THE METABOLIC CHALLENGES OF IMMUNE CELLS IN HEALTH AND DISEASE

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THE METABOLIC CHALLENGES OF IMMUNE CELLS IN HEALTH AND DISEASE

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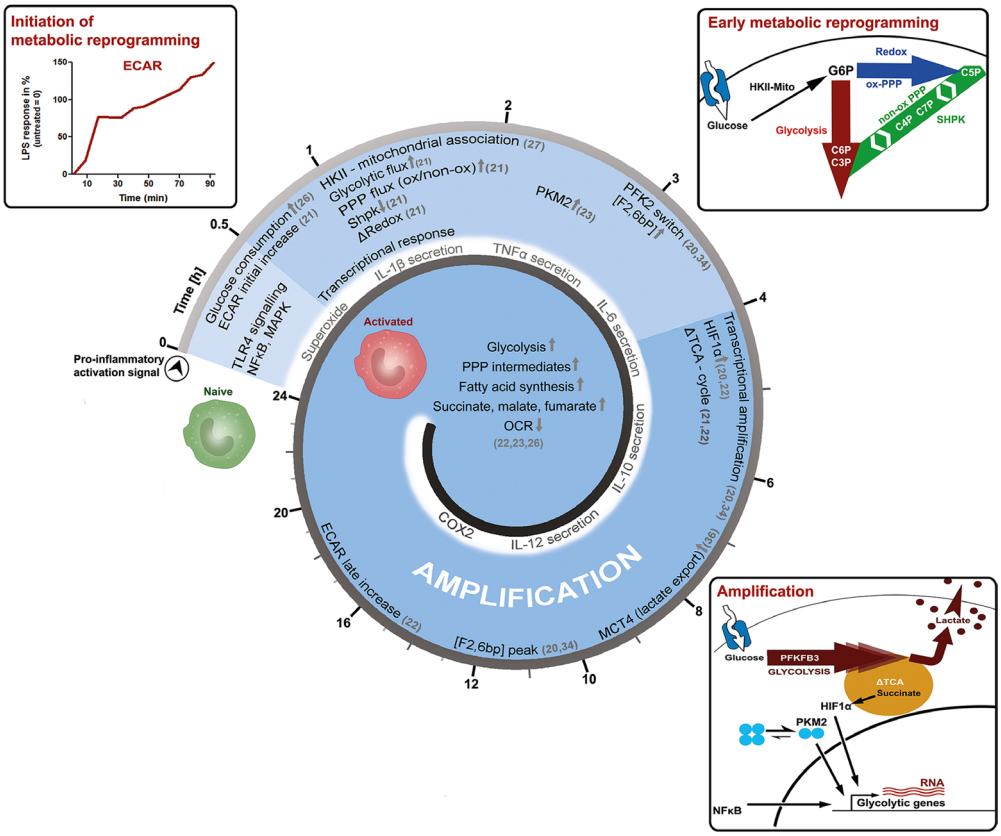
Obesity and its co-morbidities, including atherosclerosis, insulin resistance and diabetes, are a world-wide epidemic. Inflammatory immune responses in metabolic tissues have emerged as a universal feature of these metabolic disorders. While initial work highlighted the contribution of macrophages to tissue inflammation and insulin resistance, recent studies demonstrate that cells of the adaptive immune compartment, including T and B lymphocytes and dendritic cells also participate in obesity-induced pathogenesis of these conditions. However, the molecular and cellular pathways by which the innate and adaptive branches of immunity control tissue and systemic metabolism remain poorly understood.

To engage in growth and activation, cells need to increase their biomass and replicate their genome. This process presents a substantial bioenergetic challenge: growing and activated cells must increase ATP production and acquire or synthesize raw materials, including lipids, proteins and nucleic acids. To do so, they actively reprogram their intracellular metabolism from catabolic mitochondrial oxidative phosphorylation to glycolysis and other anabolic pathways. This metabolic reprogramming is under the control of specific signal transduction pathways whose underlying molecular mechanisms and relevance to physiology and disease are subject of considerable current interest and under intense study. Recent reports have elucidated the physiological role of metabolic reprogramming in macrophage and T cell activation and differentiation, B- and dendritic cell biology, as well as in the crosstalk of immune cells with endothelial and stem cells. It is also becoming increasingly evident that alterations of metabolic pathways play a major role in the pathogenesis of chronic inflammatory disorders.

Due to the scientific distance between immunologists and experts in metabolism (e.g., clinicians and biochemists), however, there has been limited cross-talk between these communities. This collection of articles aims at promoting such cross-talk and accelerating discoveries in the emerging field of immunometabolism.



Time and Demand in Immunometabolism. This schematic figure illustrates molecular events during immune cell activation (macrophages are shown in the schematic as an example) as dynamic processes, which together constitute the required cellular metabolic-reprogramming that is required to sustain specific bioenergetic- and precursor-demand during the various stages of immune cell activation. Time and demand appear not only as critical factors to understand the complex interplay of cellular processes like protein signaling, transcriptional regulation and the rearrangements of metabolic-flux but also to understand the combined mechanisms of metabolism and immunology in the entire organism in health and disease.

Nagy C and Haschemi A (2015) Time and demand are two critical dimensions of immunometabolism: the process of macrophage activation and the pentose phosphate pathway. Front. Immunol. 6:164. doi: 10.3389/fimmu.2015.00164

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EDITORIAL
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Editorial: The metabolic challenges of immune cells in health and disease

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Keywords: immunometabolism, metabolic disease, T cells, B cells, macrophages

Only few years ago, scientists had to struggle to convince audiences and editors that cell metabolism and biochemistry were not boring, let alone persuade the scientific community that alterations of the metabolic machinery could underpin human diseases (1). We hear from more senior scientists (we were only undergraduate students at the time, more often than not dreading our studies of glycolysis, Krebs cycle, electron transport chain, etc., on the "famous" Lehninger text-book) that publishing the first paper on c-Myc-mediated transcriptional control of the metabolic enzyme lactate dehydrogenase as a key mechanism for cancer transformation (2) or lymphocyte survival and activation via TCR-dependent regulation of nutrient uptake and utilization (3, 4) was not easy at all. Indeed, they had to overcome the preconception of a well-established scientific community that, for the last few decades, had believed in the supremacy of molecular biology and genetics as experimental tools for understanding cellular mechanisms and disease processes.

In 2015, metabolism is the heart of an ever growing body of studies, spanning the fields of cancer, stem cells, and, as highlighted in this series of review articles, immunology and metabolic diseases. This unexpected renaissance in the field of metabolism stands on the shoulders of giants. Indeed, scientists of the caliber of Warburg, Krebs, and Mitchell, just to name a few, spent their entire lives exploring the intricacies of cell metabolism. Not only had they elucidated the pathways for utilization of glucose and other nutrients for the generation of ATP but had also initiated the modern and fashionable concept of integration of metabolic processes with diseases and immune-regulation. As described by Nagy and Haschemi (5), Kempner and Peschel proposed the idea of a tight link between metabolism and inflammation, the modern so-called immunometabolism, as far back as 1930s. Unfortunately, the whole field of metabolism was relegated to the margins of modern research for long time, being considered irrelevant for addressing more important questions, such as how proliferation, differentiation, and cell death, are regulated in the cell. This obnubilation lasted until the realization that all these processes have distinct metabolic requirements and that impairing metabolism could perturb them. We now know that signaling pathways directly control specific metabolic pathways and enzymes, and vice versa, and even more astonishingly, that intermediates of metabolism, such as lactate or succinate, or metabolic enzymes (i.e., GAPDH or PFKFB3) can regulate gene expression, protein translation, or indeed entire processes, such as endothelial sprouting.

The studies on immunometabolism that we present here encompass both cellular and systemic aspects of disease. At a cellular level, immunometabolism studies show how intracellular metabolic pathways activated downstream of growth factors and cytokines control immune cell functions. On an organismal level, immunometabolism investigates how immune cells regulate the homeostasis of metabolic tissues and how they contribute to the process of metabolic diseases, including obesity and type II diabetes. This collection contains 10 review articles that cover important and emerging aspects in both of these branches of immunometabolism.

At the cellular level, Howie et al. (6) focus on the mechanisms of nutrient sensing in T cells and how these integrate with TCR and cytokine signals via the mTOR pathway to determine distinct differentiation pathways toward effector or regulatory T cell (Treg) subsets. Going deeper into the

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biology of Treg lymphocytes, Coe et al. (7) describe recent findings on the unique metabolic needs of Treg as compared to effector T cells (Teff), with a particular focus on mTOR-mediated control of metabolism in these T cell subsets. Schurich and Henson (8) discuss the emerging view that as a consequence of viral infection and antigenic load, $CD8^+$ T cells can become senescent or exhausted. These are distinct fates of a T cell, sustained by different metabolic programs, which in turn dictate opposing outcomes during immune responses. Nagy and Haschemi (5) illustrate the metabolic changes that take place in macrophages upon LPS-induced activation and polarization. They then focus on how the pentose phosphate pathway is regulated during LPS- versus IL-4-induced polarization of macrophages and may be of importance in the provision of both nucleotide precursors and redox-equivalents determining different cell fates and types of immune response. Finally, Jones and Bianchi (9) give an overview of some recent key examples of metabolic control of biological processes beyond cellular proliferation. In particular, the roles of intermediates of metabolism in the control of gene expression, of metabolic enzymes in the regulation of protein translation and cellular differentiation, and of aerobic glycolysis in epigenetic determination of trained immunity are discussed.

At a more systemic level, Wang et al. (10) offer their views on the possible interplay between tumor cells and immune cells in the tumor microenvironment. Tumor cells may compete for nutrients with some immune cells, thereby compromising their function. Tumor cells may also become metabolically symbiotic with other immune cells, which in turn may provide signals for tumor growth. Gerriets and MacIver (11) overview the well-established link between nutritional status and immune cell function. They pay particular attention to the signals linking nutrient stress to T cell metabolic adaptation and how this crosstalk may result in low-grade inflammation leading to metabolic syndrome as a consequence of obesity or increased risk of mortality by infectious diseases as a consequence of malnutrition. The connection between immune cells and metabolic diseases is further discussed by Capasso et al. (12), who ask questions about the possible crosstalk of B cells with the adipose tissue in homeostatic conditions and during obesity; however, this field is currently understudied, leaving us with more questions than answers. Continuing on the same issue, Mauro et al. (13) discuss the clinical evidence of the association that exists between increased incidence of obesity worldwide and increased prevalence and severity of cognitive disorders. They speculate that systemic metabolic imbalance can have direct consequences on the integrity and function of the blood–brain barrier, thereby leading to the insurgence of cerebrovascular and neurodegenerative pathologies; however, the mechanistic links are unknown at present. Finally, Tannahill et al. (14) discuss the importance of macrophages in the pathogenesis of multiple sclerosis, the metabolic changes behind macrophage polarization, and how macrophage metabolic re-education could be used in the future for the treatment of multiple sclerosis.

In conclusion, these are exciting times for the discovery of the many mechanisms of integration between metabolic and signaling pathways as ways to determine cell fates and types of immune response. These are also exciting times for those who are investigating the metabolic crosstalk between immune cells and stromal cells during homeostasis and in diseased tissues. We are hopeful that gaining deeper understanding of how metabolism and signaling pathways coordinate with each other will lead to new perspectives on disease mechanisms and, ultimately, to the development of novel therapeutic tools.

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PERSPECTIVE ARTICLE
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Time and demand are two critical dimensions of immunometabolism: the process of macrophage activation and the pentose phosphate pathway

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A process is a function of time; in immunometabolism, this is reflected by the stepwise adaptation of metabolism to sustain the bio-energetic demand of an immune-response in its various states and shades. This perspective article starts by presenting an early attempt to investigate the physiology of inflammation, in order to illustrate one of the basic concepts of immunometabolism, wherein an adapted metabolism of infiltrating immune cells affects tissue function and inflammation. We then focus on the process of macrophage activation and aim to delineate the factor time within the current molecular context of metabolic-rewiring important for adapting primary carbohydrate metabolism. In the last section, we will provide information on how the pentose phosphate pathway may be of importance to provide both nucleotide precursors and redox-equivalents, and speculate how carbon-scrambling events in the non-oxidative pentose phosphate pathway might be regulated within cells by demand. We conclude that the adapted metabolism of inflammation is specific in respect to the effector-function and appears as a well-orchestrated event, dynamic by nature, and based on a functional interplay of signaling- and metabolic-pathways.

Keywords: immunometabolism, inflammation, macrophage activation, metabolic reprograming, primary carbohydrate metabolism, pentose phosphate pathway, sedoheptulose kinase, time and demand

CONCEPTS OF IMMUNOMETABOLISM

The first concepts of immunometabolism date back to the pre-genomic age of biomedical research. As early as 1912, Levene and Meyer used dog blood-derived leukocytes to directly demonstrate that hexoses are converted into two molecules, each containing a chain of three carbons (1). They also provided further evidence that hexoses are the source of lactate and assumed that this process accounts for "synthethic purposes by the leukocytes." This period is widely recognized as the onset of modern biochemistry and furthermore of immunometabolism.

Immunometabolism is also tightly linked to research on cancer metabolism, especially with regard to the pioneering work of Otto Warburg, wherein he further developed the concept of *cellular physiology* (2). It was revealed that exudate leukocytes have high *aerobic glycolysis*, while respiration was very low and it was concluded then that white blood cells must have a *cancer metabolism* (3, 4). However, they differentiated immune cell- and cancer metabolism in that cancer cells use aerobic glycolysis to live, while aerobic glycolysis in white blood cells is a sign of *aging* or *dying off*. With this background, Walter Kempner and Ernst Peschel, both from the Bergmann'sche Institut at the Charite in Berlin, published their work with the German title: "Stoffwechsel der Entzündung" (Metabolism of Inflammation) (5). In 1930, they formulated two fundamental questions: what are the specific reactions of inflammation? Which processes lead to cell migration and subsequently to tissue swelling or necrosis? They presumed that an adapted cellular metabolism of white blood cells may play a major role in these processes. They tested their hypothesis in a human *in vivo* model of sterile-inflammation and provided fundamental new insights, which are still of relevance for today's concepts of immunometabolism. Kempner and Peschel used the beetle-juice (cantharidin)-induced skin blister model and metabolically defined the inflamed human tissue in order to examine the *physiology of inflammation*. They observed a disrupted equilibrium of oxygen, CO_2, sugar, lactate, and bicarbonate as a result of inflammation and concluded that this was induced by the metabolism found in infiltrating immune cells. They expected this to happen as a function of time. They demonstrated a drop in glucose over a period of 6–90 h and pulsed oral glucose administrations indicating that glucose replenishment from healthy tissue was also gradually declining. Within the inflamed area (the blister) also oxygen concentration declined. This was again attributed to high cellular respiration of infiltrated cells and a reduced gas-exchange with the healthy tissue. In addition to that, they measured a time-dependent increase in lactate and a decrease in the bicarbonate levels, which together could explain the decrease in pH of inflamed tissue, previously observed by Schade (6). Kempner and Peschel identified metabolic changes in inflamed tissue as a function of time, which is actively established by infiltrating "injured" immune cells with an adapted cellular metabolism (5). Thereby, they delineated a complex interplay between cellular metabolism and the physiology of inflammation. In 2011, the cantharidin-induced skin blister was re-evaluated and recommended as an excellent human *in vivo* model to study inflammation (7). This report also reveals that the infiltrating cells in this model are mainly neutrophils and monocytes/

macrophages; these cells were probably also the cause for the observation by Kempner and Peschel.

Since then, a new school of immunobiology has started to reveal the molecular mechanism behind the observed metabolic-adaptation in various immune cells and models of immunology. As an example, the action of the pentose phosphate pathway (PPP) and the power of redox-biology, including superoxide production, were identified as essential in forming the respiratory-burst of phagocytes (8, 9). Also amino acid and lipid metabolism, as well as their adaptations, were characterized as fundamental to properly fuel the function of an immune response (10, 11). In recent years, however, new concepts in immunometabolism have evolved and further mechanistic-details have surfaced that enable us to better understand how these metabolic-adaptions are reached and regulated.

TIME RESOLVED METABOLIC-ADAPTATIONS DURING MACROPHAGE ACTIVATION

Macrophages are important immune cells, which regulate tissue homeostasis by sensing and interpreting cell injury and infection, the classic triggers of an inflammatory response (12). Today, macrophages are classified according to the activation stimuli into at least two polarization states, the classic M1 (representing a pro-inflammatory phenotype) and the alternative M2 macrophage (representing an anti-inflammatory or homeostasis inducing phenotype), in order to discriminate between the effector phenotypes resulting from the distinct activation signals (13). However, *in vivo* macrophages rather appear to blend into various "shades of activation," while retaining some of their plasticity (14–18). Furthermore, macrophage populations and phenotypes can dramatically change over time, as exemplified by the finding that the inflammatory response is a spatially and temporally coordinated process. Recently, the polarization process of macrophages has been further associated with the reprograming of cellular metabolism (19–25). Information processing by signal-transduction pathways starts shortly after activation and is temporally coordinated, reflected by the phosphorylation and de-phosphorylation of signal transducers and effector molecules. The question arises how the reprograming of primary carbohydrate metabolism is timed in the process of macrophage activation. We would like to present more detailed and more importantly time-resolved information on key events, which appear to establish a pro-inflammatory M1-like metabolic-phenotype induced by lipopolysaccharide (LPS, **Figure 1**).

After only 20 min of *in vitro* LPS-stimulation, simultaneously with prime signaling events, the glucose uptake of cells approximately doubles (26). At the same time, the extracellular acidification rate (ECAR), an indirect measure of aerobic glycolysis, also increases until reaching a certain plateau-state, to then adapt, and further increase (21). This response indicates that LPS leads to a rapid induction of glycolytic flux, which is modulated and amplified in multiple steps (**Figure 1**). The extension phase of ECAR is accompanied by a slow and marginal decrease in the oxygen consumption rate (OCR). The molecular mechanisms leading to these immediate early metabolic events, however, are not known and acidification may also result from sources other than the formation of lactic acid.

However, 1 h after LPS stimulation, the mRNA of the glucose transporter (GLUT1) is induced and the uptake of glucose further increases (26). After uptake of glucose, it becomes phosphorylated by hexokinases (HK) to glucose 6-phosphate (G6P), which can then be diverted into various catabolic and anabolic pathways. Non-stationary metabolic flux analysis, tracking the fate of intracellular glucose during macrophage activation, reveals that already 1 h after LPS-exposure a considerable amount of glucose is used by both, glycolysis and the PPP (21). In rat-Kupffer cells, which are specialized liver macrophages, as well as murine dendritic cells, HK-II was shown to associate with mitochondria within an hour after LPS stimulation (27, 28). A similar mechanism is observed in cancer cells, where mitochondrial matrix derived ATP is channeled to HK-II and thereby augmenting the glycolytic flux (29). Recently, the sedoheptulose kinase (Shpk, formerly known as CARKL) was characterized as a unique heptose kinase, phosphorylating sedoheptulose (a ketoheptose) to sedoheptulose 7-phosphate (S7P), which can then act as a reaction partner of glyceraldehyde 3-phosphate (G3P) in the non-oxidative PPP (21, 30–32). In macrophages, the mRNA of Shpk is rapidly down-regulated by LPS but not by interleukin (IL)-4 stimulation (21). Regulation of Shpk will be further discussed in the next section. Also, approximately after 1 h, LPS specifically induces pyruvate kinase M2 (PKM2) protein expression and phosphorylation, which becomes further augmented in the late phase of macrophage activation (23). Phosphorylation of PKM2 favors dimeric configuration and PKM2 translocation into the nucleus, where it acts together with hypoxia-inducible factor 1-alpha (HIF1α) as a transcriptional inducer of interleukin 1-beta (IL-1β) and more importantly of glycolytic genes like PFK, constituting an amplification loop in the intermediate and late phase of macrophage activation (23, 33). Within 2–4 h after activation by LPS, an isoform switchs from the liver-type 6-phosphofructo-2-kinase (PFKFB1 aka PFK2) to the ubiquitous and more active PFKFB3 occurs (34). This is also observed when LPS is used in combination with interferon gamma (IFNγ) to induce a pro-inflammatory macrophage activation (20). PFKFB3 produces augmented levels of fructose 2, 6-bisphosphate (F2,6bP), which then functions as an allosteric activator of 6-phosphofructo-1-kinase (PFK1) to further sustain the pro-glycolytic program (**Figure 1**). Interestingly in yeast, PFK1 derived F1,6bP allosterically activates PKM2, indicating the presence of metabolic feedback loops (35).

