finally, this study was not designed to address the potential effects of metformin on MS disease activity in the absence of clinically defined metabolic syndrome. Taken together, there is weak evidence to support the use of pharmacologic agents commonly used to manage hypernutritional states for the management of MS. Furthermore, the increasingly complex relationship between metabolism and inflammation questions the utility, not to mention feasibility, of overtly impinging on the enzymatic activity of metabolic machinery to destabilize, for example, the Th17 lineage in a disease like RRMS.

Pyruvate kinase: at the crossroads of metabolism and inflammation

As in the case of GAPDH, certain glycolytic enzymes have the potential to impact the nature and quality of an inflammatory response via functions or features unrelated to their enzymatic capabilities[267]. The current status of research into the non-metabolic functions of glycolytic enzymes in immunity was the topic of my review published in 2017[267] (**Appendix B**). Using the C57BL/6 EAE model, I recently elucidated isoforms of metabolic machinery enriched in T cells of the demyelinating CNS. While I observed higher expression patterns of many metabolically relevant genes in T cells localized to the spinal cord compared with those isolated from quiescent peripheral tissues, most interestingly, I identified selective enrichment of the M2 isoform of pyruvate kinase (PKM2)[226]. PKM2 is a multi-functional protein with non-metabolic pro-

inflammatory roles that are becoming increasingly appreciated, especially in the context of macrophage biology[268-273], and more recently in T cells[274].

Pyruvate kinase isoforms in glycolysis

Pyruvate kinase catalyzes the conversion of phosphoenolpyruvate (PEP) into pyruvate and, in T cells, can exist as two alternatively spliced isoforms, pyruvate kinase isoform M1 (PKM1) and PKM2. PKM2 is the major isoform expressed at the protein level by murine lymphocytes[275], though in human lymphocytes, PKM1 is the predominant isoform expressed at baseline with selective PKM2 upregulation occurring upon activation[276]. PKM1 and PKM2 differ by inclusion of a single exon (exon 9 for PKM1 versus exon 10 for PKM2), of which only 24 amino acid residues differ[277]. The structures of PKM1 and PKM2 are extremely similar[278], but the minute differences in amino acid sequence result in important functional differences, particularly with respect to their ability to take on different oligomeric states (**Figure 3**). The tetrameric state of both isoforms is the glycolytically active form. Whereas PKM1 exists solely as a tetramer, PKM2 can dissociate into dimers and monomers that are less able to convert PEP to pyruvate, but serve other non-metabolic functions in the cell. From the perspective of glycolysis, this dynamic feature of PKM2 reduces its overall efficiency as a glycolytic enzyme which allows for the accumulation of upstream

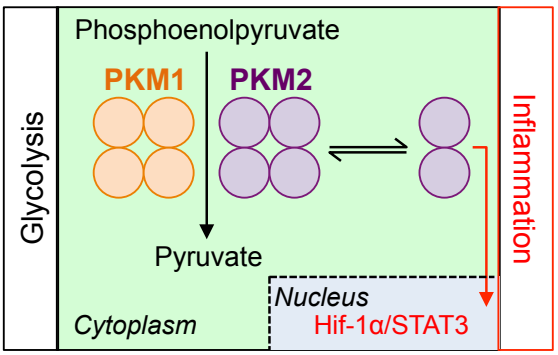


Figure 3: *Oligomeric state regulates PKM2 but not PKM1 functions*

glycolytic intermediates, thereby promoting *de novo* amino acid and lipid biosynthesis – processes that are critical for the production of a daughter cell[206]. Interestingly, many cancer cell lines also exclusively express PKM2[279], and cancer researchers have likewise identified many pro-proliferative and non-canonical functions that are specifically attributed to this particular isozyme[280-289].

Regulation of PKM2 oligomeric state

Unlike PKM1, PKM2 can adopt non-tetramer oligomeric states that alter its contributions to the cell. The major signal enforcing PKM2 tetramerization is fructose 1,6 bisphosphate (FBP), the glycolytic intermediate formed during the phosphofructokinase-catalyzed step in glycolysis. The 24 distinct amino acid residues in PKM2 exon 10 contribute to forming a single binding pocket for FBP in each monomer of PKM2 that is not present in PKM1[278]. It is not clear what structural alterations induced by FBP binding favor formation of the PKM2 tetramer. Serine binding to histidine 464 also increases PKM2 activity by an unknown mechanism[206]. In addition to these endogenous PKM2 activators, synthetic PKM2 tetramerizing agents, TEPP-46 and DASA-58, have been developed[288,290] and are frequently used to interrogate the effects of blocking the non-metabolic functions of PKM2, which are apparently mediated primarily by PKM2 dimers and monomers.

There are numerous signals that can trigger dissociation of the PKM2 tetramer. Growth factor signaling triggers phosphorylation of proteins involved in signal transduction, and phosphor-tyrosine residues can bind to PKM2 at lysine

433 (K433) and destabilize the tetramer by preventing FBP binding[285]. PKM1 contains a glutamate at residue 433 (E), and introducing K443E mutation in PKM2 blocks p-Tyr binding and impairs cancer cell proliferation through mechanisms potentially related to failed accumulation of upstream metabolites for anabolic processes or impaired induction of cell cycle regulators[282,283,286]. Interestingly, acetylation of PKM2 at K433 by p300 acetyltransferase also stabilizes PKM2 in the dimer state, and a significant amount of nuclear PKM2 is acetylated at K433[284]. Thus, the K433 residue, which is present in PKM2 but not PKM1, serves as an important integrator of signals that leads to PKM2 tetramer dissociation. Reactive oxygen species can also favor dimerized PKM2 by oxidizing cysteine 358, a modification that destabilizes the PKM2 tetramer but for unclear reasons either does not occur or does not affect C358 in PKM1[287]. There are numerous other post-translational modifications that favor dissociation of the PKM2 tetramer, including phosphorylation, methylation, and glycosylation (reviewed in [291]). In each case, the dissociation of PKM2 and subsequent reduction in glycolytic rate is believed to support cell proliferation by allowing for the accumulation of upstream metabolites necessary for anabolic processes. In addition, PKM2 dimers have their own non-metabolic functions that serve critical roles in proliferative programs.

Non-metabolic functions of PKM2 and their relationship to immunity

Besides passively altering the flux of material through the glycolytic cascade, PKM2 can also directly impact cell biology in non-metabolic ways. The

current understanding by which this occurs is through the ability of PKM2 to function as a protein kinase and as a co-activator of transcription.

As a protein kinase, PKM2 can transfer the phosphate group from PEP to other substrates. Substrate binding to the nucleotide-binding pocket of PKM2 is believed to be important for this kinase activity. A K367M kinase-dead mutant of PKM2 prevents the PEP-dependent phosphorylation of histone H3 at threonine 11[282]. Interestingly, studies in U87/EGFR glioblastoma cells reveal that K367M and K433E mutants of PKM2 both prevent EGF-induced β-catenin-dependent upregulation of pro-proliferative factors myc and cyclin D1[283]. In this system, in order for PKM2 to serve its non-metabolic role, it must be able to bind phospho-tyrosine residues and phosphorylate histone H3 using PEP as a substrate. This series of reports beautifully illustrates how the PKM2 can act as a protein kinase as well as a co-activator of transcription to direct downstream transcriptional responses.

A preponderance of evidence suggests that the non-metabolic functions of PKM2, but not PKM1 can affect gene signatures important to Th17 cells. The protein kinase function of PKM2, but not PKM1, is essential for the phosphorylation of STAT3[281]. Likewise, hydroxylation of PKM2, but not PKM1, by prolyl hydroxylase 3 (PHD3) facilitates its function as a co-activator for Hif-1α gene signatures[280]. Recently, escalating interest in PKM2 in immunity has revealed that many of its non-metabolic functions in cancer translate to inflammatory immune cells. For example, targeting the non-metabolic functions of PKM2 using TEPP-46 or DASA-58 reduces the LPS-mediated engagement of

Hif-1α gene signatures, including IL-1β[268]. Likewise, elevated glycolytic signatures and ROS accumulation in macrophages isolated from people with coronary artery disease drives PKM2 dimerization, nuclear translocation, and engagement of P-STAT3 inflammatory signatures[269]. Thus, while predominantly studied in cancer cells and macrophages, the non-metabolic functions of PKM2 overlap substantially with lineage specifying factors involved in Th17 differentiation and may play a role in their functions.

Summary

Upon activation immune cells must change their metabolism in order to provide biosynthetic and bioenergetic support to downstream inflammatory programs. This altered metabolic state, reminiscent of the metabolism of highly proliferative cancer cells, must be engaged before a T cell can divide or gain the capacity to secrete certain pro-inflammatory mediators, and as a result, could be targeted for anti-inflammatory gains. A major feature of T cell metabolic adaptation is an elevated flux through glycolysis that is especially apparent in *in vitro* settings. Many groups have attempted to target inflammation by limiting the glycolytic capabilities of immune cells. Despite this, there is a large body of evidence to suggest that glycolytic inhibition alone is unlikely to be sufficient to quell inflammatory responses *in vivo*. On the other hand, metabolic adaptation is achieved, in part, by the expression of new sets of metabolic isozymes that, in some cases, confer non-metabolic benefits to the inflammatory program. One of these, the M2 isoform of pyruvate kinase (PKM2), can directly influence

downstream inflammation independent of its function as a glycolytic enzyme. The major feature of PKM2 that distinguishes it from PKM1 is its ability to translocate into the nucleus and serve as a transcriptional co-activator or protein kinase. These unique functions seem particularly important for Hif-1α and STAT3-mediated inflammatory responses and may thus be exploitable mechanisms by which to destabilize the Hif-1α/STAT3-dependent Th17 lineage of cells.

III. THESIS RATIONALE

Relapsing remitting multiple sclerosis (RRMS) is a common debilitating disease that afflicts millions of people worldwide. RRMS is characterized by episodic bouts of neurologic disturbance followed by periods of near complete recovery superimposed on insidious disability accumulation. As a disease that arises in young adulthood with limited treatment options and no cure, a diagnosis of RRMS is a lifetime sentence to a progressively worsening debilitation. There is a critical need to develop a greater understanding of and novel therapeutic strategies to treat RRMS.

The main pathologic feature of RRMS is multifocal demyelination commonly associated with immune cell infiltration of lesioned areas. T cells are agents of the immune system that play critical if not orchestrating roles in this pathology, and efforts to target their contributions to disease are under active investigation. T cells defined by their ability to produce the cytokine IL-17A (Th17 cells) are believed to be highly pathogenic in RRMS. Genetic and pharmacologic strategies targeting the Th17 response can profoundly attenuate disease in murine models of RRMS and serve as the basis for ongoing clinical trials aimed at defusing their contributions to disease. Thus, understanding the mechanisms by which Th17 cells achieve their pathologic functions in RRMS is a valuable line of research. Metabolism is a critical regulator of inflammatory T cell functions. Interventions that impose metabolic constraints, particularly with respect to glucose utilization, prevent the development of T cells that have the capacity to

drive inflammation in the CNS. However, there remains great controversy as to the different metabolic proclivities and vulnerabilities of T cells in the context of inflammation.

In the first half of my thesis work, I characterize the effects of 3-bromopyruvic acid (3-BrPa), an inhibitor of GAPDH, on T cell functions relevant to the experimental autoimmune encephalomyelitis (EAE) model of RRMS. The results of this work were published in the *Journal of Immunology* under the title "Lineage specific metabolic properties and vulnerabilities of T cells in the demyelinating central nervous system." In this study, we show that T cells isolated from the spinal cords of mice at the peak of EAE disease are more metabolically active than those isolated from uninvolved peripheral spleens. We further demonstrate that this metabolic potentiation expands beyond glycolytic machinery and includes machinery involved in mitochondrial metabolism. We reveal that metabolic inhibition with 3-BrPa is effective at dampening antigen-induced IFN-γ, but surprisingly, is much less effective at limiting antigen-induced IL-17A production in T cells isolated from the EAE spinal cord. Finally, we show that 3-BrPa can be used to subvert the differentiation of IFN-γ producing T cells that drive disease in adoptive transfer EAE models. Together, this study identifies enrichment of certain pieces of metabolic machinery in T cells isolated from the site of inflammation in the EAE model, and also demonstrates that different effector responses (e.g. IFN-γ versus IL-17A) may be differentially susceptible to metabolic perturbation.

In the second part of my thesis studies, I build on work from the first by elucidating the functions of an isoform of metabolic machinery enriched in T cells of the EAE spinal cord, the M2 isoform of pyruvate kinase (PKM2), and in particular, how the non-metabolic functions of PKM2 might be manipulated to disrupt a Th17 response seemingly refractory to glycolytic inhibition. This study was promising given the preexisting availability of small molecules TEPP-46 and DASA-58 that prevent PKM2 from performing non-metabolic functions hypothesized to be important for Th17 differentiation. As hypothesized, I found that TEPP-46 and DASA-58 block differentiation of IL-17A-competent T cells during Th17 differentiation. However, this treatment unexpectedly promoted their differentiation into GM-CSF producers, known for their propensity to drive brain inflammation during EAE. In addition, these drugs also potently blocked the differentiation of TGFβ-induced Tregs. Using genetic strategies, I discovered that the effects of these drugs on T cell functions were PKM2-independent, and instead, related to inhibition and potentiation of TGFβ and P-STAT5 signaling pathways respectively. Finally, I demonstrate that TEPP-46 is not therapeutic in the EAE model, perhaps related to the observed effects on GM-CSF-producing T cells and Tregs.

Taken together, the work submitted in this dissertation contribute a greater understanding of the metabolic state of T cells involved in CNS demyelination during EAE, and the effects of two pharmacologic strategies (enzymatic inhibition of GAPDH versus inhibition of the non-metabolic functions of PKM2) on T cell functions relevant to this model. Collectively, they lay the groundwork for future

studies aimed at investigating the lineage-specific metabolic vulnerabilities of T cells *in vivo*, and how these may be exploited to curtail inflammation for the treatment of RRMS.

CHAPTER II: MATERIALS AND METHODS

Animals

Mice were housed in accordance with the guidelines of the University of Virginia Institutional Animal Care and Use Committee. Mice with PKM2 deleted in T cells (PKM2-KO) were developed by obtaining and crossing two commercially available strains from Jackson Laboratories: CD4-cre (#022071) and $Pkm2^{fl/fl}$ (#024048). 2d2 (#006912), $Rag1^{-/-}$ (#002216), and C57BL/6 (#000664) mice were also obtained from Jackson Laboratories and maintained in-house. All studies were performed in both male and female mice at least 8 weeks old unless otherwise indicated.

Tissue Staining

Mouse tissues for immunohistochemistry (IHC) were paraffin-embedded and mounted by members of the University of Virginia School of Medicine Research Histology Core. For staining, sections were deparaffinized in xylene and ethanol gradient. Luxol Fast Blue staining was performed according to manufacturer instructions. PKM2 staining was performed using Cell Signaling Technologies antibody against PKM2 (D784) and the Vector Labs ImmPRESS Excel Rabbit IgG Peroxidase Staining Kit for (#MP-7601). CD45 and CD3 IHC was performed by members of the UVA Biorepository and Tissue Research Facility (BTRF). For immunofluorescent (IF) studies, frozen tissue sections were sectioned to 30μM and stained with PKM2 (D784) followed by Donkey anti-Rabbit Cy3, and CD3-e660 (eBioscience #50-0032-82). Isotype controls were

used for both PKM2 (Rabbit IgG, Cell Signaling Technologies #3900S) and CD3 (Rat IgG eBioscience #50-4031-80) stains and concentrations equal to respective primary antibody. IHC images were captured on the EVOS FL Auto. IF images were captured on the SP8 Leica Confocal Microscope.

$CD4^+$ T cell isolation and differentiation

$CD4^+$ T cells were isolated from spleen and lymph node homogenates using the EasySep™ mouse CD4+ T cell isolation Kit (StemCell Technologies #19852). For *in vitro* differentiation of sorted CD4+ T cells, cells were plated on platebound anti-CD3 (2C11, 1µg/mL) anti-CD28 (2µg/mL CLONE) at a concentration of $2x10^6$ cells/mL. Th17 differentiation was performed in Iscove's Modified Dubelcco's Medium (Gibco #12440-053) supplemented with 10% FBS, non-essential amino acids (Gibco #11140-050), 1mM sodium pyruvate (Gibco #11360-070), HEPES (25mM), β-mercaptoethanol (Fisher Scientific #O3446I-100), and 100U/mL penicillin/streptomycin (Gibco #15140122). Th17 skewing cocktail is as follows: murine IL-6 (20ng/mL), IL-23 (10ng/mL), TGFβ1 (0.3ng/mL), anti-IFN-γ (10µg/mL, XMG1.2) and anti-IL-4 (10µg/mL, 11B11). Th1 skewing was performed in RPMI-1640 (Gibco #11875-093) supplemented exactly the same as IMDM, except with 10mM HEPES (Gibco #15630-080). Th1 skewing cocktail is as follows: human IL-2 (100U/mL), murine IL-12 (10ng/mL), anti-IL-4 (10µg/mL). Blocking antibodies were purchased from BioXCell and cytokines were purchased from Thermo Fisher Scientific. Treg differentiation was achieved by culture with TGFβ1 (5ng/mL) in XVIVO-10 (Lonza 04-743Q)

supplemented with penicillin/streptomycin. For both Th17 and also Treg differentiation, the effects of TEPP-46 and DASA-58 were measured out to 2 days or approximately 48 hours. 3-BrPa was obtained from Acros Organics (#AC325690250) and used at 10-20μM in saline. 6-Diazo-5-oxo-L-norleucine (DON) was purchased from Cayman Chemical (#17580). TEPP-46 and DASA-58 were obtained from Cayman Chemical Company or MedChemExpress and used at concentrations of 50μM and 25μM respectively in <0.1% DMSO. Effects of STAT5 inhibition were assessed using STAT5-IN-1 obtained from MedChemExpress (#HY-101853) at 100μM in 0.1% DMSO.

Experimental Autoimmune Encephalomyelitis (EAE)

Transfer EAE studies were performed by harvesting splenocytes from $\geq$ 8 week old 2d2 mice and transferring them into immunodeficient *Rag1*$^{-/-}$ mice. For assessing the effects of 3-BrPa on Th1 transfer EAE, 2d2 splenocytes were skewed toward the Th1 lineage for 3 days in the presence of 50μg/mL MOG_{35-55}. Cells were treated with 10-20μM 3-BrPa or saline vehicle on days 0 and 2 of skew. On day 3, cells ($5x10^6$) were injected i.p. into *Rag1*$^{-/-}$ recipients. 250ng pertussis toxin was administered on the day of and 2 days after transfer. For assessing the effects of TEPP-46 on Th17 transfer EAE, 2d2 splenocytes were skewed toward the Th17 lineage in the presence of 50μg/mL MOG_{35-55} $\pm$ TEPP-46 for 2 days. $4x10^6$ cells were then transferred intravenously into *Rag1*$^{-/-}$ recipients. On d.0 and d.2 post transfer, recipient mice were given 200 and 400ng of pertussis toxin respectively. Mice were scored out to at least 16 days

post transfer. A blinded observer confirmed atypical EAE when apparent. For active immunization based EAE studies, female C57BL/6 mice aged 8 weeks were subcutaneously immunized with 50µg MOG_{35-55} emulsified in Complete Freund's Adjuvant with 2mg/mL mycobacteria. Pertussis toxin was administered as in transfer EAE studies. To determine the *in vivo* effects of TEPP-46 during EAE, TEPP-46 was suspended at 5mg/mL in 0.5% carboxymethylcellulose with 0.1% Tween 20 and delivered at 50mg/kg by oral gavage every 18 hours either prior to or after disease onset. For all EAE studies, classic disease severity was measured as follows 0- no disease, 1- tail paralysis, 2- gait abnormality on grate walk, 3- hindlimb weakness on grate flip, 4- bilateral hindlimb paralysis or failure of plantar placement, 5- moribund. Scoring by 2-3 blinded observers was used to determine the effects of oral TEPP-46 on active EAE.

Cell harvest from central nervous system tissues

For flow cytometric determination of immune invasion of the CNS, mice were euthanized by CO2 asphyxiation then perfused with 0.9% sterile normal saline + 5U/mL heparin. Brain, spinal cord, and cerebellum were harvested into HBSS with Ca^{2+}/Mg^{2+} (Thermo Fisher Scientific #14025126) and digested in 4mg/mL collagenase IV (Worthington LS004188) + 50U/mL DNAse (Worthington LS002139). Digestion was achieved by shaking tissues at 180RPM, 37C for three rounds of 15 minutes. To assist with digestion, CNS tissues were gently triturated through a 5mL pipette before each round of shaking. Complete RPMI was used to stop digestion. Undigested CNS leftovers were filtered out through a

70μM strainer, and samples were pelleted at 1200RPM, RT, 8min, accel=9, decel=3 to remove digestion buffer. Debris generated during the digestion was removed by Percoll density gradient (GE Healthcare #17-0891-01). Isolated cells were subsequently washed in complete RPMI, and used for downstream studies.

For studies interrogating the biology of T cells in the demyelinating CNS, active EAE was induced and immune cells were harvested from the spinal cords and spleens of mice at peak disease (d.14-18). The resulting cell suspension was then stained with a PE-conjugated antibody against TCRβ (eBioscience #12-5961-83) and T cells were positively sorted with Miltenyi MACS anti-PE Microbeads (#130-048-801). Splenic T cells were isolated similarly after tissue homogenization. MACS purifications yielded ~90% purity. Results obtained with magnetically sorted cells were confirmed by analysis of $TCR\beta^+CD4^+$ and $TCR\beta^+CD8^+$ T cells sorted on the BD Biosciences Influx Fluorescence Activated Cell Sorting machine at the University of Virginia Flow Cytometry Core Facilities.

Seahorse metabolic flux analyses

To assess the functional metabolic capacity of T cells, cells were sorted as described and subjected to glycolytic or mitochondrial stress tests using the Seahorse XFp. For studies assessing the function of unstimulated freshly sorted $CD4^+$ T cells, 3-4x10^5 cells/well. For studies assessing the metabolic state of T cells activated *in* vitro or *ex vivo* after harvesting from EAE tissues, 1x10^5 cells were used per well. All Seahorse studies were performed according to manufacturer instructions.

Antigen recall assays in CNS tissues of mice with EAE

Cells were isolated from the EAE CNS as described above. $5x10^5$ cells were stimulated in 200 µL of complete RPMI with 50 µg/mL MOG35-55 and 10 µM 3-BrPa or 1mM 6-Diazo-5-oxo-L-norleucine (DON) for 48 hours, and IFN-γ (BioLegend #517902 and #505704) and IL-17A (eBioscience #eBio17CK15A5 and #eBio17B7) secretion were measured by ELISA. Post-stimulation cell viability was determined by flow cytometry. In some cases, spinal cord meninges were separated from the spinal cord parenchyma prior to recall as described in the text.

Flow Cytometry

Flow cytometry was performed using the Beckman Coulter Gallios 10 color flow cytometer available through the Brain Immunology and Glia Center at the University of Virginia, Department of Neuroscience. Antibodies used are as follows: TCRβ (H57-597), CD4 (RM4-5), CD8 (53-6.7), CD19 (eBio1D3), CD11b (M1/70), MHC-II (M5/114.15.2), CD45 (30-F11), CD25 (PC61.5), FoxP3 (FJK-16s), RORγT (B2D), T-bet (eBio4B10), IL-17A (TC11-18H10.1), IL-2 (JES6-5H4), IFN-γ (XMG1.2), GM-CSF (MP1-22E9), and CD16/32 Fc Block (93). All antibodies were purchased from eBioscience/Thermo Fisher Scientific except IL-17A, which was obtained from BioLegend. Live/Dead discrimination was performed using Ghost Dye™ Violet 510 from Tonbo Biosciences (#13-0870) or Zombie Aqua Fixable Viability kit (BioLegend #423101). For macrophage/myeloid

stains, cells were used for compensation controls. For T cell stains, OneComp eBeads (Thermo Fisher Scientific 01-1111-42) were used. Staining was performed in PBS + 2% FBS + 2mM EDTA. For intracellular cytokine staining, the eBioscience™ Intracellular Fixation and Permeabilization Kit (#88-8824-00) was used. For intranuclear staining, the eBioscience™ Foxp3/Transcription Factor Staining kit (#00-5523-00) was used. All stains were performed according to manufacturer recommendations.

RNA isolation and qRT-PCR

For gene expression studies, RNA was isolated using the Bioline Isolate II RNA Mini Kit (BIO-52073) and quantified using the BioTek Epoch Microplate Spectrophotometer. Equal amounts of RNA were then converted to cDNA using the Bioline SensiFAST™ cDNA synthesis kit (BIO-65054). qRT-PCR was performed using Bioline SensiFAST™ kits (BIO-98005, BIO-86005) and the Thermo Fisher Scientific Pikoreal96 or BioRad CFX384 Touch™ systems. Primers used listed in Supplementary Table 1.

Western Blotting

For assessment of STAT phosphorylation, cells were lysed in ice-cold RIPA containing protease inhibitors (MedChemExpress HY-K0010) and 1mM sodium orthovanadate (Sigma Aldrich #450243). Protein concentration was determined using the Pierce™ BCA assay (Thermo Fisher Scientific #23225) and Epoch BioTek Spectrophotometer. Lysates were then denatured in SDS with

100μM 1,4-Dithiothreitol (Fisher Scientific # BP172-5) and electrophoresed on BioRad Mini-PROTEAN® TGX™ Any KD™ precast gels. The BioRad TransBlot® Turbo™ system was used to transfer protein onto BioRad Immun-Blot PVDF membranes. Antibodies used are as follows: Phospho-Tyr694 STAT5 (C11C5), Phospho-Tyr705 STAT3 (D3A7), polyclonal Total STAT5 (#9363S), Total STAT3 (124H6), PKM1 (D30G6), PKM2 (D784), Lamin B1 (D4Q4Z), Actin (AC-74). All Western Blotting antibodies were purchased from Cell Signaling Technologies except Actin, which was purchased from Sigma Aldrich. Secondary antibodies used were as follows: anti-Mouse IgG HRP-linked whole Ab (NXA931) and anti-Rabbit IgG HRP-linked whole Ab (NA934) both purchased through Sigma Aldrich. Chemiluminescence was achieved with Perkin Elmer LLC Western Lighting Plus-ECL, Enhanced Chemiluminescent Substrate (Thermo Fisher Scientific #50-904-9325) and captured using the GE Healthcare ImageQuant LAS4000mini. For subcellular fractionation studies, the NE-PER™ Nuclear and Cytoplasmic Extraction Kit was used (Thermo Fisher Scientific #78833).

Statistics

All statistical analyses were performed with Prism 7 (GraphPad software). Specific tests used to determine significance are described in the figure legends.

CHAPTER III: GLYCOLYTIC INHIBITORS VERSUS ACTIVATORS AND THE T CELL RESPONSE IN EAE

I. METABOLIC REGULATION OF T CELLS IN THE EAE SPINAL CORD

The results presented herein are excerpted from my 2017 publication entitled "Lineage-Specific Metabolic Properties and Vulnerabilities of T Cells in the Demyelinating Central Nervous System" in the *Journal of Immunology*. (**APPENDIX A**).

Research Question

What are the metabolic peculiarities and vulnerabilities of T cells that contribute to disease in the EAE model?

Rationale

Metabolic processes like glycolysis are critical regulators of T cell functions. In fact, the very stimuli that elicit a T cell effector response also activate the unique metabolic programs that are required to sustain them. Thus, intervening at the level of metabolism holds the potential to subvert inflammatory or pathogenic T cell responses. There is a great body of evidence to suggest that inflammatory T cells, such as IFN-γ-producing Th1 cells and IL-17A-producing Th17 cells, contribute to pathological outcomes in the EAE model[116,156]. Both of these cell lineages appear to rely on glucose for their induction and maintenance, as deletion of the major glucose transporter expressed on T cells hinders the development of Th1 and Th17 responses in

colitis and graft versus host disease (GVHD) models[209]. The major studies on auto-inflammatory effector T cell responses *in vivo* have been performed in GVHD, colitis, and rheumatoid arthritis (RA) models[209,238-240,242,243,253,254]. At the time the studies presented in this section were performed, the major metabolic processes supporting T cell functions in the inflamed central nervous system (CNS) were largely unexplored. It was conceivable that the metabolic processes supporting T cells involved in GVHD, colitis, RA, and EAE may be different for the following reasons: (i) the completely dichotomous therapeutic efficacy of TNF-α blockade in GVHD/colitis/RA versus MS suggest that while all are inflammatory diseases with prominent T cell involvement, they are likely governed by distinct inflammatory cues which may likewise invoke distinct metabolic patterns in the responding T cells; (ii) the different localization of T cells in GVHD/colitis/RA tissues (massive tissue infiltration) versus RRMS/EAE (predominantly perivascular) may result in a differential access to nutrients that can profoundly impact the metabolic state of responding cells; and (iii) this differential migratory pattern of T cells may indicate the presence of a disease-specific stimulus affecting the highly metabolic process of cell migration and invasion[255]. Thus, it was my hope that performing the studies herein would shed light on the metabolic nature of T cells in the inflamed CNS, and contribute to efforts aimed at manipulating T cell metabolism to modulate their function in a diverse set of contexts.

Main findings

Metabolic characterization of T cells at the site of inflammation in the EAE model

For all metabolic characterizations of T cells in EAE, we compared T cells isolated from the site of inflammation in the EAE model (spinal cord) to those isolated from a peripheral tissue not actively involved in inflammation (spleen) of the same mouse at peak disease. T cells isolated from the spinal cord compared with the spleen at this stage of EAE are more directly involved in disease, as evidenced not only by their localization but also by their capacity to produce cytokines upon MOG_{35-55} restimulation (**Figure 4A**). Studying the metabolic idiosyncrasies of these spinal cord T cells may identify targetable vulnerabilities by which those engaged in pathology can be selectively destabilized without necessarily impacting homeostatic T cell functions. To assess the functional metabolic capabilities of T cells activated during EAE and successfully trafficked to the EAE spinal cord, and how they differ from relatively uninvolved peripheral T cells, we subjected isolated cells to a glycolysis stress test on the Seahorse Extracellular Flux XFp (**Figure 4B**). These studies revealed that T cells isolated from the EAE spinal cord had a much greater utilization of glucose, as measured by media acidification (an accepted proxy for lactate production), at baseline and after glucose stimulation (**Figure 4C**). Basal mitochondrial respiration, as measured by oxygen consumption rate (OCR) was also elevated in spinal cord T cells, indicating elevated flux through the TCA cycle in addition to cytoplasmic glycolytic flux (**Figure 4C**).

To determine whether specific subsets of machinery were selectively responsible for this metabolic induction, we performed targeted transcriptional analyses of metabolic machinery. We identified enriched expression of glycolytic machinery (**Figure 5A**) in both $CD4^+$ and $CD8^+$ T cells from the EAE spinal cord, and of particular interest, in the case of pyruvate kinase, this was only observed for the M2 isoform (PKM2) (**Figure 5B**). We further probed machinery used during metabolism of glutamine, amino acids, lipids, and fatty acids (**Figure 5C**). We similarly found that these were expressed at higher relative quantities in T cells isolated from the EAE spine compared with the periphery (**Figure 5D**). Interestingly, the site-specific enrichment of non-glycolytic machinery was less evident in $CD8^+$ compared with $CD4^+$ T cells. Taken together, our transcriptional studies indicated that T cells of the EAE CNS are enriched for a distinct set of metabolic machinery that differs greatly from that of peripheral T cells not directly engaged in the pathologic response, and likely contributes to their heightened metabolic state at least with respect to glycolytic flux and mitochondrial respiration.

Differential susceptibility of IFN-γ and IL-17A-producers to the glycolytic inhibitor 3-BrPa

T cells of the EAE spinal cord exhibit elevated glycolytic flux compared with peripheral T cells isolated from the same mouse. To determine how this unique metabolic activity affects their responses at the site of inflammation, we harvested cells from the spinal cords of mice with ongoing EAE and stimulated

them with MOG_{35-55} in the presence or absence of the metabolic inhibitor, 3-BrPa. The exact mechanism of action of 3-BrPa is unclear, though it is reported to be an inhibitor of HK[292] and GAPDH[293] at millimolar and micromolar concentrations respectively. At the concentrations used in our studies (10-20μM), we found 3-BrPa to be a robust inhibitor of GAPDH, and less so HK in immune cells (**Figure 6A**). Addition of 3-BrPa to EAE spinal cord homogenates restimulated with MOG_{35-55} robustly suppressed IFN-γ production in this assay, but was much less effective at blocking IL-17A production (**Figure 6B**). Unlike 3-BrPa, we found that the glutaminase inhibitor 6-Diazo-5-oxo-L-norleucine DON was equally effective at blocking IL-17A and IFN-γ production in this assay (**Figure 6C**). These data demonstrate that metabolic perturbation with the glycolytic inhibitor 3-BrPa is differentially effective at curtailing IFN-γ compared with IL-17A production in EAE spinal cords restimulated with MOG antigen.

3-BrPa prevents the acquisition of an encephalitogenic Th1-skewed response

Having determined that 3-BrPa treatment was much more successful at preventing MOG_{35-55}-elicited IFN-γ compared with IL-17A production in *ex vivo* cultures of EAE spinal cords, we sought to determine its therapeutic potential in the EAE model. Systemic administration of 3-BrPa at concentrations used in the cancer literature (10mg/kg)[294] resulted in severe toxicity when administered at the onset of EAE. We thus shifted our focus to determining how 3-BrPa treatment affected the differentiation of encephalitogenic Th1-skewed cells. To do this, we harvested splenocytes from the 2d2 strain of mice, cultured them in cognate

antigen (MOG_{35-55}) under Th1 skewing conditions in the presence or absence of 3-BrPa. 3-BrPa did not affect the viability of T cells produced (**Figure 7A**), or their expression of T-bet (**Figure 7B**). However, 3-BrPa did reduce the amount of IFN-γ produced in culture supernatants (**Figure 7C**). We found that immunodeficient *Rag1*$^{-/-}$ recipients of cells cultured in saline vehicle developed severe EAE, whereas those that received 3-BrPa-treated cells developed a profoundly attenuated disease (**Figure 7D**). Whereas recipients of saline-treated cells exhibited severe demyelination and T cell infiltration of the spinal cord, 3-BrPa-treated cells were much less able to infiltrate the CNS and induce a demyelinating response (**Figure 7E,F**). Thus, 3-BrPa can prevent the generation of T cells capable of driving a Th1-mediated form of adoptive transfer EAE.

Discussion & Conclusions

From these studies, we conclude that a glycolytic inhibitor can prevent T cells from acquiring a Th1-skewed encephalitogenic program, and relatedly, T cells isolated from the site of disease in the EAE model exhibit a heightened metabolic state characterized in part by elevated glycolytic flux. We show that, T cells in the EAE spinal cord are metabolically distinct from their peripheral, quiescent, counterparts at the transcriptional and functional levels. This perhaps indicates that T cells participating in the destruction of CNS tissues during EAE may have different, and thus targetable, metabolic vulnerabilities compared with those in peripheral tissues. In testing the effects of glycolytic inhibition on *ex vivo* cultures of EAE spinal cord, we found that the small molecule glycolytic inhibitor

3-bromopyruvic acid (3-BrPa) potently suppressed MOG_{35-55}-induced IFN-γ production, but was much less effective at blocking IL-17A production. On the other hand, the glutaminase inhibitor DON was able to block both IFN-γ and IL-17A production with equal efficacy. This observation led us to the intriguing conclusion that distinct cytokine-directed responses may be fueled and thus differentially affected by distinct metabolic processes. More broadly, it is conceivable that organ-specific microenvironments fine-tune local immune responses by regulation of metabolic pathways. These exciting ideas are pursued further in Chapter V: Future Directions.

In addition, we found that 3-BrPa treatment prevented the generation of Th1-skewed cells capable of driving EAE in adoptive transfer models. There are a few possible interpretations of this outcome. It could be that 3-BrPa treatment selectively blocks the *in vitro* expansion of MOG_{35-55}-specific 2d2 T cells, and thus, while approximately the same number of T cells were transferred in saline versus 3-BrPa conditions, the latter contained less antigen-specific cells capable of driving disease. This could be due to effects of metabolic inhibition on the T cell or the antigen presenter. This interpretation of the results suggests that the acquisition of encephalitogenic programs by T cells responding to antigen can be selectively blocked by metabolic inhibition. Interestingly, the transferred T cells, regardless of drug treatment, still expressed T-bet and were capable of producing IFN-γ, albeit to a lesser degree. Though Th1 cells are highly effective at transferring disease in passive EAE models[116], including the one described here, IFN-γ is not widely considered to be the major cytokine driving EAE. In fact,

genetic deletion of IFN-γ results in a severe form of EAE[95,295] characterized by ataxia, and likely mediated by exuberant Th17 responses[296]. This raises the possibility that 3-BrPa is able to block Th1-transfer EAE by a mechanism impacting the Th1 program, but independent from production of IFN-γ. One IFN-γ-independent encephalitogenic attribute that may be impacted is the migratory capability of these cells after 3-BrPa treatment. Indeed, the wealth of evidence that signaling pathways such as PI3K-AKT-mTOR control both metabolic and also migratory responses has spurred investigation into the crosstalk between cell metabolism and migration in immunity[297,298].

It is not very surprising that T cells involved in inflammation (i.e. in the inflamed spinal cord in the EAE model) are more metabolically active than those that are uninvolved (i.e. in the spleens). However, simplistic as this finding was, it had not yet been reported in the literature, and in addition, through targeted transcriptional studies, it identified specific isoforms of metabolic machinery enriched in T cells of the EAE spinal cord. A follow up study that more broadly and deeply investigates the expression of isoforms of metabolic machinery in T cells of the EAE spinal cord, perhaps by RNA-seq, could prove to be very informative. Furthermore, combining these types of studies with lineage tracing approaches has the potential to identify aspects of metabolic machinery that are unique to specific subsets of T cells (e.g. Th17, Th1, Treg), perhaps facilitating the development of more targeted metabolic interventions for inflammatory conditions. As we experienced with systemic administration of 3-BrPa, systemic administration of an overt inhibitor of glycolysis is not likely to be the most

promising therapeutic approach for a disease of chronic inflammation. As an alternative, approaches targeting specific isoforms of metabolic machinery, especially those that interact uniquely with inflammation, may be more immediately translatable. In our targeted transcriptional studies, we identified enrichment of the M2 isoform of pyruvate kinase (PKM2) transcript in T cells isolated from the EAE spinal cord, but intriguingly, no such regulation of its alternative counterpart PKM1. PKM2 is capable of serving non-metabolic functions relevant to inflammation in immune cells, and it is thus possible that its selective induction could serve metabolic as well as non-metabolic purposes. This possibility forms the basis for my follow-up study on the non-metabolic properties of PKM2 and how these contribute to the effector responses of T cells.

Figures

Figure 4: T cells in the EAE spinal cord are metabolically active

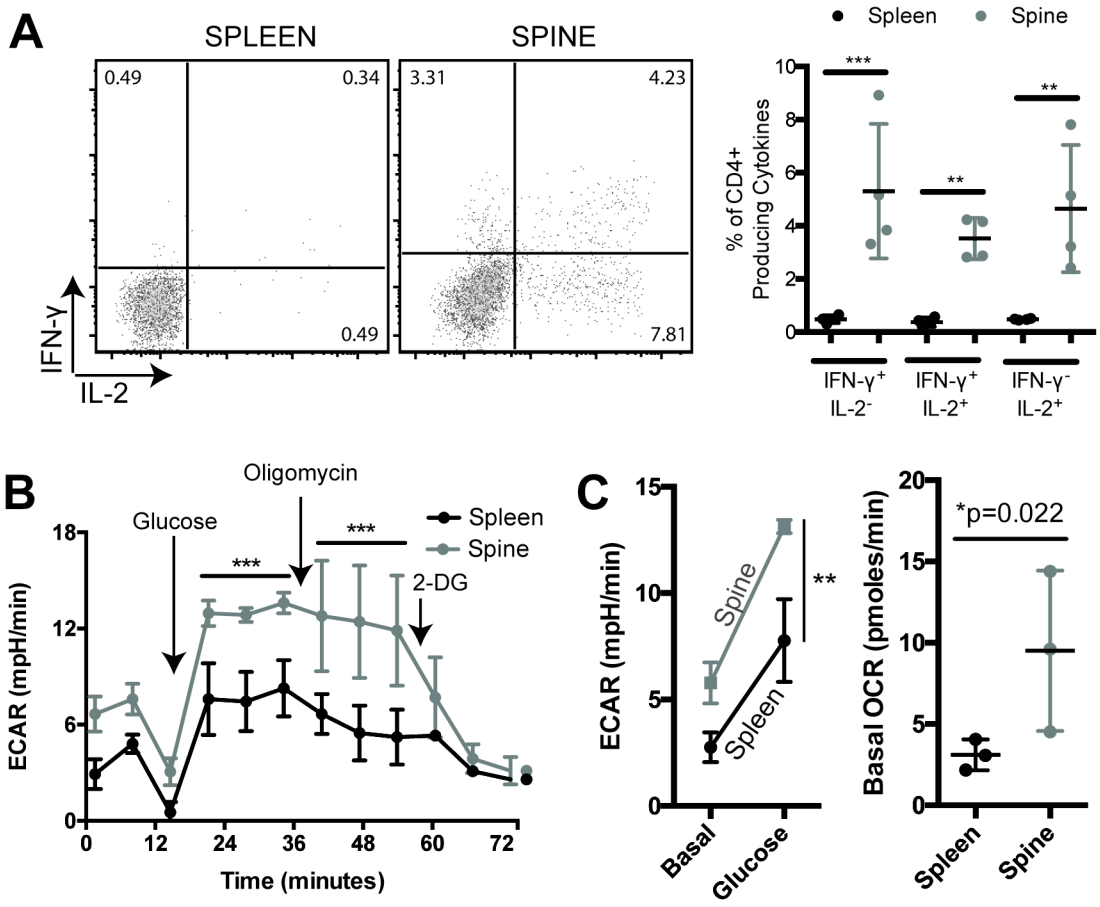


Figure 4: (A) MOG_{35-55}-induced IFN-γ and IL-2 production in splenic and spinal cord homogenates isolated from mice at peak EAE; gated on live, singlet, $CD45^+CD11b^+TCR\beta^+CD4^+$. Data are representative of N=2 independent experiments. **(B)** Glycolysis stress test and extracellular acidification rate (ECAR) of $TCR\beta^+CD4^+$ T cells isolated from the spleens and spinal cords of mice at peak EAE (n = 3 biological replicates per group). ***p=0.001, two-way ANOVA with Sidak post test. **(C)** Average glycolytic rates at baseline (basal) and after glucose stimulation (glucose) and basal oxygen consumption rate (OCR) from Seahorse data in **(B)**. **p=0.001, Two Way ANOVA, *p=0.05, paired Student T test. Data shown in **(B-C)** are from N=1 independent experiment. Data are mean ± SEM.

Figure 5: Transcriptional regulation of metabolic machinery in T cells isolated from the EAE spinal cord versus spleen

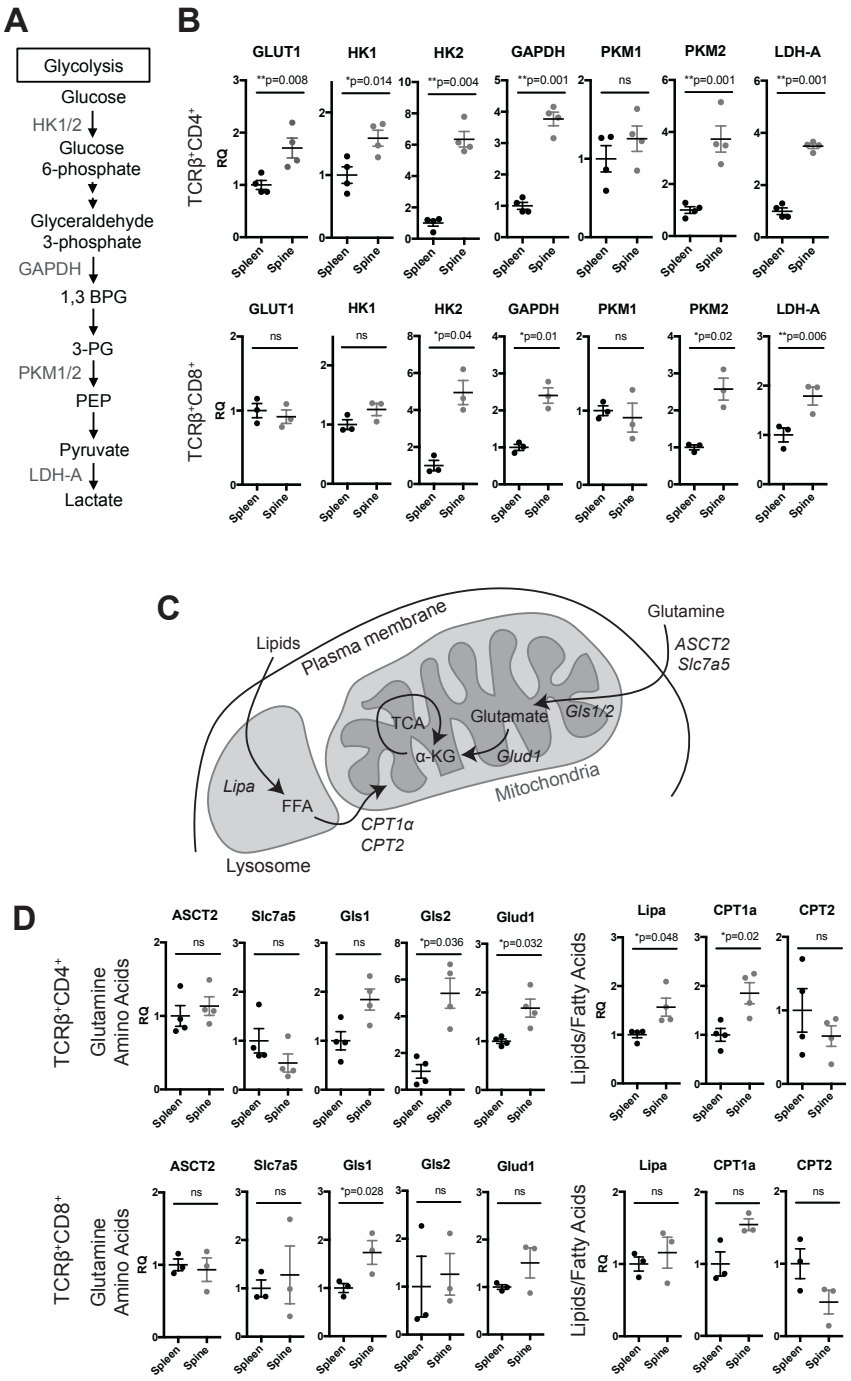


Figure 5: (A) Glycolysis diagram and **(B)** qRT-PCR for mRNA expression of glucose transporter 1 (GLUT1), hexokinase 1/2 (HK1/2), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), pyruvate kinase isoform M1/2 (PKM1/2), and lactate dehydrogenase A (LDH-A) in $TCR\beta^+CD4^+$ and $TCR\beta^+CD8^+$ T cells flow sorted from the spleens and spinal cords of mice at peak EAE. **(C)** Diagram of non-glycolytic machinery and their functions. **(D)** qRT-PCR for mRNA

expression of ASC Amino Acid Transporter 2 (ASCT2), Amino acid transporter light chain, L-system (Slc7a5), glutaminase 1 and 2 (Gls1/Gls2), glutamate dehydrogenase 1 (Glud1), carnitine palmitoyltransferase 1a and 2 (CPT1a/CPT2), and lysosomal acid lipase (Lipa) in $CD4^+$ and $CD8^+$ T cells ($CD45^+CD11b^-$ $TCR\beta^+$ $CD4/8^+$) isolated from spleen and spinal cord from mice at peak EAE. *$p<0.05$ by paired Student's T test RQ = relative quantity. Actin is used as the housekeeping gene. Each dot represents one mouse. Data are mean ± SEM from N=1 independent experiment

Figure 6: Differential efficacy of 3-BrPa in limiting IFN-γ versus IL-17A production in ex vivo *cultures of EAE spinal cord*

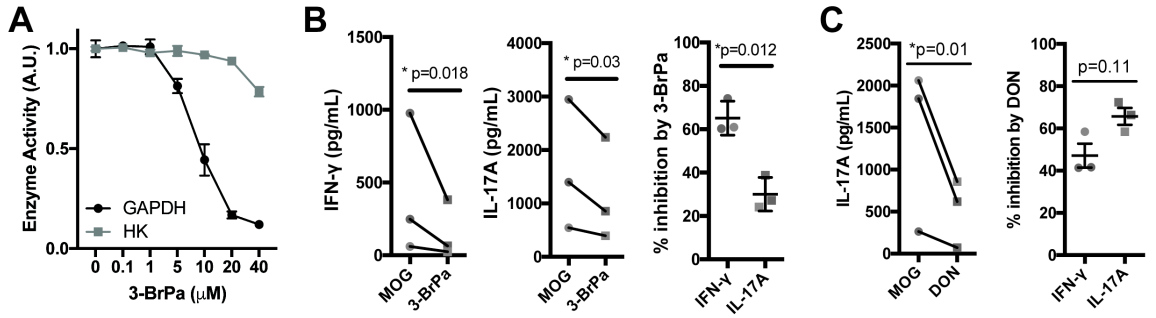


Figure 6: ***(A)*** *Dose dependent inhibition of GAPDH and hexokinase activities in a lymph nodes single cell suspension by 3-BrPa. N=2 experiments with n=1-2 mice per experiment.* ***(B)*** *Effect of 3-BrPa on MOG_{35-55}-induced IFN-γ and IL-17A production in spinal cord homogenates from mice at peak EAE; a representative of N=3 experiments with 3-5 mice is shown. Each dot is a mouse.* ***(C)*** *Effect of DON (1 mM) on IFN-γ and IL-17A production in spinal cord homogenates from mice at peak EAE; N=1 experiment with n=3 mice per group.*

Figure 7: 3-BrPa prevents the acquisition of a Th1-skewed encephalitogenic program

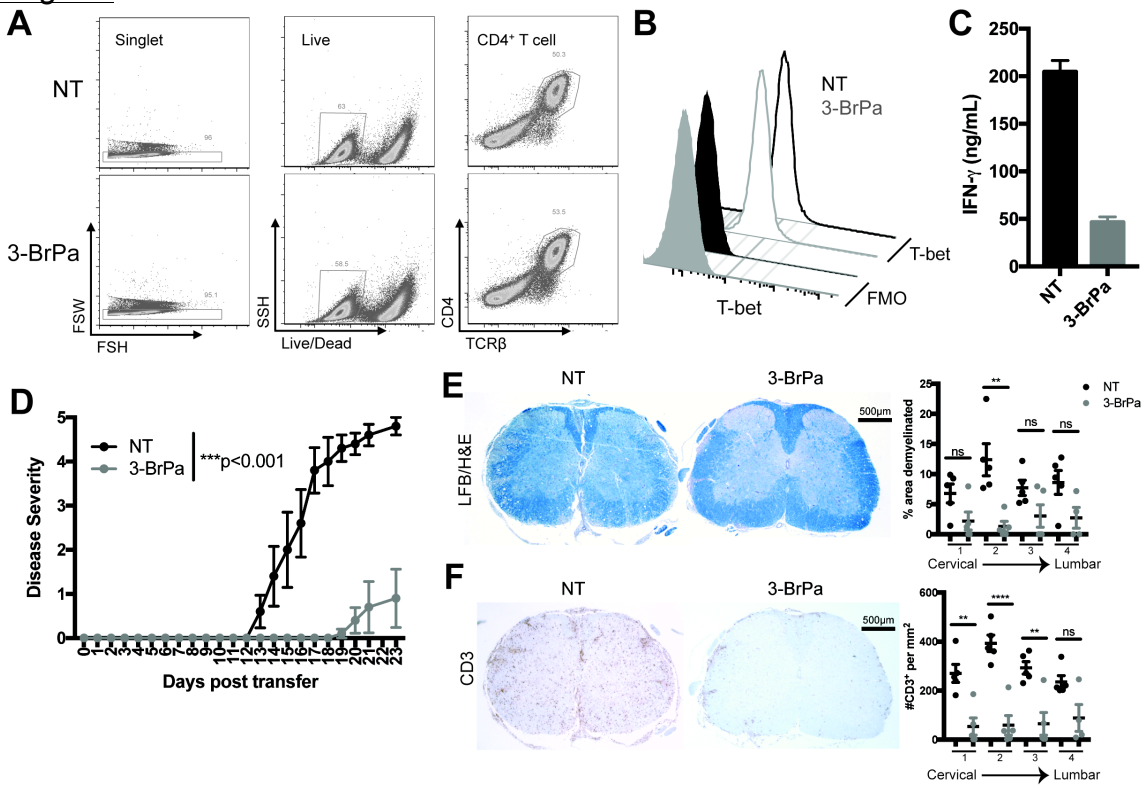


Figure 7: *Splenocytes were isolated from 2D2 mice and maintained in Th1 promoting conditions in the presence of MOG_{35-55} peptide (50 μg/ml) for 3 days. 3-BrPa or saline was added at day 0 and day 2.* ***(A)*** *Cell viability,* ***(B)*** *T-bet expression and* ***(C)*** *IFN-γ production by flow cytometry and ELISA respectively were measured prior to adoptive transfer at day 3.* ***(D)*** *Disease severity and incidence of EAE in $Rag1^{-/-}$ mice (n=5 mice group, 2 independent experiments) ***p<0.001 by Two Way ANOVA with Sidak Post Test. Spinal cord sections of the saline and 3-BrPa group were stained with* ***(E)*** *Luxol Fast Blue and* ***(F)*** *CD3 (n=5 for each group). A blinded researcher manually quantified percentage of demyelination and cell number. **p<0.01, ****p<0.0001 by One Way ANOVA with Sidak Post Test. Data are presented as mean ± SEM.*

II. PKM2, THE DRUGS THAT TARGET IT, AND T CELLS IN EAE

The results herein are excerpted from a manuscript in preparation entitled "Unexpected consequences of PKM2-activators in T cells lacking PKM2."

Research Question

How do the non-metabolic functions of PKM2 impact T cell functions relevant to the EAE model?

Rationale

In our preceding study, we observed enrichment of PKM2 transcript in T cells isolated from the EAE CNS compared with those isolated from the periphery. Interestingly, we did not observe enrichment of PKM1, raising the possibility that PKM2 may be selectively induced to perform an isoform-specific function. Whereas both PKM1 and PKM2 can serve as glycolytic enzymes in their tetrameric forms, only PKM2 can dissociate into dimers and monomers that, while becoming much less effective at catalyzing PEP to pyruvate conversion in glycolysis, gain unique non-metabolic activities. Interestingly, the non-metabolic functions of PKM2 overlap with processes engaged and required during Th17 differentiation. Namely, the PKM2 dimer is critical for PEP-mediated phosphorylation of substrates like STAT3 as well as the co-activation of canonical DNA-binding proteins such as Hif-1α. Both STAT3 and Hif-1α are required for Th17 differentiation. In our previous study, we identified the Th17 response in the EAE spinal cord as being uniquely resistant to glycolytic inhibition, a finding confirmed in a separate study on Th17 cell metabolism in a different model of inflammation. Thus, we decided to study the non-metabolic

roles that PKM2 plays in the function of Th17 cells. It was my belief at the onset of this project that I might identify PKM2 as a major regulator of inflammatory responses important for the EAE disease process, with respect to Th17 functions and beyond. I further anticipated that commercially available drugs that enforce PKM2 tetramerization (PKM2 activators, TEPP-46 and DASA-58) without affecting PKM1 activity represented unique avenues to target the relationship between metabolism and inflammation while avoiding the side effects of systemic metabolic inhibition we observed with 3-BrPa. As I was performing these studies, numerous other groups were beginning to identify TEPP-46 (the agent of choice for *in vivo* work) as therapeutic in a diverse array of inflammatory settings. Thus, there was substantial evidence to support my hypothesis that targeting PKM2 could impact the immune response, and particularly Th17 functions, to elicit anti-inflammatory outcomes in the EAE model.