Approximately 4–6 h after macrophage activation, the export of glycolytic lactate appears to become mandatory for the activation process as indicated by the increased expression of monocarboxylate transporter 4 (MCT4) (36). Knockdown of MCT4 results in enhanced intracellular lactate accumulation, a decreased expression of LPS-induced glycolytic enzymes and an attenuated secretion of tumor necrosis factor-alpha (TNFα) and IL-6. Accumulating intracellular lactate might decrease glycolytic activity by inhibiting PFK1, an enzyme which may reach maximal activity in the later phase, as indicated by peaking F2,6bP concentrations and PFKFB3 mRNA levels at 6–12 h (34, 37). Also, approximately 4 h after initiation of macrophage polarization by LPS, the tricarboxylic acid (TCA) cycle changes its operational mode from a catabolic pathway to a partly anabolic system (21, 22). The TCA-cycle metabolite succinate accumulates in a

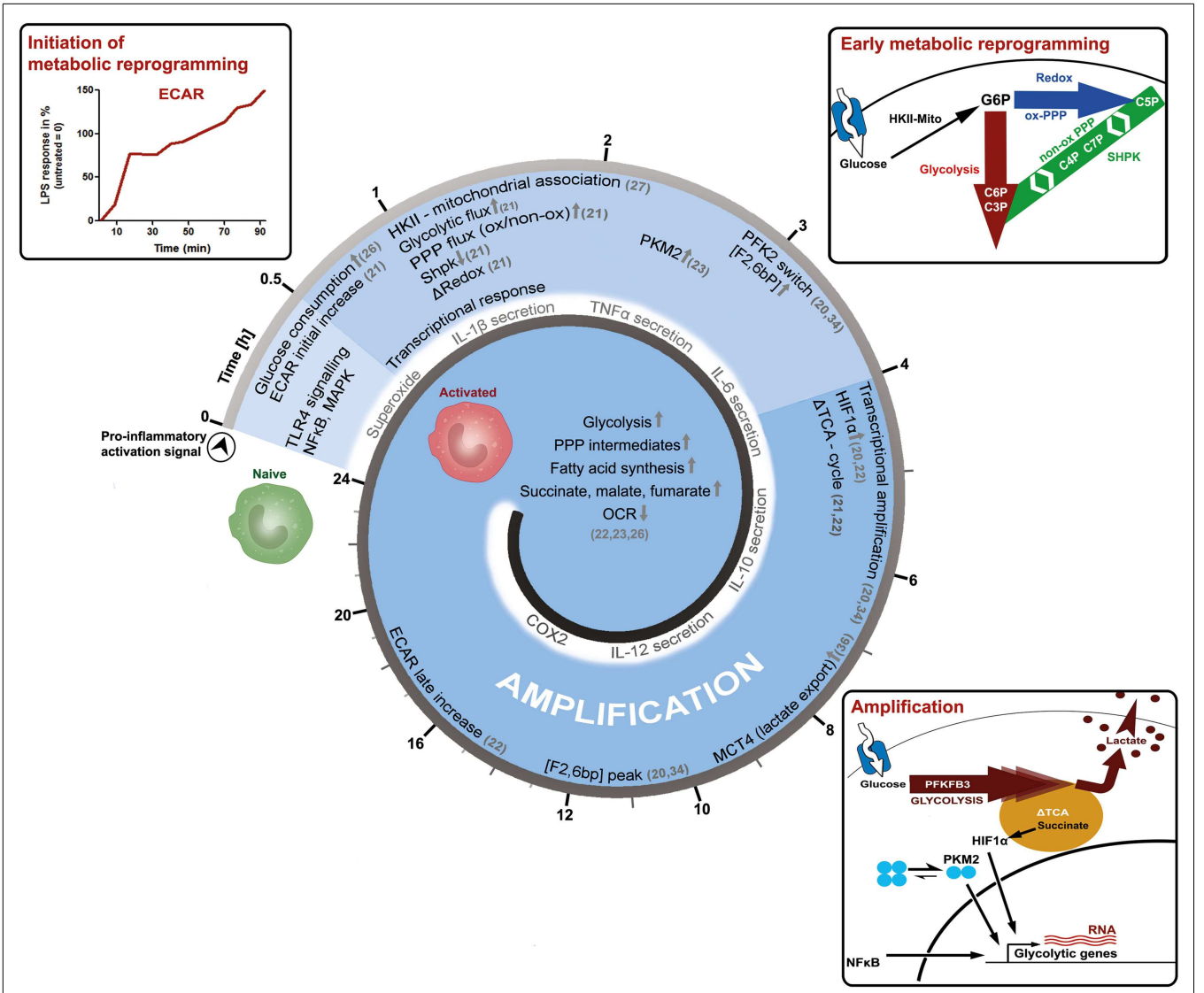

FIGURE 1 | Time-resolved metabolic reprograming during pro-inflammatory macrophage polarization. This model illustrates the activation of a macrophage as a function of time and is based on the literature discussed in the main text. LPS-induced activation can be grouped into an initiation-, an early metabolic-reprograming,- and an amplification-phase. The initiation phase of the metabolic response is characterized by an increase in glucose consumption and in the extracellular acidification rate (ECAR). The early metabolic reprograming phase depicts the increase and rerouting of carbon flux through glycolysis and the PPP, events which also regulate the cellular redox-state. In this setting, the mitochondrial association of hexokinase-II (HKII) appears to provide sufficient levels of glucose 6-phosphate (G6P), while the downregulation of sedoheptulose kinase (Shpk, previously known as CARKL) appears to be necessary to maintain appropriate carbon flux at the interface of glycolysis and the PPP. During the amplification phase, this pro-glycolytic metabolic-phenotype is further strengthened. A switch toward the more active 6-phosphofructo-2-kinase (PFK2) enzyme PFKFB3 produces higher levels of fructose 2-bisphosphate [F2,6bP], thus allosterically activating PFK1 and enhancing glycolytic flux. Dimers of the pyruvate kinase M2 (PKM2), as well as accumulating succinate further augment metabolic reprograming by supporting HIF-1α dependent transcriptional induction of glycolytic genes. In the amplification phase, also the export of intracellular glycolysis-derived lactate through monocarboxylate transporter 4 (MCT4) becomes obligatory, which may otherwise inhibit PFK1. These initial events lead to more prominent metabolic changes observed 24 h after macrophages have encountered the pro-inflammatory stimuli. However, further time-resolved data is required to refine these processes and our current perspective, how cellular metabolism of macrophages adapts during activation.

macrophage cell line and bone marrow derived macrophages (BMDMs) (21, 22). Succinate, derived by glutamine-dependent anerplerosis and gamma-aminobutyric acid (GABA)-shunt, was shown to inhibit the prolyl hydroxylase-dependent degradation of HIF1α and to enhance IL-1β production (22). Increased succinate levels may also increase succinylation of metabolic enzymes such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), transaldolase (TALDO), and lactate dehydrogenase (LDH) A-chain, possibly further shaping late phase metabolic adaptations (22, 38). Succinate dependent HIF1α stabilization as well as increased succinylation are both suppressed by the inhibition of glycolysis, indicating that these processes are dependent on increased glycolytic

flux (22, 23). Approximately 24 h after LPS-stimulation, metabolic reprograming is firmly established: glycolytic gene expression and metabolites are increased, as well as lactate and ECAR (22, 23). The TCA-cycle supports increased fatty acid synthesis as well as the formation of cycle intermediates (succinate, malate, fumarate), while OCR is reduced, indicating a significant decline in oxidative metabolism (22, 23).

To briefly summarize the overall consequences of the discussed adaptations: LPS stimulated macrophages increase aerobic glycolysis and PPP activity, reduce mitochondrial respiration, and reconfigure the TCA-cycle. In order to replenish NAD^+ for glycolysis, lactate production and secretion are enhanced, leading to acidification of the environment. Such a pro-inflammatory metabolism is important for the generation of redox-equivalents as well as precursor molecules such as amino acids, lipids, and nucleotides, sustaining a burst in pro-inflammatory mediator production (39, 40). In reference to protein-signal-transduction leading to the observed metabolic adaptions, nuclear factor kappa-B (NFκB) and HIF1α are two well-characterized transcription factors, which increase the expression of glycolytic genes (41, 42). In contrast to an pro-inflammatory activation of macrophages, alternative activation (i.e., by IL-4) is associated with mitochondrial biogenesis as well as increased fatty acid oxidation and oxidative phosphorylation, primarily driven by lysosomal lipolysis of endocytosed lipoprotein particles (24, 43). In general, the M2 metabolic program mainly relies on STAT6, PPARγ, and its co-activator PGC1β to promote oxidative metabolism. The manifold metabolic changes during macrophage activation as well as their regulatory mechanisms have been recently discussed in detail in some excellent reviews (44–46).

THE PPP SUSTAINS THE METABOLIC DEMAND OF MACROPHAGES DURING POLARIZATION

The PPP represents a prime example on how increased carbon-flux can contribute to mount the specific effector functions of LPS-activated macrophages by complementing their appropriate demands through supplying both redox-power and ribose moieties either at the same time or independently from each other. The PPP is divided into the oxidative (oxPPP) and non-oxidative branch (non-oxPPP). Briefly, the oxPPP, with glucose 6-phosphate dehydrogenase (G6PD) as its rate-limiting enzyme, is highly active in macrophages (21, 47), decarboxylates G6P, and forms ribose 5-phosphate (R5P) through three irreversible reactions, while simultaneously reducing two molecules of $NADP^+$ to NADPH and liberating one molecule of CO_2. The non-ox PPP consists of reversible reactions, which can either recycle R5P to glycolytic intermediates or use the latter to generate pentose phosphates (C5P) through reverse flux. The general aspects of PPP architecture and function have been reviewed elsewhere in great detail (48–50).

Oxidative PPP derived NADPH serves as a cofactor for NADPH-oxidase dependent reactive oxygen species (ROS) production, while also reducing oxidized redox-couples to simultaneously sustain an anti-oxidant response (i.e., glutathione and thioredoxin systems), thereby partly controlling the redox balance during macrophage activation (51, 52). Also, the function of many redox-sensitive signaling proteins, which are associated with the process of activation, are potentially dependent on increased flux through the oxPPP (53–59). Furthermore, NADPH is also critical for reductive biosynthesis serving activation associated membrane expansion and the production of lipid mediators such as prostaglandins. Remarkably, it was reported that NADPH levels undergo periodic oscillations in macrophages and neutrophils, which are tightly linked to superoxide oscillations and adapt, upon LPS stimulations to a higher frequency (60–63). These oscillations may depend on periodic glucose influx and PPP activity, and appear to encode information in their amplitude and frequency (64, 65). Severe G6PD deficient leukocytes have been associated with impairments in their oxidative burst, their bactericidal activity (66–68), their resistance to oxidative stress (69), as well as modified cytokine responses (70–72). Overexpression of G6PD in a macrophage cell line enhanced the activation of NFκB and p38-MAPK signaling pathways and potentiated the expression of pro-inflammatory cytokines as well as ROS production (73). In contrast to IL-1β, the production of TNFα and IL-6 does not appear to be directly dependent on aerobic glycolysis, as recently suggested by the activation of PKM2 and 2-deoxyglucose (2-DG) treatment, respectively (22, 23). Notably, 2-DG is a glycolytic inhibitor downstream from hexokinase and can therefore become phosphorylated to 2-deoxyglucose 6-phosphate, which is partly metabolized by the oxPPP in red blood cells (74). Whether this also occurs in macrophages or not remains to be investigated. Inhibition of G6PD or LDH, however, was shown to decrease TNFα and IL-6 levels, implicating that these cytokines are rather regulated by redox-state than simply by the increased glycolytic-flux (21). Apart from redox-power, the macrophage activation process also demands a large amount of pentose phosphates probably to sustain *de novo* nucleotide synthesis for their characteristic transcriptional response. In contrast to M2, M1 macrophages drastically change their transcriptional profile (40). Isotope distribution analysis of a non-stationary metabolic flux experiment with asymmetrically labeled glucose after 1 h of LPS-induced macrophage activation indicated that both ox- and non-oxPPP flux rates increase, while most of the pentose phosphates (C5) are derived from the non-ox branch (21).

The non-oxPPP relies on transketolase (TK) and TALDO catalyzed reversible transfer of keto-groups to various aldose acceptors. TK uses thiamine pyrophosphate as cofactor to transfer two carbon (C2)-units, while TALDO can transfer C3-units by forming Schiff base intermediates (75, 76). Thereby, this pathway interconverts carbohydrate-phosphates of different chain length (C3P to C7P), without the need of energy in form of ATP (carbon scrambling, **Figure 2A**). The regulation of non-oxPPP is complex due to its reversible nature and still not fully understood. The flux-rate and its direction are generally thought to depend on thermodynamics, which impose a major constraint on the structure of metabolic pathways (77). However, the recent identification of Shpk indicates additional regulatory mechanism, which was previously not considered (**Figure 2B**) (78). In contrast to TK or TALDO, Shpk is reported to be regulated differently during LPS- and IL-4 induced polarization (21). LPS stimulation leads to a rapid down-regulation of Shpk mRNA in the early phase of macrophage activation in mice and humans likewise and *in vitro* as well as *in vivo*. In contrast to LPS, IL-4 stimulation

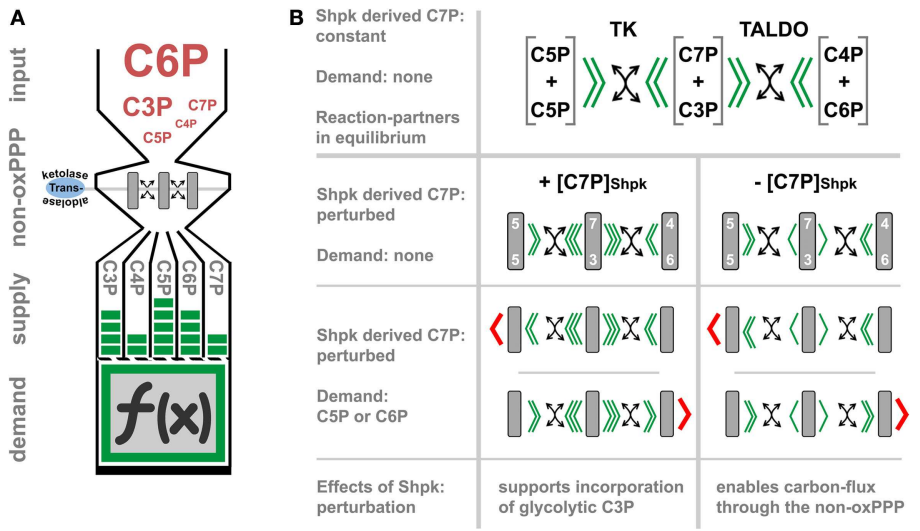

FIGURE 2 | The function and regulation of the non-oxidative PPP. **(A)** represents a simplified model, which illustrates how transketolase (TK) and transaldolase (TALDO) may interconvert carbohydrate-phosphates of three to seven carbon-atoms in length (C3P to C7P) without the need of energy (carbon-scrambling) to account for the cellular demand, which in part defines cell function (indicated by the symbol $f_{(x)}$). In **(B)**, we theoretically evaluate the regulatory effect of Shpk-derived sedoheptulose 7-phosphate ($[C7P]_{Shpk}$) on non-oxPPP flux in the presence of TK and TALDO. Flux through the non-oxPPP, by its reversible reactions, is dependent on the stoichiometry of the reactants (indicated by green arrowheads). In contrast to TK- and TALDO-derived S7P, the phosphorylation of free sedoheptulose to S7P by Shpk requires energy in form of ATP. Assuming a constant contribution by Shpk, this additional source of S7P may therefore act as a thermodynamic buffer, which can be actively regulated to induce a non-equilibrium. In theory, perturbation of Shpk can either increase the resistance (increased $[C7P]_{Shpk}$) or lower it (decreased $[C7P]_{Shpk}$) to support shunting through the non-oxPPP. However, in the presence of TK or TALDO, an increased $[C7P]_{Shpk}$ will promote the incorporation of glycolytic-G3P into the PPP. This model further illustrates that non-oxPPP flux-direction is also dependent on the demand of respective molecules (indicated by red arrowheads).

maintains or even slightly increases Shpk levels (21). Counterbalancing LPS-induced down-regulation of Shpk by overexpression in a macrophage cell line resulted in an accumulation of pentose phosphates and an imbalance of the cellular redox system, as indicated by the accumulation of oxidized redox couples as well as blunted LPS-induced intracellular superoxide production (21). In theory, Shpk, by the formation of rate-limiting S7P, should increase the shunting of glycolysis-derived G3P into the non-oxPPP (78) and regulate oxPPP activity through the formation or recycling of pentose phosphates (79). So far, we have no confirmed mode-of-action, how Shpk activity actually regulates carbon-flux through the non-oxPPP, and no information on its activity and local distribution during macrophage activation. Therefore, we can only speculate on the consequences of Shpk regulation for the process of metabolic-adaptation (**Figure 2B**). Shpk-derived S7P may act as a thermodynamic buffer to support a stable non-equilibrium, which drives (low S7P) or inhibits (high S7P) carbon-flux through the non-oxPPP. However, flux-direction seems to be determined by demand and by the presence of TK and TALDO (**Figure 2B**). In addition to that, high S7P levels can directly modulate glycolytic flux through the inhibition of hexose phosphate isomerase, as well as by competitively inhibiting fructose 6-phosphate (F6P) phosphorylation by PFK (80, 81). Therefore, the consequences of Shpk regulation appear as strictly context dependent, which is defined by the demand of metabolites (i.e., C5P) and the presence or absence of other enzymes. We know that Shpk only partially colocalizes with G6PD in the cytoplasm of cells, which points out that there are instances where the ox- and the non-oxPPP are coupled to and uncoupled from each other (21). Information on the function of TK and TALDO in the process of macrophage activation is rare; however, both enzymes were tightly linked to oxidative stress-defense in other cell types (82–84). Notably, yeast seems to lack a Shpk homolog but utilizes a specific sedoheptulose–bisphosphatase [dephosphorylates sedoheptulose 1,7-bisphosphate (S1,7bP) to S7P] for riboneogenesis when the demand for nucleotide precursors is high (85). S1,7bP was previously reported to also exists in rat liver tissue (86, 87); however, there appear to be some major differences in the architecture of heptose metabolism (heptolysis) between fungi and vertebrates (78, 85).

In summary, these findings indicate that during macrophage activation the cellular demands are covered by a precisely coordinated interplay of many pathways to sustain such profound polarization events. The PPP appears as a versatile hub to reroute carbon moieties within the network of primary carbohydrate metabolism while independently controlling cellular redox-states.

CONCLUSION

This collection of findings may support our perspective that time and demands are critical to understand the molecular events important to mount an immune response. Immunometabolism demonstrates its consequences for physiology at various levels including cells, tissues, organisms, and entire populations, as we currently experience with diseases like cancer, cardiovascular diseases, obesity, and diabetes to name but a few. Already, Kempner and Peschel considered diabetic patients in their investigations

and noted a sustained glucose supply together with a prolonged inflammatory response compared to non-diabetics. Since then, many excellent studies further delineated the complex interplay of metabolism, the immune system and tissue function, and malfunction. At the molecular level, macrophages adapt their metabolism very early in the polarization process, which then become amplified over time. This highlights that we need to strongly consider the process leading to activation and not only the phenotypic "endpoints." A macrophage located within a complex tissue microenvironment may go through multiple, subsequently occurring, activation events, which then may amplify or antagonize each other. It will be interesting to test *in vivo* if, and importantly how, subsequent or parallel cross-presentation of multiple activation-stimuli (i.e., pro- and anti-inflammatory signals such as LPS, IFNγ, TNFα, IL-6, IL-4, or IL-10) may skew and define the process of metabolic reprograming in macrophages.

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Nutrient sensing via mTOR in T cells maintains a tolerogenic microenvironment

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We have proposed that tolerance can be maintained through the induction, by Treg cells, of a tolerogenic microenvironment within tolerated tissues that inhibits effector cell activity but which supports the generation of further Treg cells by "infectious tolerance." Two important components of this tolerogenic microenvironment depend on metabolism and nutrient sensing. The first is due to the up-regulation of multiple enzymes that consume essential amino acids, which are sensed in naïve T cells primarily via inhibition of the mechanistic target of rapamycin (mTOR) pathway, which in turn encourages their further differentiation into FOXP3$^+$ Treg cells. The second mechanism is the metabolism of extracellular ATP to adenosine by the ectoenzymes CD39 and CD73. These two enzymes are constitutively co-expressed on Treg cells, but can also be induced on a wide variety of cell types by TGFβ and the adenosine generated can be shown to be a potent inhibitor of T cell proliferation. This review will focus on mechanisms of nutrient sensing in T cells, how these are integrated with TCR and cytokine signals via the mTOR pathway, and what impact this has on intracellular metabolism and subsequently the control of differentiation into different effector or regulatory T cell subsets.