Main Findings

PKM2 protein is enriched at sites of inflammation in the EAE model, including in T cells

To take our initial step toward determining the therapeutic efficacy of targeting the non-metabolic functions of PKM2 in the EAE model, we harvested spinal cords from mice with EAE and performed IHC. Specifically we sought to determine the abundance and localization of PKM2 protein with respect to areas of demyelination, as delineated by luxol fast blue (LFB) myelin staining. We

found that PKM2 protein is enriched in areas of spinal cord demyelination (**Figure 8A**). Immunofluorescence (IF) studies confirmed an enrichment of PKM2 staining in areas of ongoing pathology, and further revealed PKM2 staining in T cells (identified by the marker CD3) (**Figure 8B**). Attempts at quantifying the percentage of T cells expressing PKM2 were complicated by the abundant PKM2 staining in the EAE spinal cord, but preliminary analyses suggest at the very least that T cells express a wide range of PKM2 and that non-T cells abundantly express PKM2 as well.

PKM2 activators, TEPP-46 and DASA-58, block the in vitro *differentiation of IL-17A, but not GM-CSF-producing T cells during Th17 differentiation*

We hypothesized that the non-metabolic functions of PKM2 would be important for the differentiation of Th17 cells (**Figure 9A**). Using standard *in vitro* differentiation protocols (see "Methods"), we discovered that PKM2 activator treatment significantly delays induction of the Th17 lineage-specifying transcription factor RORγT (**Figure 9B**). To gain a broader picture of how PKM2 activator treatments were affecting acquisition of the Th17 differentiation program, we performed qRT-PCR for genes associated with Th17 cells. These studies revealed that PKM2 activators delay induction of STAT3-dependent genes IL-17A, RORγT (Rorc), IL-21, IL-1R1, and IL-23R, but not the aryl hydrocarbon receptor-dependent target IL-22[299] (**Figure 9C**). Intriguingly, this delay in acquisition of Th17 lineage identity did not correlate with changes in overall metabolism (**Figure 9D**), suggesting that PKM2 activators achieve this

effect independent of sustained metabolic disruptions. To investigate the effect of PKM2 activators on the functionality of differentiating Th17 cells, we assessed cytokine production capacity two days post treatment, a time point when we see robust IL-17A production under normal conditions. As we expected from our transcriptional studies, PKM2 activator treatment blocked the differentiation of IL-17A-producing T cell subsets, but to our surprise potentiated that of GM-CSF producers (**Figure 9E**). In general, TEPP-46 treatment was associated with a 2-3-fold increase in the total frequency of GM-CSF- versus IL-17A-competent T cells during Th17 differentiation (data not shown). Taken together, these data reveal that PKM2 activators TEPP-46 and DASA-58 prevent T cells from acquiring the ability to produce IL-17A during Th17 differentiation, but are ineffective at blocking the differentiation of GM-CSF producers, and may even potentiate the generation of such.

PKM2 activators do not inhibit in vitro *differentiation of Th1 cells*

Our data thus far reveal that PKM2 activators can block $CD4^+$ T cells from acquiring pro-inflammatory functions that are transcriptionally induced by STAT3. These findings are in line with the outcomes we hypothesized, given the known role of PKM2 in regulating STAT3-controlled transcriptional programs. We further hypothesized that, because STAT3 is particularly important for differentiation of Th17 cells, but not that of other lineages, PKM2 activators would selectively inhibit Th17 cell differentiation. To test this hypothesis, we harvested $CD4^+$ T cells from C57BL/6 mice and skewed them toward the Th1 lineage in the

presence or absence of TEPP-46. We observed that, in stark contrast to what was observed with Th17 cells, TEPP-46 consistently promoted the differentiation of T cells capable of producing IFN-γ (**Figure 10**). Very similar results were obtained with DASA-58 (data not shown).

Development and characterization of mice with PKM2 deleted in T cells (PKM2-KO)

To gain further insight into the mechanism of action of PKM2 activators in Th17 cells, we used existing tools to generate a mouse lacking PKM2 specifically in $CD4^+$ T cells. This was achieved by using an available strain of mice that has the M2-defining exon of PKM2 (exon 10) flanked by loxP sites such that Cre-mediated recombination drives the expression of PKM1 at the expense of PKM2 (**Figure 11A**). For studies involving these mice, PKM2-WT mice are those with the genotype *CD4-Cre*$^-$ *Pkm2*$^{fl/fl}$, whereas PKM2-KO are those with the genotype *CD4-Cre*$^+$ *Pkm2*$^{fl/fl}$. In line with our findings of T cells sorted from the EAE spinal cord, we found that freshly isolated $CD4^+$ T cells from PKM2-WT mice predominantly expressed PKM2 at the protein level whereas, as expected, $CD4^+$ T cells from PKM2-KO mice expressed PKM1 (**Figure 11B**). Replacement of PKM2 with PKM1 in PKM2-KO T cells did not drive changes to baseline functional metabolism as measured by the Seahorse Extracellular Flux analysis (**Figure 11C**). The development of T cell precursors in the thymus (**Figure 11D**) and their naïve/memory phenotypes in secondary lymphoid tissues (**Figure 11E**) were also unchanged by Cre expression. Looking at more differentiated T cell

subsets *in vivo*, in the spleen, we observed a minute increase in FoxP3$^+$ regulatory T cell (Treg) frequency in PKM2-KO mice that is not recapitulated in pooled lymph nodes (**Figure 11F**) or in small intestinal lamina propria lymphocytes (SI-LPL) known to house Tregs (**Figure 11G**). Similarly, and surprisingly, we also did not observe any change to the prevalence of RORγT-expressing CD4$^+$ T cell subsets in small intestine lamina propria lymphocytes (LPL) (**Figure 11G)**. Likewise, PKM2-KO T cells proliferated similarly to WT littermate controls and were not defective in their ability to differentiate into various lineages of helper T cell subsets, including IL-17A-producers (**Figure 11H**). The functional metabolism of these PKM2-KO T cells skewed toward the Th17 lineage was indistinguishable compared to WT littermate controls (**Figure 11I**). Importantly, qRT-PCR studies confirmed deletion of PKM2 and compensatory induction of PKM1 throughout the course of these studies (**Figure 11J**). These data demonstrate existing tools can be used to replace PKM2 with PKM1 at early stages of T cell development, and this genetic manipulation does not overtly impact T cell development *in vitro* or *in vivo*, including their differentiation into IL-17A-producers. This latter finding is particularly intriguing giving the effects of PKM2 activators on Th17 differentiation, and the convincingly and repeatedly demonstrated specificity of these drugs as selective modulators of PKM2 enzymatic activity.

Effects of PKM2 activator treatment on differentiating Th17 cells are PKM2-independent

Minute structural differences in PKM2 and PKM1 are believed to confer the former with non-metabolic functions that are selectively perturbed by TEPP-46 and DASA-58[288]. Thus, it was extremely unexpected to observe that PKM2 activators were still able to affect Th17 differentiation by PKM2-KO T cells in a manner indistinguishable from WT (**Figure 12A**). Possible explanations for this observation include reacquisition of PKM2 protein expression in PKM2-KO T cells during *in vitro* culture or, given that the major non-metabolic functions of PKM2 are presumed to relate to its subcellular localization, an effect of PKM2 activator treatment on compensatory PKM1 subcellular localization. However, we do not observe detectable levels of PKM2 at the protein level in PKM2-KO T cells after 48 hours of Th17 differentiation (**Figure 12B**). Furthermore, subcellular fractionation studies consistently showed that while TEPP-46 extrudes PKM2 from the nucleus to the cytoplasm for 4-8 hours of treatment, it does not prevent PKM1 nuclear localization (**Figure 12C**). Taken together, these data identify two novel aspects of PKM2 activators highly relevant to their development as therapeutics for inflammatory pathologies: (i) they block the generation of IL-17A-producing T cells, but not that of GM-CSF-producers, and (ii) do so in a PKM2-independent manner.

PKM2 activators affect T cell differentiation by a PKM2-independent mechanism involving TGFβ1

There are many instances in the literature in which genetic or pharmacologic interventions that disturb cell metabolism control the balance between development of Th17 cells and a related, anti-inflammatory regulatory T cell subset (Treg) defined by their expression of the forkhead box P3 (FoxP3) [171,202,300]. However, in our system, despite blocking IL-17A-producing T cells from developing, we did not observe PKM2 activators to induce FoxP3 expression during Th17 differentiation (data not shown). We thus reasoned that PKM2 activators might inhibit a lineage-driving factor common between Treg and Th17 cells. Transforming growth factor β1 (TGFβ1) is important for both Treg and also, alongside STAT3 activating signals (e.g. IL-6), Th17 differentiation (**Figure 13A**)[177,301,302]. To test this hypothesis, we determined the effects of PKM2 activators on the TGFβ1-dependent generation of inducible Tregs (iTregs) *in vitro*. We found that after two days of stimulation, TGFβ1 treatment induced a large population of FoxP3-expressing T cells as expected, and that both TEPP-46 and also DASA-58 blocked this effect in PKM2-WT and PKM2-KO T cells (**Figure 13B**). Given the consistently overlapping effects of TEPP-46 and DASA-58 in PKM2-WT and PKM2-KO T cells, and the reported unsuitability of DASA-58 for *in vivo* studies[288], we focused on TEPP-46 for the remaining studies. Downstream effects of TGFβ1 signaling are mediated in part by receptor activated Smads 2 and 3[303] (**Figure 13C**). We thus assessed the effects of TEPP-46 on Smad2/3-dependent and independent targets[181]. In both PKM2-WT

and PKM2-KO T cells, we found that TEPP-46 blocked induction of the Smad2/3 dependent target interferon regulatory factor 8 (Irf8) but not that of the Smad2/3 independent target C-C motif chemokine receptor 8 (CCR8) (**Figure 13D**). FoxP3 transcript was not induced appreciably at this time point (24hrs), though protein expression of this Smad2/3-dependent target was suppressed by PKM2 activators at 48hrs (**Figure 13B**) in the WT and KO setting. To identify how altered TGFβ1 signaling might relate to our observations with Th17 cells, we differentiated PKM2-WT and PKM2-KO T cells under Th17 conditions in the presence or absence of TEPP-46 in a range of TGFβ1 concentrations. We found that the dose of TGFβ1 inversely correlated with differentiation of GM-CSF-competent T cells and directly with IL-17A producers (**Figure 13E**). Indeed, lowering the concentration of TGFβ1 used during Th17 differentiation (from 0.3ng/mL) reduced the generation of IL-17A but not GM-CSF producers, mirroring observed effects of PKM2 activator treatment. These experiments convincingly demonstrate that PKM2-independent effects of PKM2 activators are related to impacts on TGFβ1-signaling.

PKM2-independent effects of TEPP-46 on Th17 functions are also mediated in part by potentiation of STAT5 signaling

In the absence of exogenous TGFβ1, TEPP-46 was still able to suppress the differentiation of IL-17A producers, and potentiate that of GM-CSF-producers (**Figure 13E**). This could be related to the effects of endogenous Smad2/3 activators (e.g. serum factors or autocrine TGFβ1) in the *in vitro* system. An

alternative explanation is that TEPP-46 impacts an additional pathway important for IL-17A and GM-CSF production by differentiating Th17 cells. Signal transducer and activator of transcription (STAT) family proteins are known to play important roles in T cell functions and differentiation[304]. With few exceptions, receptor-mediated signaling drives phosphorylation of STAT proteins, conferring them the ability to translocate to the nucleus and bind DNA. STAT3 and 5 are known to reciprocally impact Th17 functions, with STAT3 enhancing and STAT5 blocking many aspects of the Th17 differentiation program[305,306]. The balance of P-STAT5/P-STAT3 signaling thus plays a critical role in the function of Th17 cells, and importantly, STAT5 is one the major transcription factors associated with GM-CSF induction[127,152] (**Figure 13F**). We thus assessed the effects of TEPP-46 on P-STAT5/P-STAT3 levels in differentiating Th17 cells. We consistently observe that TEPP-46 increases P-STAT5, with little effect on P-STAT3 at 24hrs of differentiation (**Figure 13G**). Similar results were observed in PKM2-KO T cells (data not shown). We then assessed how STAT5 inhibition affected TEPP-46-mediated changes to the Th17 cytokine profile. We found that a selective STAT5 inhibitor (STAT5-IN-1)[307] partially normalized both GM-CSF$^+$ and IL-17A$^+$ production disruptions associated with TEPP-46 treatment (**Figure 13H**). Thus, PKM2 activators exert their effects on T cell differentiation in part by disrupting TGFβ1 and P-STAT5 signaling in a PKM2-independent manner. Further investigations are required to determine how these observations are related.

Evidence against TEPP-46 as a therapeutic candidate for RRMS

Having discovered that PKM2 activators block the generation of IL-17A-, but not GM-CSF-producing T cell subsets, block iTreg differentiation, and achieve these outcomes via a PKM2-independent mechanism involving TGFβ1 and STAT5 signaling, we sought to assess the functional consequences of these observations in experimental autoimmune encephalomyelitis (EAE), a mouse model of MS. Toward this end, we skewed splenocytes isolated from the 2d2 strain of mice toward the Th17 lineage in the presence or absence of TEPP-46 and transferred these into immunodeficient (*Rag1*$^{-/-}$) recipients (**Figure 14A**). Prior to transfer, splenocytes differentiated in DMSO vehicle were mostly IL-17A producers, whereas those differentiated in TEPP-46 were predominantly GM-CSF producers (**Figure 14B**), consistent with our results obtained with purified T cells. Mice receiving the vehicle-treated cells developed a severe ascending paralytic syndrome that is classically associated with EAE. However, mice receiving the TEPP-46-treated cells presented differently. While some animals developed a classic EAE, many developed ataxic syndromes commonly described as atypical disease (**Figure 14C**)[111]. Flow cytometric analyses revealed that TEPP-46-associated atypical EAE correlated with potent accumulation of immune cells in the brain and cerebellum/brainstem, and relatively fewer in the spinal cords (**Figure 14D**). In line with these findings, histological studies identified accumulation of immune cells, particularly in the periventricular regions of TEPP-46-treated mice (**Figure 14E**). We observed

accumulation of $CD45^+$ cells in the periventricular regions of four out of eight sick TEPP-46-recipients analyzed, but only one out of seven DMSO-recipients. Recent studies implicate GM-CSF as a potent driver of atypical EAE[111], and are likely responsible for the observed effects of TEPP-46 in this system. Furthermore, these results help to explain why daily administration of TEPP-46 (50mg/kg oral) failed to ameliorate disease in active immunization-based models of EAE (**Figure 15**).

Discussion & Conclusions

In summary, the data presented in this study do not support the use of PKM2 activators in the treatment of MS. However, they provide a path toward gaining a better understanding of Th17 lineage specification, particularly with respect to the identification of regulators of IL-17A and GM-CSF production. These discoveries may aid in future efforts to target these cytokines in their associated disease states. In addition, our studies reveal that the potent effects of PKM2 activators on the acquisition of cytokine production capabilities by T cells occurs in a manner that is independent of PKM2 protein expression. While previous reports in non-T cells use knockdown or the same genetic knockout strategies as we employed to convincingly demonstrate the target specificity of PKM2 activators for PKM2[268,308], our studies in T cells demonstrate that PKM2 protein expression is not required for at least some of the activities of these compounds. In our numerous studies assessing the non-metabolic functions of PKM2 in Th1, Th17, and Tregs, we did not identify any activities of TEPP-46 and

DASA-58 that were not identically recapitulated in the complete absence of PKM2 protein expression, as induced by available genetic strategies. Given that TEPP-46 and DASA-58 are two structurally distinct compounds exhibiting similar "off-target" effects, it is reasonable to hypothesize that they are able to bind to and disrupt non-metabolic activities of compensatory PKM1 that (i) overlap with those of PKM2 and (ii) are uniquely salient to the function of T cells. Indeed, we are able to detect PKM1 in nuclear fractions of T cells, and there is precedent in the literature for PKM1 serving non-metabolic functions in the cell, and particularly in the nucleus[309]. Further study on the targets of TEPP-46 and DASA-58 are warranted and underway.

The near exclusive expression of PKM2 in adult murine T cells is interesting, particularly given the reported protein expression of both PKM1 and PKM2 in murine macrophages. It is unclear what aspects of T cell functions benefit from the exclusive expression of PKM2, especially given that our numerous studies failed to elicit any PKM2-specific functions that were not redundantly performed by PKM1. We currently do not have any data to suggest any isoform-specific functions of PKM2 in the context of T cell functions.

In attempting to identify the PKM2-independent mechanism of action of TEPP-46 and DASA-58, we revealed TGFβ1 as a critical regulator of GM-CSF and IL-17A production by differentiating Th17 cells. As an extension of these findings, we found that many outcomes related to PKM2 activator treatment could be reversed by inhibition of STAT5. At present, it is unclear how the effects of PKM2 activators on TGFβ1 and STAT5 are related, if at all. Preliminary

studies do not support the hypothesis that increasing TGFβ1 dose during Th17 differentiation affects levels of P-STAT5. Perhaps the simplest explanation for our findings is that by preventing the TGFβ1-mediated differentiation of IL-17A-producing T cell subsets, PKM2 activators passively result in the STAT5-dependent generation of GM-CSF producers. This fits our data showing that while TEPP-46 and DASA-58 have very similar effects on Th17 cytokine production profiles, as well as the regulation of Smad2/3-dependent and independent targets, only TEPP-46 increases P-STAT5. This model, where the major PKM2-independent mechanism of action of TEPP-46 and DASA-58 is the inhibition of TGFβ1 signaling, also fits the observation that PKM2 activators prevent TGFβ1-mediated iTreg differentiation, a process that would otherwise be potentiated by elevated P-STAT5. Experiments with inhibitors of endogenous TGFβ1 signaling are required to further understand how inhibition of TGFβ1 relates to observed outcomes with PKM2 activators during Th1, Th17, and Treg differentiation.

Interestingly, we found that while TEPP-46 potentiated the differentiation of GM-CSF- and IFN-γ-competent Th17 and Th1 cells respectively, qRT-PCR studies showed that prior to restimulation, Th17 and Th1 cells differentiated in the presence of TEPP-46 actually contained lower levels of these transcripts. There are a few possible explanations for this divergence between transcript and protein levels. It is possible that TEPP-46 increases P-STAT5 levels but prevents its ability to bind DNA until restimulation with calcium ionophores. This interpretation of the data would also explain why the P-STAT5-responsive

expression of Il2ra is also reduced with TEPP-46 treatment. In addition, it may be that TEPP-46 promotes increased translation and secretion of proteins including GM-CSF, thereby reducing transcript levels. This interpretation of the data is consistent with the observation that, despite reduced GM-CSF transcript levels, Th17 cells differentiated in the presence of TEPP-46 drive a form of EAE associated with T cell hyper-production of GM-CSF.

Thus, there are many open questions regarding how PKM2 activators achieve their effects during T cell differentiation. The major takeaway from these studies thus far, however, is a critical and conclusive contribution to the field of immunometabolism: that PKM2 activators function in the absence of PKM2.

Figures

Figure 8: PKM2 is highly expressed in the EAE lesion

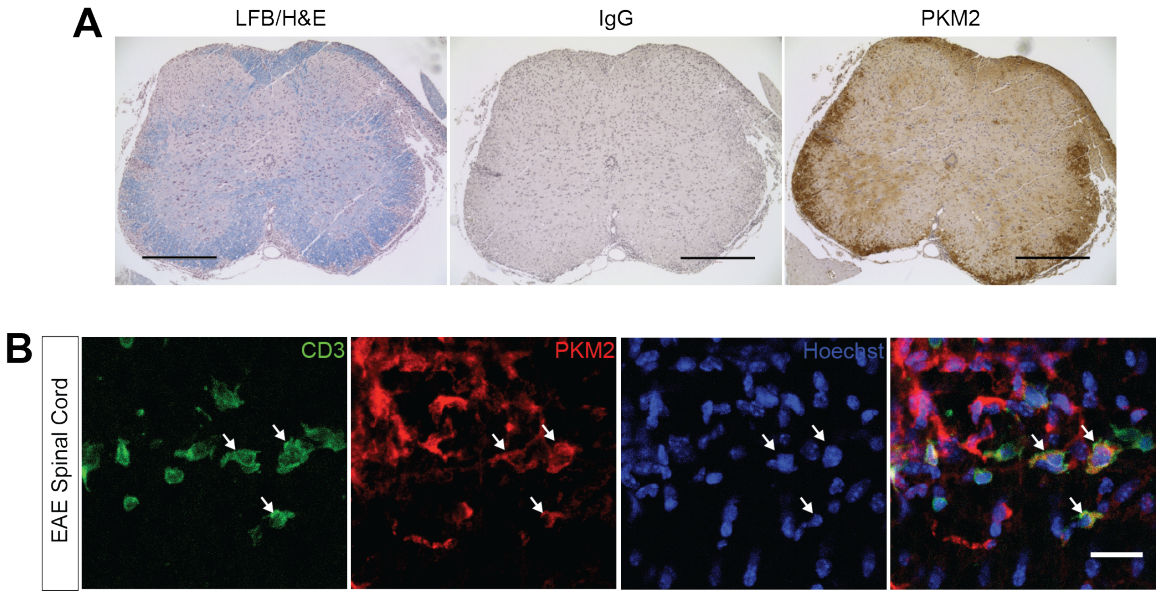


Figure 8: (A) Immunohistochemistry for luxol fast blue (LFB) with hematoxylin and eosin (H&E) counterstain to identify sites of demyelination in spinal cord slices of mice with EAE. Staining with IgG isotype control or PKM2 performed in adjacent sections. Scale bar is 400μM; **(B)** Immunofluorescence localizing PKM2 staining to $CD3^+$ T cells. Arrows identify T cells with prominent PKM2 staining. Scale bar is 20μM.

Figure 9: PKM2 activators prevent differentiation of IL-17A but not GM-CSF producing T cells

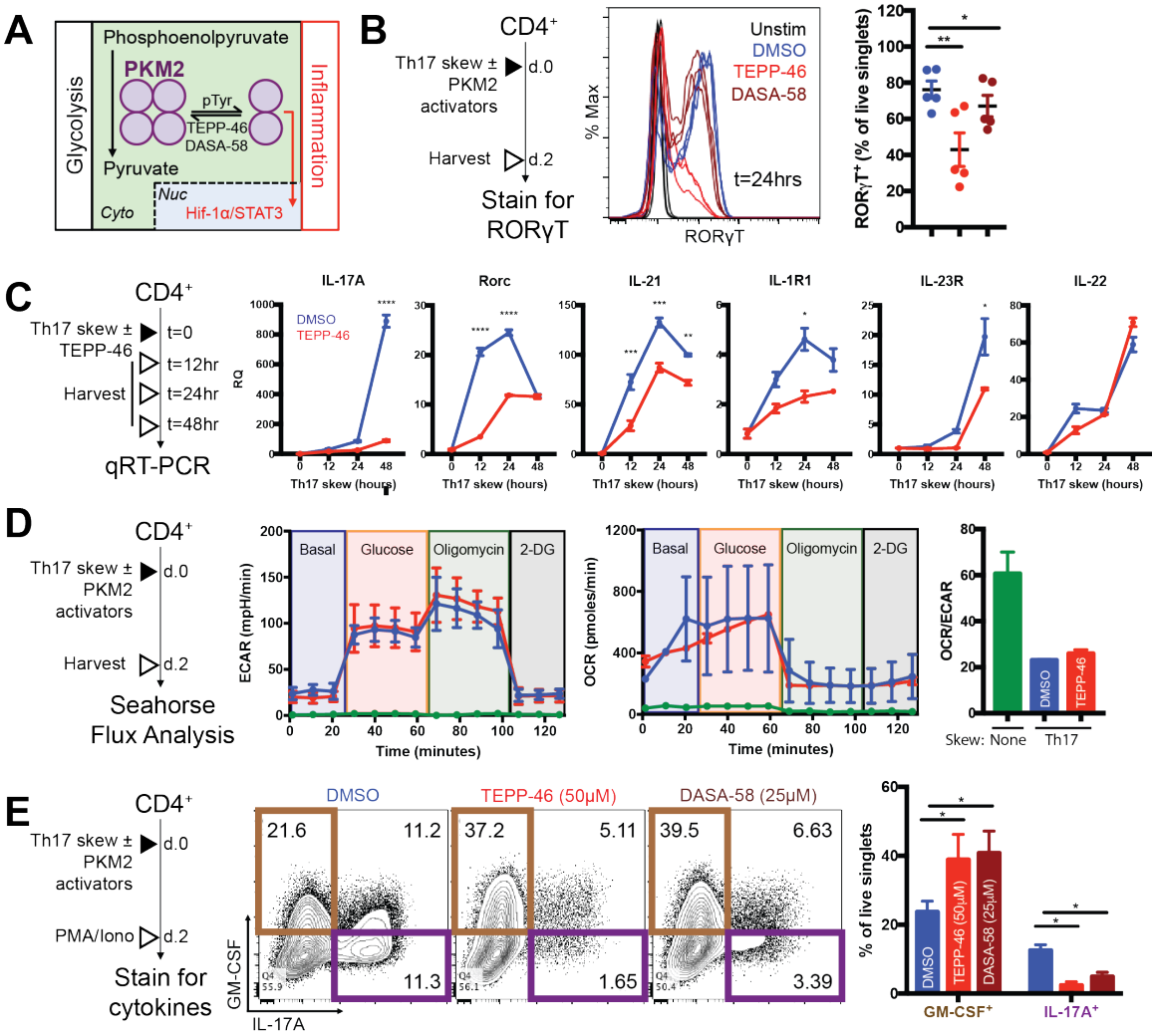


Figure 9: (A) Accepted mechanism of action of PKM2 activators; **(B)** Effect of PKM2 activators on RORγT protein expression after 24 hours of Th17 differentiation. Repeated-measures One Way Anova with Tukey Post Test **(C)** qRT-PCR studies for transcripts related to the Th17 lineage. Repeated Measures Two Way Anova with Sidak Post Test. **(D)** Seahorse Extracellular Flux Analysis on unstimulated CD4$^+$ T cells (green) as well as those skewed for 2 days toward Th17 lineage in the presence (red) or absence (blue) of TEPP-46. Quantification of OCR/ECAR in each condition also shown; **(E)** IL-17A/GM-CSF production by CD4$^+$ T cells differentiated toward the Th17 lineage for 2 days in the presence of DMSO vehicle, TEPP-46, or DASA-58 with quantification. Repeated Measures One Way Anova with Tukey Post Test. All experiments performed at least N=2 independent times with n=1-3 mice per experiment. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 10: TEPP-46 does not prevent Th1 differentiation

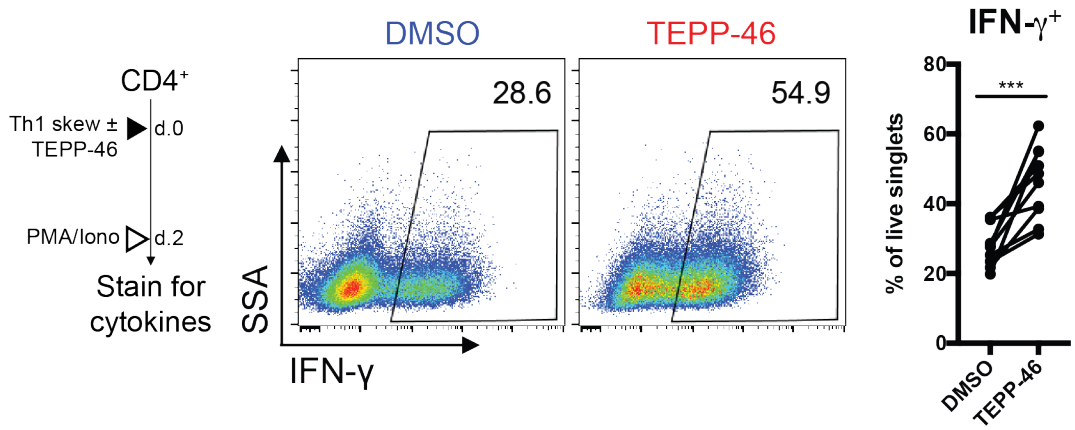


Figure 10: WT CD4$^+$ T cells were differentiated toward the Th1 lineage in the presence of TEPP-46 (50μM) and restimulated after 2 days to assess acquisition of IFN-γ-producing capabilities. Representative flow cytometry plots are shown. Quantification is data pooled from N=4 independent experiments with n=2-3 mice per experiment. ***p<0.001 by Paired Student's T-test.

Figure 11: Deletion of exon 10 in PKM2 drives compensatory PKM1 expression

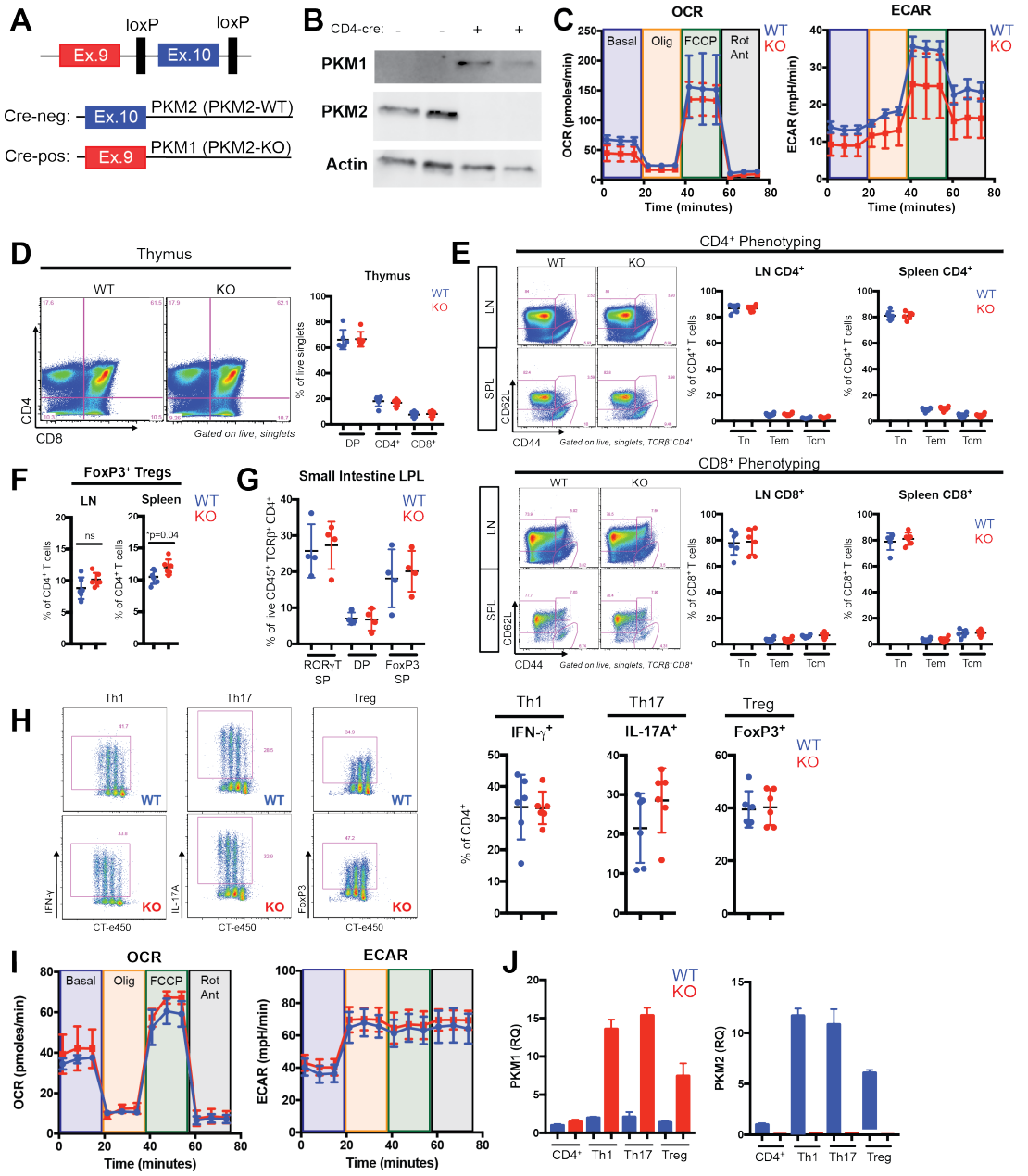


Figure 11: (A) Available genetic model for Cre-mediated PKM2 deletion. PKM2-specifying exon 10 is flanked by loxP sites such that Cre-mediated excision both deletes PKM2 while also enforcing PKM1 expression in Cre-expressing cells; **(B)** Protein expression of PKM1 and PKM2 in CD4$^+$ T cells sorted from *Cd4-cre*$^-$ *Pkm2*$^{fl/fl}$ (PKM2-WT) and *Cd4-cre*$^+$*Pkm2*$^{fl/fl}$ (PKM2-KO) controls; **(C)** Seahorse Extracellular Flux Analysis assay for functional metabolism in freshly isolated PKM2-WT and PKM2-KO CD4$^+$ T cells subjected to mitochondrial stress test; **(D-F,H)** comparisons of PKM2-WT and PKM2-KO T cell development *in vivo* and *in vitro*. Data are pooled from N=2 independent experiments with T cells from n=3 mice per experiment; **(D)** representative flow cytometry for CD4 and CD8

expression in thymic cells from PKM2-WT and PKM2-KO mice with quantification; **(E)** Prevalence of memory and naïve $CD4^+$ and $CD8^+$ T cell subsets based on expression of CD44 and CD62L in secondary lymphoid structures – organized as in **(C)**; **(F)** $FoxP3^+$ regulatory T cell frequency in secondary lymphoid tissues in PKM2-WT and PKM2-KO mice; Students T Test; **(G)** T cell subsets in the lamina propria of the small intestine pooled from N=2 independent experiments; **(H)** *In vitro* differentiation of PKM2-WT/KO T cells down Th1, Th17, and Treg lineages; **(I)** Seahorse Extracellular Flux analysis on PKM2-WT/KO T cells skewed down Th17 lineage for 2d and subjected to mitochondrial stress test; **(J)** Confirmation of PKM2 deletion in PKM2-KO T cells skewed toward Th1, Th17, and Treg lineage. **(C,I)** are representative of N=3 independent experiments with T cells from n=3 mice per experiment. **(B)** Representative of at least N=3 independent experiments with n=2-3 mice per experiment. **(J)** N=1 independent experiment with T cells from n=3 mice per experiment. Data are expressed as mean $\pm$ SEM.

Figure 12: Effects of PKM2 activators during Th17 differentiation are recapitulated in the absence of PKM2 protein expression

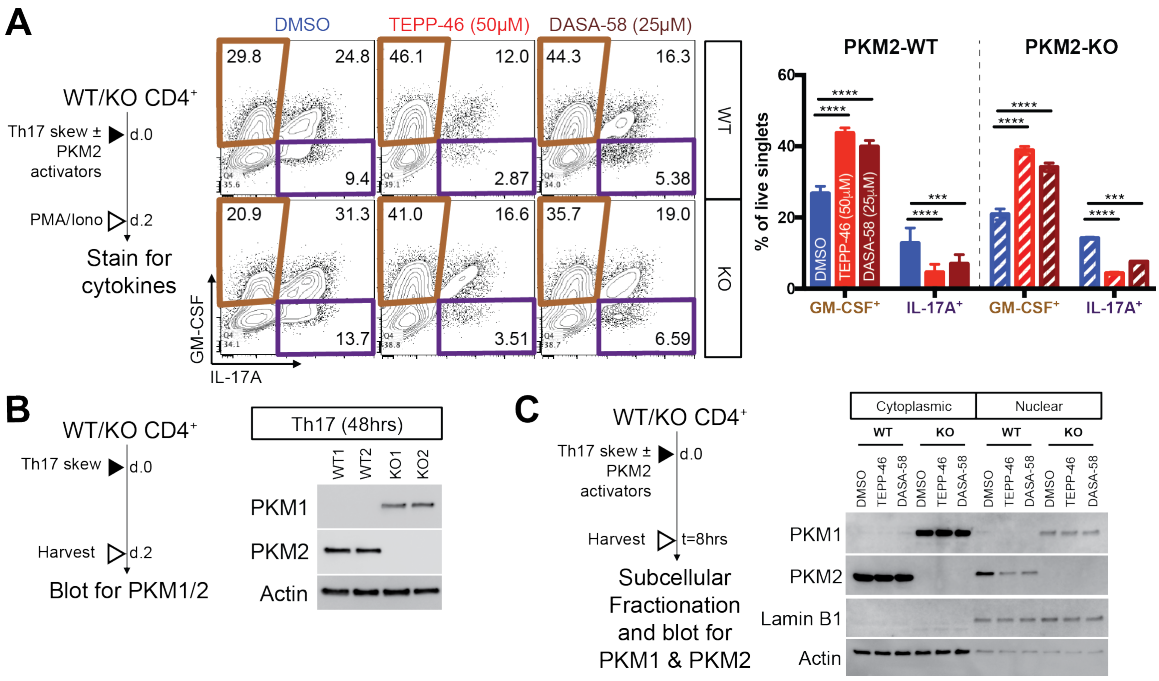


Figure 12: (A) Effects of PKM2 activators on GM-CSF and IL-17A production in PKM2-WT/KO T cells skewed toward the Th17 lineage for 2 days. Quantification is data pooled from N=2 independent experiments with T cells from n=5 total mice per genotype and expressed as mean ± SEM. Significance is as follows: ***p<0.001, ****p<0.0001 by Repeated Measures One Way Anova with Tukey Post Test; **(B)** PKM1 and PKM2 western blot on Th17 skewed for 2 days from PKM2-WT and PKM2-KO; **(C)** Effect of TEPP-46 on PKM1 and PKM2 subcellular localization in PKM2-WT and PKM2-KO T cells skewed toward Th17 lineage for 8 hours (Results are representative of N=4 independent experiments with n=1-2 mice per experiment per genotype for TEPP-46. Results with TEPP-46 successfully replicated in N=1 independent experiment with n=2 mice per genotype for DASA-58 (shown).

Figure 13: PKM2-independent mechanism of action of PKM2 activators

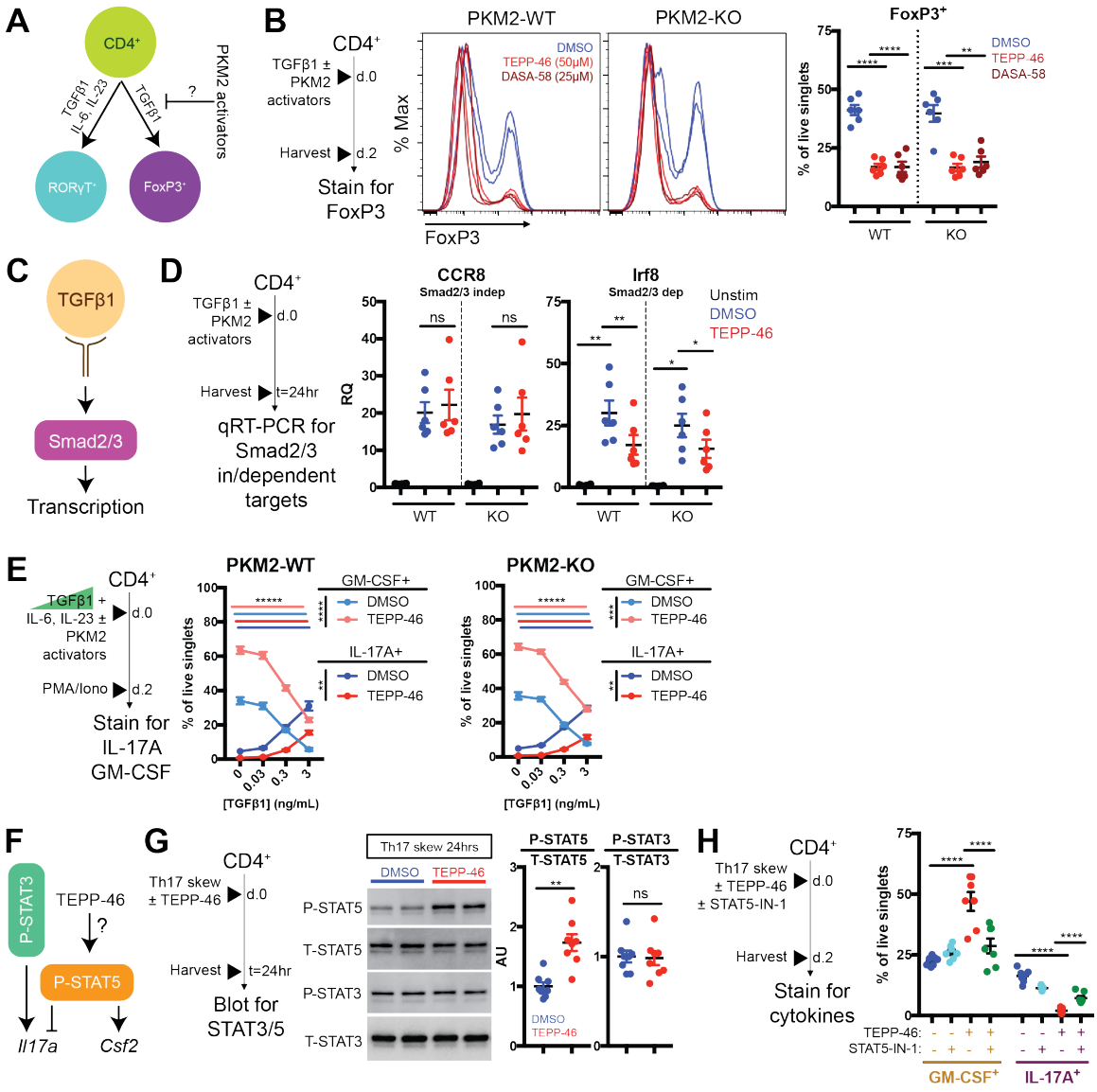


Figure 13: (A) Schematic of TGFβ1, its impact on Th17 and iTreg differentiation, and the hypothesized effect of PKM2 activators; **(B)** Flow cytometry to determine effects of PKM2 activators on TGFβ1-mediated induction of FoxP3 with pooled quantification from N=3 independent experiments with T cells from n=2 mice per genotype per experiment. Repeated Measures One Way Anova with Tukey Post Test; **(C)** Schematic of TGFβ1 signaling through Smad2/3; **(D)** qRT-PCR for the effects of PKM2 activator treatment on TGFβ1-mediated induction of Smad2/3-independent (CCR8) and Smad2/3-dependent (Irf8) targets after 24 hours of stimulation. Data are pooled from N=3 independent experiments with n=2 mice per genotype per experiment. Repeated Measures One Way Anova with Tukey Post Test; **(E)** Effects of PKM2 activators on IL-17A and GM-CSF producing T cells (defined as in **Figure 9E/12A**) with varying doses of TGFβ1 during Th17 differentiation. Data are pooled from N=2 independent experiments with n=2 mice per genotype; Repeated Measures Two Way Anova with Tukey Post Test;

(F) Schematic of competing effects of P-STAT3 and P-STAT5 at the *Il17a* locus, the role of P-STAT5 as a potentiator of GM-CSF (*Csf2*) transcription, and the hypothesized effect of TEPP-46 on this signaling axis; **(G)** Representative western blots showing effects of TEPP-46 on P-STAT5 and P-STAT3 at 24 hours of Th17 differentiation. Quantification is pooled densitometric analysis from N=3 independent experiments with T cells from a total of n=7 mice. Paired Students T-test; **(H)** Effects of STAT5 inhibition on TEPP-46-associated changes to IL-17A and GM-CSF cytokine production profiles in differentiating Th17 cells. Data are pooled from N=3 independent experiments with T cells from n=7 total mice. Repeated Measures One Way Anova with Tukey Post Test. Significance is as follows: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are presented as mean $\pm$ SEM.

Figure 14: TEPP-46 promotes a highly encephalitogenic T cell

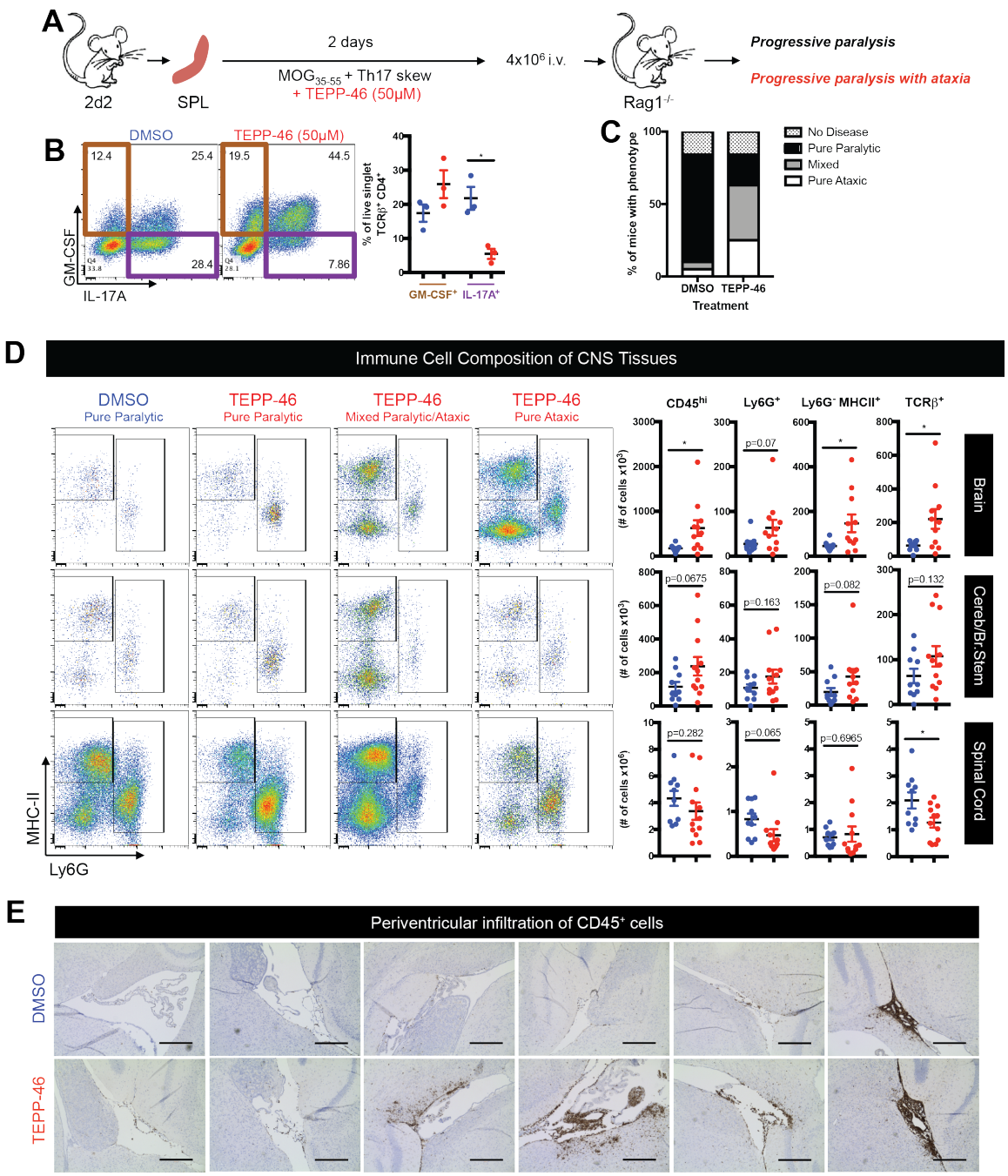


Figure 14: (A) Schematic of transfer EAE paradigm in which splenocytes are harvested from 2d2 mice, stimulated with cognate antigen MOG_{35-55} in Th17 skewing conditions ± TEPP-46, and transferred into immunodeficient (*Rag1*$^{-/-}$ recipients); **(B)** Example flow plot of IL-17A and GM-CSF production by TCRβ^{+}CD4^{+} cells on the day of transfer. Significance is as follows: *p<0.05 by Repeated Measures One Way Anova with Tukey Post Test; **(C)** Outcomes (at least 16 days post transfer) for mice receiving DMSO and TEPP-46-treated T

cells pooled from N=4 independent experiments with n=19 DMSO and n=24 TEPP-46; **(D)** Example flow plots from mice receiving DMSO or TEPP-46-treated cells with differing disease outcomes. Shown are MHC-II and Ly6G expression on myeloid cells (defined as live, singlet, $CD45^{hi}CD11b^{+}$ cells) infiltrating into brain, cerebellum, and spinal cord in each of the outcomes. Quantifications are pooled from N=2 independent experiments and expressed as mean $\pm$ SEM. Significance determined by two-tailed Welch's T-Test. *p<0.05; (**E)** IHC for CD45 in the periventricular brain regions of recipients of DMSO and TEPP-46-treated T cells. From N=2 independent experiments. Scale bar is 400μM.

Figure 15: TEPP-46 is not therapeutic in vivo *during EAE*

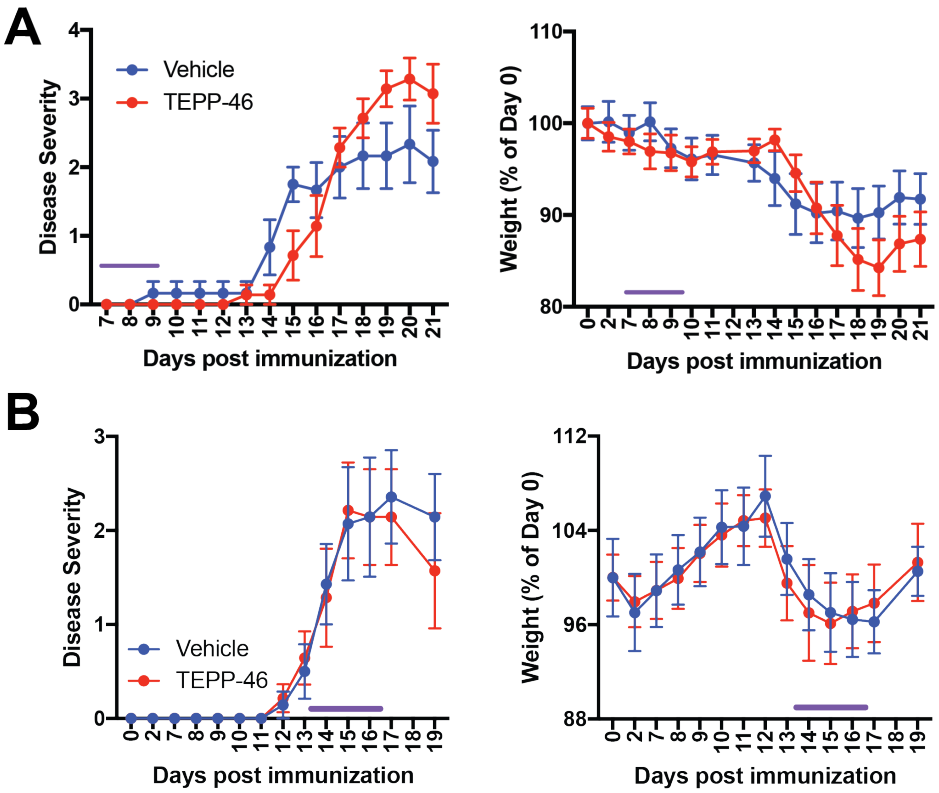


Figure 15: *Oral TEPP-46 (50mg/kg in 0.5% carboxymethylcellulose with 0.1% Tween 20) every 18 hours is not therapeutic in immunization-based active EAE.* **(A)** Prophylactic treatment schedule (n=7 female mice per group); **(B)** Therapeutic treatment schedule (n=8 female mice per group)

CHAPTER IV: OVERARCHING CONCLUSIONS

The major goal of this thesis is to provide a greater understanding of the pathophysiologic mechanisms that contribute to disease in multiple sclerosis. To achieve this goal, I focused on T cells, agents of the adaptive immune system believed to play critical if not orchestrating roles in MS. More specifically, my thesis work centered on the metabolism of T cells involved in CNS inflammation and how metabolic activity (e.g. glycolysis) and metabolic machinery (e.g. PKM2) contribute to their pathologic programs in a mouse model of multiple sclerosis.

My studies confirm earlier findings that inhibition of glycolysis can prevent the differentiation of a T cell capable of infiltrating the CNS and orchestrating EAE. I determined this through my experiments with 3-BrPa and its ability to prevent T cells from acquiring a Th1-skewed encephalitogenic program. In addition, I observed elevation in glycolytic flux as well as transcriptional enrichment for glycolytic machinery in T cells isolated from the EAE spinal cord versus matched spleens. Together, these findings suggest that T cells need to perform glycolysis in order to gain an encephalitogenic phenotype, and also that elevations in glycolytic flux are characteristic of T cells contributing to disease in the spinal cord. Despite these two complementary observations, it is not entirely clear that glycolysis is the main driver of pathologic T cell responses in EAE. Production of IL-17A by immune cells in the EAE spinal cord, as an example, seems particularly resilient to metabolic perturbations with 3-BrPa, especially when compared with that of IFN-γ. Furthermore, even if glycolysis does fuel encephalitogenic T cell responses during RRMS, systemic administration of

glycolytic inhibitors for a chronic condition is unlikely to be feasible. From the perspective of my own studies, despite blocking T cells from acquiring an encephalitogenic phenotype *in vitro*, *in vivo* administration of 3-BrPa during EAE was lethal. Thus, in addition to identifying the metabolic vulnerabilities of T cells contributing to EAE disease pathogenesis, it will also be important to elucidate more nuanced ways of intervening at the level of metabolism to control their responses.

One such nuanced way to approach metabolic interventions is to target specific isoforms of metabolic machinery, especially those that share a unique relationship with inflammation. This was the rationale behind my attempts to selectively target PKM2 with available compounds TEPP-46 and DASA-58. However, despite many preceding reports demonstrating the isoform-specific contributions of PKM2 to biological processes with relevance to inflammation, I observed, in T cells, total compensation for loss of PKM2 by expression of PKM1. In fact, in my *in vitro* and *in vivo* studies, the only isoform-specific difference I observed between PKM2 and PKM1 was the susceptibility of the former to nuclear extrusion by TEPP-46 and DASA-58 (a distinction with no obvious functional outcome). I have found that TEPP-46, the PKM2 activator of choice for *in vivo* work, is unlikely to be a good therapeutic candidate for MS given its propensity for potentiating GM-CSF-mediated inflammation and its suppression of TGFβ1-mediated induction of Tregs.

Given the studies presented, I conclude that efforts to target metabolism to treat Multiple Sclerosis will likely require an approach that is more nuanced

and targeted than blunt metabolic manipulations. In my research, the candidacies for both a metabolic inhibitor (3-BrPa) and also a metabolic activator (TEPP-46/DASA-58), as therapies for MS were likely put to rest. Nevertheless, as these studies contribute to the field of immunometabolism, particularly with respect to the context of MS, and pave the way for novel avenues of research that will be the subject of my next chapter.

CHAPTER V: FUTURE DIRECTIONS

I. SHORT TERM

My short-term future directions are focused on resolving outstanding questions related to PKM2 activators and their precise mechanisms of action. I anticipate that the results of these studies will significantly contribute to the understanding of PKM1, PKM2, and the drugs that target them. These are targeted experimental plans that are underway, and I am hoping to complete these goals during my final two years of clinical training in the MD/PhD program.