Keywords: mTOR, metabolism, immune regulation, T cell differentiation, tolerance

INTRODUCTION

The mechanistic target of rapamycin (mTOR) signaling acts as a principle integrator of nutrient-sensing pathways that control and coordinate the metabolism of the cell according to its need to proliferate or functionally differentiate (1, 2). When a naïve or resting T cell recognizes its cognate antigen, the activation process involves synthesis of many new proteins, the induction of rapid cell proliferation, cytokine driven differentiation toward a range of effector functions, and chemokine induced cell movement to any site of inflammation. All these processes require a rapid increase in the main source of energy for the cell, which is ATP. While oxidative phosphorylation (OXPHOS) by the mitochondria is the most efficient means to generate large amounts of ATP, there seems to be a switch from primarily OXPHOS in resting T cells to an aerobic form of glycolysis, known as the "Warburg effect" (3), during activation and proliferation (4). This may be because glycolysis can use glucose as the basic source of carbon to generate many of the fundamental building blocks of the proliferating cell, such as amino acids, lipids, complex carbohydrates, and ribonucleotides (5). The mTOR pathway is strongly implicated in this metabolic switch because its activation up-regulates the surface expression of the glucose transporter, Glut1, probably as a result of TCR and CD28 signaling through phosphatidylinositide 3-kinase (PI3K) and protein kinase B (PKB also known as AKT) (6). AKT signaling via mTOR also leads to higher expression of amino acid and other nutrient transporters, such as the transferrin receptor (7). Signaling downstream of mTOR via ribosomal S6 kinase and 4E-BP1 is also required to initiate protein synthesis from mRNA at the ribosome (8). Rapamycin is a drug (trade name sirolimus) that inhibits mTOR by forming a complex with FKBP12, which binds to raptor and disrupts the activity of the mTORC1 complex. Rapamycin is used clinically as an immunosuppressive agent, particularly in allogeneic transplantation, and has over recent years gained interest as a potential alternative to calcineurin inhibitors, which not only have renal toxicity but are also thought to block the induction of regulatory T cells (9).

MECHANISMS OF PERIPHERAL TOLERANCE

REGULATORY T CELLS ARE ENRICHED WITHIN TOLERATED TISSUES

It has recently become clear that tolerance is associated with Treg cells that act within a highly localized microenvironment to maintain a state of acquired immune privilege (10, 11). Tolerance to skin grafts can be induced using a short course of non-depleting CD4 antibodies in mice expressing a TRC transgenic, monoclonal population of CD4$^+$ T cells such that every T cell recognizes the male antigen presented by MHC-II on the graft (12). This tolerance is not due to clonal deletion as the graft recipients contain normal numbers of male specific T cells, including a proportion that show evidence of recent activation, by expression of CD44 and IL-2. As these mice are on a RAG$^{-/-}$ background, there are no FOXP3$^+$ Treg cells present in the naïve animal pre-grafting, but after tolerance induction, peripherally induced, FOXP3$^+$ Treg cells are found gradually increasing over time (up to 50%) within the tolerated graft tissue, but only in small numbers (1–2%) in the lymph nodes or spleen (12). This suggests that the Treg cells are acting to control the response of effector T cells primarily within the graft itself.

This can be demonstrated where alloantigen specific tolerance has been induced to a skin graft (e.g., by a short period of coreceptor blockade with non-depleting anti-CD4 and CD8

monoclonal antibodies), and then that tolerated graft has been removed and re-transplanted onto a secondary recipient with no immune system of its own (e.g., a recombinase activating gene 1 knock out mouse). This skin graft is accepted by the secondary recipient as it has no T cells to cause any rejection. If, however, we treat the recipient at the time of graft transfer with monoclonal antibodies that inactivate or deplete FOXP3$^+$ Treg cells (e.g., anti-CD25, or if the original recipient carries the hCD2.FOXP3 knock in reporter, anti-hCD2), the transferred skin grafts are rapidly rejected (11, 13). This shows that the re-transplanted, originally tolerated skin graft carried over within it perfectly functional effector T cells, but that it also contained FOXP3$^+$ Treg cells that were actively blocking the ability to cause rejection. By studying the changes in gene expression between tolerated and rejecting skin grafts, and comparing dendritic cells (DCs) when they interact with Treg cells in the presence or absence of antigen (14–16), it was found that while co-stimulatory ligands and antigen presentation by DCs were down-regulated, there was also an up-regulation of a number of enzymes that either catabolize or utilize essential amino acids (EAAs) (17). In the context of a restricted microenvironment within tissues, where there may not be free exchange of amino acids and other nutrients with plasma in the vasculature, the local depletion of EAAs by these enzymes could be an effective mechanism to control the immune response via the mTOR nutrient-sensing pathway (**Figure 1**). Conversely, edema and breakdown of the vasculature may provide an excess of amino acids that would promote T cell activation and graft rejection. This regulation by amino acid availability might be particularly effective if regulatory T cells were more resistant to the effects of amino acid starvation. It has been shown that the intracellular concentration of leucine, a particularly strong activator of mTOR, is controlled by a TCR induced expression of the neutral amino acid transporter slc7a5 in Th1 and Th2 effector T cells, where it is required for their activation and differentiation, but regulatory T cells seem not to depend on this particular transporter (18).

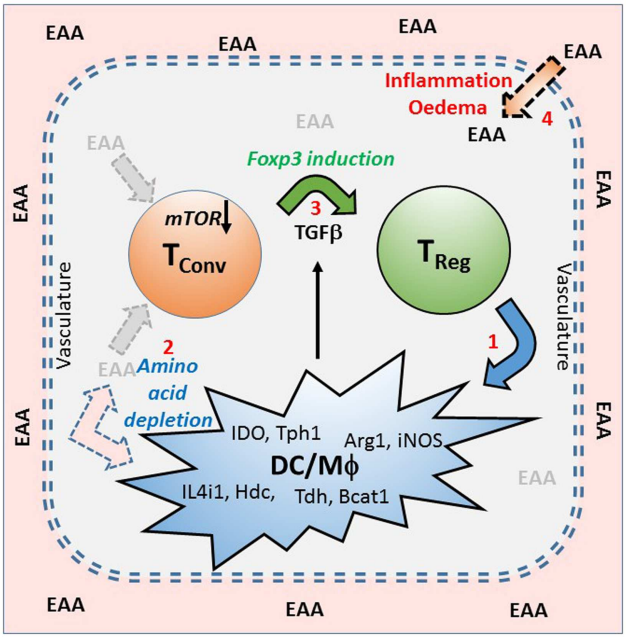

FIGURE 1 | A model of infectious tolerance that depends on a nutrient depleted microenvironment maintained by Treg cells within tissues. This model proposes that immunological tolerance is maintained within tissues by the localized depletion of nutrients, particularly the essential amino acids (EAA), which are required for the proliferation and effector function of conventional T cells (Tconv). Amino acid depletion is primarily as a result of regulatory T cells (Treg) inducing (1), in dendritic cells (DC) and macrophages (Mϕ), a range of enzymes that catabolize (2) or utilize EAA (examples are shown). This lack of EAA is sensed via the mTOR pathway which, in the presence of TGFβ, encourages the expression of FOXP3, and the induction of further Treg (3). Under conditions of tolerance the intact vasculature maintains a barrier between the blood and the tissues, but if there is inflammation or damage to the vasculature, causing edema, then EAA can leak into the tissues (4) and contribute to a breaking of the tolerant microenvironment.

IDO MEDIATED TRYPTOPHAN CATABOLISM AS A MECHANISM OF IMMUNE REGULATION

The maternal immune response to paternal alloantigens expressed on the developing fetus is in many ways similar to that seen in transplantation. The expression of the enzyme indoleamine 2,3 dioxygenase (IDO) in the placenta during pregnancy was shown to be important for avoiding that immune response by the finding that a specific inhibitor, 1-methyl tryptophan, could induce spontaneous abortion of semi-allogeneic, but not syngeneic, conception (19). *In vitro* experiments showed that IDO seemed to act primarily through depletion of tryptophan, although there is some evidence that the kynurenine products of tryptophan catabolism may also play a role (20). The tryptophan depletion is sensed, at least in part, by general control non-repressed 2 (GCN2), which is one of the initiators of the integrated stress response, activation of which leads to a block in the proliferation of CD8 effector T cells (21). GCN2 is also required for the survival of T cells, including CD4$^+$ Treg cells, during periods of amino acid starvation (17), but it was not essential for T cells to sense the absence of other EAAs and halt their proliferation *in vitro* (17). The *in vitro* induction of forkhead box P3 (FOXP3) as a result of stimulating naïve CD4$^+$ T cells in the presence of low doses of TGFβ was also unaffected by activating the GCN2 pathway with histidinol (an inhibitor of histidyl-tRNA synthetase) while in contrast, inhibition of the mTOR pathway with rapamycin gave a synergistic increase in FOXP3 expression (17). It has recently been found that tryptophan levels can be sensed via mTOR and PKCθ signaling (22).

DEPLETION OF ESSENTIAL AMINO ACIDS MAINTAIN AN IMMUNE PRIVILEGED MICROENVIRONMENT WITHIN TOLERATED TISSUES

Indoleamine 2,3 dioxygenase may have been the first example of immune regulation due to amino acid catabolism because tryptophan is thought to be present at the lowest concentration of all the EAAs, at least in the plasma. Recently, it has been shown that mast cells that seem to be specifically associated with tolerated skin grafts, express the enzyme tryptophan hydroxylase (TPH1) (23), which utilizes tryptophan to synthesize serotonin. TPH1 knockout mice, unlike wild type controls, could not be made tolerant of allogeneic heart grafts using costimulation blockade, but this could be reconstituted with wild type mast cells. Providing

5-hydroxytryptophan to bypass the defect in serotonin synthesis in TPH1 knockout mice was not sufficient to allow the induction of tolerance, suggesting that the mechanism was dependent on tryptophan depletion rather than serotonin synthesis (24). Similarly, arginase (ARG1) expression has been implicated in regulating the immune response during pregnancy (25, 26) and has also been associated with a presumed protective, type 2, population of macrophages within tissues (27). Arginine is the substrate for the inducible form of nitric oxide synthase (iNOS), which is normally associated with classically activated macrophages and a Th1 effector cell response, but under limiting concentrations of arginine *in vitro*, both arginase and iNOS can cause sufficient depletion of arginine to cause mTOR inhibition and subsequently block T cell proliferation (17). Another enzyme called IL4-induced 1 (IL4i1) for its induction in myeloid cells under Th2 conditions, depletes EAAs with hydrophobic side chains such as phenylalanine (28). IL4i1 was also found to be induced in DC when co-cultured with Treg cells (17).

Expression of many of these EAA consuming enzymes could be induced within skin grafts *in vivo* (17) and in DCs *in vitro* (17) by a cognate interaction with antigen specific Treg cells, either by specific cytokines such as TGFβ, IL4, or interferon-γ (IFN-γ) or via cell surface interactions such as CTLA4 (17). In addition, catabolic enzymes specific for threonine (threonine dehydrogenase – TDH) and the branched chain amino acids (branched chain amino acid aminotransferase – Bcat1) were more closely associated with the inflammation and wound healing even when skin was grafted onto recipients with no adaptive immune system (17). This suggests that tissues such as skin have a built in nutrient-sensing mechanism for protecting themselves against immune attack that might be important for maintaining self-tolerance, which might explain why long-term surviving, fully healed in syngeneic skin grafts also had higher levels of these particular enzymes, as well as an increased infiltration by FOXP3$^+$ Treg cells (16).

All these observations led us to propose that tolerance may be maintained by regulatory T cells that induce a tolerogenic microenvironment within tissues that is, at least in part, dependent on the induction of many different enzymes that deplete the local pool of EAAs. This lack of EAAs is sensed by T cells via the mTOR pathway, which inhibits the generation and function of effector T cells, while encouraging the development of further FOXP3$^+$ Treg cells (**Figure 1**). This mechanism may explain the phenomenon known as "infectious tolerance" where it was shown that naïve T cells that co-existed with regulatory T cells in a tolerant environment acquired all the properties of the original tolerant T cells within 3 weeks, such that tolerance was maintained if the original cohort of tolerant T cells were subsequently depleted (29). The question then arises as to how the consequent inhibition of mTOR regulates the activation and differentiation of different functional T cell subsets.

mTOR INTEGRATES NUTRIENT SENSING AND ACTIVATION SIGNALS IN T CELLS

THE mTOR PATHWAY IN T CELLS

The mTOR pathway (**Figure 2**) acts generally to coordinate many aspects of cell growth and metabolism, including the response to hypoxia and the biogenesis and oxidative capacity of mitochondria

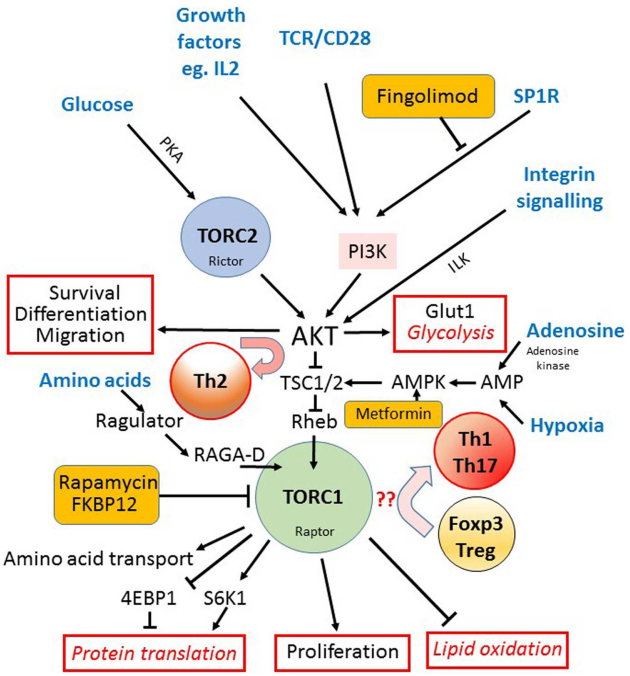

FIGURE 2 | The mTOR pathway in T cells. The mechanistic target of rapamycin (mTOR) is a component of both the TORC1 and TORC2 signaling complexes. The TORC1 complex acts as the main integrator of many different signals (input signals shown in blue text) from nutrients such as glucose, via TORC2, the TCR, costimulation and growth factors, via PI3K and AKT, and amino acids via the regulator complex. Hypoxia and AMP levels are also sensed via AMPK and TSC1/2. AKT activation downstream of TORC2 is important for cell survival, drives the expression of the glucose receptor (Glut1) and glycolytic metabolism, and is required for the differentiation into Th2 cells (outputs of signaling shown outlined in red). TORC1 is important for the initiation of mRNA translation via S6K1 mediated phosphorylation of the ribosomal protein S6, and the up-regulation of amino acid transporters at the cell surface. TORC1 also activates lipid oxidation and cell proliferation while it inhibits the expression of FOXP3 and Treg differentiation in favor of Th1 and Th17 cells. The sites where three different clinically available drugs (rapamycin, fingolimod, and metformin) impact on the mTOR pathway are indicated (orange boxes).

(30). mTOR forms two distinct complexes that seem to have different signaling functions (TORC1 and TORC2) (31). TORC1 is thought to be the main nutrient-sensing complex and is composed of the serine/threonine kinase mTOR itself, the scaffolding protein raptor, the positive accessory proteins FKB12, deptor, and mLST8, and a regulatory subunit PRAS40 that is a target of AKT downstream of PI3K signaling (32). Most signals, which eventually lead to activation of the TORC1 complex, including glucose, cytokines, growth factors, and costimulation in T cells, do so via PI3K signaling, which eventually phosphorylates mTORC1 via the tuberous sclerosis (TSC) 1/2 complex and the ras homolog expressed in brain (Rheb). Rheb is localized within the cell in a Rab7$^+$ lysosomal compartment and the interaction between TORC1 and Rheb is entirely dependent on the sensing of sufficient amino acids. Although the molecular sensor of amino acids in mammals remains unclear, downstream signaling requires the four ras-related GTP binding (or RAG GTPase – RRAG) proteins

(A–D) together with the ragulator complex (33, 34), so that a lack of available amino acids acts as a potent inhibitor of TORC1 activity. The immunosuppressive drug rapamycin binds to FKB12 and disrupts the formation and function of the TORC1 complex (35) and therefore has a similar effect on cells as does amino acid starvation. Conversely, TORC1 activation drives protein synthesis via phosphorylation of S6K1, which phosphorylates the ribosomal protein S6 and initiates the translation of messenger RNA. At the same time, 4E-BP1, an inhibitor of protein translation, is also deactivated by mTOR-mediated phosphorylation.

Much less is known about how the TORC2 complex is regulated: there is some evidence that it senses reactive oxygen species and is involved in sphingolipid homeostasis at the plasma membrane (36), while it also seems to sense glucose availability via a cAMP/PKA pathway (37). TORC2 is thought to be negatively regulated by TORC1 activity via Sin1 phosphorylation (38). Rapamycin therefore indirectly activates TORC2 in the short term, but chronic long-term inhibition (over hours to days) of TORC1 (39) or by amino acid starvation (40) seems to eventually reduce the activity of TORC2. TORC2 controls various spatial aspects of cell growth, in particular cell polarity and responses to chemotactic signals via G protein coupled activation of RAS (41).

mTOR SIGNALING INHIBITS FOXP3 EXPRESSION

It has long been known that mTOR inhibition by rapamycin is potently immunosuppressive, partly because it blocks the ability of T cells to respond to interleukin 2 (IL-2) signaling via PI3K and consequently their ability to proliferate in response to antigen (42). More recently, it is has become clear that mTOR signaling also controls the differentiation of $CD4^+$ T helper cell subsets (43), and in particular, the expression of the "master" transcription factor for regulatory T cells, FOXP3. mTOR activation downstream of the TCR, CD28 costimulation and cytokine mediated PI3K signaling is generally required for the proliferation and differentiation of effector T cells but this is inhibitory for FOXP3 expression (44, 45). Signaling downstream of the sphingomyelin phosphate receptor (S1PR), which is required for lymphocyte trafficking and exit from the lymph nodes, can also activate mTOR (46). Interestingly, this pathway is the target of the immunosuppressive drug known as Fingolimod/FTY720 (47), which also has the potential to promote Treg cell development (48). Although the exact mechanism by which mTOR inhibition enhances FOXP3 expression has not been clarified, there is some evidence that implicates a number of different pathways. These could act via poorly defined effects on FOXP3 translation via inhibition of S6K1 and reduced phosphorylation of the ribosomal protein S6. Additionally, mTOR could act either indirectly via suppressor of cytokine signaling 3 (SOCS3) (49, 50) or directly on signal transducer and activator of transcription 3 (STAT3) downstream of IL-6 and the satiety hormone leptin (51). Phospho-STAT3 may then compete for the IL-2 driven STAT5 enhancement of FOXP3 transcription (52). In addition, FOXO3a (53, 54) and the TGFβ signaling component SMAD3, two transcription factors promoting FOXP3 expression, are negatively regulated by AKT downstream of TORC2 (55). Evidence from mice with T cell targeted deficiencies in either raptor (TORC1) or rictor (TORC2) suggests that TORC1 tends to promote Th1 differentiation (43) while TORC2 may bias toward Th2 via AKT and PKCθ (56). Inhibition of both complexes seems to be required for the optimal induction of $FOXP3^+$ Treg cells while Th17 cell development seems to be independent of TORC2, but is inhibited by rapamycin in favor of $FOXP3^+$ Treg cells (57).