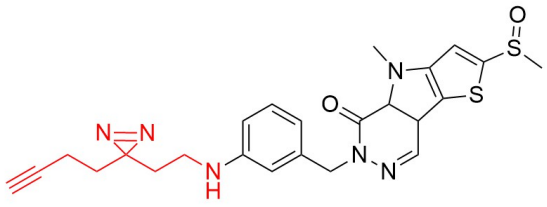
Identification of proteins targeted by TEPP-46

TEPP-46 has gained prominence among the cancer and immunology communities as a potent inhibitor of tumor growth as well as inflammatory macrophage responses. It is the PKM2 activator of choice for *in vivo* work due to its reportedly favorable pharmacokinetic profile[288]. DASA-58 is another commercially available PKM2 activator often used in *in vitro* settings. In my experience, both TEPP-46 and also DASA-58 are very similar in their ability to potently modulate Th1, Th17, and Treg functions, and they do so by a PKM2-independent mechanism. Given that TEPP-46 and DASA-58 are structurally distinct compounds, it is difficult to envision that they have the exact same off-target effects, unless they both affect PKM1. Studies demonstrating the target specificity of TEPP-46 and DASA-58 for PKM2 versus PKM1 did so via enzymatic assays[288,290]. Both compounds increase the ability of PKM2 to convert phosphoenolpyruvate (PEP) to pyruvate, presumably by enforcing PKM2

tetramerization, but have no effect on the rate of this reaction when performed by the constitutively tetramerized PKM1. In addition, the resolved crystal structure of PKM2 bound to TEPP-46 indicates that the binding site for these small molecules is in the PKM2-specific exon 10 region[288]. Finally, I have found that PKM2 activators relocalize PKM2, but not PKM1, from the nucleus to the cytoplasm during T cell activation. While these findings convincingly demonstrate PKM2-specific activities of TEPP-46 and DASA-58, they do not rule out the possibility that these compounds can bind to PKM1 and alter any non-metabolic functions it may have (to be discussed). Both PKM2 and PKM1 form complexes with non-metabolic proteins, suggesting that both perform non-metabolic functions in the cell. It is therefore possible that PKM1 and PKM2 perform an overlapping non-metabolic function in Th1, Th17, and Treg cells that is disrupted by binding of PKM2 activators. On the other hand, there are other pyruvate kinase isoforms besides PKM1 and PKM2. The liver type pyruvate kinase (PKL) and the red blood cell type (PKR) are splice variants that, while not expressed highly in immune cells, may become induced during activation and be targeted by TEPP-46 and DASA-58. Thus, in the literature and in my own studies, it remains unclear if PKM2 activators are mediating their PKM2-independent effects via disruption of other pyruvate kinase isoforms. In addition to the overlapping PKM2-independent effects of TEPP-46 and DASA-58, I also observed non-overlapping effects. Treatment of differentiating Th17 cells with TEPP-46 increases phosphorylation of STAT5, whereas DASA-58 does not have this effect. These findings suggest to me that TEPP-46 and DASA-58 may interact

with different proteins beyond pyruvate kinase isoforms. Thus, a major outstanding issue identified in my studies is that PKM2 activators are likely able to target proteins other than PKM2. I believe addressing this issue will provide much greater insight into their mechanisms of action, which will be an important contribution to the many fields in which they are being evaluated as therapeutics.

Because TEPP-46 is commonly used for *in vivo* work, I have chosen to study its targets. To do so, I have partnered with members of Dr. Ku-Lung Hsu's lab in the Department of Chemistry at the University of Virginia. These collaborators have functionalized TEPP-46 with a diazirine group (**Figure 16**). The added diazirine group makes it possible to photo-crosslink TEPP-46 to any proteins to which it is bound, as well as the subsequent enrichment of these proteins via click chemistry[310]. First, I will ensure TEPP-46-diazirine achieves similar outcomes on Th17 and Treg differentiation as the native compound. Following this, I will treat unstimulated, Th17 and Treg cells from PKM2-WT and PKM2-KO animals with the TEPP-46-diazirine and perform mass spectrometry to identify all proteins pulled down. I anticipate that the modified TEPP-46 will pull down both PKM1 and also PKM2, indicating its ability to bind and



TEPP-46 Diazirine

Figure 16: Functionalization of TEPP-46 for target identification. *The native structure of TEPP-46 (black) has been modified with a diazirine functional group (red) that will allow for identification of drug-protein interactions. Reproduced with the permission of Rebecca McCloud, Ph.D. Candidate, Hsu Lab, Department of Chemistry, University of Virginia.*

potentially disrupt the protein-protein interactions of both isoforms. In addition, I expect TEPP-46 to target proteins related to the stability of P-STAT5. These experiments are ongoing, and I anticipate completion by the end of 2019. If successful, these findings will provide significant insight into the therapeutic potential and mechanism of action of PKM2 activators in general, and particularly that of TEPP-46.

The PKM1 and PKM2 Interactome

The premise of PKM2 activators is that the unique ability of PKM2 to exist in multiple oligomeric states is the major reason why it, as opposed to PKM1, is able to perform non-metabolic functions in the cell. For example, the PKM2 dimer, but not the PKM2 tetramer or PKM1, can phosphorylate STAT3[281]. However, there are also incidences in the literature in which PKM1 performs non-metabolic functions. PKM1 was recently shown to translocate into the nucleus and co-activate transcriptional programs mediated by hepatocyte nuclear factor-4α (HNF-4α)[309]. In addition, there is growing dissatisfaction[275,311,312] with the idea that PKM2, due to its non-metabolic functions, is the pyruvate kinase isoform that is exclusively important for the tumorigenic programs of cancer cells as originally proposed[279,286]. Taken together, a major open question in the field of cancer and immune cell metabolism is the significance of PKM2 versus PKM1 expression. Addressing this question will be a major contribution to efforts to target pyruvate kinase isoforms in various disease states. In addition, doing so will likely reveal new players in metabolism, tumorigenesis, and inflammation.

I am particularly interested in the binding partners of PKM1 and PKM2 in T cells. Using the CD4-Cre mediated excision of PKM2-specifying exon 10, I can generate T cells that exclusively express PKM1 (PKM2-KO) and compare these with wild type T cells that nearly exclusively express PKM2 (PKM2-WT). I have already used these T cells to determine that the major functions of TEPP-46 and DASA-58 on Th17 and Treg cells are PKM2-independent. My presumption, as stated earlier, is that these PKM2 activators target PKM1. In addition to using these cells, I am currently in the process of using CRISPR-Cas9 technology to generate a line of mice in which PKM2 should predominantly exist in its dimeric form. This has been accomplished by inserting a point mutation to modify amino acid residue 433 in exon 10 of PKM2 converting it from a lysine to a glutamine (PKM2-K433Q). It has been reported that this K433Q variant of PKM2 prevents fructose 1,6 bisphosphate binding, is predominantly dimeric, and accumulates in the nucleus of cells[284]. Thus, I plan to use immunoprecipitation and mass spectrometry approaches to identify binding partners of native PKM2, PKM1, and dimeric PKM2 in PKM2-WT, PKM2-KO, and PKM2-K433Q T cells respectively. I also plan on using TEPP-46 to identify which of these binding partners are sensitive to inhibition by PKM2 activator treatment. I will perform these studies in unstimulated freshly isolated $CD4^+$ T cells to get an understanding of the baseline PKM1/2 interactome in T cells. I also hope to do this in $CD4^+$ T cells flow-sorted from an inflamed tissue *in vivo* (e.g. the EAE spinal cord), or if cell number is a limiting factor, with *in vitro* activated T cells. My major expectation for these studies is the identification of PKM1 and PKM2 binding partners that are

directly associated with TGFβ signaling pathways. My studies reveal the dominant effects of TEPP-46 and DASA-58 to be mediated, at least in part, by inhibition of a TGFβ1-transduced signal. It is my belief that TEPP-46 and DASA-58 achieve this overlapping outcome by affecting an aspect of the TGFβ1 signaling cascade that intersects in some way with both PKM1 and also PKM2. I am currently in the process of characterizing PKM2-K433Q T cells and anticipate significant progress to have been made on this short-term goal by the end of 2020.

Identifying the* in vivo *regulation of PKM1 and PKM2 in T cells

I have found that WT $CD4^+$ T cells almost exclusively express the M2 isoform of pyruvate kinase at baseline. Upon *in vitro* activation, these T cells further upregulate PKM2 protein expression with minimal induction of PKM1. Thus, $CD4^+$ T cells activated *in vitro* suppress expression of the M1 splice variant of pyruvate kinase, instead favoring induction of the basally expressed PKM2. The reasons for this selective expression are unclear, especially given that my studies with T cells expressing either isoform of pyruvate kinase strongly suggest redundancy in their functions. Regardless of why selective expression occurs, it is likely that upregulating PKM2 is beneficial for a T cell requiring heightened glycolytic flux for its functions. However, my studies of T cells in the EAE spinal cord revealed a surprising degree of variation in PKM2 expression (**Figure 17**). This has led me to conclude that functional T cell heterogeneity may be imparted by metabolic consequences of differential PKM2 expression. Alternatively, unlike

in the *in vitro* setting, *in vivo* conditions may be more favorable for PKM1 protein expression. To resolve this question, I plan to use flow cytometry to assess the expression of PKM1 and PKM2 in different populations of T cells *in vivo*. In particular, I am interested in how PKM1/PKM2 expression levels change when T cells exit quiescence and become activated in the periphery and CNS of the EAE model. This study will inform future efforts to target pyruvate kinase species to manage RRMS.

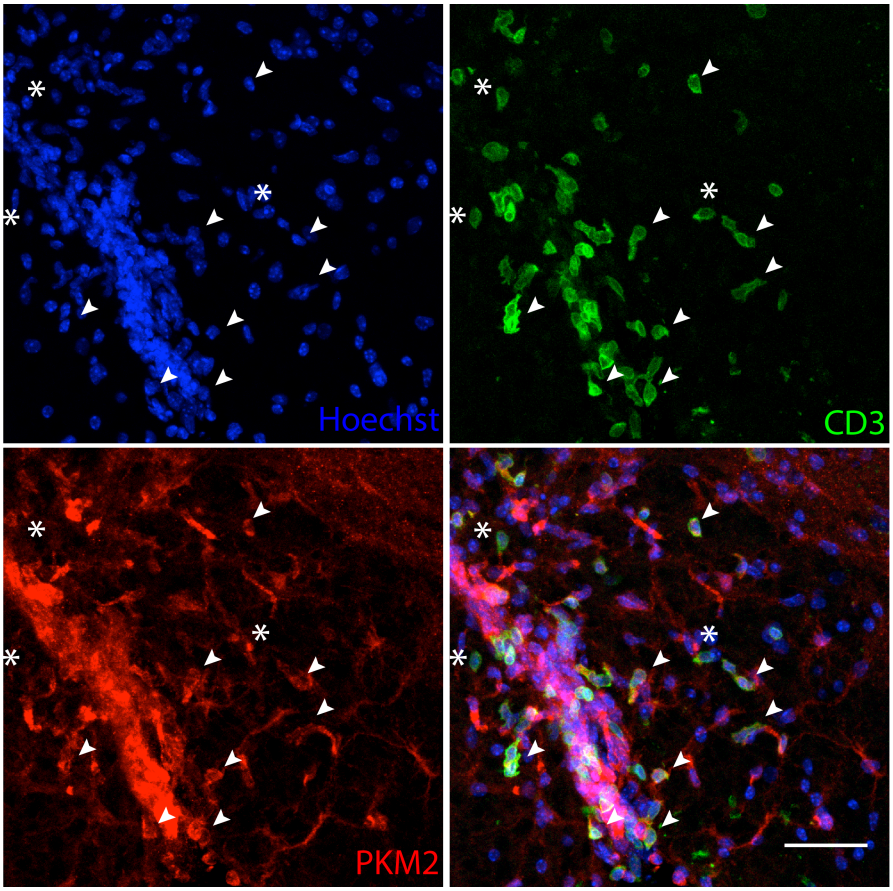


Figure 17: PKM2 expression by CD3$^+$ cells in the EAE spinal cord. *Arrowheads indicate CD3$^+$ cells with robust PKM2 staining. Asterisks identify CD3$^+$ cells with low levels of PKM2 staining. Scale bar is 40μM. Staining and imaging performed by Anthony Fernández-Castañeda, Ph.D. candidate, Gaultier Lab, Department of Neuroscience, University of Virginia.*

Finally, I would like to know how administration of TEPP-46 affects T cell responses *in vivo*. There is evidence from the cancer literature that TEPP-46 can be used in *nu/nu* mice (that lack an immune system) to prevent cancer growth[288]. I would like to extend these observations to include an understanding of how the endogenous immune system is affected by TEPP-46. In my EAE studies, I found that *in vivo* administration of TEPP-46 did not produce therapeutic outcomes expected of an anti-inflammatory agent. I hypothesized this to be related to my *in vitro* findings that TEPP-46 potentiated the development of GM-CSF-producing T cells and blocked Treg differentiation. Both of these outcomes, while detrimental in the EAE setting, may be desirable in the context of anti-tumor immunity. I am currently performing experiments to assess the *in vivo* effects of TEPP-46 on active T cell responses in the EAE model, and I believe that these findings will be translatable to cancer immunology. These experiments are ongoing and will be completed by the end of 2018.

Summary of Short-term Future Directions

My overarching goal for these studies is to identify aspects of T cell biology regulated by PKM1 and PKM2. Primarily, I anticipate these ongoing efforts will help to clarify the mechanism by which TEPP-46 and DASA-58 control Th1, Th17, and Treg differentiation in PKM2-WT and PKM2-KO T cells. By extension, I further anticipate this work to elucidate critical roles for novel proteins and pathways in the regulation of inflammatory T cell responses relevant to CNS autoimmunity. Finally, I expect these studies to provide a greater understanding

of the therapeutic potential for PKM2 activators such as TEPP-46, which are currently being tested in myriad disease models including cancer, diabetic nephropathy, and inflammation.

II. LONG-TERM

The major area of interest for my long-term follow up studies is in understanding how different organ-specific microenvironments can direct different flavors of immune cell responses via metabolic regulation. These studies have the potential to provide significant insight into mechanisms of controlling immune responses in a tissue-specific manner. These are more ambitious plans that I would like to pursue if I were to begin my post-doctoral work today.

Metabolic regulation of P-STAT5

My experiments with PKM2 activator treatment of differentiating Th17 cells revealed that TEPP-46, by an unknown mechanism, increases the phosphorylation of STAT5. In addition, TEPP-46 treatment results in an increase in GM-CSF producing T cells during Th17 differentiation, and this is a STAT5-dependent process. I subsequently designed an experiment to elucidate how metabolic perturbations might affect STAT5 phosphorylation in T cells. I assessed the effects of oligomycin (OLG), an ATP-synthase inhibitor, on P-STAT3 and P-STAT5 in response to treatment with IL-6 and IL-2. Surprisingly, I found that OLG potently inhibited cytokine-mediated P-STAT5 but not P-STAT3

(**Figure 18**). This implies that microenvironments that restrict ATP availability in T cells might potently modulate T cell effector functions, such as the production of GM-CSF, via effects on P-STAT5. The intriguing aspect of this model is it implies that P-STAT5, but not P-STAT3, acts as a major metabolic sensor in T cells.

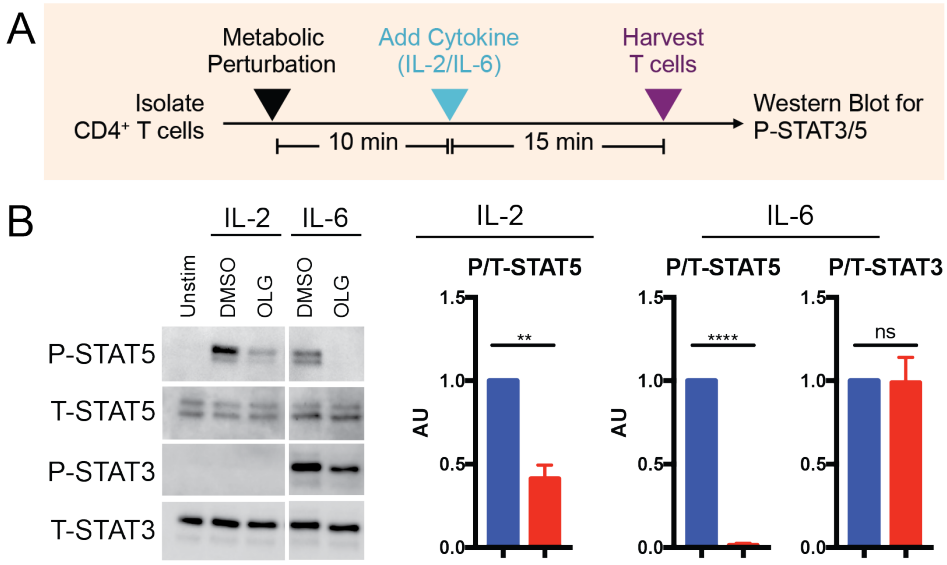


Figure 18: Metabolic control of STAT5 phosphorylation. *(A) Experimental design to determine if ATP-synthase inhibition with oligomycin (1μM) affects IL-2 (100U/mL) or IL-6 (20ng/mL)-mediated phosphorylation of STAT3 and STAT5. (B) Representative western blot with densitometric quantification. Quantification data are presented as mean ± SEM and are pooled from N=2 independent experiments with T cells from n=2 mice per experiment. DMSO-treatment is in blue. Oligomycin treatment is in red. Data are normalized such that ratio of P/T-STAT5 and P/T-STAT3 in DMSO-treated condition is set to 1. **p<0.01, ****p<0.0001 by Paired Student's T-test. AU = arbitrary units*

An initial way to address how metabolic regulation of P-STAT5 influences T cell functions would be to measure the effects of 2-DG and OLG on the generation of GM-CSF-producing T cells during Th17 differentiation in WT $CD4^+$ T cells and $CD4^+$ T cells transduced to express a constitutively active form of STAT5 (CA-STAT5). It has been shown that 2-DG suppresses development of IL-17A-producing T cells, and the inability of 2-DG treated cells to transfer EAE

suggests that GM-CSF is impacted as well[171]. Thus, I expect that 2-DG and OLG will prevent the generation of GM-CSF-producing T cells during normal Th17 differentiation, but may not be able to prevent their differentiation when T cells express CA-STAT5. If true, this would imply that the effects of metabolic perturbations on T cell functions are translated into functional outcomes through regulation of P-STAT5.

More broadly speaking, I am interested to know if the metabolic restriction in different microenvironments supports different levels of P-STAT5 in T cells, thus fine-tuning local immune responses. Anti-tumor immunity is a good system in which to test this idea, as this is a situation where site-specific metabolic deficits are known and defined[228]. My belief is that the tumor microenvironment will restrict the ability of T cells to access nutrients that contribute to ATP levels, such as glucose, and as a result, impair survival signals transmitted by cytokine-mediated phosphorylation of STAT5. One way to test this would be to measure P-STAT5 after IL-2 stimulation in tumor infiltrating $CD4^+$ and $CD8^+$ T cells compared with unstimulated naïve T cells, as well as matched effector T cells generated in a synchronous immunization-based paradigm. This comparison will reveal if tumor infiltrating lymphocytes (TIL), known for their metabolic deficiency, lose their ability to phosphorylate STAT5 downstream of IL-2, IL-7, or IL-15 compared with either their naïve precursors or “age-matched” effectors that are capable of driving immune responses. Anti-tumor immunity is often attributed to the function of $CD8^+$ T cells, and it seems that the effector functions of these cytotoxic lymphocytes are particularly sensitive to manipulations affecting P-

STAT5[313]. A way to test if metabolic regulation of STAT5 can affect T cell functions *in vivo* would be to use a solid tumor model and measure the ability of WT and CA-STAT5 $CD8^+$ T cells to maintain their cytokine production profile in the tumor after adoptive transfer. If metabolic regulation of P-STAT5 is important for T cell functions in this context, then one might expect transferred WT TIL to adopt an exhausted-appearing profile secondary to metabolic constraints placed on STAT5 signaling, whereas CA-STAT5 TIL would be resilient to this outcome. In fact, a similar study has already been performed in $CD8^+$ T cells, where it was discovered that CA-STAT5 promotes $CD8^+$ invasion and tumor regression in a melanoma model[314]. In this study, tumor antigen-specific $CD8^+$ expressing CA-STAT5 were able to maintain their functionality despite their upregulation of co-inhibitory receptor PD-1. PD-1 is known to achieve its inhibitory effects in TIL, in part, via inhibition of T cell metabolism[229,315]. While the mechanism for the potent anti-tumor immunity elicited by CA-STAT5 $CD8^+$ T cells was completely unexplored in this study, I believe this genetic modification may have rendered these TIL insensitive to the metabolic constraints of the tumor microenvironment.

Taken together, from my own offshoot studies of metabolic perturbations and cytokine-mediated phosphorylation of STAT5 as well as reports from the cancer immunology literature, there is evidence to suggest that STAT5 plays a role as a metabolic sensor in T cells. Intuitively, this makes sense given that the IL-2→P-STAT5 axis is a major proliferation signal for T cells, and it may be important to regulate the amount of signal that gets through based on the ability of the microenvironment to support proliferation or effector function. The clinical

setting where understanding the relationship between metabolism and STAT5 may be particularly beneficial is in anti-tumor immunity. CAR-T cells demonstrate robust efficacy in the setting of hematologic malignancy, but are much less reliable in the treatment of solid tumors[316]. Identification of STAT5 as a major integrator of metabolic cues in TIL may allow for the development of CAR-T cells that can maintain their functionality in the hostile microenvironment of the solid tumor. Thus, I believe that studying the relationship between T cell metabolism and P-STAT5 will yield important basic science insight that can then be leveraged for translational benefit.

Tissue microenvironment and T cell metabolism

An interpretation of my studies with 3-BrPa is that glycolytic inhibition impairs the acquisition of a pro-migratory phenotype required for Th1-mediated encephalitogenesis. In considering this possibility, I have become very interested in how metabolic restrictions affect the migration of T cells into different tissues. More broadly, I am intrigued by the idea that different tissue microenvironments may be able to influence or even direct local immune responses based on the metabolic constraints they impose. It is already known that metabolism can direct T cell fate *in vivo*[223,224]. Likewise, a rich body of evidence supports the notion that the distinct identities of various tissue-resident macrophages are imprinted by nuances in their organ-specific microenvironments[317-319]. I hypothesize the existence of an additional layer of regulation whereby tissue-specific cues can

refine local immune responses through enforcing the adoption of a specific metabolic signature.

From the cancer literature, it is clear that the microenvironments that T cells are exposed to can potently and durably modulate the quality of their effector functions[228]. I am interested to know if the brain imposes any such metabolic restrictions that allows for a finely tuned T cell response that, for example, can control a latent virus without triggering a demyelinating immune response. To achieve this, I think it would be informative to study the metabolism of an IL-17A-producing T cell in the arthritic joint compared with the inflamed CNS during EAE. The differing patterns of tissue invasion of $CD4^+$ T cells in the RA joint (diffuse invasion of synovium[145]) versus in the RRMS CNS (predominantly perivascular localization with minimal parenchymal invasion[42]) indicates to me, at the very least, that the physiology of the joint and CNS support different levels of T cell invasion. While there are certainly non-metabolic mechanisms by which T cell invasion of the CNS is limited (e.g. the CXCR4-CXCL12 axis that traps T cells in the perivascular space[139,141]), I still wonder if the acquisition of a tissue-invasive phenotype goes hand-in-hand with metabolic alterations. Migration is a highly metabolic process, particularly with respect to mitochondrial metabolism[255] and, by extension, maybe invasion is as well.

To determine the difference between the metabolic basis for migration and invasion, it may be useful to combine lineage-tracing methods with single cell RNA-seq to study subset-dependent expression of metabolic machinery in T cells with different localizations in the EAE spinal cord. Of particular interest are

comparisons of $CD4^+$ T cells in the perivascular space, which are known to actively migrate along blood vessels[142], versus those that have either successfully (or passively) invaded the CNS parenchyma. Another way to study the metabolic distinction between tissue invasion and migration along vessels in perivascular spaces is through leveraging CXCR4 antagonists to facilitate invasion of $CD4^+$ T cells into the EAE spinal cord. Blood brain barrier expression of CXCL12 and its interaction with CXCR4 on T cells is known to regulate T cell invasion of the CNS[139,141]. Whereas $CD4^+$ T cells tend to accumulate in perivascular spaces of wild type mice, with pharmacologic or genetic CXCR4 antagonism, these cells gain the capacity to penetrate the CNS[139,142]. With such an approach, more cells can be used to compare the metabolic distinction between migratory versus invasive behaviors of T cells and, as a result, functional metabolic assays can be performed. I envision that a better understanding of the metabolic nuances associated with migratory and invasive phenotypes may yield important therapeutic insight into how to best generate a T cell that can penetrate the defenses of solid tumors, or to hamstring one that is aiming to penetrate a target organ during auto-inflammation.

From a broader perspective, I would like to know if an antigenically identical immune response, triggered in different areas of the body, elicits T cells with different metabolic profiles and perhaps different functionalities as a result. A way in which this could be accomplished would be to inducibly express an exogenous antigen, such as ovalbumin (OVA), under tissue specific promoters in OT-II (or OT-I) mice that are populated by T cells genetically engineered to target

OVA. Immunization with OVA can then be used to target an autoinflammatory response based on the tissue-specific promoter used. Downstream assessments of functional metabolism of T cells will reveal if responding T cells in the brain, as an example, are metabolically distinct from those attacking the joint. Combined use of lineage tracing approaches can more specifically identify metabolic differences between T cells with similar cytokine production profiles. Furthermore, *ex vivo* assessment of metabolic perturbations on cytokine responses would reveal if effector T cells have distinct metabolic vulnerabilities based on the tissue from which they are derived. If tissue-specific metabolic divergence is observed, adoptive transfer studies could be performed to assess if T cells harvested from one tissue are more or less equipped to orchestrate immune responses in an organ that supports T cells with a different metabolic signature.

An alternative and exciting study would be to determine how organ-specific tissue microenvironments affect the metabolic characteristics of invading myeloid cells of different pathogenicities. A recent report details the ability of peripheral monocyte-derived cells to reconstitute resident macrophage populations in the brain[320]. In this breakthrough study, brain-engrafting macrophages (beMφ) derived from monocyte precursors were found to repopulate microglia (MG) depleted via a variety of manipulations. RNA-sequencing revealed distinct gene signatures between beMφ and MG, with a major altered function being an upregulation in lipid metabolism in the engrafted cells. Importantly, the behavior of reconstituted mice was indistinguishable from

controls, suggesting repopulation to not be an inflammatory or pathogenic process. I would be interested to extend the observations made by Cronk and colleagues by comparing engrafting Mφ that do not drive overt disease with those that do. In the Becher study using GM-CSF overexpressing immune cells, monocyte-derived cells are found to invade numerous organs, including the brain, but exhibit distinct transcriptional profiles depending on the organ from which they are isolated[111]. Intriguingly, this study also revealed that a gene signature indicative of heightened lipid metabolism distinguished GM-CSF-mediated infiltrating Mφ found in the CNS versus those found in peripheral organs also undergoing inflammation. It would be very intriguing to compare these findings with infiltrating Mφ in homozygous Yeti mice, which exhibit an IFN-γ-dependent myeloproliferative autoimmune syndrome[233]. I hypothesize that comparing the organ-specific transcriptional and metabolic signatures of monocyte-derived cells that infiltrate organs in these three models (repopulating engrafting Mφ, GM-CSF-driven infiltrating Mφ, and IFN-γ-driven infiltrating Mφ) would reveal overlapping phenotypes, that might be attributed to a dominant effect of the tissue microenvironment as well as distinctions that might be attributable to the differing disease states.

If successful, these types of studies would provide insight into the extent to which organs imprint a tissue-specific metabolic signature onto infiltrating immune cells, and the salience thereof. Besides being of basic scientific interest, these studies have the potential to elucidate targeted ways to control aberrant immune responses in specific organs. This would be a major advance over

current blanket immunosuppressive approaches. I am particularly interested in how the brain microenvironment controls immunity via metabolic regulation. In addition to the differing invasiveness of T cell responses in the brain and in peripheral tissues, I am intrigued by the ability of the brain to withstand the constant immune pressure needed to suppress the latent viruses and parasites that reside there. To me, this implies that the brain is a space where T cells are routinely performing their effector functions, but to a degree which does not induce a full immune attack. Perhaps there is a metabolic basis for this. As a follow up to these studies, it would then be pertinent to identify the tissue specific effectors (e.g. neurotransmitters) that directly program distinct metabolic signatures in infiltrating immune cells.

Summary of Long-Term Future Directions

My overarching goal for these long-term studies is to identify how the distinct metabolic microenvironments of different tissues, particularly of the brain, are able to influence the types of immune responses that occur in them. I believe I have identified STAT5 as an integrator of environmental cues (e.g. glucose availability) that can then modulate T cell responses. In addition to testing this belief, my proposed future studies would extend this observation to identify the metabolic signatures of T cells and monocyte-derived cells responding in different tissue microenvironments. If successful, these studies will be an important contribution to the numerous fields touched by immunometabolism, including but not limited to autoimmunity and cancer immunotherapy.