WHILE mTOR INHIBITION IS REQUIRED FOR FOXP3 EXPRESSION, mTOR ACTIVATION IS NEEDED FOR REGULATORY FUNCTION

Mechanistic target of rapamycin inhibition therefore seems to be associated with tolerance and $FOXP3^+$ Treg cell induction, and this appeared to be confirmed by T cell specific mTOR knockout mice, which develop an excess of $FOXP3^+$ Treg cells over Th1 and Th2 effector cells (43). Recent data, however, from FOXP3-Cre.$Raptor^{fl/fl}$ mice where TORC1 activity has been specifically knocked out in $FOXP3^+$ Treg cells, indicates that TORC1 activation is still required for Treg cells to function, as evidenced by the development of an autoinflammatory condition very similar to scurfy or FOXP3 deficient mice (58). CD4–$Cre.Raptor^{fl/fl}$ mice, lacking TORC1 activity in all T cells, however, did not develop disease, presumably because this also compromised the effector T cells. This raises the possibility that the optimal induction and expansion of $FOXP3^+$ Treg cells takes place in the nutrient depleted microenvironments associated with tolerance, but the Treg cells only become fully active and proliferative when there is inflammation that needs to be controlled, which requires a re-activation of their mTOR pathway. Interestingly, it had previously been postulated that the optimal functional induction of $FOXP3^+$ Treg cells required alternate cycles or oscillations of mTOR inhibition, first to promote induction, and subsequently mTOR activation to promote proliferation (59).

MODULATION OF FOXP3 EXPRESSION BY ADENOSINE AND HYPOXIA

Hypoxia induced factor (HIF) 1α, another downstream target of TORC1, has also been implicated either as a positive (60, 61) or a negative (62, 63) regulator of FOXP3 expression. HIF1α is a BHLH-Pas transcription factor that has an essential role in the response of cells to hypoxia and which is able to bind directly to FOXP3 protein to target it for proteosomal degradation (62). The level of HIF1α transcription is controlled by NFκβ (64), but its activity is mainly controlled post-translation by an oxygen mediated ubiquitination and degradation controlled by the von Hippel-Lindau tumor suppressor complex with additional positive regulation via a TORC1 mediated phosphorylation (65). Activation of naïve T cells under hypoxic conditions has also been suggested to enhance FOXP3 expression and the differentiation to Treg cells (60), but it is not clear whether this is a direct effect of HIF1α on FOXP3 expression, or whether it is an indirect effect of HIF1α feedback inhibition of mTOR (66). Hypoxia is associated with raised levels of AMP within the cell, and the enzyme AMP activated protein kinase (AMPK) causes inhibition of mTOR via TSC1/2 (67, 68).

AMP and adenosine are particularly relevant to immune regulation, as TGFβ is able to induce in a range of hematopoietic cells the co-expression of two ectoenzymes, CD39 and CD73 (69) that are also constitutively expressed on Treg cells (70). These two enzymes (**Figure 3**) act at the cell surface to convert extracellular sources of ATP, which is associated with inflammation and cell necrosis, into the anti-inflammatory product adenosine.

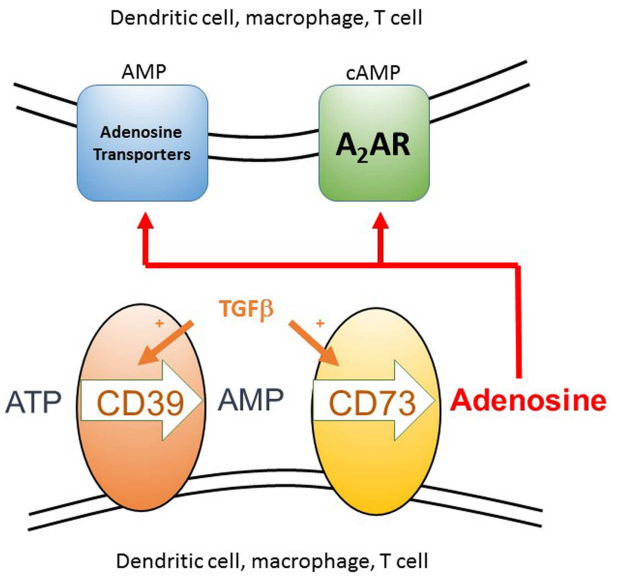

FIGURE 3 | The generation of extracellular adenosine as a component of an anti-inflammatory microenvironment. Extracellular ATP arises as a result of cell death, either from the host or pathogen, as can itself be inflammatory. The two ectoenzymes CD39 and CD73 are normally co-expressed constitutively on Treg cells, but can be induced on the surface of many different cell types, including conventional T cells, dendritic cells, and macrophages, in the presence of a source of TGFβ. These enzymes sequentially convert extracellular ATP to AMP and then adenosine. Adenosine can then act either by binding to the A2A receptor on T cells and DC, which signals via cAMP or it can be taken up via adenosine transporters where it is rapidly converted to intracellular AMP by adenosine kinase. These two signaling pathways act to inhibit inflammation and T cell proliferation. AMP activated kinase mediated inhibition of the mTOR pathway can then occur downstream of either signaling pathway.

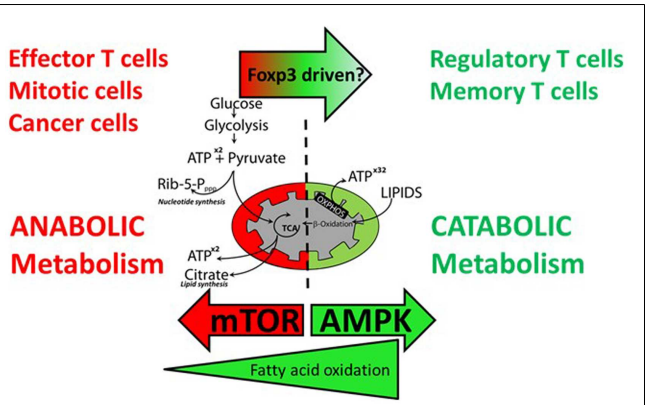

FIGURE 4 | Effector T cells and regulatory T cells have different requirements for anabolic and catabolic metabolism. Effector T cells, proliferating T cells, and cancer cells favor anabolic metabolism using aerobic glycolysis to fuel energy and cell mass demands during proliferation. Activation of mTOR drives this metabolic shift. Precursors for nucleotide synthesis and lipid synthesis are supplied via the pentose phosphate pathway and the TCA cycle, respectively. Resting regulatory and memory T cells do not require glycolysis and TCA intermediates for cell growth and favor catabolic metabolism fueled by β-oxidation of fatty acids to fuel oxidative phosphorylation. Inhibition of mTOR and activation of AMPK favor a shift to catabolic metabolism.

Extracellular adenosine can generate the second messenger cAMP within the target cell via activation of specific G protein coupled receptors on the cell surface [e.g., $A_{2A}R$ on T cells (71, 72)] or it can be directly taken up by specific adenosine transporters (73) where, once inside the cell, it is rapidly converted to AMP by adenosine kinase. AMP is also generated in the cell downstream of G protein signaling via cAMP, which is subsequently broken down to AMP by phosphodiesterases. Although there is evidence that this pathway is relevant to tumors escaping immune surveillance (74, 75), it remains, however, to be resolved whether adenosine is as an important component of the anti-inflammatory microenvironment within tolerated tissues.

T CELLS DISPLAY METABOLIC AND FUNCTIONAL PLASTICITY IN RESPONSE TO DIVERSE ENVIRONMENTAL CUES

T cells not only adapt to their environment by changing metabolic mode, but in addition their chosen fuel source and metabolites, to a large extent, affect their fate and function (**Figure 4**). T cells use glucose and glutamine as their primary source of energy but can switch to ketone bodies and fatty acid use under certain circumstances (76). Glucose is the primary substrate for ATP production in T cells (77, 78). During glycolysis, glucose is converted to two molecules of pyruvate and two molecules of ATP in an oxygen-independent process. Pyruvate generated from glycolysis is oxidized in the TCA cycle yielding NADH and FADH2, which is used to fuel OXPHOS. OXPHOS is oxygen dependent and efficiently yields as much as 36 molecules of ATP per molecule of glucose. In order to mount an effective immune response T cells must expand rapidly and can achieve doubling times as low as 2–6 h (79). To fuel this expansion T cells undergo a major metabolic shift from primarily catabolic fatty acid oxidation (FAO) driven OXPHOS to anabolic glycolysis and glutaminolysis during activation, then revert back to FAO in the memory phase. Glycolysis is an amphibolic process that although less efficient in net ATP production, fuels rapid T cell growth by providing NADPH and ribose from the pentose phosphate pathway for reductive biosynthetic reactions and nucleotide synthesis and fuels lipid synthesis via citrate from the TCA cycle. During this process, glucose is incompletely oxidized and is fermented to lactate while glutamine is converted to glutamate, aspartate, and ammonia. This shift to oxygen-independent glycolysis in the context of normoxia has been termed aerobic glycolysis and is a feature of cancer cells, where the process is termed Warburg metabolism, reviewed in Ref. (80).

Multiple environmental nutritional signals are integrated by T cells via mTOR and AMPK to control their choice of metabolism and function. These signals include glucose and glutamine concentration, oxygen tension, amino acid concentration, lipids, salt concentration (NaCl), leptin concentration, and ATP:ADP ratio. In addition, immune-specific inputs such as T cell receptor triggering and co-stimulatory/inhibitory signals and cytokines are integrated by T cells to change their metabolic mode.

GLUCOSE IS REQUIRED FOR T CELL ACTIVATION

Glucose is critical for T cell activation. It is the primary carbon source for macromolecules such as lipids and nucleotides in T cells

and can fuel the pentose phosphate pathway to generate NADPH reducing equivalents. During activation the T cell increases its rate of glucose metabolism and up-regulates cell surface Glut1 receptors to transfer glucose from the extracellular space (81, 82). Concomitant with the increase in surface Glut1, hexokinase is also upregulated (83, 84) after T cell activation. Hexokinase phosphorylates glucose thus trapping it in the cytoplasm and maintaining a glucose gradient across the plasma membrane to maintain facilitated diffusion of glucose. Indeed, even in the presence of glutamine, in the absence of glucose T cell survival and proliferation is severely impaired (77). Effector T cells (Teff) specifically Th17, Th1, and Th2 are particularly glycolytic and dependent on glucose and accumulate preferentially in Glut1 transgenic mice at the expense of Treg cells. Treg cells preferentially use fatty acids as a fuel, their development is enhanced in the presence of excess fatty acids (78).

GLUTAMINE AS AN IMPORTANT CARBON SOURCE FOR T CELLS

Glutamine is the most abundant amino acid in serum (85) and has also been implicated in immune regulation (86). It is essential for T cell activation as a source of nitrogen and as a key anapleurotic substrate enabling nucleotide synthesis and redox control in addition to fueling metabolism via the TCA cycle following conversion to α-ketoglutarate. T cells consume glutamine at an equivalent rate to glucose (87, 88). Activation of T cells triggers a rapid 5- to 10-fold increase in SNAT1 and SNAT2 (89) (sodium dependent neutral amino acid transporter) glutamine transporter expression and glutamine import via a CD28 and ERK/mitogen-activated protein kinase (MAPK) dependent mechanism. Naïve T cells transport glutamine into the cell via ASCT2 (slc1a5) (90) where the concentration of glutamine becomes sufficient to act as an efflux substrate to drive the system L neutral amino acid transporter slc7a5 in complex with CD98 (slc3a2) to import neutral amino acids into the cell. Sustained neutral amino acids and glutamine are essential for TCR/CD28 driven mTORC1 activation, but not other TCR signaling pathways such as MAPK or IKK (90). Activated TORC1 subsequently prolongs glutamine anapleurosis by activating glutamate dehydrogenase via indirectly inhibiting transcription of its inhibitor, SIRT4 (91). Glutamine can fuel the TCA cycle for anabolic and catabolic metabolism in the presence or absence of glucose and in the presence of hypoxia (92). Cells require acetyl coenzyme A for lipid synthesis. During hypoxia or active proliferation where aerobic glycolysis is engaged glucose carbons are converted to lactate and diverted away from the TCA cycle. Under these conditions cells can use reductive metabolism of α-ketoglutarate as an alternative anaplerotic route to produce acetyl co-A for the synthesis of lipids (93).

FATTY ACID METABOLISM

Resting naïve T cells, memory CD8 T cells and resting regulatory T cells share FAO as a common default metabolic mode (78, 94–96). This metabolic state enhances ATP production through mitochondrial OXPHOS while minimizing anabolic processes required for increased cell mass during proliferation. Environmental lipid concentration has a role in determining the fate of differentiating T cells. Treg cell homeostasis requires environmental lipids, which activate the nuclear receptors peroxisome proliferator activated receptor (PPAR)α and PPARγ that function as fatty acid sensors and regulators of lipid metabolism. These receptors promote FOXP3 expression by CD4 T cells in response to TGFβ (97). Clinically, PPARγ agonists downregulate the production of pro-inflammatory cytokines such as IL-6, TNFα, and leptin (98). Provision of fatty acids to T cells *in vitro* promotes differentiation to Treg cells while inhibiting effector differentiation (78). These observations may explain the severe acute immunosuppression associated with calorific starvation observed in humans.

DIETARY NaCL AND INFLAMMATION

Recent evidence suggests that dietary sodium chloride concentration may play a role in controlling inflammation by inhibiting induction of peripheral Treg cells and favoring the induction of Th17 and Th2 cells (99, 100). Elevated levels of NaCl by just 40 mM have been shown to activate p38 MAPK signaling, resulting in activation of the osmosensitive form of NFAT5 (also known as TONEBP) leading to activation of serum glucocorticoid kinase 1 (SGK1), an AGC serine/threonine kinase (99). SGK1 has been shown to govern salt transport and salt homeostasis in multiple cell types dependent on TORC2 activity (101). Raised levels of salt were shown to turn on SGK1 expression, enhance IL-23R expression, and augment TH17 cell differentiation (99, 100). Powell and colleagues showed that after activation by mTORC2, SGK1 promoted T helper type 2 (T_H2) differentiation by negatively regulating degradation of the transcription factor JunB mediated by the E3 ligase Nedd4-2 (102). The same group also showed that SGK1 turns off IFN-γ via TCF-1. Sodium chloride concentrations vary anatomically, the concentration in plasma is approximately 140 mM, whereas in insterstitium and lymph nodes it is much higher ranging from 160 to 250 mM (103, 104). Thus, it is possible that sodium concentration limits pro-inflammatory activation of T cells in the blood while allowing Th17 differentiation in tissues and lymph nodes.

LEPTIN AS A PRO-INFLAMMATORY CYTOKINE

Leptin is an IL-6-like cytokine produced by adipocytes (termed an "adipokine"), which acts directly on the hypothalamus as a satiety hormone and also has effects on metabolism and T cell functions (105, 106). Adipokines are hormones or cytokines secreted by adipocytes, which have pleiotropic effects on the nutritional status and immune system of the organism. These include the cytokines IL-1, IL-6, IFN-γ, TNFα as well as leptin and adiponectin. Leptin is produced at high levels constitutively by regulatory T cells, which also express the leptin receptor (ObR) (107). Leptin is required for activated T cell proliferation and cytokine production in part via inducing up-regulation of mRNA and surface expression of the Glut1 receptor and glucose uptake (108). Leptin in combination with T cell receptor triggering induces activation of $CD4^+CD45RA$ naïve T cells but inhibits activation of $CD4^+CD45RO^+$ memory T cells in humans (109). It skews these cells to produce pro-inflammatory cytokines including IFN-γ and TNFα and leptin itself. Leptin functions to negatively regulate Treg cell activity and proliferation. Leptin deficient mice (*ob/ob*) and leptin receptor deficient mice (*db/db*) have decreased susceptibility to autoimmunity and increased numbers of Treg cells (107, 110, 111). Neutralization of leptin in Treg cell cultures enhances

their IL-2 dependent proliferation while maintaining suppressive function. Thus, leptin appears to function as a feedback control mechanism to control Treg cell activity in response to nutrient availability.

SIGNALING MECHANISMS REGULATING T CELL METABOLISM

mTOR COORDINATES METABOLISM AND T CELL DIFFERENTIATION

The activation of naïve T cells has been primarily associated with glucose metabolism, even under aerobic conditions, as this not only provides a source of ATP for energy and effector cell activity but also generates the precursors for nucleotide synthesis and lipogenesis that are required for cell proliferation (5). Under conditions of nutrient restriction and mTOR inhibition, however, it would be expected that T cells would switch to more efficient pathways of energy generation, such as OXPHOS and FAO, both of which require active mitochondria. Indeed, it has been shown that Treg cells have higher levels of AMPK activity, which as we have seen leads to mTOR inhibition, and this in turn reduces the expression of the glucose transporter (Glut1) and enhances lipid oxidation, effects, which can be reversed in Glut1 over-expressing transgenic mice (78). Multiple intracellular signaling pathways control the choice of metabolic activity engaged by T cells.

c-Myc

The proto-oncogene c-Myc is a critical positive regulator of both gylcolysis and glutaminolysis and as such has a potentially important role in T cell plasticity. c-Myc and its binding partner max binds to over 10,000 sites in mammalian genomes at a consensus E-box sequence CAGCTG (112, 113). c-Myc has a fundamental role in controlling metabolism. It increases transcription of all the glycolysis genes (114) and also the glutamine transporters ASCT2 and SN2 by binding to their promoters (114–116). c-Myc increases usage of pentose phosphate pathway, glycolysis, and glutaminolysis (84) and also augments mitochondrial biogenesis via up-regulation of PGC1 (117–119) and the transferrin receptor TFRC (120), which is necessary to provide iron for the heme containing proteins of the electron transport chain. Thus, c-Myc drives cells toward anabolic metabolism, at the same time it promotes cell division via glutaminolysis providing the anaplerotic substrate aKG needed for synthesis of polyamines required for T cell proliferation (115). Myc deletion in T cells using inducible tamoxifen cre-lox systems leads to inhibition of glycolysis and glutaminolysis (84). Glutamine deprivation inhibits T cell activation induced growth and proliferation (84).

ESTROGEN RECEPTOR RELATED RECEPTOR α

Estrogen receptor related receptor α (ERRα) is an orphan receptor, one of three members of a family α, β, and γ, which bind to a DNA consensus site termed the ERR response element (TNAAGGTCA) in multiple genes (121). Despite its name it is not activated by estrogen or related hormones, but seems to be constitutively active, having an active ligand binding region in the absence of ligand (122). ERRα is important in immune reprograming as it seems to function to "rewire" cells to use glucose for anabolism (123). The glucose transporter glut1 and glucose uptake are inhibited in ERRα null T cells, and by chemical inhibition of ERR (123). ERRα physically interacts with PGC1α and PGC1β (124), which act as co-activators to activate transcription of a number of genes important for FAO (MCAD, CPT1B), TCA cycle (IDH3A, AC02), and OXPHOS (CYCS, ATP5B) in multiple cell types (125). Interaction of ERRα with the transcriptional co-repressor RIP140 results in down regulation of many of the genes, which PGC1α/β activates (126–128). Acute inhibition of ERRα in T cells results in their inability to proliferate or differentiate into Th subsets, an effect, which is rescued for proliferation and Treg cell differentiation, but not Teffector differentiation, by addition of long chain fatty acids (123). Thus, ERRα functions in T cells to enable them to prepare for the metabolic demands of proliferation and differentiation into effector subsets by enhancing glucose uptake and mitochondrial biogenesis.

PGC1α

Peroxisome proliferator activated receptor γ co-activator 1α (PGC1α) is a transcriptional co-activator, a protein with ability to enhance transcription factor binding to genes, which has no specific DNA binding capability of its own. It has a central role in metabolism being the co-activator for multiple transcription factors involved in mitochondrial biogenesis (129) and glucose and fatty acid metabolism (130) and gluconeogenesis (131).

LIVER X RECEPTOR

Liver X receptors (LXRs) are receptors of the nuclear receptor family, which bind to endogenous oxysterols. LXRs have two isoforms, α and β. Both isoforms are expressed by CD4 T cells. These receptors heterodimerize with the retinoid X receptor (RXR) and function to modulate cholesterol homeostasis by controlling genes involved in cholesterol and lipid metabolism including sterol regulatory element binding protein (SREBP) (132, 133). LXRs have potent effects on T cell function including inhibition of lymphocyte proliferation (132). Ectopic expression of LXR was also shown to inhibit T cell differentiation into Th17 cells via induction of srebp-1 a protein capable of inhibition of aryl hydrocarbon receptor (Ahr) binding to the IL-17 gene (134). Agonists of LXR have been shown to ameliorate experimental autoimmune encephalomyelitis (EAE) (134). SREBP-1 has recently been shown to be essential for coordinating T cell receptor activation and lipid anabolism in dividing CD8 T cells (135). In the absence of SREBP-1, CD8 T cells can enter G1 phase of cell cycle but fail to continue to mitosis due to a lack of sufficient cellular cholesterol.

Ca^{2+} SIGNALING

Calcium levels in the mitochondrial matrix are tightly regulated by mitochondrial Ca^{2+} uniporters (MCU), which transport Ca^{2+} across the inner mitochondrial membranes (136). Mitochondrial sequestration of Ca^{2+} ions results in positive feedback leading to activation of plasma membrane CRAC channels and sustained T cell activation (137, 138). Ca^{2+} concentration in the mitochondrial matrix also has important effects on the rate of the TCA cycle as three calcium dependent TCA enzymes: 2-oxyglutarate dehydrogenase, NAD^{+}-isocitrate dehydrogenase, and pyruvate dehydrogenase are activated by Ca^{2+} ions leading to increased mitochondrial metabolism (139).

MITOGEN-ACTIVATED PROTEIN KINASE

The MAPK family of serine/threonine/tyrosine kinases plays a central and pleitropic role in transducing diverse signals from the environment into nuclear transcription factor activation. They are involved in the cellular responses of T cells to inflammatory cytokines, mitogens, insulin, heat shock, and osmotic stress [reviewed in Ref. (140)]. MAPK is required for glucose and glutamine uptake and metabolism in T cells in a CD28 dependent manner and is required for glutaminase activity (89, 141).

AMP ACTIVATED PROTEIN KINASE

AMP activated protein kinase is important in energy homeostasis within the cell and the sensing of hypoxia due to the increase in AMP to ATP ratio under these conditions [reviewed in Ref. (142)]. The binding of AMP or ADP allows the phosphorylation of AMPK, which activated its serine/threonine kinase activity. AMPK phosporylates acetyl-Co-A carboxylase (ACC1) to inhibit its lipogenic activity and ACC2 to promote expression of carnitine palmitoyltransferase (CPT1A), which is the rate limiting factor for the uptake and oxidation of lipids in the mitochondria. PGC1α activity (see above) is also promoted by AMPK activation. AMPK also inhibits TORC1 signaling by phosphorylating TSC1/2 and enhances autophagy, glucose uptake, and mitochondrial biogenesis. The net effect of AMPK activation is to shut down energy intensive processes and to activate pathways that replenish ATP levels within the cell.

METABOLIC FEEDBACK AND "MOONLIGHTING" FUNCTIONS OF METABOLIC ENZYMES

In addition to carrying out metabolic activities, many enzymes of the glycolytic, pentose phosphate, TCA, and fatty acid metabolism pathways have dual function and "moonlight" as RNA binding proteins, transcriptionally controlling their targets in a metabolite dependent fashion (**Figure 5**). This area has been well reviewed (96, 143, 144) so only a few key examples will be highlighted here.

The most thoroughly investigated example of a metabolic enzyme having a second function in RNA translational control is cytosolic aconitase/iron regulatory protein 1 – IRP1 (145, 146). This enzyme functions to convert citrate to isocitrate in the TCA cycle. It is dependent on replete levels of cellular iron for enzymatic function as it contains a cubane 4fe–4S iron sulfur cluster responsible for its catalytic activity. In conditions of low iron this cluster disassembles and converts to IRP1, which is capable of binding to iron responsive elements (IREs), conserved hairpin structures in the 3′ UTR of RNAs responsible for iron homeostasis (147). IRP binds with very high affinity (Kd 5pM) to transferrin receptor RNA (148) to protect the RNA from degradation and increase translation. It simultaneously binds to an IRE in ferritin, an iron storage protein, causing it to become translationally repressed (149). In this way, the enzymatic activity of the protein, dependent on iron senses cellular iron levels and acts as a rheostat for iron by adjusting the translation of RNA encoding proteins involved in iron homeostasis. Uptake of iron is also essential for the heme containing proteins in the electron transport chain, and would be required for resting Treg cells preferentially engaging FAO and OXPHOS.

Several enzymes of the glycolysis pathway have been shown to have RNA binding activity. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH), aldolase, lactate dehydrogenase (LDH), phosphoglycerate kinase (PGK), and glucose 6 phosphate dehydrogenase (G6PDH) have been shown to regulate the translation of immunologically relevant mRNA targets (150–152). GAPDH, LDH PGK, and G6PDH all share a dinucleotide binding region termed the Rossman fold (153). This RNA binding region consists of two βαβ folds each of which binds a mononucleotide. The Rossman fold RNA binding activity is competed for by the dinucleotides NAD^+, NADH, and ATP. In the case of GAPDH, the cofactor NAD^+ is required for glycolytic activity and inhibits target RNA binding (150) In this way, when GAPDH is required for glycolysis and cofactors are abundant its RNA binding ability is competed for by cofactors and shut off. GAPDH has been shown to target many RNAs including mRNA (150), tRNA (154), rRNA (155), and viral RNAs (156). GAPDH mRNA targets include IL-2, GM-CSF, IFNα (150), GLUT1 (151), and IFN-γ (95) where it binds to an AU rich region in the 3′ untranslated region. In the case of IFN-γ, GAPDH comprises a component of the gamma interferon-activated inhibitor of translation (GAIT) complex (95). If activated T cells are deprived of glucose, and instead provided with galactose, then glycolysis cannot take place, and yet the T cells still activate and proliferate (because galactose provides alternative precursors for nucleotide synthesis via the pentose phosphate

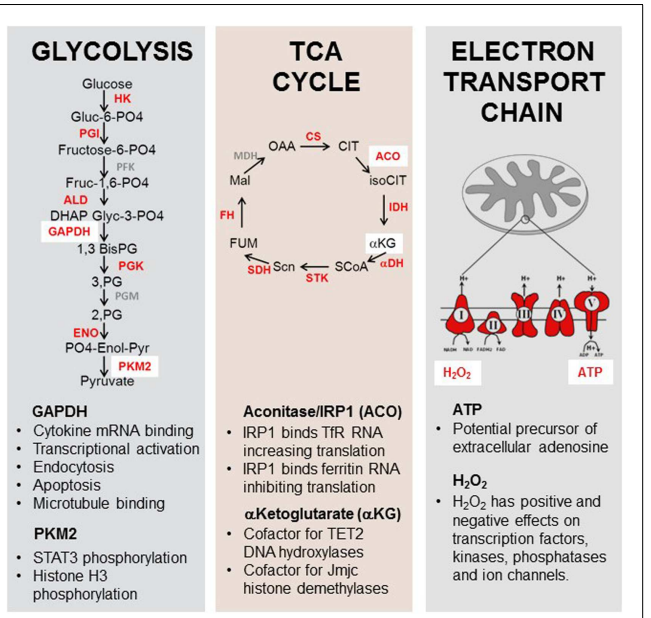

FIGURE 5 | Moonlighting functions of metabolic enzymes and metabolites. Enzymes and metabolites of the glycolysis, TCA cycle, and electron transport chain play roles in immune function. Enzymes and substrates in red have been shown to have additional non-metabolic functions in eukaryotes [reviewed in Ref. (173–176)]. GAPDH can bind to the 3′UTR of some cytokine genes, inhibiting their translation. Pyruvate kinase M2 has been shown to have kinase activity for the pro-inflammatory transcription factor STAT3. Aconitase functions as a rheostat for cellular iron in addition to its role in the TCA cycle. α-Ketoglutarate is an essential cofactor for the enzymes TET2 and jumonji-C histone demethylases. ATP may act as a precursor for extracellular adenosine production and H_2O_2 has been shown to possess a signaling role in multiple cytoplasmic and nuclear pathways.

pathway), but now because GAPDH has no substrate, it blocks the translation of IFN-γ. Under these conditions the T cells also then express other markers of T cell exhaustion such as programed death 1 (PD-1) (95). The corollary of this is that inducing glycolysis, for example, by mTOR activation, will tend to promote effector cell differentiation. There are also suggestions that there may be other examples where metabolic enzymes, for example, hexokinase (157) and IDO (51) can have a secondary, signaling role, in DC differentiation.

Pyruvate kinase (PK) catalyzes the final step of glycolyis; phosphoenolpyruvate to pyruvate. It exists in two differentially spliced forms in most cells PKM1 and PKM2 (158). Highly proliferating cells including embryonic cells tumor cells and activated T cells preferentially express the less efficient form PKM2, which may support accumulation of glycolytic intermediates necessary for production of amino acids and nucleotides during proliferation (96, 159, 160). PKM2 also "moonlights" as a kinase for STAT3 using PEP instead of ATP as a phosphate donor (161). This finding suggests that the metabolic status of the T cell, anabolic or catabolic, indirectly controls activity of a transcription factor known to transduce pro-inflammatory signals downstream of diverse cytokines including interferons, IL-5, IL-6, and leptin. Effector T cells in anabolic mode might be predicted to enhance transcriptional programs normally associated with these pro-inflammatory signals.

Treg CELL EPIGENETICS AND METABOLISM

Constitutive expression of FOXP3 has been shown to be essential for continued Treg cell maintenance of functional tolerance *in vivo* (11, 162–164). However, FOXP3 expression on its own does not seem to be sufficient to enable full differentiation into the Treg cell lineage. Mature Treg cells have a characteristic epigenetic "fingerprint" of demethylated genes associated with Treg cell function, which includes five genes termed the "Treg.me"; FOXP3, CTLA4, Ikzf2 (Helios), Ikzf4 (Eos), and Tnfrsf18 (GITR) (165, 166). In addition, several hundred proteins have been found to be associated with FOXP3 in mass spectroscopy screens (167) several of which are important for maintaining FOXP3 transcription. Several important transcription factors including Runx1, NFAT, and GATA3, which are necessary for FOXP3's function also associate with FOXP3. Importantly, progression along the Treg cell lineage seems to occur prior to the induction of FOXP3 as several characteristic Treg cell gene sets can be observed in T cells from mice with a targeted disruption of the FOXP3 gene into which GFP has been inserted under the control of the FOXP3 promoter. FOXP3 is thought to amplify the pre-existing gene profile (168). Characteristic epigenetic modifications have been shown to be associated with the FOXP3 gene in mature Treg cell, which are not present in naïve T cells. An intronic conserved non-coding element (CNS2) was discovered to be demethylated preferentially in stable Treg cells (169, 170). Histones surrounding this region were also shown to have characteristic modifications of open chromatin (H3K4me3 and acetyl H4 high) (169). DNA methylation and histone modification by methylation, acetylations, and phosphorylation via the action of methyl transferases, acetyl transferases, and kinases, respectively, requires cellular metabolites as enzymatic substrates (171). For example, the sirtuins (histone deacetylases) and poly ADP ribose polymerases (PARPs) require the coenzyme NAD^+ to function (172). In addition, the TET2 DNA hydroxylases, involved in demethylation of DNA and the jumonji-C (JmjC) histone demethylases involved in histone demethylation belong to a group of enzymes called the α-ketoglutarate dependent enzymes, which require the TCA cycle metabolite α-ketoglutarate to function in addition to ascorbate, oxygen, and iron. Both these classes of enzymes are inhibited by the TCA cycle intermediates fumarate and succinate. It is conceivable that epigenetic changes in Treg cells required for stability and reprograming to the Treg cell lineage may be influenced by the metabolic program that the cell adopts in response to environmental stimuli such as glucose or fatty acid availability.

ARE REGULATORY T CELLS ADAPTED METABOLICALLY FOR CURRENT AND FUTURE MICROENVIRONMENTS?

The experimental data of Treg cell metabolism are predominantly derived from *in vitro* observations of resting Treg cells. These cell cultures are usually performed in media with a vast molar excess of EAAs, 10-fold physiological levels of glucose and glutamine and oxygen concentrations in excess of physiological norms. However, it is well known that Treg cells proliferate vigorously *in vivo* and presumably require glycolytic intermediates to fuel anabolic demand during multiple rounds of mitosis. The question remains, what would be the advantage to non-proliferating Treg cells of adopting FAO and OXPHOS as a default metabolic mode? We hypothesize that Treg cells are uniquely adapted to their current and future *in vivo* environments. While Treg cells interact with DCs in a tolerant microenvironment they induce EAA-catabolizing enzyme expression in the DC, forming a zone of acute EAA starvation. In this situation their catabolic mode preferentially protects them from the effects of amino acid starvation, and presumably limiting glucose concentrations in inflamed environments. In addition, they receive survival signals in the form of IL-2 from effector T cells, yet produce little IL-2 themselves, thus, inhibiting further effector T cell proliferation. It is possible that shifting metabolism to catabolic mode in this situation frees up "moonlighting" glycolytic enzymes to suppress translation of pro-inflammatory cytokines by the Treg cells. Expansion of Treg cell numbers in the draining lymph nodes of inflammatory sites would elicit a switch to anabolic metabolism under conditions of sufficient glucose and glutamine, enabling increase in cell mass and mitosis. The ability to shift from an anabolic expansion mode to a catabolic suppression mode may be key to Treg cell function, and presents an attractive target for therapeutic intervention.

CONCLUDING REMARKS

Nutrient sensing and the coordination of metabolism seem to be inherently associated with the mechanisms of immune regulation *in vivo*. The question that then arises is – can any of these metabolic processes be specifically targeted for manipulating immune responses in transplantation, the treatment of autoimmune diseases and cancer immunotherapy? Many of these pathways are common to many different cells in the body and relying on the immunosuppressive effects of available drugs such as the mTOR inhibitors may therefore have a variety of unwanted side effects. Consequently, we need to look either for potential

target components of these metabolic pathways that are restricted primarily to immune cells or for ways to amplify the effects of metabolic inhibitors such that they can be used at doses well below that which have effects outside the immune system. One way to achieve this might be to concentrate on the period of immune reconstitution after lymphocyte depletion when the metabolic needs of homeostatic proliferation of a small number of residual T cells could be biased in favor of regulatory T cells.

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Metabolic regulation of regulatory T cell development and function

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It is now well established that the effector T cell (T_{eff}) response is regulated by a series of metabolic switches. Quiescent T cells predominantly require adenosine triphosphate-generating processes, whereas proliferating T_{eff} require high metabolic flux through growth-promoting pathways, such as glycolysis. Pathways that control metabolism and immune cell function are intimately linked, and changes in cell metabolism at both the cell and system levels have been shown to enhance or suppress specific T cell effector functions. Furthermore, functionally distinct T cell subsets require distinct energetic and biosynthetic pathways to support their specific functional needs. In particular, naturally occurring regulatory T cells (T_{reg}) are characterized by a unique metabolic signature distinct to that of conventional T_{eff} cells. We here briefly review the signaling pathways that control T_{reg} metabolism and how this metabolic phenotype integrates their differentiation and function. Ultimately, these metabolic features may provide new opportunities for the therapeutic modulation of unwanted immune responses.

Keywords: metabolism, regulatory T cells, T cell differentiation, T cell function, mTOR pathway

METABOLIC FEATURES OF REGULATORY T CELLS

T cell differentiation and fate are orchestrated by signaling events involving the T cell receptor (TCR), co-stimulatory or co-inhibitory receptor stimulation, and cytokines. In addition, a variety of other environmental factors can also contribute to this decision. T cells switch between highly proliferative states (i.e., developing thymocytes and activated proliferating T cells) and quiescent states (i.e., naive, memory, and anergic T cells), characterized by the activation of different intracellular metabolic pathways (1). T cells use glucose as their primary fuel source for generation of adenosine triphosphate (ATP) and it is necessary for cell survival, growth, activation, proliferation, and cytokine production (2, 3).

T cell receptor stimulation is accompanied by signals from growth factors and cytokines such as interleukin (IL)-2 or IL-7, and co-stimulatory molecules, such as CD28, which lead to an increase in glucose uptake and glycolysis through induction of phosphoinositide-3-kinase (PI3K)-dependent activation of Akt (4). Akt induces glucose metabolism by facilitating glucose uptake via the upregulation of glucose transporter 1 (Glut1) on the T cell membrane (5). Failure of T cells to up-regulate glucose metabolism results in decreased cytokine production, proliferation, and ultimately to apoptosis (6–8) or anergy (9). An increase in the rate of protein synthesis also occurs following T cell activation and is regulated via Akt, which controls the activation of the mammalian target of rapamycin (mTOR), which is a key regulator of protein synthesis in T cells (10, 11).

Naturally occurring regulatory T cells (T_{reg}), defined as $CD4^+CD25^+Foxp3^+$ T cells, play a non-redundant role in the maintenance of physiological tolerance to self-antigens and prevention of autoimmune responses (12, 13). T_{reg} generation in the thymus is promoted by recognition of self-peptides with intermediate affinity (14). T_{reg} cells are characterized by a specific metabolic signature regulating their responsiveness to antigenic stimulations when compared to other $CD4^+$ T cell subsets (15–18). Specifically, Th1, Th2, and Th17 cells express high surface levels of Glut1 and are highly glycolytic. T_{reg}, in contrast, express low levels of Glut1 and have high lipid oxidation rates *in vitro* (19). Furthermore, blocking glycolysis promotes T_{reg} cell generation through the transcription factor hypoxia-inducible factor 1α (HIF1α), whose induction required mTOR pathway activation (20). In turn, HIF1α enhances Th17 development through direct transcriptional activation of RORγt, and concurrently, it attenuates T_{reg} development, by binding FoxP3 and targeting it for proteasomal degradation.

Collectively, these observations underscore the key role of metabolic cues and regulatory pathways in defining T cell differentiation and function (**Figure 1**).

mTOR INHIBITION AND T_{reg} DIFFERENTIATION

The mTOR is a key regulator of T cell metabolism, that serves to integrate nutrient sensing pathways with signaling pathways involved in differentiation, growth, survival, and proliferation (21). TCR and co-stimulatory signals along with cytokines tweak the mTOR pathway via the upstream PI3K/Akt signaling networks to match the energy requirements associated with T cell activation (22, 23). Conventional $CD4^+$ and $CD8^+$ T cells, upon stimulation, utilize the mTOR pathway to meet the increased metabolic demands of T cell activation by switching from primarily oxidative phosphorylation, seen in resting T cells, toward a state of enhanced aerobic glycolysis, a phenomenon popularly described as the Warburg effect (3, 24, 25). The importance of this phenomenon in determining T cell fate was first noticed using the selective inhibitor of mTOR, rapamycin, which prevented the generation

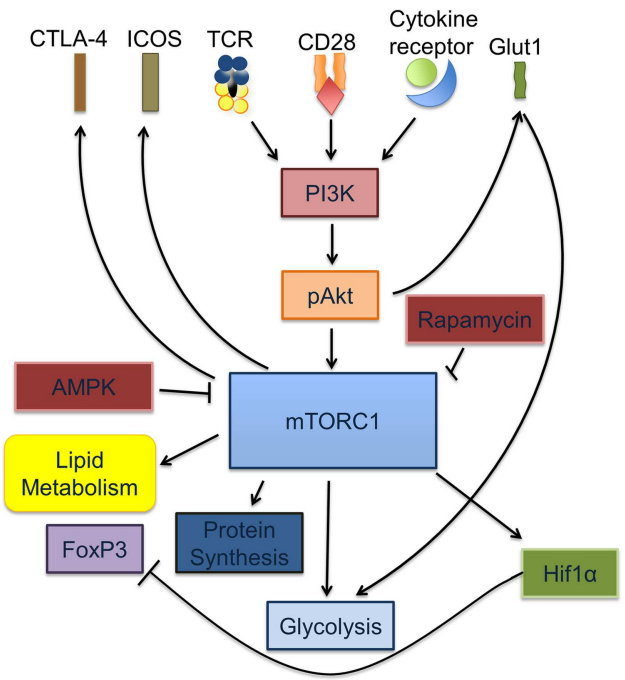

FIGURE 1 | mTORC1 is a key regulator of T_{reg} and T_{eff} activation and differentiation. Signaling through the TCR is accompanied by cytokine signals from IL-2, IL-7, and leptin as well as co-stimulation via CD28. Together, these signals cause increased glycolysis through the induction of PI3K-dependent activation of Akt, which in turn increases glucose transport via the upregulation of Glut1 on the T cell surface. Activated pAkt increases the rate of protein synthesis via activation of mTORC1. Activated mTORC1 activates HIF1α, regulates lipid metabolism and glycolysis.

of T_{eff} responses and promoted the generation of T_{reg} cells (9, 26–28). Additionally, T cell-specific mTOR knockouts were shown to have poor T_{eff} responses and defaulted toward a more T_{reg} phenotype (29). These studies not only revealed the importance of mTOR as a critical regulator in the differentiation of T_{reg}, but also highlighted the importance of the metabolic pathways that predominate within functionally different T cell subsets.