Supplementary Table 1

Supplementary Table 1: Primer list for qRT-PCR studies

Metabolism Studies		
Target	**Forward (5'-3')**	**Reverse (5'-3')**
ASCT2	TGCTTTCGGGACCTCTTCTA	TGATGTGTTTGGCCACACCA
CPT1a	AGATCAATCGGACCCTAGACAC	CAGCGAGTAGCGCATAGTCA
CPT2	TGTCTTCCAAGCACTTCTGG	TGGATAGGCTGCAATGTCTC
Gls1	GGGAATTCACTTTTGTCACGA	GACTTCACCCTTTGATCACC
Gls2	AGCGTATCCCTATCCACAAGTTCA	GCAGTCCAGTGGCCTTCAGAG
Glud1	CCCAACTTCTTCAAGATGGTGG	AGAGGCTCAACACATGGTTGC
Glut1	GCTGCCCCTGAGGAGGAA	AGAGGCCACAAGTCTGCATT
HK1	AGGATGACCAAGTCAAAAAGATTG	TCTTGAAGCGTGTCAGGATATCTA
HK2	TGATCGCCTGCTTATTCACGG	AACCGCCTAGAAATCTCCAGA
LDH-A	GCTCCCCAGAACAAGATTACAG	TCGCCCTTGAGTTTGTCTTC
Lipa	CTAGAATCTGCCAGCAAGCC	AGTATTCACCGAATCCCTCG
PKM1	GCTGTTTGAAGAGCTTGTGC	TTATAAGAGGCCTCCACGCT
PKM2	TCGCATGCAGCACCTGATT	CCTCGAATAGCTGCAAGTGGTA
SDHd	TGGTCAGACCCGCTTATGTG	GGTCCAGTGGAGAGATGCAG
Slc7a5	CTGGATCGAGCTGCTCATC	GTTCACAGCTGTGAGGAGC
GAPDH: Taqman Mm99999915_g1		

Th17/Treg Studies		
Target	**Forward (5'-3')**	**Reverse (5'-3')**
Ccr8	TGACCGACTACTACCCTGATTTCTT	GCTGCCCCTGAGGAGGAA
IL-17A	ATCCCTCAAAGCTCAGCGTGTC	GGGTCTTCATTGCGGTGGAGAG
IL-1R1	TGGAACAGAGCCAGTGTCAG	CAGGAGAAGTCGCAGGAAGT
IL-21	CGCCTCCTGATTAGACTTCG	TGGGTGTCCTTTTCTCATACG
IL-22	TCGTCAACCGCACCTTTATG	CCCGATGAGCCGGACAT
IL-23R	GCCAAGAAGACCATTCCCGA	TCAGTGCTACAATCTTCTTCAGAGGACA
Irf8	CGTGGAAGACGAGGTTACGCTG	GCTGAATGGTGTGTGTCATAGGC
RORc	CACGGCCCTGGTTCTCAT	CAGATGTTCCACTCTCCTCTTCTCT

Reference		
Target	**Forward (5'-3')**	**Reverse (5'-3')**
β-Actin	AGGTGTGCACCTTTTATTGGTCTCAA	TGTATGAAGGTTTGGTCTCCCT

APPENDIX A

Lineage-specific metabolic properties and vulnerabilities of T cells in the demyelinating central nervous system

Lineage-Specific Metabolic Properties and Vulnerabilities of T Cells in the Demyelinating Central Nervous System

Scott M. Seki,*,†,‡ Max Stevenson,* Abagail M. Rosen,* Sanja Arandjelovic,§ Lelisa Gemta,¶ Timothy N. J. Bullock,¶ and Alban Gaultier*

Multiple sclerosis (MS) is a disease that is characterized by immune-mediated destruction of CNS myelin. Current MS therapies aim to block peripheral immune cells from entering the CNS. Although these treatments limit new inflammatory activity in the CNS, no treatment effectively prevents long-term disease progression and disability accumulation in MS patients. One explanation for this paradox is that current therapies are ineffective at targeting immune responses already present in the CNS. To this end, we sought to understand the metabolic properties of T cells that mediate ongoing inflammation in the demyelinating CNS. Using experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice, a well-studied model of MS, we showed that the $CD4^+$ and $CD8^+$ T cells that invade the EAE CNS are highly glycolytic. Elevated glycolytic rates in T cells isolated from the EAE CNS correlate with upregulated expression of glycolytic machinery and is essential for inflammatory responses to myelin. Surprisingly, we found that an inhibitor of GAPDH, 3-bromopyruvic acid (3-BrPa), blocks IFN-γ, but not IL-17A, production in immune cells isolated from the EAE CNS. Indeed, in vitro studies confirmed that the production of IFN-γ by differentiated Th1 cells is more sensitive to 3-BrPa than is the production of IL-17A by Th17 cells. Finally, in transfer models of EAE, 3-BrPa robustly attenuates the encephalitogenic potential of EAE-driving immune cells. To our knowledge, these data are among the first to demonstrate the metabolic properties of T cells in the demyelinating CNS in vivo. ***The Journal of Immunology*****, 2017, 198: 4607–4617.**

Multiple sclerosis (MS) is a devastating disease of the CNS with high prevalence, early age of onset, and no cure (1, 2). MS is a disease with prominent autoimmune features that result in the debilitating destruction of CNS myelin, or demyelination (3).

There is a critical need for novel strategies to treat MS. Nearly all therapies that are approved by the U.S. Food and Drug Administration to treat MS target the immune system, with the most effective being those that prevent peripheral immune cell infiltration into the CNS. Although this therapeutic strategy robustly diminishes relapse rates, it paradoxically fails to prevent MS disease progression (4, 5). A better understanding of the mechanisms regulating in situ inflammation in the MS CNS may reveal new targets for therapeutic intervention. There are no treatments specifically designed to target self-propagating immune responses in the MS CNS, and the development of such may have profound therapeutic benefit as a stand-alone or combinatory treatment.

A great body of evidence suggests that T cells, agents of the immune system that normally direct inflammatory responses against invading pathogens, play a critical role in MS pathogenesis (6, 7). In vitro studies have revealed that activated T cells require upregulated glycolytic metabolism to fuel inflammatory programs, leading to speculation that glycolytic blockade may block a wide range of inflammatory T cell functions in autoimmune disease (8–10). Related studies showing that glycolysis is required for the in vitro differentiation of T cells further support this hypothesis (11). More recent studies have begun to show that glycolysis is also detected in vivo during experimental autoimmune encephalomyelitis (EAE) (11–14). It is becoming increasingly clear that in vitro metabolic status of immune cells is not always recapitulated in vivo, thus necessitating a closer look at the metabolic pathways fueling autoimmunity in vivo (10).

In this study, we use EAE, a murine model of CNS demyelination, to address major questions in MS research: are the glycolytic properties of T cells in the demyelinating CNS unique from peripheral T cells, and how does this glycolysis contribute to their proposed role as orchestrators of demyelination? Currently, the metabolic state of T cells in the EAE spinal cord is unclear. Furthermore, it remains unclear whether distinct metabolic pathways fuel different inflammatory programs and, likewise, whether intervention along all points of a metabolic pathway will produce the same effects. Using two models in which encephalitogenic T cells are generated in vivo, active immunization-based EAE and spontaneous EAE in the 2D2 strain of mice, we identified the metabolic properties of T cells in the EAE CNS. Our studies implicate glucose and glutamine as primary metabolic substrates for inflammatory T cell responses during CNS demyelination

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Abbreviations used in this article: 3-BrPa, 3-bromopyruvic acid; cat. no., catalog number; DON, 6-diazo-5-oxo-L-norleucine; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein peptide; MS, multiple sclerosis; qRT-PCR, quantitative RT-PCR.

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in vivo. Given the therapeutic potential of targeting metabolic pathways to attenuate inflammatory disease, we characterized the effects of 3-bromopyruvic acid (3-BrPa), an inhibitor of GAPDH and the glycolytic cycle, on inflammatory responses in the EAE spinal cord. We found that 3-BrPa potently blocked IFN-γ production by immune cells from the EAE spinal cord, but it was not as efficient at blocking IL-17A production. Subsequent studies confirmed that in vitro–differentiated IFN-γ–producing Th1 cells are indeed more susceptible to 3-BrPa than are IL-17A–producing Th17 cells. Likewise, we found that 3-BrPa was effective in ameliorating disease onset and severity in EAE induced by the adoptive transfer of Th1 cells. These data provide novel and important insights into the therapeutic potential of targeting T cell glycolysis to treat MS.

Materials and Methods

Mice

All studies were performed with C57BL/6 mice (8–16 wk) obtained from the Jackson Laboratory (catalog number [cat. no.] 000664). 2D2 mice have been described previously (7) and were obtained from the Jackson Laboratory (cat. no. 006912). All animal experiments complied with regulations of the Institutional Animal Care and Use Committee of the University of Virginia.

Reagents

3-BrPa was purchased from Acros Organics (cat. no. AC325690250). 6-Diazo-5-oxo-L-norleucine (DON) was purchased from Cayman Chemical (cat. no. 17580).

Experimental autoimmune encephalomyelitis

Active EAE was induced as described (15). Briefly, male or female (sex matched within experiments) C57BL/6 mice were immunized s.c. with 50 μg of myelin oligodendrocyte glycoprotein peptide $(MOG)_{35–55}$ (cat. no. CS0681; CS Bio) emulsified at a 1:1 ratio in CFA (cat. no. F5881; Sigma-Aldrich). On days 0 and 2, 250 ng of pertussis toxin (cat. no. 180; List Biological) was administered i.p. Transfer EAE was induced based on previously described protocols (16, 17). Immune cells were isolated from spleens of 8–12-wk-old 2D2 male mice and differentiated into Th1 lineage–promoting conditions for 3 d, as previously described (14). Cells were treated with 10–20 μM 3-BrPa or saline vehicle on days 0 and 2 of skew. On day 3, cells (5×10^6) were injected i.p. into $Rag1^{-/-}$ immunodeficient hosts, and the pertussis toxin was administered as for active EAE. Disease severity was scored using a standardized scale: 0, no disease; 1, complete tail paralysis; 2, loss of hindlimb coordination on grate walk; 3, hindlimb weakness; 4, complete bilateral hindlimb paralysis; and 5, moribund or death due to EAE.

T cell purification from the EAE spinal cord and spleen

To determine the glycolytic profile of T cells isolated from the demyelinating CNS, active EAE was induced, and T cells were harvested from the spinal cords and spleens of mice at peak disease (days 14–18). Harvesting T cells from the spinal cord was achieved by first digesting the tissue in HBSS supplemented with 2 mg/ml Collagenase IV (cat. no. LS004188; Worthington) and 50 U/ml DNase (cat. no. 10104159001; Sigma-Aldrich). Debris generated during the digestion was removed by Percoll density gradient (cat. no. 17-0891-01; GE Healthcare). The resulting cell suspension was stained with a PE-conjugated Ab against TCRβ (cat. no. 12-5961-83; eBioscience), and T cells were positively sorted with MACS anti-PE MicroBeads (cat. no. 130-048-801; Miltenyi Biotec). Splenic T cells were isolated similarly after tissue homogenization. MACS purifications yielded ∼90% purity. Results obtained with magnetically sorted cells were confirmed by analysis of $TCR\beta^+CD4^+$ and $TCR\beta^+CD8^+$ T cells sorted on a BD Biosciences Influx Fluorescence Activated Cell Sorting machine at the University of Virginia Flow Cytometry Core Facilities.

Transcriptional analysis of T cells isolated from the EAE CNS and spleen

Transcriptional analyses were performed by quantitative real-time PCR on a Bio-Rad CFX384 Touch Real-Time PCR Detection System. Purified T cells were snap-frozen at −80°C. RNA was extracted using a Bioline Isolate II RNA Mini Kit (cat. no. BIO-52073), according to the manufacturer's instructions. RNA was converted into cDNA using a Bioline SensiFAST cDNA synthesis kit (cat. no. BIO-65053). Quantitative RT-PCR (qRT-PCR; see Supplemental Table I for primer list) was performed using Bioline SensiFAST kits (cat. no. BIO-98005 and BIO-86005).

Seahorse metabolic flux analysis

To assess the glycolytic capacity of T cells isolated from the EAE spinal cord and spleen, sorted T cells were subjected to a glycolytic stress test and simultaneously assessed for glycolytic activity using a Seahorse XFp Analyzer. T cells were seeded at 1×10^5 per well in XF Base Medium Minimal DMEM (cat. no. 103193-100) supplemented with 2 mM L-glutamine (cat. no. 25030-081; Life Technologies) and subjected to the glycolytic stress test (cat. no. 103017-100; Seahorse Bioscience). All Seahorse experiments were performed according to the manufacturer's instructions.

Ag-recall assays in CNS tissues of mice with EAE

Cells were isolated from the EAE CNS as described above. A total of 5×10^5 cells was stimulated in 200 μl of complete RPMI 1640 with 50 μg/ml $MOG_{35–55}$ and 10 μM 3-BrPa or 1 mM DON for 48 h, and IFN-γ (cat. no. 517902, cat. no. 505704; BioLegend) and IL-17A (cat. no. eBio17CK15A5, cat. no. eBio17B7; eBioscience) secretion was measured by ELISA. Poststimulation cell viability was determined by flow cytometry. In some cases, spinal cord meninges were separated from the spinal cord parenchyma prior to recall, as described in the text.

Effects of 3-BrPa on T cell proliferation and cytokine production

To assess the effects of 3-BrPa on T cell proliferation, T cells were isolated from spleens and lymph nodes of C57BL/6 mice using a $CD3^+$ T Cell Enrichment Column (cat. no MTCC-10; R&D Systems), stained with 20 μM Cell Proliferation Dye eFluor 450 (cat. no. 65-0842-85; eBioscience), and stimulated to divide in complete RPMI 1640 with plate-bound anti-CD3 (cat. no. BE0001-1; 1 μg/ml) and soluble anti-CD28 (cat. no. BE0015-5; 10 μg/ml; both from Bio X Cell) Abs for 72 h. After 24 h of stimulation, a time when T cells have engaged glycolysis to fuel activation (18) but have not yet divided, 10 μM 3-BrPa or saline vehicle was added to the cells. Division was monitored for an additional 48 h and determined every 24 h by flow cytometric analysis of eFluor 450 dye dilution. The same T cell–isolation procedure used for the proliferation assay was used to determine how 3-BrPa affects T cell cytokine production. Enriched T cells were stimulated with anti-CD3 and anti-CD28 in the presence of 3 μg/ml Brefeldin A (cat. no. 00-4506-51; eBioscience), as well as 10 μM 3-BrPa or saline vehicle, for 6 h at 37°C, 5% CO_2 and assessed for viability and cytokine production by flow cytometry.

Flow cytometry

Flow cytometric analyses were performed on a 10-color Beckman Coulter Gallios flow cytometer. The following Abs were used: TCRβ (H57-597), CD4 (RM4-5), CD8 (53-6.7), CD19 (eBio1D3), CD45 (30-F11), CD11b (M1/70), MHC-II (M5/114.15.2), IL-2 (JES6-5H4), TNF-α (MP6-XT22), IFN-γ (XMG1.2), IL-17A (TC11-18H10.1; BioLegend), T-bet (eBio4B10), and a Zombie Aqua Fixable Viability kit (cat. no. 423101; BioLegend). An eBioscience Intracellular Fixation and Permeabilization Buffer Set (cat. no. 88-824-00) was used for intracellular cytokine staining, and an eBioscience FoxP3/Transcription Factor Staining Buffer Set was used for intranuclear staining (cat. no. 00-5523-00). All Abs for flow cytometry were purchased from eBioscience, unless otherwise noted.

GAPDH and hexokinase assay

Single-cell suspensions were prepared from the lymph nodes of C57BL/6 mice and incubated with increasing concentrations of 3-BrPa for 30 min at 37°C. Cells were next lysed in the assay extraction buffer, and the enzymatic activity of both enzymes was determined according to the manufacturer's instructions (GAPDH Activity Assay Kit [cat. no. K680], Hexokinase Colorimetric Assay Kit [cat. no. K789]; BioVision).

Immunohistochemistry and histology

Slides were deparaffinized using xylene and an ethanol gradient and stained with Luxol Fast Blue to assess demyelination. Adjacent sections were submitted to the University of Virginia Biorepository and Tissue Research

Facility for CD3 staining. An investigator blinded to the status of the groups performed histological analyses.

Statistics

All statistical analyses were performed with Prism 7 (GraphPad). Analyses involving two groups were performed using a two-tailed *t* test.

Results

T cell glycolytic activity and inflammation are potentiated in the EAE spinal cord

Because our primary interest is in understanding the fuel sources driving in situ inflammation in the demyelinating CNS, we explored the metabolic properties of T cells in the EAE spinal cord. To this end, we flow sorted $CD4^+$ and $CD8^+$ T cells from the CNS of mice at peak EAE and assessed their metabolic characteristics. Using Seahorse Extracellular Flux Analyses, we determined that $CD4^+$ T cells from the EAE spinal cord had elevated basal and maximal glycolytic rates in comparison with those isolated from the spleens of the same animals (Fig. 1A, 1B). Basal mitochondrial respiration of spinal cord $CD4^+$ T cells was also elevated (Fig. 1B). Results from our transcriptional studies indicated that the elevated glycolytic rates in these spinal cord $CD4^+$ T cells might be, in part, fueled by increased expression of glycolytic enzyme transcripts (Fig. 1C). Basal expression of IFN-γ, a Th1 cell–derived cytokine associated with EAE and MS pathology (19, 20), and T-bet, the transcription factor that drives differentiation of Th1 cells, was also increased in $CD4^+$ T cells in the EAE spinal cord (Fig. 1D) and correlated with increased IFN-γ and IL-2 production upon restimulation (Fig. 1E). Transcriptional and Seahorse studies were confirmed using $TCR\beta^+$ cells magnetically sorted from the diseased spinal cord (Supplemental Fig. 1). These results indicate that, during EAE, pathogenic T cells at the site of inflammation are more glycolytic than peripheral T cells in more quiescent tissues. This metabolic observation is likely to be tightly linked to the activation state of T cells in the EAE CNS, which, as we found, are robust expressers of the glycolytically regulated cytokines IFN-γ and IL-2.

Regulation of nonglycolytic metabolism in $CD4^+$ T cells in the EAE CNS

To gain a more comprehensive understanding of the metabolic properties of T cells in the EAE spinal cord, we also explored the transcriptional regulation of nonglycolytic metabolism in these T cells (Fig. 2A). We observed significant increases in glutaminase 2, glutamate dehydrogenase 1, lysosomal acid lipase, and carnitine palmitoyltransferase 1a in $CD4^+$ T cells but not in $CD8^+$ T cells (Fig. 2B). Our data suggest that, unlike their $CD4^+$ counterparts, spinal cord $CD8^+$ T cells may preferentially regulate glycolytic metabolism during EAE. Increased metabolism of glutamine and/or lipids and fatty acids may be related to the significant elevation in basal oxygen consumption rate in T cells isolated from the EAE spinal cord (Fig. 1A, Supplemental Fig. 1). Previous groups have shown that, during EAE, Ag-specific T cells leave lymphoid organs following immunization and are retained in the CNS (21–23). Our observations are likely a reflection of this CNS-proximal T cell activation and, as such, illustrate a profound divergence from what would be predicted based on in vitro observations, where activated T cells, in comparison with resting state T cells, are known to uniformly increase amino acid transporter expression while shutting down lipolytic pathways. Taken together, our data provide important insights into the metabolic basis of the site-specific perpetuation of the T cell response in the EAE spinal cord in vivo.

Immune cells in the EAE spinal cord require glycolysis for inflammatory functions

Our data suggest that numerous substrates could fuel $CD4^+$ T cell–mediated inflammation in the CNS (Fig. 2). As a result, we hypothesized that T cells in the EAE spinal cord may maintain metabolic flexibility or, in other words, the capacity to adapt when discrete energy resources are limiting.

We sought to determine whether glycolysis was essential for T cell effector functions in the EAE spinal cord. We first examined the effects of a glycolytic inhibitor, 3-BrPa, on T cell functions (24, 25). 3-BrPa has been described as an inhibitor for GAPDH and hexokinase (26–29). Using a single-cell suspension prepared from lymph nodes, we found that 3-BrPa was inhibiting GAPDH activity at a dose that was not affecting hexokinase activity (Fig. 3A). We discovered that 3-BrPa is a potent suppressor of T cell proliferation (Fig. 3B) without impacting cell viability at the end of the assay (Fig. 3C). We also observed that production of the cytokines TNF-α and IL-2 was affected by 3-BrPa in a dose-response manner (Fig. 3D, left panel), independently of cell survival (Fig. 3D, right panel). To understand whether 3-BrPa could impact TCR signaling events, we examined activation of CD69 and phosphorylation of ERK. 3-BrPa did not block TCR-mediated upregulation of CD69 expression (Fig. 3E) or impact viability (Fig. 3F). Similarly, the phosphorylation of MAPK ERK was not affected by 3-BrPa (Fig. 3G). Using the same assay, cell death and impaired TCR signaling were observed at a higher dose of 3-BrPa (100 μM; data not shown).

We next induced EAE in C57BL/6 mice and performed Ag-recall assays on harvested spinal cord homogenates in the presence or absence of 3-BrPa to analyze the effects of glycolysis inhibition on cytokine production. Stimulation of EAE spinal cord homogenates with $MOG_{35–55}$ potently elicited production of IFN-γ, and this induction was profoundly sensitive to treatment with 3-BrPa (Fig. 4A). Strikingly, production of IL-17A, another cytokine associated with autoimmune demyelination (30, 31), was largely refractory to 3-BrPa treatment (Fig. 4A). There was no effect of 3-BrPa on the viability of APCs, $CD4^+$ T cells, or $CD8^+$ T cells in these assays (data not shown). Unlike 3-BrPa, the glutaminase inhibitor DON potently inhibited IFN-γ and IL-17A production, indicating that IL-17A production in this assay may be preferentially facilitated by glutamine metabolism (Fig. 4B). In these recall assays, no cytokine production was detected in the absence of MOG stimulation (data not shown).

T cells that drive spontaneous EAE in 2D2 mice require glycolysis

Our results show that T cells require glycolysis for specific inflammatory functions in active immunization-based EAE. To confirm these findings in a model of adjuvant-free EAE, we repeated these studies in 2D2 mice, a strain on the C57BL/6 background that is predisposed to developing spontaneous EAE. $CD4^+$ T cells from 2D2-transgenic mice express a TCR that recognizes $MOG_{35–55}$ (7). $MOG_{35–55}$ restimulation of splenocytes from 2D2 mice during EAE potently induced IL-2 and IFN-γ production by $CD4^+$ T cells (Fig. 4C). This pattern of cytokine secretion is not observed prior to the development of the spontaneous disease (data not shown). We further found that MOG-induced IFN-γ and IL-2 production can be potently inhibited with 3-BrPa (Fig. 4C). This is in line with literature showing that production of IFN-γ and IL-2 is regulated by glucose availability (32). In subsequent studies of spinal cord tissues isolated from 2D2 mice during EAE, we found that IFN-γ was profoundly sensitive to treatment with 3-BrPa, whereas IL-17A was only

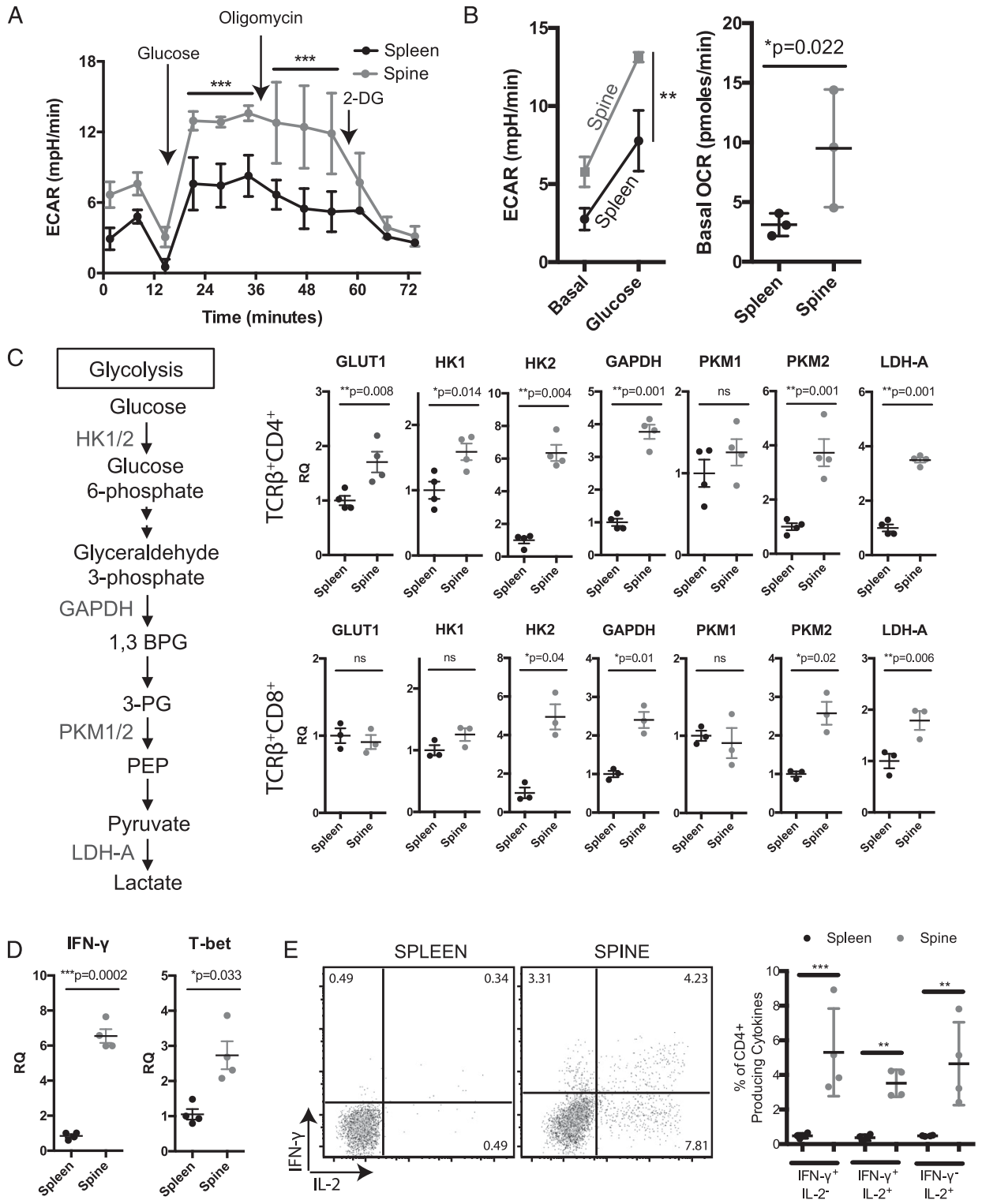

FIGURE 1. T cell glycolytic activity and inflammation are potentiated in the EAE CNS. (**A**) Glycolysis stress test and extracellular acidification rate (ECAR) of TCRβ$^+$ CD4$^+$ T cells isolated from the spleens and spinal cords of mice at peak EAE (n = 3 biological replicates per group). ***$p < 0.001$, two-way ANOVA with Sidak posttest. (**B**) Average glycolytic rates at baseline (basal) and after glucose stimulation (glucose) and basal oxygen consumption rate (OCR) from Seahorse data in (A). **$p < 0.001$, two-way ANOVA, *$p < 0.05$, paired Student t test. (**C**) Glycolysis diagram and qRT-PCR for mRNA expression of glucose transporter 1 (GLUT1), hexokinase 1/2 (HK1/2), GAPDH, pyruvate kinase isoform m1/2 (PKM1/2), and lactate dehydrogenase A (LDH-A) in TCRβ$^+$CD4$^+$ and TCRβ$^+$CD8$^+$ T cells flow sorted from the spleens and spinal cords of mice at peak EAE. Significance was determined using a ratio-paired Student t test. Actin is used as the housekeeping reference. Each dot represents one mouse. Data shown in (A)–(C) are from n = 1 experiment. (**D**) IFN-γ and T-bet expression in cells from (*Figure legend continues*)

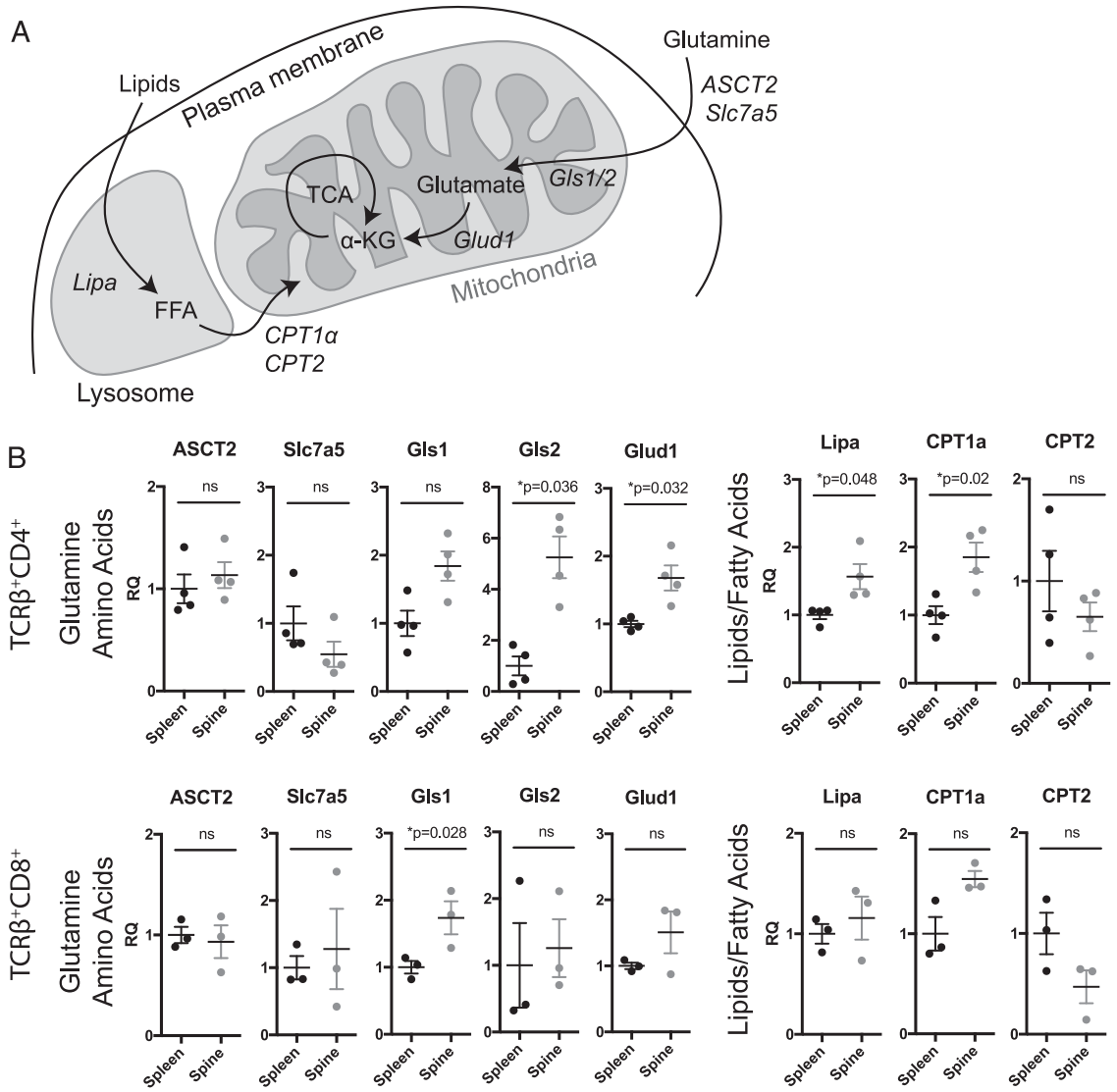

FIGURE 2. Regulation of nonglycolytic metabolism in T cells in the EAE spinal cord. (**A**) Schematic of nonglycolytic machinery assessed and their functions. (**B**) qRT-PCR for mRNA expression of ASC Amino acid transporter 2 (ASCT2), amino acid transporter L chain, L-system (Slc7a5), glutaminase 1 and 2 (Gls1/Gls2), glutamate dehydrogenase 1 (Glud1), carnitine palmitoyltransferase 1a and 2 (CPT1a/CPT2), and lysosomal acid lipase (Lipa) in $CD4^+$ and $CD8^+$ T cells ($CD45^+CD11b^-$ $TCR\beta^+$ $CD4/8^+$) isolated from spleen and spinal cord from mice at peak EAE. $^*p < 0.05$, paired Student *t* test Actin is used as the housekeeping gene. Each dot represents one mouse. ns, not significant; RQ, relative quantity.

moderately affected (Fig. 4D). These data suggest that, in two mouse models of MS, IFN-γ production by T cells during active disease is inhibited by 3-BrPa, but 3-BrPa is a less effective modulator of IL-17A production.