Consistent with the above findings, T_{reg} display higher levels of AMP kinase activity and preferential lipid oxidation for their energy requirements (19). The AMP-activated kinase acts as a sensor of the AMP/ATP ratio, which is increased during hypoxia and inhibits mTOR kinase to promote mitochondrial oxidative metabolism rather than glycolysis (30, 31). Interestingly, activation of AMP kinase via Metformin, a drug used to treat diabetes mellitus, increased the T_{reg} population in the $CD4^+$ T cell compartment in an *in vivo* murine model of asthma (19). In this study, mice sensitized by aerosol to ovalbumin in the presence of metformin, and challenged 21 days later showed an increase in the frequency and number of $CD4^+Foxp3^+$ T cells in the draining lymph nodes as compared to mice immunized in the absence of metformin. However, no change in airway responsiveness was noted even though there were fewer lymphocytes recovered in the bronchial alveolar lavage in the metformin treated animals. Additionally, inhibition of mitochondrial lipid uptake and oxidation pathways by Etomoxir, an inhibitor that prevents long chain fatty acid uptake to the inner mitochondrial membrane for beta oxidation, abrogated the generation of T_{reg} without altering T_{eff} differentiation (19). Furthermore, T_{reg} were shown to express lower levels of the glucose transporter Glut1 as compared to T_{eff}, and transgenic $CD4^+$ T cells overexpressing Glut1 were shown to develop fewer T_{reg}. Overall, these studies indicate that fatty acid oxidation is the dominant metabolic process utilized for the generation of energy in T_{reg}.

mTOR AND T_{reg} FUNCTION

While inhibition of mTOR enhances T_{reg} generation during an immune response, mTOR activity is known to be required to maintain their suppressive capabilities. In this section, we review recent findings that investigated this apparent dichotomy in the function of mTOR in T_{reg} biology. mTOR exists as two structurally distinct complexes (mTORC1 and mTORC2). Both complexes localize within different subcellular compartments and have different functions in the cell; rapamycin-sensitive mTORC1 forms the fundamental nutrient sensing complex that is activated by Akt kinase downstream of PI3K signaling induction (via the TCR, co-stimulatory receptors, and cytokines) whereas the rapamycin-insensitive mTORC2 controls spatial aspects of cell growth through activation of cytoskeletal components (32, 33). The mTORC2 complex also, in turn, activates the kinase Akt (34, 35). Thus, Akt lies both upstream and downstream of mTOR. In mice, $CD4^+$ T cells lacking both mTORC1 and mTORC2 complexes fail to differentiate into any T_{eff} lineage (Th1, Th2, or Th17) and instead differentiate toward the T_{reg} cell phenotype, consistent with the $CD4^+$ population of mTOR null mice (36). However, recent findings by Hu Zheng et al. indicate a crucial role of the mTORC1 complex to the suppressive activity of T_{reg} (29). Indeed, mTORC1 activity was shown to be higher in T_{reg} than naive T cells under steady state conditions. Impairment of the mTORC1 pathway in T_{reg} via selective genetic deletion of Raptor, an obligatory component of mTORC1, in the $CD4^+$ $FOXP3^+$ compartment, led to the early onset of a fatal autoimmune disease in mice (29). Moreover, the disease mimicked the autoimmune disease seen in Scurfy mice that bear a loss-of-function mutation in the FoxP3 transcription factor, indicating impaired T_{reg} function. Mechanistically, the mTORC1 pathway in T_{reg} was shown to be necessary to initiate the upregulation of surface CTLA-4 and ICOS, key intrinsic receptors for T_{reg}-mediated suppression. In addition, mTORC1 was shown to induce cholesterol and lipid metabolism as well as proliferation in the T_{reg} population (29). Finally, recent investigations have revealed a non-redundant role of mTORC1 in mitochondrial metabolism (37). Collectively, these investigations imply a differential use of mTOR in T_{reg} as compared to conventional effector cells.

A MODEL OF T_{reg} DIFFERENTIATION BASED ON mTOR ACTIVATION

From the aforementioned studies, it is clear that the metabolic cues from the environment and subsequent mTOR activity play a key role in T_{reg} differentiation. Powell et al. have proposed a model of T_{reg} differentiation based on mTOR activity that mimics that seen in conventional T cell differentiation. Briefly, naïve T cells, receiving strong mTOR activation upon antigen recognition (through

environmental cues, TCR, cytokine, and co-stimulatory stimulation), differentiated into short-lived T_{eff} cells exhibiting high glycolytic activity, while those receiving weak mTOR activation developed into long-lived memory T cells dependent on oxidative phosphorylation to meet their energy needs (38). One can suggest that the high level of mTOR activity in T_{eff} cells would be necessary to sustain higher demand for energy via glycolytic pathways while the opposite would hold true for quiescent memory T cells. A similar model can be applied to induced T_{reg} where naïve T cells in the presence of TGF-β receiving either high or low mTOR activating signals could result in the differentiation of "effector" and "memory" Foxp3$^+$ T_{reg} respectively. As such, CD4$^+$ Foxp3$^+$ T cells that traffic to activating lymph nodes and become robustly stimulated (mTOR^{hi}) generate short-lived "effector" T_{reg}. These effector T_{reg} would then home to the tissues and control immune responses. This model can explain why T cells stimulated *in vitro* with high doses of peptide in the presence of exogenous TGF-β develop into T_{reg}. These mTOR^{hi} T_{reg} exhibit high glycolytic activity similar to that of conventional T_{eff} cells (**Figure 2**). Consequently, this model can also be applied to natural T_{reg} cells differentiation into effector or memory T_{reg} arising through associated mTOR hi or low activity upon antigen recognition (38).

OSCILLATING mTOR ACTIVITY PROMOTES PROLIFERATION IN T_{reg}

A hallmark feature of T_{reg} cells is their ability to proliferate abundantly *in vivo* while remaining anergic and poorly proliferative *in vitro* (39, 40). This anergic *in vitro* state was shown to be reversible via activation in the presence of supra-physiologic concentrations of IL-2 (41). In addition, short-term treatment of T_{reg} with rapamycin preceding activation in the presence of supra-physiologic quantities of IL-2 was shown to promote proliferation

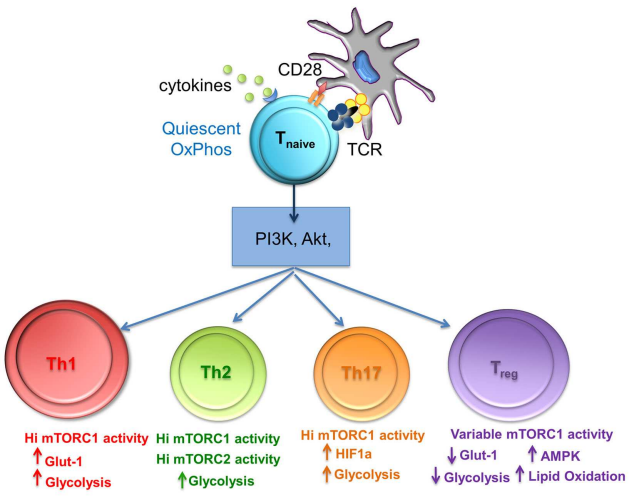

FIGURE 2 | The level of mTORC1 activity and glycolysis is important for differentiation of T cell subsets. mTORC1 integrates nutrient sensing and signaling pathways to match the energy requirements of activated T cells. Th1, Th2, and Th17 cells require high levels of glycolysis that is mediated by high mTORC1 activity, whereas T_{reg} differentiation requires variable mTorc activity, reduced glycolysis, and lipid oxidation.

in vitro at much higher levels than those induced by IL-2 alone. This posed a conundrum as to how two signals having opposite effects on mTOR activity can converge to enhance proliferation of T_{reg}. To explain this phenomenon, a model was put forward (18), which postulates that mTOR activity in T_{reg} is highly dynamic, oscillating between low and high activation states. As mentioned before, mTOR activity in T_{reg} was shown to be higher at resting states when compared to naïve T_{eff}. According to this model, the intermittent reduction in mTOR signaling followed by its enhanced activation by means of TCR triggering and IL-2 stimulation promotes T_{reg} proliferation. However, T_{reg} requirement for down-regulation of mTOR signaling was shown to be short-lived as protracted incubation with rapamycin ablated their proliferation. This model also identified the adipocyte hormone leptin as a key signal that regulates mTOR activity *in vivo*, promoting T_{reg} proliferation. Within the immune system, leptin has been seen to activate pro-inflammatory cells while diminished leptin levels can lead to immunosuppression (42). Leptin produced by T_{reg} cells was shown to contribute to the activation of the mTOR pathway in an autocrine manner. Other mechanisms through which mTOR activity is maintained in its oscillating state to overcome their hypo-responsiveness and enter the cell cycle continue to be investigated.

METABOLIC REGULATION OF T_{reg} AND Th17 DIFFERENTIATION

Interleukin-17 (Th17) producing and induced regulatory T cells (iT_{reg}) differentiate from naïve CD4$^+$ T cells and mediate diverse and often opposing effects in lymphoid and peripheral tissues. Under the influence of TGFβ and IL-2, naive T cells are induced to express the transcription factor FoxP3, and differentiate into tissue-resident iT_{reg}, which support a suppressive environment. However, in the presence of IL-6, naive T cells stimulated with TGFβ express the transcription factors STAT3 and Rorγt, secrete IL-17, and produce an inflammatory environment.

It has recently emerged that metabolic factors can modulate the balance of Th17 and iT_{reg} cells resulting in inflammation or actively maintained tolerance.

Commitment to the Th17 lineage, like other T_{eff}, requires increased mTORC1 activity to sustain differentiation and function. As the presence of TGFβ is required for the development of both Th17 and T_{reg} cell subsets, the relative differentiation of each cell type can be influenced by the level of mTORC1 activation. This interconnectivity is especially significant because of the opposing functions of the two cells types. The metabolic regulation and influence on the Th17:T_{reg} ratio has been articulately reviewed by Barbi, Pardoll, and Fan-Pan (43) and so is briefly summarized here.

The activation of mTOR, and the subsequent switch to aerobic glycolysis, is essential for Th17 development; IL-1 enhances Th17 cell differentiation and proliferation via mTOR activation (44) whereas mTOR inhibition prevents Th17 differentiation (45, 46) and ameliorates Th17-dependent symptoms in a murine EAE model (47). Concomitantly, in these experiments, mTOR inactivation increases T_{reg} cell numbers and function and sensitizes T_{reg} to TGFβ (45, 48).

As well as mTOR, hypoxia-inducible factor (HIF1α), a transcription factor activated during inflammation and in response to

low oxygen levels, is a critical regulator of metabolism. In T cells, HIF1α plays a role in inducing aerobic glycolysis even in the presence of plentiful oxygen (49). Elevated glycolysis in Th17 cells is dependent on HIF1α, and indeed, the transcription factor is essential for their differentiation and function (20). HIF1α activation, under aerobic conditions, is modulated by mTORC1 and therefore the concerted actions of HIF1α and mTORC1 preferentially guide Th17 cell development and effector functions. Furthermore, HIF1α directly binds to FoxP3 and targets it for proteosomal degradation while also increasing the transcription of the Th17-related transcription factor Rorγt. Mice with HIF1α-deficient T cells are resistant to Th17-dependent EAE with a response that is characterized by a decrease in Th17 cells and an increase in T_{reg} cells (50). Thus, HIF1α and mTOR represent important mediators of the Th17:T_{reg} balance in hypoxic and inflamed tissues, and as such are potentially important targets for clinical interventions.

VISCERAL ADIPOSE TISSUE-ASSOCIATED T_{reg}

Metabolic stress is also known to influence the development of T_{reg}, and specifically to affect adipose-tissue-resident T_{reg} cells. This population of T_{reg} produces high levels of IL-10 and is characterized by the expression of GATA3, CCR2, KLRG1, and lack of CD103 expression (51). Visceral adipose tissue (VAT) T_{reg} are thought to be important for the maintenance of responsiveness to insulin, by regulating adipokine release. In obese humans and mice, VAT T_{reg} are progressively replaced by a pro-inflammatory T_{eff} cell infiltrate, which accumulates in adipose tissue and produces cytokines that causes systemic low grade chronic inflammation (52–54), subsequently leading to insulin resistance and other obesity-related morbidities. VAT resident T_{reg} negatively regulate inflammation and represent a tissue specific T_{reg} population that express a distinct T cell repertoire (52) and a unique transcription factor, peroxisome-proliferator-activated receptor γ (PPARγ) (51). Obesity in mice and humans causes a reduction in VAT-associated T_{reg} differentiation (55). Moreover, removal of VAT resident T_{reg} by conditional knock-out of PPARγ, or activation, by treatment with pioglitazone, modulates levels of inflammatory cell subsets and insulin sensitivity (51). Leptin, a class I cytokine, is produced in higher amounts by adipocytes in obese individuals and inhibits rapamycin-induced proliferation of T_{reg} via increased activation of mTORC1 (18, 56). Leptin, secreted in the VAT, therefore, represents a potential regulator of the function of adipose-tissue-resident T_{reg}. In contrast to leptin, adiponectin, an anti-inflammatory adipokine, retains insulin-sensitizing properties and negatively correlates with body mass index while positively correlating with T_{reg} cell representation in VAT (57).

AMINO ACID CONCENTRATION REGULATES T_{reg} DIFFERENTIATION AND FUNCTION

Regulatory T cell differentiation and function are also controlled by the availability of amino acids in the local milieu. The essential amino acids arginine, glutamine, and tryptophan are essential for T cell activation (58–61) and their depletion from the local microenvironment results in T_{reg} generation. For example, Tryptophan is catabolized by indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO), which are present on many suppressive cell types including regulatory dendritic cells (DC) and some tumors. Low concentrations of tryptophan inhibits T cell growth but enhances T_{reg} generation (62–64) through an mTOR-dependent mechanism (65). The depletion of arginine by arginase (ARG1) and nitric oxide synthase (iNOS) also inhibits T cell activation via mTor inhibition. ARG1, iNOS, and IDO can be induced by T_{reg} in actively tolerant skin grafts *in vivo* (66) providing a feed-back loop by which T_{reg} can influence amino acid availability via autocrine mTOR activation and subsequently control T_{eff} activation and function. The influence of amino acid metabolism on T_{reg} differentiation and function has been reviewed elsewhere (67).

THERAPEUTIC IMPLICATIONS

The metabolic pathways influencing T_{reg} differentiation and function are amenable for modulation in therapeutic settings, thus providing the clinician with potentially valuable tools in the fight against immune-mediated diseases. As the mechanisms by which Rapamycin affects T_{reg} function are elucidated, more areas of clinical intervention will be opened for this FDA approved, well tolerated, and bio-available drug. To this end, it has recently been demonstrated by Makki et al. (68) that the metabolic effects of Rapamycin can protect against insulin resistance, increase energy expenditure, and reduce weight gain in diet-dependent obese mice. These phenotypic effects correlate with an increase of T_{reg} and myeloid derived suppressor cells in the adipose tissue (68). These finding will certainly fuel the debate over the use of Rapamycin beyond organ transplantation.

Proglisterone, which is currently licensed as a drug for the treatment of Type II diabetes, provides another potential target to modulate T_{reg} metabolism. Proglisterone is known to stimulate PPARγ and when used to treat mice fed a high fat diet, it restores the number and function of visceral adipose specific T_{reg} and this effect appears to be PPARγ specific (51). Therefore, Proglisterone can potentially target pathologies related to VAT T_{reg} with no bystander effects on other T_{reg} populations. The regulation of accumulation and function of PPARγ^{+} T_{reg} by leptin and adiponectin represents a potentially valuable therapeutic pathway that may, in the future, be targeted in order to regulate obesity-related pathologies. Moreover, the role of leptin and T_{reg} in the progression of obesity-related diabetes is yet to be fully elucidated and may provide even more targets for future drug research.

On another note, and potentially related to T_{reg} dependence on fatty acid metabolism, short-chain fatty acids (scFA), of bacterial origin (i.e., propionate, butyrate, and acetate), can restore the T_{reg} compartment in the gut of germ-free mice that had been treated with irradiation or antibiotics. This re-population is partially dependent on the expression of free fatty acid receptor 2 (FFAR2) on colonic T_{reg}, which physiologically express higher levels of FFAR2 than other T_{reg} sub-populations. This observation opens up the tantalizing possibility that colonic T_{reg} may be specifically targeted, in clinical settings, using synthetic scFA to treat gut-related problems in immunocompromised individuals (69).

CONCLUDING REMARKS AND PERSPECTIVES

The recent ground breaking research in how metabolism effects T_{reg} biology has provided the scientific and medical community

with a plethora of novel mechanistic insights that will inevitably lead to a better understanding of disease and a host of therapeutic targets. However, we still need to understand how the varying tissue-specific transcription factors found in T_{reg} sub-populations are influenced by their environment, external and internal metabolic factors. The expression of PPAR-γ by VAT T_{reg} suggests that the metabolic environment can influence the expression of transcription factors not only in resident cells but also in new migrants to the tissue. A future challenge will involve extending this concept to establish whether the metabolic microenvironment, which characterizes different tissues, can determine the balance of regulation versus inflammation *in situ*. If true, this possibility will pave the way for organ-selective immune-metabolic therapy.

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The many unknowns concerning the bioenergetics of exhaustion and senescence during chronic viral infection

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The immune system cannot be continuously reactivated throughout the lifetime of an organism; there is a finite point at which repeated antigenic challenge leads to the loss of lymphocyte function or the cells themselves. Antigen-specific T cells can be compromised in two ways through the distinct processes of replicative senescence and exhaustion. Senescence is initiated by a DNA damage response whereas exhaustion triggers inhibitory receptors to dampen the immune response. These two distinct pathways not only differ in their initiation but also growing evidence suggests that their biogenergetics is also different. Here, we review recent findings uncovering the metabolism of these unique states.

Keywords: exhaustion, senescence, chronic viral infection, bioenergetics, metabolism

METABOLISM OF THE IMMUNE RESPONSE TO VIRAL INFECTION

During viral infections $CD8^+$ T cells undergo clonal expansion and produce effector molecules. In order to meet this bioenergetic demand, the cells switch their metabolism from a mitochondrial-dependent oxidative and fatty acid metabolism (1, 2), toward glycolysis and glutamine oxidation (3, 4), even in the presence of sufficient oxygen, a process termed aerobic glycolysis. Activated T cells use this metabolism, although it is less energy efficient, as it allows for the increase in biomass through the biosynthesis of fatty acids and nucleotides (3). Cytokines and co-stimulatory signals via CD28 help promote the metabolic switch (5, 6). This acute phase of the viral response is then followed by a contraction phase, characterized by a decrease in mitochondrial membrane potential and high levels of reactive oxygen species (ROS), which triggers the majority of the antigen-specific effector cells to be cleared by apoptosis (7). The remaining antigen-specific cells differentiate into long-lived memory cells that protect against recurrent infection. The metabolism also changes within the memory population; memory T cells revert back to fatty acid oxidation and preferentially use the TCA cycle to fuel oxidative phosphorylation (OXPHOS) (2). These changes appear to be governed by IL-15, an important cytokine for $CD8^+$ memory T cells, as it promotes mitochondrial biogenesis and regulates the mitochondrial spare respiratory capacity, the extra capacity available in cells to produce energy (2, 8).

Although numerous infections are successfully cleared by the acute immune response, certain viral infections are not resolved and result in chronicity. The function of virus-specific T cells during chronic infections is often characterized by varying degrees of impairment, leading to defects in the ability of the host to eliminate the pathogen. Depending on the antigenic load generated by the infectious agent the impaired memory formation can result in T cells becoming either senescent or exhausted (9). Thus, immune senescence arises as a consequence of low-grade antigenic stimulation with the resulting inflammation determining the rate of senescence, as seen with CMV or EBV infection and not age *per se* (10, 11). The quantity of the lifelong antigenic load and the resulting inflammation determines the rate of immune senescence. Whereas a high-antigen load, caused by HIV, HCV, and HBV infection, leads to the formation of exhausted T cells (12). In this present review, we will discuss the current understanding of the metabolic requirements of antigen-specific $CD8^+$ T cells in chronic infections.

SENESCENCE AND EXHAUSTION ARE DISTINCT PROCESSES

As introduced above two different cellular processes can lead to T cell dysfunction, namely, senescence and exhaustion (**Figure 1**). Numerous mechanisms have been proposed to cause cellular senescence, including repeated cell division, telomere shortening, and damage by ROS (13, 14). The ensuing DNA damage triggers the recruitment of a complex of proteins that are involved in DNA repair, which is commonly referred to as the DNA damage response (DDR) that inhibits cell cycling until the DNA is repaired (13, 15, 16). Senescence manifests itself in T cells as the loss of the co-stimulatory molecule CD28 and the acquisition of innate markers such as killer-cell lectin-like receptor G1 (KLRG-1), while senescent T cells lose proliferative capacity they retain their cytotoxic activity and secretion of TNFα and IFNγ (17–19).