In vitro–derived Th1 and Th17 cells are differentially responsive to 3-BrPa

Defining the properties of glycolysis inhibitors toward Th1 and Th17 cells is critical, especially in the case of MS, in which patient stratification based on whether their disease is driven by Th1 or Th17 responses has been proposed as an important diagnostic criterion influencing subsequent treatment options (33). Our work with 3-BrPa suggests that glycolysis inhibition may manifest uniquely in different T cell types, a phenomenon that has been observed with other glycolytic inhibitors (14). To determine whether 3-BrPa differentially affects Th1 versus Th17 responses, we generated Th1 and Th17 cells in vitro using established protocols (16) and, after terminal differentiation was achieved, restimulated these T cells with anti-CD3 and anti-CD28 in the presence or absence of 3-BrPa. We found that 3-BrPa blocked Th1 and Th17 production of IFN-γ and IL-17A, as determined by ELISA and flow cytometry (Fig. 5A, 5C). Furthermore, this inhibition correlated with reduced expression of the transcripts for these cytokines (Fig. 5B). Interestingly, 3-BrPa treatment did not

(A)–(C). (**E**) MOG-induced IFN-γ and IL-2 production in splenic and spinal cord homogenates isolated from mice at peak EAE; gated on live, singlet, $CD45^+CD11b^-$ $TCR\beta^+$ $CD4^+$. Data are representative of $n = 2$ independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ by paired Student *t* test. RQ, relative quantity.

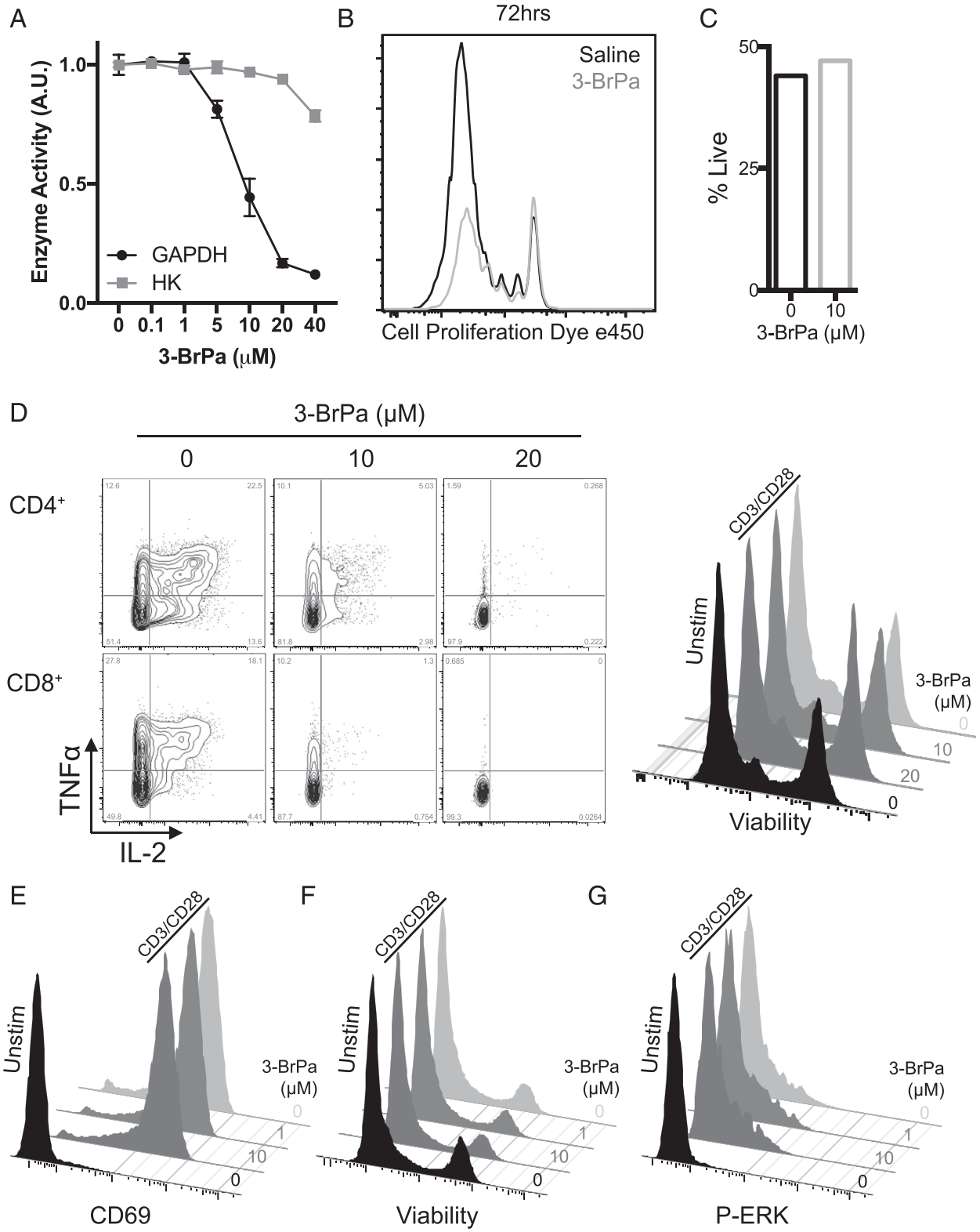

FIGURE 3. 3-BrPa is an inhibitor of T cell effector functions. (**A**) Dose-dependent inhibition of GAPDH and hexokinase activities in a lymph node single-cell suspension by 3-BrPa. (**B**) Effect of 3-BrPa on T cell proliferation. Lymph node cells were stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (10 μg/ml). At 24 h, a time when glycolysis is engaged but no cell divisions have occurred, 3-BrPa was added to a final concentration of 10 μM, and subsequent divisions were assessed after 48 additional hours. Proliferation of $CD8^+$ T cells is shown. Gated on live, singlet $CD8^+$ cells. (**C**) Effect of increasing concentrations of 3-BrPa on TNF-α and IL-2 production in T cells stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml). Gated on live, $CD11b^-$ $CD19^-$ $TCR\beta^+$ $CD4^+$ or $CD8^+$ cells. (**D**) Effect of 3-BrPa on CD69 induction on T cells after 6 h of activation with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml). Gated on live, singlet, $CD11b^-$ $B220^-$ $CD4^+$ cells. (**E**) Effect of 3-BrPa on CD69 induction, (**F**) viability, and (*Figure legend continues*)

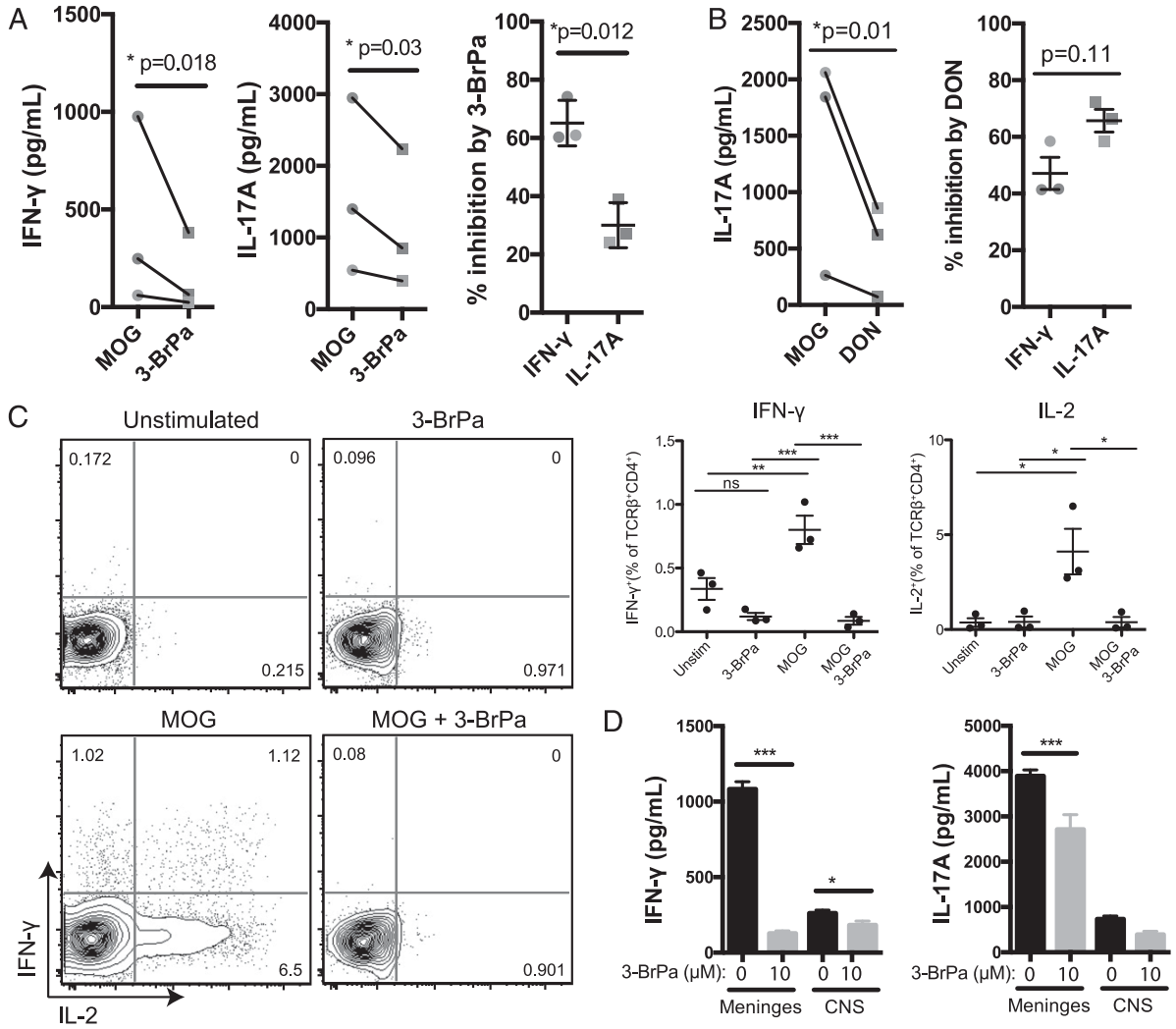

FIGURE 4. 3-BrPa is a potent inhibitor of IFN-γ, but not IL-17A, production in EAE spinal cords. (**A**) Effect of 3-BrPa on MOG-induced IFN-γ and IL-17A production in spinal cord homogenates from mice at peak EAE; a representative of $n = 3$ experiments with three to five mice is shown. Each dot is a mouse. (**B**) Effect of DON (1 mM) on IFN-γ and IL-17A production in spinal cord homogenates from mice at peak EAE; $n = 1$ experiment with $n = 3$ mice per group. (**C**) Flow cytometry of IFN-γ and IL-2 production by splenic $CD4^+$ T cells from 2D2 mice with spontaneous EAE restimulated with MOG peptide. $n = 1$ experiment with $n = 2$–3 mice per group. Each dot is a mouse. Gated on live, singlet, $TCR\beta^+$ $CD4^+$ cells. (**D**) Effects of MOG-induced IFN-γ and IL-17A production in meningeal and spinal cord parenchymal homogenates from 2D2 mice that developed spontaneous EAE. $n = 1$ experiment with $n = 1$ mouse. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA with Tukey posttest. ns, not significant.

result in altered regulation of T-bet or RORγt transcript or protein levels over the course of our study (data not shown). Importantly, in these experiments, we also observed that 3-BrPa–mediated inhibition of IL-17A production by Th17 cells was less effective than the inhibition of IFN-γ production by Th1 cells. These data support our earlier observations and provide further evidence for a direct and differential effect of 3-BrPa on $CD4^+$ T cells of different lineages.

3-BrPa limits the pathogenicity of encephalitogenic Th1 cells

Having determined that T cells in the demyelinating CNS have an elevated glycolytic profile and that inflammatory responses are sensitive to 3-BrPa treatment, we wanted to determine how 3-BrPa would affect the engagement of encephalitogenic programs in immune cells. To do this, we used the adoptive transfer model of EAE in immunodeficient hosts ($Rag1^{-/-}$). We prepared immune cells from the spleen of 2D2 mice and skewed them to promote generation of IFN-γ–producing Th1 cells. Cells were treated at days 0 and 2 with 10 μM 3-BrPa or vehicle (saline) and transferred at day 3 to $Rag1^{-/-}$ mice. This treatment paradigm with 3-BrPa did not affect T cell viability (Fig. 6A), but it was sufficient to significantly inhibit IFN-γ production (Fig. 6B). Importantly, we did not observe a difference in T-bet expression, suggesting that Th1 differentiation was not affected (Fig. 6C). Mice receiving saline-treated cells were uniformly moribund by day 20 post-transfer (Fig. 6D). Treatment with 3-BrPa reduced disease inci-

(**G**) phosphorylation of ERK in $TCR\beta^+$ $CD4^+$ T cells stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) for 60 min. Gated on singlet, $TCR\beta^+$ $CD4^+$ cells. The same results were observed in $CD8^+$ T cells. (A, E, and F) $n = 2$ experiments with $n = 1$–2 mice per experiment. Data in (B)–(D) are representative of $n = 2$–3 independent experiments. (G) $n = 1$ experiment.

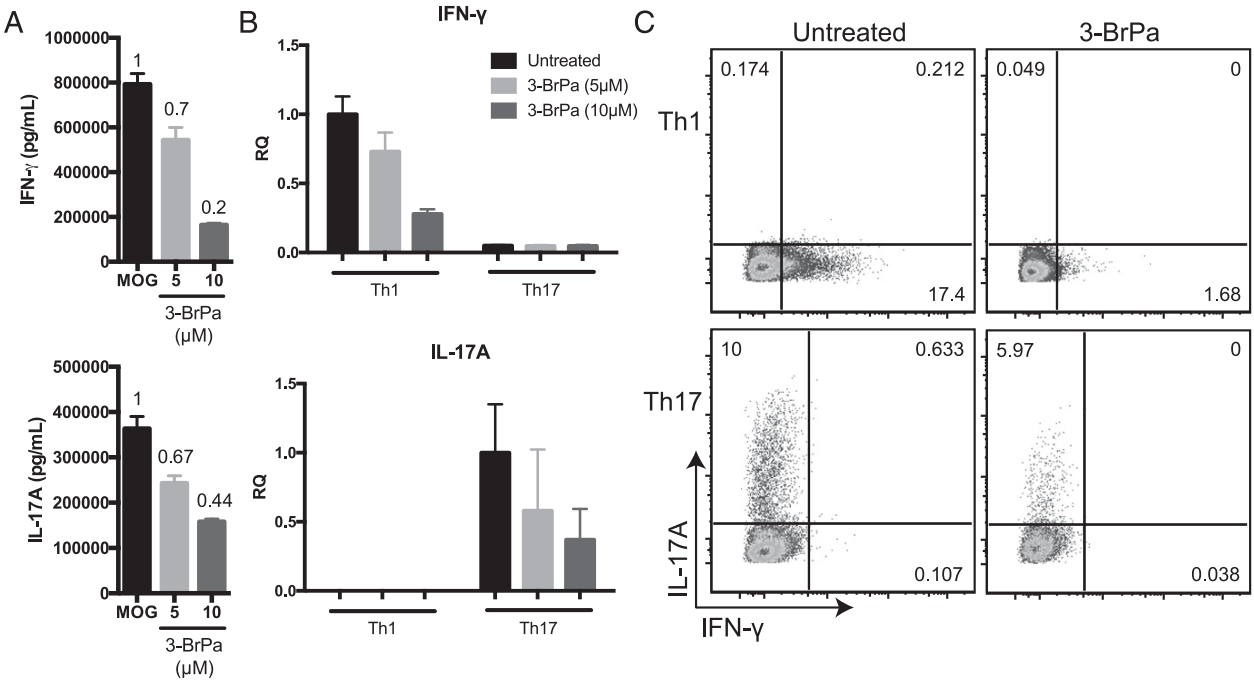

FIGURE 5. 3-BrPa is a more selective inhibitor of Th1 compared with Th17 cytokine production. ELISA (**A**) and qRT-PCR (**B**) for IFN-γ and IL-17A production by in vitro–derived Th1 and Th17 cells stimulated with anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml) in the presence of 3-BrPa (10 μM) for 20 h. (**C**) Flow cytometry for IFN-γ and IL-17A production by in vitro–derived Th1 and Th17 cells stimulated as above for 6 h in the presence of brefeldin A. Gated on live, singlet, TCRβ$^+$, CD4$^+$ cells. All data are from $n = 1$ experiment with $n = 1$–2 mice per group. Numbers above bars in (A) represent fold cytokine concentration relative to untreated condition. ns, not significant; RQ, relative quantity.

dence, and those mice that did develop EAE experienced a delayed disease course (Fig. 6D). We performed Luxol Fast Blue staining at day 20 and found a decrease in demyelination in mice from the 3-BrPa group (Fig. 6E). We noticed a significant spread in the 3-BrPa group that is probably linked to the decrease in disease incidence observed within this group (Fig. 6D). Logically, we found a decrease in CD3$^+$ cells in the 3-BrPa group at all of the spinal cord levels examined (Fig. 6F). To test whether 3-BrPa could be used to reduce EAE severity, we administered it at disease onset. We discovered that systemic administration of 3-BrPa (10 mg/kg) at the onset of the EAE was associated with severe mortality and hypothermia (data not shown). These data collectively show that pharmacologic inhibition of encephalitogenic immune cells could ameliorate the onset and severity of multifocal demyelinating events, highlighting the potential for developing a means of continuously targeting immune cell glycolysis as a therapy for autoimmune demyelination, especially in the context of IFN-γ–driven disease.

Discussion

Blocking immune infiltration of the CNS is a common and effective strategy to lower the relapse rate in MS. However, this approach fails to block ongoing responses in the CNS. Perhaps for this reason, all U.S. Food and Drug Administration–approved MS treatments lower relapse rates, yet none prevent disease progression (4, 5). Therefore, understanding what fuels the immune response in the demyelinating CNS is of critical importance in revealing the potential of metabolic-based therapeutics that could prevent disease progression.

Although many studies have shown that energy-derivation pathways are important regulators of immune responses, reliance on individual nutrient requirements in disease-specific contexts remains unclear. Publications on the metabolic status of T cells in the demyelinating CNS have established that in vitro–derived T cells require glycolysis to differentiate into a proinflammatory phenotype that can drive EAE (11–14) and that these T cells maintain some aspects of glycolytic character after reisolation from the spinal cord in transfer models of EAE and ex vivo culture (14). These seminal works have led to the speculation that pharmacologic or genetic manipulation of T cell glycolytic metabolism could lead to improved patient outcomes in MS. However, no study has actually determined the metabolic state of T cells isolated from sites of ongoing CNS demyelination. Given that the metabolic characteristics of T cells generated in vitro can be strikingly different from those generated in vivo (10), this knowledge gap is significant.

In this article, using multiple models of EAE, we provide a comprehensive metabolic characterization of T cells orchestrating CNS demyelination. T cells isolated from the CNS of mice with actively induced EAE have a robustly elevated glycolytic transcriptional and metabolic profile in the CD4$^+$ and CD8$^+$ T cell compartments. Interestingly, CD4$^+$ T cells seem to more strongly regulate their ability to consume nonglucose metabolic substrates, indicating that CD8$^+$ T cells may be more vulnerable to glycolytic inhibition. We have also made similar observations through analysis of T cells isolated from the spinal cords of 2D2 mice that develop spontaneous EAE (S.M. Seki and A. Gaultier, unpublished observations). It is important to note that the goal of our study is not simply to compare the metabolic state of active versus inactive T cells generated in vivo but, rather, to describe the unique metabolic properties and vulnerabilities of T cells that are specifically found in the EAE spinal cord.

Aerobic glycolysis or the "Warburg effect" is also a hallmark of cancer cell metabolism, and the pursuit of novel cancer therapeutics has identified a plethora of nonclassical metabolic inhibitors (34, 35). However, in many cases, experience with these

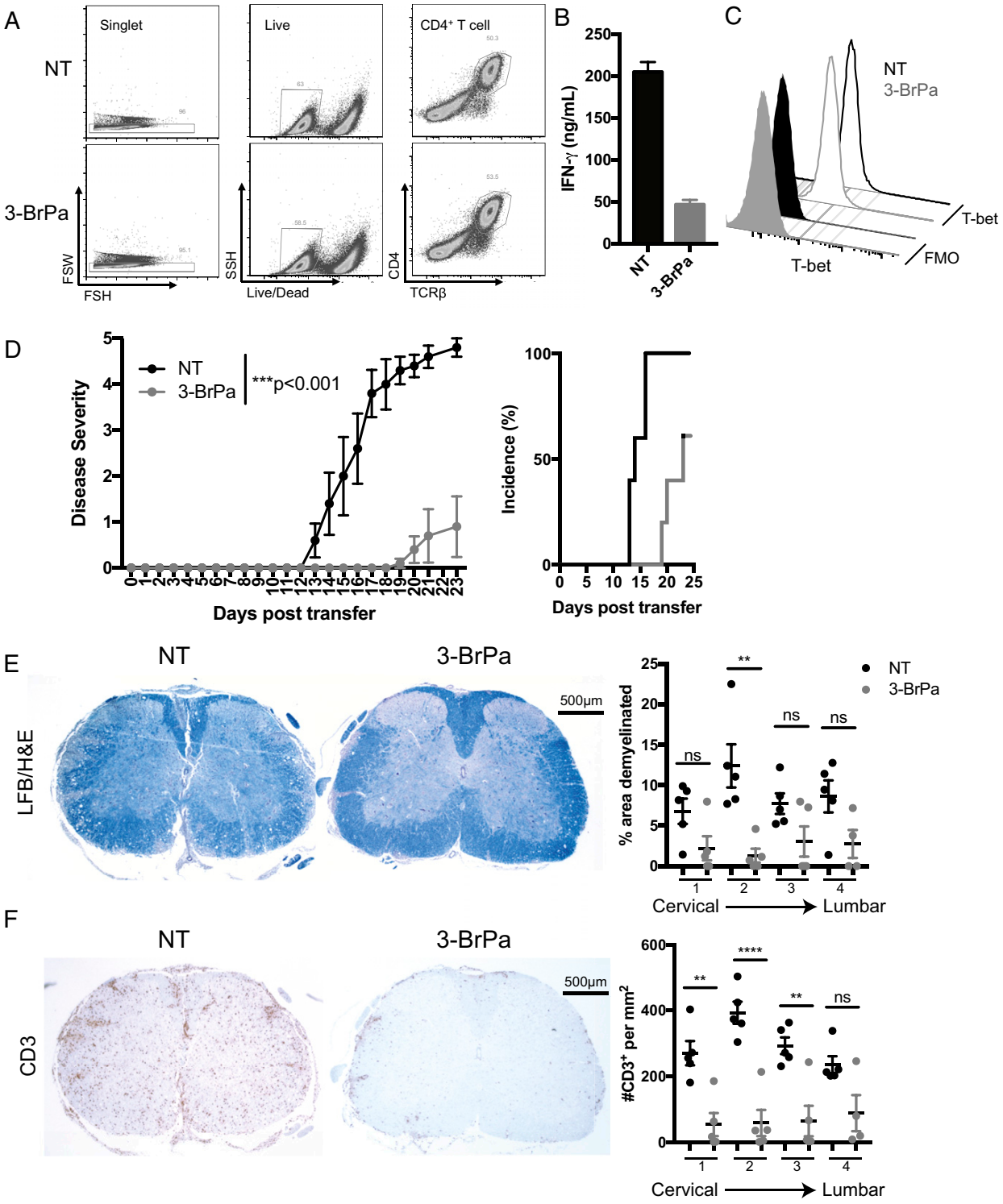

FIGURE 6. 3-BrPa attenuates the encephalitogenic programming of immune cells. Splenocytes were isolated from 2D2 mice and maintained in Th1-promoting conditions in the presence of $MOG_{35–55}$ peptide (50 μg/ml) for 3 d. 3-BrPa or saline was added at days 0 and 2. Cell viability (**A**), IFN-γ production (**B**), and T-bet expression (**C**) were measured prior to adaptive transfer at day 3. (**D**) Disease severity and incidence of EAE in $Rag1^{-/-}$ mice (n = 5 mice group, two independent experiments). $***p < 0.001$, two-way ANOVA with Sidak posttest. Spinal cord sections of the saline and 3-BrPa groups were stained with Luxol Fast Blue (**E**) and CD3 (**F**) (n = 5 for each group). Percentage of demyelination and cells number were quantified manually (right panel). $**p < 0.01$, $****p < 0.0001$, one-way ANOVA with Sidak posttest. ns, not significant.

drugs in immunological systems is lacking. One such drug is 3-BrPa, a robust GAPDH inhibitor that is currently being tested in preclinical cancer research (28, 36). We found that 3-BrPa is a potent inhibitor of T cell GAPDH and, unexpectedly, that this inhibition had lineage-specific effects on T cell populations. Production of TNF-α and IL-2 by Ag-inexperienced T cells is

robustly inhibited by 3-BrPa, as is IFN-γ production by Th1 cells. In contrast, IL-17A production by Th17 cells is only mildly affected by 3-BrPa. Given that MS patients can be stratified into those with IFN-γ–driven disease and those with IL-17A–driven disease and that treatment regimens may soon be tailored according to this stratification (33), our experiences with 3-BrPa may have important implications. The thorough evaluation of diverse antiglycolytics, such as 3-BrPa, and how they impact immune cells will be critical for developing a diverse array of metabolically focused MS therapeutics that can be rationally deployed to accommodate patient-specific heterogeneity in disease. Further studies will be needed to understand the metabolic status of in vivo–derived pathogenic Th17 cells and their reliance on GAPDH activity and glycolysis for effector functions.