Continuous T cell stimulation in the setting of a high-antigenic load induces a state termed T cell exhaustion, characterized by the loss of effector functions in a hierarchical manner (20). IL-2 production and proliferation are the first functions to be lost, followed by TNFα production and cytotoxic activity. At late stages of exhaustion, IFNγ production is eventually compromised. When the antigen persists long-term at high-levels exhausted T cells are ultimately removed by apoptosis. So in

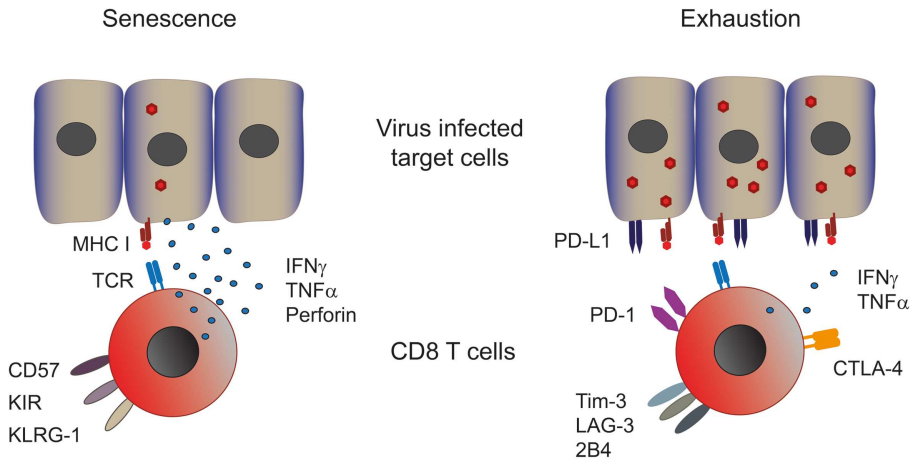

FIGURE 1 | Phenotype and function of senescent and exhausted T cells. Senescent T cells are present in chronic viral infections with a low viral and antigenic load. Senescent T cells express CD57, the late activation marker killer-cell lectin-like receptor subfamily G member 1 (KLRG-1) and killer-cell immune globulin-like receptors (KIR) and are capable of producing significant amounts of effector cytokines such as perforin, IFN-γ, and TNF-α (left panel). Exhausted CD8 T cells are found in persistent infections with a high viral and antigenic load. These cells express various co-inhibitory receptors such as PD-1, CTLA-4, Tim-3, LAG-3, and 2B4, which dampen their effector response consequently exhausted T cells produce low amounts of effector cytokines (right panel). Both T cell types show impaired proliferative capacities.

contrast to senescent T cells, which can produce high amounts of effector cytokines, exhausted T cells do not. Both groups have low-proliferative potential in common. Furthermore, functional exhaustion is accompanied by a marked change in T cell phenotype. Expression and maintenance of the co-inhibitory receptors programed cell death-1 (PD-1) at high levels is a hallmark of exhausted T cells, concomitantly other inhibitory receptors such as cytotoxic T lymphocyte antigen-4 (CTLA-4), lymphocyte activation gene-3 (LAG-3), T cell immunoglobulin and mucine domain containing molecule-3 (Tim-3), CD160 (21), and the natural killer-cell receptor 2B4 can also be significantly increased (12). T cell dysfunction in exhaustion is, at least in part, mediated by these inhibitory receptors, since multiple studies have demonstrated that their blockade results in functional recovery of exhausted T cells, examples include PD-1 blockade in HIV, HBV, and HCV (22–25) and CTLA-4 and Tim-3 blockade in HBV (26, 27).

Both senescent and exhausted T cells show a differentiation profile distinct from memory or effector cells. Senescent human $CD8^+$ T cells express high levels of T-bet but only a moderate amount of EOMES (28). Exhausted T cells express high levels of the transcriptional repressor Blimp-1, responsible for the increased expression in co-inhibitory receptors (29). NFATc1, is also increased and surprisingly is associated with poor cytokine expression (12), while T-bet expression in exhausted T cells seems to be important in supporting their persistence and sustenance of any residual functionality (12). However, exhausted T cells do help to control viral levels both in chronically infected patients and shown experimentally in elegant T cell transfer studies in murine chronic lymphocytic choriomeningitis virus (LCMV) (30). It is tempting to speculate that both T cell senescence and exhaustion are mechanisms by which viral infections are kept under control in order to avoid extensive on going immune damage.

METABOLISM IN SENESCENT AND EXHAUSTED CD8 T CELLS

Senescent and exhausted T cells display a metabolic phenotype distinct from memory cells but also, due to their differing functionality, from each other (**Figure 2**). Senescent human $CD8^+$ T cells isolated from healthy donors stimulated through the TCR have been shown to preferentially utilize glycolysis and also exhibit mitochondrial dysfunction and impaired mitochondrial biogenesis, which may explain their dependence on glycolysis for energy (17). The ability of a T cell to undergo mitochondrial biogenesis leads to an increased capacity of the cell to respond to metabolic stress, a characteristic termed spare respiratory capacity (2). It has been demonstrated that, unlike other memory subsets senescent $CD8^+$ T cells have a substantially reduced spare respiratory capacity making them energetically unstable (17).

Information regarding the metabolism of exhausted T cells is limited and, to date, restricted to mouse models where alterations in metabolism have been reported. During acute infection with the LCMV Armstrong clone, virus-specific CD8 T memory cells develop. In contrast, during chronic infection with the LCMV clone 13, repetitive antigen-stimulation leads to the loss of memory cells. Virus-specific cells detectable in chronically infected animals were either still naïve or functionally exhausted (31). Wherry et al. have demonstrated using exhausted $CD8^+$ T cells following chronic infection with LCMV that genes involved in glycolysis and oxidative metabolism were significantly altered compared to effector and memory CD8 T cells (32), suggesting that exhaustion is a distinct state.

Information regarding the metabolic requirements of HIV is hampered by the toxicity of many of the antiretrovirals (33). However, removal of mitochondrial content from a cell line leads to the attenuation of HIV infection (34). Furthermore, cells isolated from HIV^+ patients may up-regulate OXPHOS owing to an increased mitochondrial mass (33). However, the methodology used in this

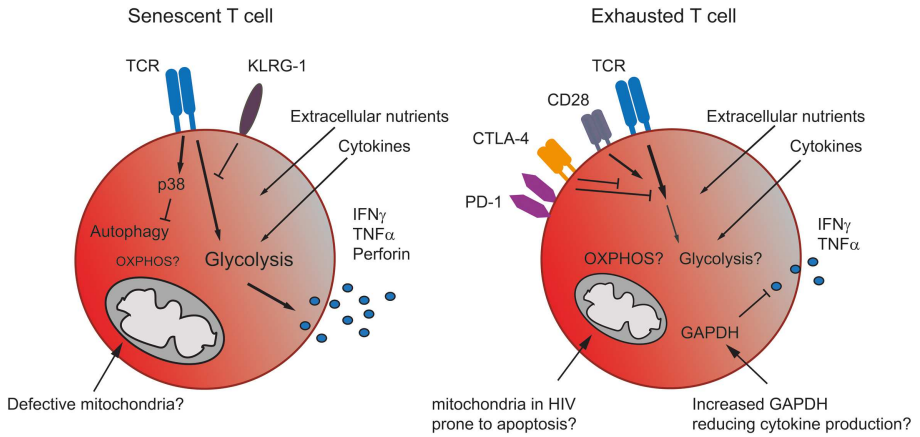

FIGURE 2 | The metabolism in senescent and exhausted T cells. Senescent T cells rely more on glycolysis than mitochondrial respiration due to the accumulation of giant non-functional mitochondria in these terminally differentiated cells. The inhibitory receptor KLRG-1 prevents signaling through the TCR, while the activation of p38 blocks autophagy, which senescent T cells use as an energy source. Nonetheless, senescent T cells generate sufficient energy to produce significant amounts of effector cytokine (left). Exhausted T cells express co-inhibitory receptors, which interfere with TCR and co-stimulatory signaling, thereby likely blocking any increase in metabolic activity. Cells may also be prone to mitochondrial induced apoptosis. Additionally, an increase in GAPDH, caused for example by lower levels of glycolysis, might also dampen effector cytokine production (right).

study cannot distinguish whether the observed increase is due to an increased number of functional mitochondria or the appearance of giant non-functional mitochondria (35). From the limited available data, it would appear that T cells utilize OXPHOS during infections with both LCMV and HIV; however, it remains to be investigated whether T cells specific to different pathogens being primed in different locations in the body establish distinct metabolic profiles. Furthermore, when examining human infections the length of time post-infection may also be a deciding factor in the metabolic fate of T cells, as repeated turnover may lead to mitochondrial dysfunction and a switch toward extra-mitochondrial metabolism (36).

One hallmark of immune senescence is the accumulation of late-differentiated effector T cells characterized by the loss of CD28 expression (9, 37, 38). Exhausted T cells have been described to also display an effector memory T cell phenotype (39). Signaling through CD28 has been demonstrated to increase glycolysis and therefore effector function in $CD4^+$ T cells. Both PD-1 and importantly CTLA-4, a direct competitor of CD28, interfere with CD28 signaling (40, 41), their increased expression on exhausted T cells is therefore likely to dampen down a metabolism suitable to sustain effector functions. While the CD28 co-stimulation pathway is considered to be important for T cell activation, alternative co-stimulatory pathways belonging to the TNF/TNF receptor (TNFR) family have been described. Signaling through CD137:CD137L (4-1BB) has been demonstrated to promote proliferation of $CD8^+CD28^-$ T cells and is an important co-stimulator for human anti-viral CD8 T cells, as a CD137 agonist given alongside PD-L1 blockade resulted in an enhanced and stable expansion of LCMV-specific $CD8^+$ T cells (42).

Furthermore, while not directly examining exhaustion Chang et al. have shown using mice infected with *Listeria* that forcing $CD4^+$ T cells to use OXPHOS induced elevated PD-1 expression, a loss of proliferation and defective IFN-γ production (43). However, what is hard to reconcile from this study is quiescent memory T cells, which utilize OXPHOS do not express PD-1 (2, 32), therefore, the elevated levels of PD-1 observed in this scenario may be controlling activation. Additionally, *in vivo* it is questionable whether T cells will find themselves in a situation where they are forced to utilize OXPHOS. T cell exhaustion and senescence is pathogen specific with generally little impact on the overall response to other infections and T cells specific to various different viruses can accumulate in the same location (44). Finally, memory, effector, and dysfunctional T cells can share the same environment, while displaying distinct metabolism, suggesting that a regulation through predominantly T cell intrinsic rather than extrinsic factors. So the question of what drives the metabolic phenotype of dysfunctional cells remains.

INFLUENCE OF METABOLIC REGULATORS ON SENESCENT AND EXHAUSTED CD8 T CELLS

Metabolism can be controlled by many different immunological and metabolic signals [reviewed in Ref. (45)], how chronic infections influence these metabolic checkpoints remains to be determined (**Figure 2**). Cytokines have been shown to regulate metabolism, indeed IL-2 can induce the expression of glucose transporter 1 (glut1) and enhance glycolysis (46). Both exhausted and senescent T cells show no or reduced production of IL-2. In line with this human senescent $CD8^+$ T cells show a reduced expression of glut1 (17). The glut1 level on exhausted T cells has not been examined. The homeostatic cytokines IL-7 and IL-15 also regulate metabolism, IL-7 promotes glycolysis in T cells (47, 48) and IL-15 has been demonstrated to regulate oxidative metabolism by enhancing mitochondrial biogenesis in memory $CD8^+$ T cells but not effectors (2). Type 1 IFN, a prevalent cytokine during viral infections induces the production of IL-15 (49), which may also add to the oxidative switch during viral infections. Additionally, type 1 IFN has also been demonstrated to modulate lipid

metabolism (50), while lipid metabolism is crucial for memory T cells, a role for this cytokine in the control of metabolism during senescence and exhaustion, where effector T cells dominate is unclear.

The distinct nature of the metabolic changes seen during chronic infection suggests additional regulatory steps in the metabolic reprograming of T cells. The transcription factor mammalian target of rapamycin (mTOR), as well as being a critical regulator of $CD8^+$ memory formation (51), is a key molecule sensing intracellular amino acids and ATP (52), and regulates fatty acid metabolism in memory T cells (1). The pro-inflammatory cytokine IL-12 increases mTOR expression in antigen-stimulated $CD8^+$ T cells, promoting $CD8^+$ T effector differentiation and metabolism. Blockade of mTOR by rapamycin led to inhibition of IL-12 induced expression of the transcription factor T-bet and skewed the CD8 response toward eomesodermin dependent memory formation (53). T-bet is important in maintaining the limited effector capacity of T cells in chronic infections (54) and IL-12 enhances functionality of exhausted T cells in chronic HBV by increasing T-bet (55). It has recently been shown that senescent $CD8^+$ T cells display very little mTOR activity and predominantly use mTOR-independent pathways to control their metabolic requirements (17). This is in line with the concept that the transition to a memory phenotype is associated with a metabolic switch from anabolism to catabolism (41, 52) via the inhibition of mTOR (51). This limited mTOR activity also corresponds to the lower level of glut 1 observed on senescent T cells compared to other memory populations (17).

This review has focused on the changes occurring to glycolysis and oxidative metabolism during chronic infections. However, alternative energy sources, such as the β-oxidation of fatty acids or autophagy may be utilized differentially by senescent and exhausted T cells. A recent article by the Pearce group has demonstrated that the fatty acids required by memory T cells are produced *de novo* via the non-classical lysosomal acid lipase (LAL) pathway to mobilize fatty acids for β-oxidation (56). This study uses the mouse OVA system together with cytokines to generate effector and memory cells, but the observed pathways have not yet been shown to occur in humans. Furthermore, while both senescent and exhausted T cells are not highly proliferative, like memory cells, they do make cytokine and senescent T cells also express high levels of effector molecules necessitating a higher catabolic demand. Macroautophagy (autophagy) is another alternate nutrient source when extracellular nutrient uptake is insufficient to meet the cellular energy demands (57). The lysosomal digestion of organelles and other materials by autophagy can generate the required metabolic precursors for metabolism (57). Furthermore, mouse models have shown autophagy to support glycolysis and autophagy competence is required for cells to proliferate and expand (58). Senescent human $CD8^+$ T cells display low-autophagic activity (17, 59), which was regulated via p38 MAPK independently of mTOR (17). However, the role of both fatty acid metabolism and autophagy during T cell exhaustion remains to be determined.

SUMMARY

Although the interest in metabolism has grown significantly and our understanding of the specific requirements of T cells with it, there is still very little understanding of how T cells fuel their energy demand during chronic viral infections. We have highlighted here the distinct phenotypes and functions of exhausted and senescent CD8 T cells and have outlined the current knowledge of their metabolic requirements. However, many questions remain open: it remains to be seen, how much influence the environment the T cell is located in at any given stage during the infection has. Are metabolic phenotypes at least partially imprinted during T cell priming in the acute phase of the infection or do they only develop over time? How big is the influence of the antigen presenting cell and the local environment during priming? For example, HBV/HCV-specific T cells are likely to encounter their cognate antigen for the first time in the liver and be primed there, whereas EBV-specific T cells might be primed in the tonsils. The liver milieu is naturally high in suppressive cytokines (60, 61), which control and dampen T cell activation and might influence the metabolic pathway used. Furthermore, it is likely that the immune response differs when taking place in a hypoxic environment compared to one with higher oxygen supply. Finally, the infecting virus itself will influence the T cell response by manipulating cytokine, chemokine, and co-stimulatory or co-inhibitory receptor expression.

These questions have so far not been taken into account when investigating chronic infections, since most studies have utilized only the murine LCMV model to study T cell dysfunction. The limitation being that, specific changes in T cell phenotype and function caused by distinct infections have not been addressed. Ultimately, the aim will have to be to better understand human T cell responses in order to allow the development of novel immunotherapies. Albeit the difficulties of establishing reliable *in vitro* models and understanding the mechanisms governing human anti-viral immunity, studies making use of human samples need to be further encouraged.

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Aerobic glycolysis: beyond proliferation

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Aerobic glycolysis has been generally associated with cancer cell proliferation, but fascinating and novel data show that it is also coupled to a series of further cellular functions. In this *Mini Review*, we will discuss some recent findings to illustrate newly defined roles for this process, in particular in non-malignant cells, supporting the idea that metabolism can be considered as an integral part of cellular signaling. Consequently, metabolism should be regarded as a plastic and highly dynamic determinant of a wide range of cellular specific functions.

Keywords: aerobic glycolysis, non-malignant cells, cellular signalling, proliferation, immunometabolism

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The term aerobic glycolyis was coined by Otto Warburg at the beginning of the nineteenth century to explain the unconventional metabolism exhibited by tumor cells. Warburg noticed that malignant cells prefer to convert glucose to lactate even in the presence of oxygen, in contrast to the metabolism of healthy/differentiated cells, where glucose is usually converted into pyruvate and only converted to lactate in the absence of oxygen. The conversion of glucose to lactate in anaerobic conditions (absence of oxygen) was already known as anaerobic glycolysis and thus he defined the metabolism of cancer cells as aerobic glycolysis, to underline that the fate of glucose is not determined by the lack of oxygen (1). The fact that cancer cell metabolism is different from that of normal cells attracted (and still does attract) a lot of interest, since this feature can potentially facilitate selective therapeutic targeting of tumor cells. Currently, we also come across a growing interest in the metabolism of non-malignant cells, particularly of those involved in the immune response. Intriguingly, recent findings in this new field of immunometabolism showed that aerobic glycolysis can be a metabolic choice of normal cells and that its function is not limited to supporting proliferation. It appears that cells can modulate their metabolism to adapt to different energy requirements and signaling events in physiological situations (see **Figure 1**). Thus, changes in cellular metabolism are not only restricted to nutrient deprivation or pathological conditions. These studies also demonstrate that, despite our fairly detailed knowledge of the map of cellular metabolic reactions, little is known about the link between metabolism and non-metabolic cellular functions. Thus, we can pose intriguing questions: can cellular metabolism determine seemingly unrelated cellular functions and do cells use the modulation of their metabolism to accomplish and regulate these functions?