It is important to note that 3-BrPa has been also reported to inhibit hexokinase II, another enzyme of the glycolysis pathway (27). However, at the concentration that was used for our study, we were unable to detect inhibition of hexokinase activity by 3-BrPa (Fig. 3A). Furthermore, 3-BrPa has also been documented as an inhibitor of other metabolic enzymes involved in glycolysis and the TCA cycle, including 3-phosphoglycerate kinase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase (37). Finally, due to the chemical nature of the inhibitor, 3-BrPa has been documented as an alkylating agent able to target the free thiol group (28). Therefore, although we hypothesize that 3-BrPa mediates its biological effect on lymphocytes by inhibiting GAPDH, it is very likely that 3-BrPa function is not limited to this enzyme. Further studies are needed to fully understand the scope of 3-BrPa's impact on T cell biology.

Previous work has shown that sustained glycolysis inhibition during MOG-reactive T cell differentiation in vitro can prevent disease by Th17 cells (11), but it left unanswered whether glycolytic inhibition could derail a Th1 cell–mediated encephalitogenesis. In adoptive-transfer models of EAE, we have determined that 3-BrPa significantly attenuated the pathogenicity of Th1-driven EAE. Systemic administration of 3-BrPa carries the risk for toxicity, as does the administration of many other broad-spectrum glycolytic inhibitors. Indeed, we did observe severe side effects when we administered 3-BrPa systemically in EAE (mortality and hypothermia), suggesting that 3-BrPa would not be a proper choice of drug to treat MS patients. This result is in contrast with recent work demonstrating that 3-BrPa was well tolerated and beneficial in an animal model of arthritis (38), perhaps highlighting some uniqueness about the CNS. The parallel development of well-tolerated drugs that can specifically target the metabolic demands of immune cells could result in a wave of novel biologics to treat inflammatory disease.

Understanding what fuels tissue-specific autoimmune responses is a critical next step in the treatment of autoinflammatory diseases. This study definitively implicates glucose utilization by T cells in CNS tissues as a key contributor to pathology in autoimmune CNS-demyelinating processes, like MS. Studies of T cell metabolic properties in the target organs of autoimmune processes, as well as those of in vitro–derived T cells, were instrumental in helping to guide this work (39). The results of our studies build upon these by identifying the metabolic characteristics of T cells in a microenvironment long known specifically for its unique modulation of T cell responses, the CNS, specifically in the context of autoimmune demyelination.

Disclosures

The authors have no financial conflicts of interest.

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APPENDIX B

Exploring non-metabolic functions of glycolytic enzymes in immunity

frontiers in Immunology

MINI REVIEW
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Exploring Non-Metabolic Functions of Glycolytic Enzymes in Immunity

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At the beginning of the twentieth century, discoveries in cancer research began to elucidate the idiosyncratic metabolic proclivities of tumor cells (1). Investigators postulated that revealing the distinct nutritional requirements of cells with unchecked growth potential would reveal targetable metabolic vulnerabilities by which their survival could be selectively curtailed. Soon thereafter, researchers in the field of immunology began drawing parallels between the metabolic characteristics of highly proliferative cancer cells and those of immune cells that respond to perceived threats to host physiology by invading tissues, clonally expanding, and generating vast amounts of pro-inflammatory effector molecules to provide the host with protection. Throughout the past decade, increasing effort has gone into elucidating the biosynthetic and bioenergetic requirements of immune cells during inflammatory responses. It is now well established that, like tumor cells, immune cells must undergo metabolic adaptations to fulfill their effector functions (2, 3). Unraveling the metabolic adaptations that license inflammatory immune responses may lead to the development of novel classes of therapeutics for pathologies with prominent inflammatory components (e.g., autoimmunity). However, the translational potential of discoveries made toward this end is currently limited by the ubiquitous nature of the "pathologic" process being targeted: metabolism. Recent works have started to unravel unexpected non-metabolic functions for metabolic enzymes in the context of inflammation, including signaling and gene regulation. One way information gained through the study of immunometabolism may be leveraged for therapeutic benefit is by exploiting these non-canonical features of metabolic machinery, modulating their contribution to the immune response without impacting their basal metabolic functions. The focus of this review is to discuss the metabolically independent functions of glycolytic enzymes and how these could impact T cells, agents of the immune system that are commonly considered as orchestrators of auto-inflammatory processes.

Keywords: immunometabolism, inflammation, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate kinase, lactate dehydrogenase, glycolysis

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INTRODUCTION

Upon activation, T cells increase biomass, proliferate, and produce inflammatory cytokines—processes that are bioenergetically and biosynthetically demanding, and likewise, necessitate a conversion from a relatively quiescent metabolism (2–5). One mechanism by which this is accomplished is through elevated glycolytic flux. As a result, many groups are pursuing the promise of anti-glycolytic therapy for inflammatory indications (6, 7). Conversely,

there is also interest in interventions to restore T cell metabolism in diseases of pathologic immunosuppression (e.g., cancer) (8–10). Intriguingly, many glycolytic enzymes serve moonlighting functions in the cell that can impact the nature and quality of an inflammatory response. Such idiosyncrasies may represent exploitable opportunities by which immune responses may be therapeutically modulated. The goal of this review is to present non-metabolic functions of glycolysis enzymes and the ways in which these idiosyncrasies may be exploited to impact inflammatory responses, particularly those of T cells.

GLYCOLYSIS ENZYMES AND THEIR ROLES IN INFLAMMATION

Hexokinase II (HK-II)

Hexokinase is the first enzyme involved in glycolysis, catalyzing the phosphorylation of glucose to glucose 6-phosphate (G6P) (**Figure 1**). Induction of HK-II, one of four isoforms of hexokinase, appears to be tightly linked to activation of inflammatory programs in immune cells (11, 12) and tumorigenic programs in cancer cells (10). Phosphorylated AKT stabilizes the localization of HK-II to the outer mitochondrial membrane (OMM). At this location, mitoHK-II has increased access to mitochondrially derived ATP, which it can then use to phosphorylate glucose to G6P, thereby trapping glucose in the cell (13). MitoHK-II also plays an anti-apoptotic role, preventing the formation of the mitochondria permeability transition pore by Bcl-2 family proteins like Bax (14, 15). The mechanism behind this process involves PI3K-AKT-mediated phosphorylation of Thr473 in HK-II, a modification that prevents G6P-mediated dissociation of HK-II from the mitochondria (16). Thus, posttranslational modifications to HK-II both facilitate its activity as a glycolytic enzyme and promote its anti-apoptotic functions.

Upon activation, immune cells upregulate HK-II (17) as well as other HK family members (18). HK-targeted interventions block glycolysis, effector function, and survival of cells involved in driving inflammatory responses (6), and for myeloid cells, this is especially true in the context of gram-negative bacterial challenges (19). However, this may not be true of all inflammatory responses. *N*-acetylglucosamine, a peptidoglycan derivative from the cell wall of Gram-positive bacteria, has recently been shown to bind HK-II and promote its dissociation from the OMM. This dissociation results in the accumulation of mitochondrial DNA in the cytosol and NLRP3 inflammasome-dependent production of mature IL-1β and IL-18 in macrophages (20). Thus, while dissociation of HK-II from the OMM might, on the one hand, abrogate the efficiency of flux through the glycolytic cascade and thus block inflammation, on the other hand, it may potentiate signals that promote secretion of major soluble transducers of inflammation depending on context. Inflammasome components (21, 22), hexokinase (6), and mitochondrial dynamics (23) are all known modulators of T cell functions; however, whether or not HK relocalization can induce inflammasome activity in T cells, and what consequences this may have, remains unclear.

Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH)

Glyceraldehyde 3-phosphate dehydrogenase is the enzyme that catalyzes conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate in glycolysis (**Figure 1**). GAPDH is well known for its numerous non-metabolic functions. In many bacteria, GAPDH is a major component of the cell surface. Multiple mechanisms are involved in this localization of GAPDH, including active transport (24) and lysis-mediated release of GAPDH which then decorates the surface of neighboring bacterial cells (25). Cell surface GAPDH binds fibronectin,

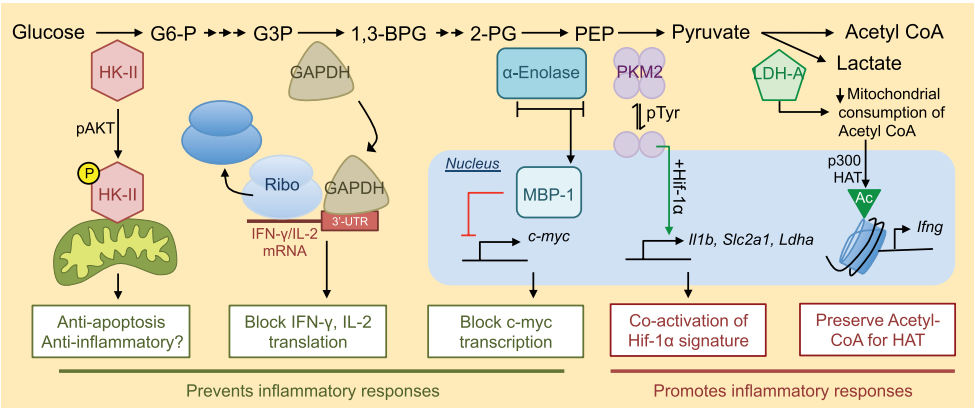

FIGURE 1 | Non-metabolic functions of glycolytic enzymes and their roles in inflammation. Many pieces of glycolytic machinery have non-metabolic functions that can contribute to the inflammatory response. An abridged version of the glycolytic cascade is listed with enzymes depicted at their appropriate level in glycolysis along with their alternative non-metabolic functions. For a more complete view of the glycolytic cascade, please see Ref. (17). G6-P, glucose 6-phosphate; G3P, glyceraldehyde 3-phosphate; 1,3-BPG, 1,3-bisphosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; Ribo, ribosome; Slc2a1, gene encoding glucose transporter 1 (Glut-1); HAT, histone acetyltransferase.

plasminogen, and other tissue components (24–26) and is an important facilitator of bacterial adherence to and invasion of host tissues. These findings translate to eukaryotic systems. In response to inflammatory cues, macrophages recruit GAPDH to the cell surface where it functions as a plasminogen receptor. In this paradigm, plasminogen bound to GAPDH digests extracellular matrix thereby facilitating macrophage migration (27). GAPDH can also localize to numerous other subcellular compartments (28). For example, oxidative stress, as occurs during neutrophil respiratory burst, drives *S*-nitrosylation of GAPDH (29), redistributing it from the cytoplasm to the nucleus and mitochondria where it is broadly implicated as a regulator of cell survival [reviewed in Ref. (28)]. GAPDH itself has been shown to have anti-inflammatory properties, as systemic administration of GAPDH prior to LPS-induced sepsis reduces cytokine storm and mortality (30), though the mechanism of this immunomodulatory effect remains unknown.

Recent work in T cells implicates GAPDH as an energy sensor that regulates translation of inflammatory cytokine mRNA in response to the availability of glucose in the cell. When glucose concentrations are low, GAPDH binds to the AU-rich elements in the 3′-untranslated region (UTR) of mRNA, including those encoding interferon gamma (IFN-γ) and IL-2 (31, 32). Binding of GAPDH to these transcripts represses their translation, thus restricting cytokine production during glucose deprivation. 3′AU-rich elements are not unique features of IFN-γ and IL-2 mRNA, and it is likely that GAPDH can regulate translation beyond these two cytokines (33). The glycolytic reaction catalyzed by GAPDH requires nicotinamide adenine dinucleotide (NAD^+), an essential indicator of cellular redox state, and intriguingly, Nagy and colleagues identified the NAD^+ binding fold of GAPDH as its RNA-binding domain (34). This finding suggests any NAD^+-dependent enzyme [in glycolysis, this is GAPDH and lactate dehydrogenase (LDH)] may be endowed with RNA-binding capabilities. Glucose deprivation, however, increases levels of intracellular NAD^+ which might be expected to compete with GAPDH for RNA binding (35). Thus, there are likely additional layers of regulation governing the role of GAPDH as a translational repressor that functions during glucose deprivation and or in response to fluctuations in NAD^+. Context-specific nuances that influence how NAD^+ affects the mRNA-binding functions of glycolytic machinery offer an intriguing line of inquiry into the interplay between metabolism and the many fundamental processes (36, 37) regulated by NAD^+.

α-Enolase

α-Enolase catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate (PEP) in glycolysis (**Figure 1**). The gene that encodes α-enolase (*Eno1*) produces a single transcript with two translational start sites. Depending on the site of translation initiation, *Eno1* can generate a full-length canonical α-enolase (48 kDa) enzyme that participates in glycolysis, or a truncated version of α-enolase (37 kDa), also known as Myc promoter-binding protein 1 (MBP-1) that represses the pro-proliferative transcription factor c-myc (38–41). Wang and colleagues identified c-myc as the master regulator of metabolic adaptation in T cells (17), demonstrating impaired growth and proliferation in c-myc deficient T cells treated with mitogenic stimuli. MBP-1 represses c-myc by binding to and inhibiting formation of the transcription initiation complex at the c-myc promoter (40, 41). Whereas α-enolase localizes to the cytoplasm, MBP-1 preferentially traffics to the nucleus where it serves these repressive functions (38). The signals that influence differential translation of α-enolase versus MBP-1 are unclear, though hypoxia may be one cue that favors translation of full-length α-enolase (42). The internal translation start site that generates MBP-1 off of *Eno1* is not present in β or γ-enolase, potentially providing an added layer of specificity for future MBP-1 modulating interventions.

Intriguingly, it seems that the induction of MBP-1 functionally impacts T cell inflammatory responses in the context of autoimmunity. A recent study (43) revealed that an anti-inflammatory population of human $CD4^+$ T cells, known as regulatory T cells (Tregs), expresses high levels of MBP-1. Moreover, MBP-1 in Tregs potentiates transcription of a specific spliced isoform of FoxP3 known to potently suppress inflammatory immune responses, particularly those mediated by the transcription factor RAR-related orphan receptor gamma T (RORγT). RORγT is a known driver of IL-17A (44) and granulocyte macrophage colony stimulating factor (GM-CSF) (45), pro-inflammatory cytokines strongly associated with auto-inflammatory diseases (46–48), and the therapeutic potential of its inhibition is under investigation for numerous inflammatory indications (49, 50). Interestingly, Tregs seem to elevate expression of both *Eno1* gene products, suggesting that the suppressive effects of MBP-1 may dominate over metabolic contributions to inflammation facilitated by full-length α-enolase or elevated glycolysis (43, 51). Thus, inducing transcriptional activity at *Eno1* may be sufficient to increase MBP-1 protein levels to immunosuppressive levels without blocking glycolysis. How the α-enolase/MBP-1 axis affects conventional T cell responses is unclear. Taken together, whereas *Hk2* encodes a single protein that can play metabolic and non-metabolic roles in a cell, *Eno1* encodes two gene products that differ drastically in their contributions to metabolism and inflammation (38, 39).

Pyruvate Kinase (PK) Isoform M2

Pyruvate kinase is the ATP-generating enzyme that catalyzes the conversion of PEP to pyruvate during glycolysis (**Figure 1**). Four isoforms of the PK enzyme exist, with the M1 (PKM1) and M2 (PKM2) isoforms being most predominant in leukocytes of the adult animal (52). PKM2 is the major isoform expressed at the protein level by lymphocytes (52). Interestingly, many cancer cell lines also exclusively express PKM2 (53), and cancer researchers have likewise identified many pro-proliferative and non-canonical functions that are specifically attributed to this particular isozyme (54–63). PKM1 and PKM2 are alternatively spliced isoforms of the PK enzyme that differ by inclusion of a single exon (exon 9 for PKM1 versus exon 10 for PKM2), of which only 22 amino acid residues differ (64). The structures of PKM1 and PKM2 are extremely similar (65), but importantly, the minute difference in amino acid sequence allows PKM2 to uniquely contribute to proliferative responses in cancer cells and inflammatory responses of immune cells (66–69). Whereas PKM1 exists solely as a tetramer that functions as a glycolytic

enzyme, PKM2 can exist as a tetramer with similar functions as PKM1 or as a dimer that loses activity as a glycolytic enzyme, but can perform numerous other non-glycolytic functions in the cell. From the perspective of glycolysis, this dynamic feature of PKM2 reduces its efficiency as a glycolytic enzyme and allows for the accumulation of upstream glycolytic intermediates, thereby promoting *de novo* amino acid and lipid biosynthesis—processes that are critical for the production of a daughter cell (70). From the perspective of inflammation, the PKM2 dimer can localize to the nucleus (58) where it is a well-known co-activator of Hif-1α gene signatures (54, 66, 67). In macrophages, this interaction is critical for the appropriate transcriptional activation of metabolic machinery, such as lactate dehydrogenase A (LDH-A) and pro-inflammatory cytokines, such as IL-1β (66). Similarly, signal transducer and activator of transcription 3 (STAT3) (55) and the aryl hydrocarbon receptor (AhR) (71) also require interaction with PKM2 for appropriate DNA binding. Thus, the PKM2 dimer seems to play a unique role as a direct modulator of proliferative and inflammatory programs. Relating to T cells, AhR, STAT3, and Hif-1α are all well-known regulators of Th17 cell differentiation perhaps implicating PKM2 as a regulator of this cell type.

Many groups in cancer research (56, 57, 60) and immunology (66–69, 72) are exploring the therapeutic potential of enforcing PKM2 tetramerization with pharmacologic compounds (62, 73). The major endogenous driver of PKM2 tetramerization is fructose 1,6 bisphosphate (FBP) (65), the product of the phosphofructokinase-catalyzed step in glycolysis. Phosphotyrosine residues generated by growth factor signaling (57, 59) can bind to PKM2 and promote release of FBP, and along with post-translational modifications, such as PKM2 phosphorylation (74), oxidation (61), acetylation (58), and succinylation (75, 76), are endogenous drivers of tetramer dissociation. Synthetic activators of PKM2 tetramerization, originally characterized in cancer models as tumor-blocking agents (62), also potently block inflammation in numerous disease models (66, 67, 77). Thus, enforcing PKM2 tetramerization shows promise as a metabolic machinery-based paradigm for controlling inflammatory responses without overtly inhibiting metabolism itself.

Lactate Dehydrogenase A

Lactate dehydrogenase is a tetrameric enzyme variably composed of A and B subunits that, when combined, form a complex with the capability of converting pyruvate to lactate (**Figure 1**). This reaction is the defining step of aerobic glycolysis (78), the form of metabolism engaged by activated immune cells, which increase their regeneration of NAD^+ consumed during glycolysis by producing lactate regardless of environmental oxygen content (2, 3). Peng and colleagues (79) recently showed that T cells almost exclusively express the A subunits of LDH, which they further upregulate upon activation, and expression of LDH-A is critical for the proper production of the inflammation-promoting cytokine IFN-γ. They found that genetic ablation of LDH-A in T cells heightened consumption of glycolysis-derived acetyl-CoA through the tricarboxylic cylic acid cycle and depleting intracellular stores of this metabolic byproduct of glucose catabolism. This acetyl-CoA depletion impaired activation-induced permissive histone acetylations that are required for opening of the *Ifng* locus during T cell activation. These findings and others (80–82) suggest that metabolic adaptations like aerobic glycolysis are important (1) as a means of generating sufficient ATP and metabolic intermediates to support anabolic processes and (2) as drivers of the epigenetic changes that are responsible for facilitating engagement of the inflammatory program [reviewed in Ref. (83)]. In addition to its ability to indirectly modulate the epigenetic landscape of the activated T cell, there is evidence to suggest that LDH-A may also be capable of directly influencing inflammatory responses. In a manner reminiscent of direct repression of IFN-γ and IL-2 mRNA translation by the glycolytic enzyme GAPDH (31, 32), LDH-A has been reported to bind to 3′AU-rich elements in GM-CSF mRNA (84). It remains unclear how the mRNA-binding properties of LDH-A affects downstream protein expression and, additionally, if this non-metabolic function is related to the level of flux through the glycolytic cascade or enzymatic activity. Inflammatory T cells are major producers of GM-CSF, a prominent driver of autoimmune responses (45–47, 85, 86), and a detailed study elucidating the metabolic requirements for GM-CSF production *in vivo*, including how it may relate to LDH-A, is warranted.

THE RELATIONSHIP BETWEEN GLYCOLYSIS AND INFLAMMATION *IN VIVO*

Seminal *in vitro* studies defined the metabolic peculiarities of inflammatory T cell subtypes (87–89) and paved the way for future works assessing the impact of glycolytic manipulations on T cell-driven inflammation *in vivo* (**Figure 2**) (10, 12, 90–94). Recent studies, however, question the strength of the relationship between glycolysis and inflammation in the *in vivo* setting. Peripheral blood T cells isolated from patients with rheumatoid arthritis show defects in glycolytic flux, rather than elevated glycolysis (95, 96). Likewise, impaired glycolysis is also detected in peripheral blood T cells isolated from multiple sclerosis patients and type 1 diabetics (43). One potential explanation for these findings may be that T cells at sites of pathology may maintain a distinct metabolism from those in circulation. Alternatively, the metabolic signatures of immune cells generated *in vitro* versus *in vivo* may be fundamentally different, and investigating the similarities and differences between these cells could reveal aspects of the metabolism–inflammation relationship that are currently being overlooked (97). The study of Treg metabolism provides a great example of the discrepancies between *in vivo* and *in vitro*-derived cells. Whereas Tregs (Tregs) generated by standard *in vitro* protocols maintain a metabolic profile that favors mitochondrial respiration over aerobic glycolysis, Tregs isolated *ex vivo* seem to be profoundly glycolytic (51, 98), and this metabolic signature is proposed to favor their transcriptional activity at *Eno1* to produce α-enolase and MBP-1 (43). Indeed, the association between glycolytic flux and inflammation is likely not as clear *in vivo* as it is *in vitro*. Nevertheless, the non-metabolic functions of glycolytic machinery, including their relationship to inflammation, have been convincingly demonstrated *in vivo*

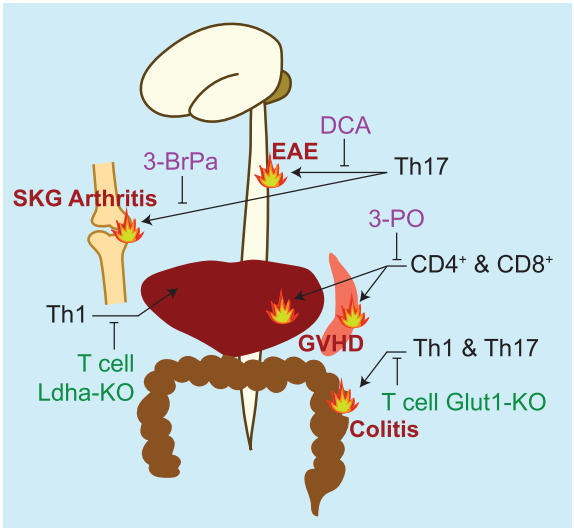

FIGURE 2 | Summary of studies targeting glycolytic machinery *in vivo* to treat pathologies with prominent inflammatory T cell contributions. Pharmacologic inhibitors of glycolysis are listed in purple. DCA, dichloroacetate, an inhibitor of pyruvate dehydrogenase kinase 1 (12); 3-BrPa, 3-bromopyruvate, an inhibitor of hexokinase and GAPDH (93); 3-PO, 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one, an inhibitor of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3, PFK2) (91). Genetic models targeting glycolytic machinery in T cells are listed in green. LDH-A, lactate dehydrogenase A (79); Glut-1, glucose transporter 1 (90). *In vivo* models of inflammation studied are experimental autoimmune encephalomyelitis (EAE)—a murine model of multiple sclerosis, SKG arthritis [a model of rheumatoid arthritis that spontaneously develops in the SKG strain of mice (99)]; graft versus host disease (GVHD), and colitis.

and represent intriguing therapeutic opportunities for the future development of metabolically focused interventions for inflammatory disease. Toward this end, it will be important to determine the non-metabolic functions of glycolytic enzymes in other systems. For example, the mitochondrial localization of HK-II is important for cardiomyocyte function and interventions at this level of glycolysis might be anticipated to impact the heart (15, 16). In the kidney, podocytes were recently shown to express high levels of PKM2, and the non-metabolic functions of the enzyme in this context appear to play essential disease-potentiating roles in the context of diabetic nephropathy (77). Indeed, an important step toward realizing the therapeutic potential of targeting the non-metabolic functions of glycolytic enzymes during inflammation is to better understand these functions within and beyond the context of immunity. Finally, just as particular inflammatory processes are often associated with a unique cytokine profile (e.g., TNF-α and rheumatoid arthritis or IL-17A and psoriasis), the metabolic proclivities and peculiarities of cells driving inflammation may also differ based on disease-specific contexts. Further investigation into the nuances of immune cell metabolism in the *in vivo* setting and how this relates to their inflammatory functions are needed to better elucidate and potentially target the relationship between metabolism and inflammation during disease.

CONCLUSION

The metabolic requirements that support immune-mediated inflammatory responses are well established *in vitro* and increasingly so *in vivo*. Elevated consumption of glucose plays an important role in inflammatory responses of T cells, where glycolytic processes can serve to generate ATP, produce metabolic intermediates that are important for anabolic processes and even alter the epigenetic landscape of the activated cell. To achieve this, activated immune cells must upregulate expression of metabolic machinery, many of which serve non-metabolic functions in the cell that are directly linked to modulating the inflammatory response. Research in cancer cells has led to the identification of many non-metabolic functions of glycolytic enzymes (100, 101), and only recently are these functions beginning to be assessed in the context of inflammation. Just as research into the metabolic activity of cancer cells provided the foundations for immunometabolic studies to identify the unique bioenergetic requirements of immune cell subsets, so too may the non-metabolic functions of glycolytic enzymes discovered in cancer cells instruct an alternative way of looking at the relationship between metabolism and inflammation. Importantly, this alternative approach may generate interventions that are more readily translatable to the clinical setting than therapies that overtly impinge on enzymatic activity of metabolic machinery.

In addition to those listed here, other isoforms of glycolytic machinery with known non-metabolic properties in cancer cells, such as phosphofructokinase-1 (102), seem to be selectively induced in immune cells in response to distinct stimuli. Determining how these contribute to the T cell inflammatory program is of interest. Conversely, activation-induced proteins that are not classically associated with metabolism, such as CD69 (103), may also play metabolic roles that are important for inflammatory immune responses. In addition, byproducts of metabolic processes, such as PEP (10), lactate (104, 105), succinate (19, 66, 106–108), citrate (109), 2-hydroxyglutarate (110), α-ketoglutarate (111), and others (102), are gaining increasing recognition for the non-metabolic roles they play as direct modulators of inflammation. Further exploration into the unique ways in which metabolic processes contribute to immune responses may reveal exploitable opportunities to destabilize the relationship between metabolism and inflammation for therapeutic benefit.

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Both the authors have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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