A Role for Aerobic Glycolysis in Vessel Sprouting

The process of vessel sprouting requires endothelial cells (ECs) to differentiate into different cell types and act in a synchronized manner. While ECs differentiated into "stalk" cells proliferate and sustain vessel sprouting, the EC differentiated into the "tip" cell is required at the forefront and has a migratory phenotype. Intriguingly, from a metabolic point of view, ECs are more similar to cancer cells than to other normal differentiated cells. ECs are highly glycolytic (it has been calculated that up to 85% of ATP in ECs is produced by glycolysis) despite the fact that they are in immediate contact with oxygen present in the blood (2). In agreement with this observation, it has been reported

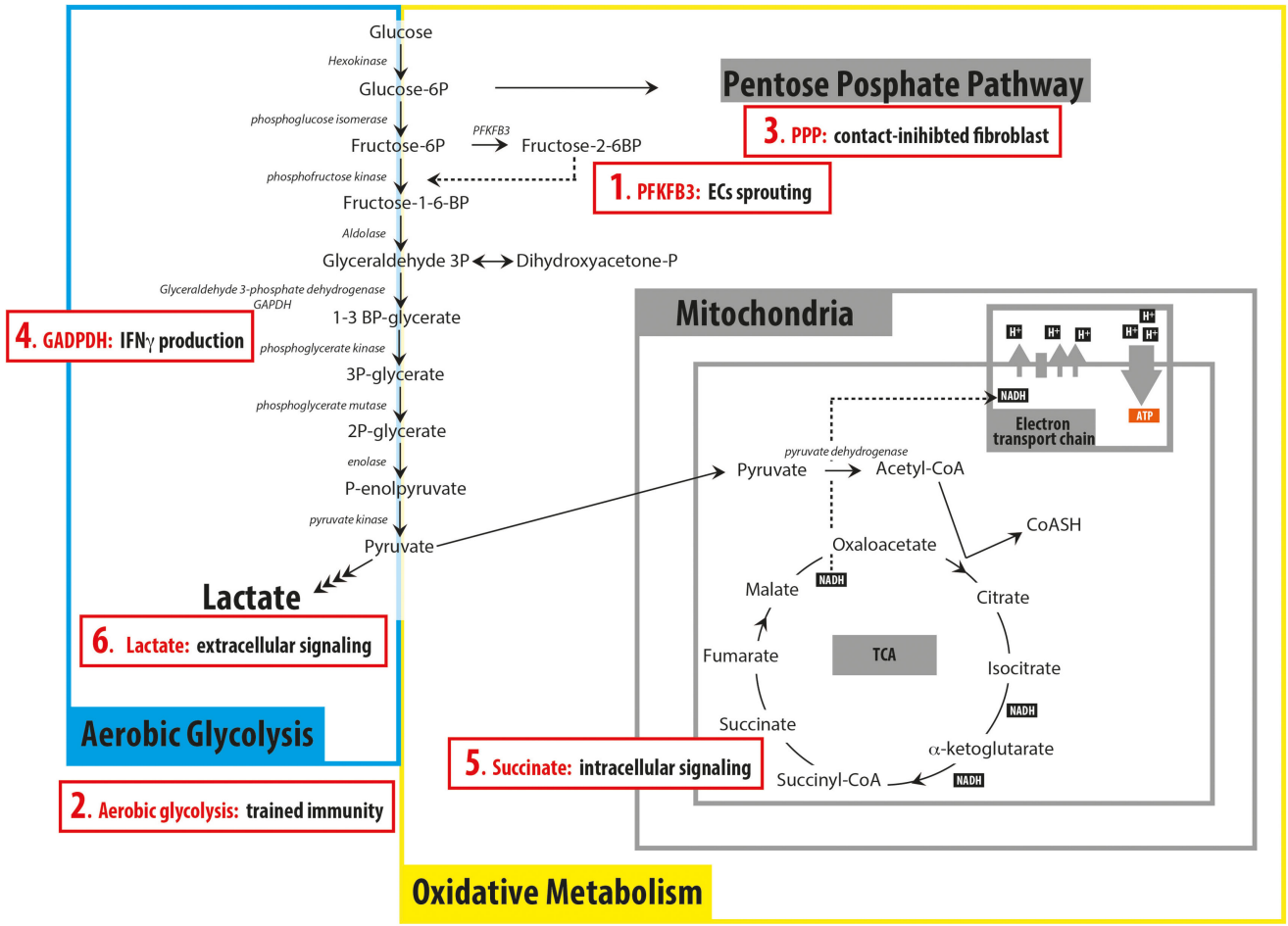

FIGURE 1 | Schematic representation of the principal pathways involved in glucose catabolism summarizing recent findings discussed in the *Mini Review* (red boxes). (1) 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 3 (PFKFB3) has a key role in regulating endothelial cells (ECs)sprouting; (2) switching to aerobic glycolysis is important for trained immunity in monocytes; (3) contact-inhibited fibroblasts rely on the pentose phosphate pathway (PPP) to produce NADPH; (4) GAPDH can function as an mRNA binding protein when not engaged in glycolysis, preventing the production of IFNγ; (5) succinate can act as intracellular signaling molecule, being involved in the production of IL-1β in BMDM; (6) Lactate secreted by cancer cells can act as extracellular signaling molecule, inducing the differentiation of macrophages into tumor-associated macrophages.

that the amount of lactate secreted by ECs does not change upon a reduction of oxygen pressure, highlighting the fact that aerobic glycolysis is the preferred metabolism of ECs (3). While vessel sprouting was primarily thought to be regulated by Notch signaling (4), recently, cellular metabolism has also been shown to play a major role in the process. In particular, an important function for 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) has been proposed (2). PFKFB3 is responsible for the formation of fructose 2,6 bisphosphate, a key regulator of glycolysis since it acts as the allosteric activator of phosphofructokinase 1 (PFK1), the rate limiting enzyme that converts fructose 6-phosphate to fructose 1-6 bisphosphate. Modulation of the level of expression of PFKFB3 by knockdown or overexpression changes the behavior of ECs growing in spheroids. Knockdown of the enzyme inhibits sprouting, while its overexpression leads to increased glycolysis, together with more and longer sprouts. Importantly, in mosaic spheroids formed of wild-type and PFKFB3 knock-down cells, predominantly wild-type cells form the tips. This preferential selection of wild-type cells is not due to the reduced proliferation rate caused by PFKFB3 silencing, since even when cell proliferation is inhibited by MitoC cells distribute with a similar pattern as before. Moreover, this distribution is maintained up to an initial ratio of wild-type/PFKFB3 knock-down of 9:1, indicating that presence of PFKFB3 is crucial to develop the tip cell phenotype. Importantly, PFKFB3 depletion also abrogates the effect of other factors promoting the tip phenotype, such as inhibition of Notch signaling. Mechanistically, PFKFB3 (and other glycolytic enzymes) has been shown to interact with actin and localize at lamellopodia, possibly to provide fast and local ATP production, where it is required to promote cell motility. Altogether, the role of PFKFB3 and glycolysis in promoting the phenotype of ECs is a clear example of how metabolism affects cellular phenotype and function beyond its role in proliferation. PFKFB3 seems to be also a promising therapeutic target. The small molecule inhibitor of PFKFB3 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) reduces the

glycolytic flux and vessel formation both *in vitro* (EC spheroids) and *in vivo* (in zebrafish and mouse retina) (5). Intriguingly, it has been recently shown that ECs have another rather unique metabolic characteristic, in that they rely on fatty acid oxidation for the production of nucleotides (6). However, this aspect of ECs metabolism is in fact linked to supporting proliferation, and thus lies outside the topic of this mini-review.

A Role for Aerobic Glycolysis in Trained Immunity

The fact that monocytes and polymorphonuclear leukocytes from peritoneal exudates rely on glycolysis as their main energy source was described in 1963 (7). However, a crucial role for glycolysis has also been recently demonstrated for the development of "trained immunity" (8). Trained immunity can be defined as "memory" of the innate immune response and is responsible for mounting a non-specific "adaptive" response, challenging the concept that the innate immune response cannot have a long-term memory. Evidence for this process has been collected in various *in vitro* and *in vivo* models. For example, monocytes exposed to β-glucan (a wall component of *Candida albicans*) *in vitro* respond to lipopolysaccharide (LPS) with greater amplitude 7 days after "priming," as the result of the activation of a pathway involving Akt/mTOR and HIF1α. Monocytes exposed to β-glucan for 24 h switch to aerobic glycolysis, and via epigenetic modification sustain this metabolic choice for at least 7 days after having being "primed." Primed monocytes are more responsive to LPS treatment as measured by the increased amount of TNFα that they secrete when compared to un-primed cells. Importantly, the switch to aerobic glycolysis during the first 24 h of priming is an absolute requirement for monocytes to undergo "training," together with epigenetic modifications and activation of the Akt/mTOR/HIF1α pathways. The amount of lactate secreted during β-glucan priming is not affected by treatment with methylthioadenosine (MTA), an inhibitor of methyltransferase, or ITF2375, a histone deacetylase inhibitor, but training is abolished by these drugs. Thus, aerobic glycolysis is not a consequence of epigenetic modifications rather both represent a crucial mechanism contributing to trained immunity. Mechanistically, it is not clear why glycolysis favors the adaptive monocyte response. It has been suggested that it can be a swift way to produce energy during infection, where a quick and robust response is required (8). An intriguing hypothesis is that metabolism could affect the epigenetic status, as demonstrated in other instances where DNA modification depends on metabolites acting as cofactors or substrates (9). Along these lines, the ratio between α-ketoglutarate (α-KG) and succinate regulates histone demethylases maintaining pluripotency of embryonic stem cells (10).

High Level of Glucose Uptake is not Equal to Proliferation

The observation that aerobic glycolysis was the preferred metabolic pathway of highly proliferative cancer cells was initially difficult to explain from an energetic point of view. Indeed, via glycolysis/OXPHOS cells generate 36 molecules of ATP for each molecule of glucose, while via aerobic glycolysis only 4 molecules of ATP are produced. This apparent paradox was resolved however, by considering the metabolic requirements for proliferation beyond ATP, including nucleotide synthesis linked to the pentose phosphate pathway (PPP), generation of amino acids for protein synthesis, and production of lipids for membrane formation. Importantly, most of these biosynthetic pathways branch out from glycolysis, explaining why this process is so crucial for cellular proliferation (11). It would therefore be logical to assume that non-proliferating or quiescent cells would require less glycolytic activity but, surprisingly, this does not always seem to be the case. Indeed, fibroblasts that are pushed into a quiescent status because of contact inhibition have a very similar glucose uptake rate to proliferating fibroblasts (12). An in depth analysis of the metabolism of quiescent vs. proliferative fibroblasts highlighted some unexpected findings. Proliferating fibroblasts rely on the PPP to generate ribose for the synthesis of nucleotides and to produce NADPH, their TCA cycle is interrupted between citrate and α-KG and they use glutamine as an anaplerotic substrate. Quiescent, contact-inhibited fibroblasts also maintain an active PPP to produce NADPH but, since they do not require nucleic acid synthesis, the carbon skeletons are recycled back to glycolysis. Their TCA cycle is completely functional and they convert pyruvate into oxaloacetate as an anaplerotic source. Unexpectedly, inhibition of the PPP with dehydroepiandrosterone (DHEA) induces cell death only in quiescent fibroblasts (albeit to a limited extent), representing a unique example of a drug that selectively targets non-proliferating cells. Thus, despite being non-proliferative, contact-inhibited fibroblasts have a very active PPP. Lemons et al. suggest three potential explanations for their findings: high levels of glucose uptake could be required to replace potentially damaged macromolecules, NADPH produced by the PPP could be used to generate GSH and thus confer protection from free radicals and, lastly, fibroblasts require high glucose levels due to their high energy consuming function, i.e., synthesizing and secreting large amounts of extracellular matrix components. Importantly, contact-inhibited fibroblasts are not a unique example of quiescent cells with high level of glucose uptake. Hematopoietic stem cells (HSCs) also prefer glycolysis to oxidative phosphorylation in a metabolic pattern driven by HIF1α, but not just consequently to the hypoxic niche were they are found (13). Moreover HSCs' metabolism (where mitochondrial activity is inhibited) is important to keep the quiescent status of these cells (14).

Aerobic Glycolysis: Regulation of Protein Translation

So far, we have described examples where aerobic glycolysis is not linked to proliferation. However, proliferating cells normally switch their metabolism to aerobic glycolysis for all the reasons we mentioned in the previous paragraph. In this section, we discuss an example where aerobic glycolysis accompanies cellular proliferation but is required for translation of signaling proteins, and not for proliferation itself. A recent study of the metabolic changes accompanying T cell activation (when they undergo a highly

proliferative phase) revealed that T cells not only increase their glycolysis rate, but they also increase their oxidative phosphorylation in comparison to naïve T cells (15). Moreover, in the initial 48 h of activation, T cells actually rely more on their mitochondria than on glycolysis, they survive and proliferate when fed with galactose (which is not a substrate of glycolysis) instead of glucose, and their proliferation and activation is extremely sensitive to mitochondrial inhibitors. Thus at a first glance, T cell activation seems to be independent of glycolysis, so why is there an increased glycolytic rate in this phase? In this respect, a detailed analysis of the full functional spectrum of activated T cells, including cytokine release, revealed some interesting novel observations. T cells fed with galactose instead of glucose secrete much less interferon γ (IFN-γ) and interleukin 2 (IL-2) compared to their glycolytic counterparts. The mechanism proposed to explain this phenotype is that a key metabolic enzyme – glyceraldehyde 3-phosphate dehydrogenase (GAPDH) – can also act as an mRNA binding protein. When glycolysis is inhibited (e.g., in galactose) the enzyme is not engaged in its main function of converting glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate, but rather binds to the 3′-UTR of the IFN-γ mRNA, thereby preventing its translation. These findings represent an intriguing example where aerobic glycolysis is not required for proliferation, but can actually regulate the synthesis of a cellular and extracellular signaling protein.

Metabolites can be used as Signaling Molecules

In this last section, we would like to underline that metabolites (either directly or indirectly linked to aerobic glycolysis) can also function as signaling molecules, reinforcing the idea that metabolism is part of the cellular signaling network. For example, succinate is used by bone marrow derived macrophages (BMDM) upon LPS stimulation to induce secretion of IL-1β (16). Indeed, as recently shown, in BMDM an increase in succinate concentration creates a condition called "pseudohypoxia," by repressing prolyl-4-hyrdoxylase (PHD) activity by product inhibition, leading to the prevention of HIF1α degradation. In turn, HIF1α can bind to the promoter of IL-1β gene inducing its expression. Importantly, secretion of different cytokines by BMDM seems to be controlled by different mechanisms since, for example, TNFα and IL-6 secretion is not dependent on succinate-mediated signaling. These data suggest that different regulatory mechanisms have evolved to control the secretion of cytokines by macrophages to ensure a specific response in different extracellular environments, such as the presence of different nutrients. Finally, while succinate functions as an intracellular signaling molecule, it appears that metabolites can also act as extracellular messengers as observed in the case of lactic acid. The Medzhitov group has recently described that lactic acid released by tumor cells induces the conversion of macrophages to tumor- associated macrophages (TAM), which show characteristics of M2 macrophages, via a HIF1α−mediated mechanism (17).

In conclusion, these are very exciting days for cellular metabolism, with old paradigms being reconsidered and different fields (such as cancer cell metabolism and immunometabolism) contributing to unravel the essential role of this complex network of reactions in cellular biology.

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The intercellular metabolic interplay between tumor and immune cells

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Functional and effective immune response requires a metabolic rewiring of immune cells to meet their energetic and anabolic demands. Beyond this, the availability of extracellular and intracellular metabolites may serve as metabolic signals interconnecting with cellular signaling events to influence cellular fate and immunological function. As such, tumor microenvironment represents a dramatic example of metabolic derangement, where the highly metabolic demanding tumor cells may compromise the function of some immune cells by competing nutrients (a form of intercellular competition), meanwhile may support the function of other immune cells by forming a metabolic symbiosis (a form of intercellular collaboration). It has been well known that tumor cells harness immune system through information exchanges that are largely attributed to soluble protein factors and intercellular junctions. In this review, we will discuss recent advance on tumor metabolism and immune metabolism, as well as provide examples of metabolic communications between tumor cells and immune system, which may represent a novel mechanism of conveying tumor-immune privilege.

Keywords: metabolism, tumor, tumor immunity, antagonism, symbiosis

INTERCELLULAR METABOLIC INTERACTION

The intercellular communication largely relies on the information exchange via soluble factors (e.g., proteins and microRNAs) and direct cell–cell contacts. Beyond this, the shuttling of metabolites may serve as an additional form of intercellular communication and a high degree of intercellular coordination in various physiopathological situations. As an intensive energy-consuming organ, brain efficiently utilizes nutrients/metabolites via organizing a complex intercellular shuttle of glucose, glutamine, glutamate, pyruvate, and lactate between neurons and astrocytes (1). Similar metabolic coordination exists in retina between glial cells and photoreceptor cells, in muscle between fast white fibers and slow red fibers, and in testis between sertoli cells and spermatogenic cells (2, 3). Also, emerging evidences have shown that various pathogen-derived metabolites mediate an intercellular host–pathogen interaction and critically impact on homeostasis and pathogenesis during pathogen invasion (4–7). Tumor microenvironment represents a dramatic example of metabolic derangement, where tumor-surrounding cells may either compromise or support highly metabolic demanding tumor cells by competing nutrients (a form of intercellular competition) or by forming a metabolic symbiosis (a form of intercellular collaboration), respectively. Amino acids, lactate, and lipids derived from stromal cells, adipocytes, mesenchymal stem cells, epithelial cells, or tumor cells from hypoxic regions can modulate tumor cell growth and their responses to therapy (8–15). Beyond this, the immune system, a pivotal cellular compartment presented in tumor microenvironment, is intimately involved in tumor initiation, progression, and responses to therapy.

TUMOR IMMUNITY

Interaction of immune system with tumor is a complex and dynamic process. As the major component of anti-tumor immunity, tumor antigen-specific cytotoxic T (CTL) and T effector (T_{eff}) cells together with antibody-producing B cells and antigen-presenting dendritic cells (DC) elicit adaptive anti-tumor activity through direct recognizing and killing tumor cells and orchestrating a plethora of adaptive and innate immune responses. Also, macrophages, natural killer (NK) cells, and NK-T cells form an important layer of non-specific innate immunity to suppress tumor progression. However, tumor often co-opts and manipulates its microenvironment favoring the development of immunosuppressive cells, such as myeloid-derived suppressor cells (MDSC) and regulatory T (T_{reg}) cells. In addition, tumor-associated macrophages (TAMs), a pivotal immune population within the tumor microenvironment, are composed of multiple distinct pro- and anti-tumoral subpopulations. Mounting evidence indicates that strengthening the amplitude and quality of T cell-mediated adaptive response is one of the most promising approaches to enhance therapeutic anti-tumor immunity (16–19).

METABOLIC REPROGRAMING IN TUMOR

The shift from glucose oxidation toward aerobic glycolysis, also termed "Warburg effect," and heightened glutamine catabolism

are characteristic hallmarks of cancer cells. The metabolic rewiring of cancer cells supporting tumor growth and survival relies on a hierarchical oncogenic cascade involved in Akt/mTOR, MAPK, and essential transcriptional factors, such as HIF1α- and Myc-dependent metabolic transcriptome. Secretion of metabolic end products, such as lactate from glycolysis and glutaminolysis and CO_2 from the pentose phosphate pathway, often result in an extracellular acidification in tumor microenvironment. Hence, the Na^+/H^+ exchanger, the H^+-lactate co-transporter, monocarboxylate transporters, and the proton pump (H^+-ATPase) that are frequently activated in cancer cells play essential role in modulating the pH and ionic compositions in tumor microenvironment (20–22). Also, elevated expression of phosphoglycerate dehydrogenase (PHGDH) and it-mediated diversion of glycolysis into serine biosynthetic pathway have been found to be essential for the proliferation of certain tumor cells, such as breast cancer cells and melanoma cells (23, 24). Beyond being key precursors for biosynthesis, metabolic products of tryptophan, cysteine, serine, and glycine also contribute to tumor homeostasis through modulating anti-oxidative response and anti-tumor immunity (8, 25–28). Together, the metabolic reprograming in tumor contributes to its growth either by directly supporting cancer cell proliferation or by shaping the microenvironment potentially favoring tumor cell survival.

METABOLIC REPROGRAMING IN IMMUNE SYSTEM

Recent studies from us and others have indicated that metabolic regulation and cell signaling are tightly and ubiquitously linked with immune responses (29–34). The distinct metabolic profiles of lymphocytes are intimately linked to their status and function (**Table 1**). Naïve T lymphocytes rely mainly on fatty acid oxidation and some glycolysis to fulfill their energy demand for survival. Upon stimulation, activated T lymphocyte "reprograms" its metabolism, by dramatically increasing aerobic glycolysis and glutaminolysis meanwhile decreasing lipid oxidation to meet its requirements for cell size growth, cell division, and cytokine production (35–38). In contrast, stimulation of B lymphocytes leads to a balanced increase in aerobic glycolysis and oxygen consumption (39, 40). As T lymphocytes begin to proliferate, they also undergo differentiation into functional subsets in response to extracellular signals, and these subsets determine the nature of the immune response. According to the nature of initial antigen challenge and specific cytokine signals, activated CD4 T cells differentiate into T_{eff}, including T helper Th1, Th2, Th9, and Th17, follicular helper Tfh, and T_{reg}. Th1 cells mediate responses to intracellular pathogens. Th2 cells control responses to extracellular bacteria and helminthes. Th9 cells play a role in the pathogenesis of asthma and resolution of parasitic infections. Th17 cells are important in anti-fungal defense and inflammation. Tfh cells are the specialized B cell helper. T_{reg} cells dampen immune responses by suppressing T cell activation and inflammatory response. The predominant metabolic program in T_{reg} cells is mitochondrial-dependent oxidation of lipid and potentially other mitochondrial-dependent metabolites. It has been indicated that forcing proliferating T cells to utilize free fatty acids for energy tends to drive enhanced T_{reg} differentiation (41). In contrast, increased aerobic glycolysis is seen in Th1, Th2, and Th17 cells, and partially due to activation of PI3K/Akt/mTOR pathway. The transcription factor HIF1 (hypoxia-inducible factor 1) has also been characterized as a key regulator of the anabolic metabolism in differentiating Th17 cells (42, 43). Similar to CD4 T cells, CD8 T cells also switch from fatty acid oxidation to aerobic glycolysis upon activation. The glycolysis and anabolic metabolism are essential for CD8 T cell growth and differentiation into cytotoxic T cells (34). After the peak of the primary T cell response, the metabolic state in CD8 T cells shifts from glycolysis back to lipid oxidation, which is pivotal for cell survival and the generation of CD8 T memory cells (44, 45).

Dendritic cells and macrophages are first-line effectors of innate immunity. DC maturation is concomitant with a metabolic switch to aerobic glycolysis (46, 47). Aerobic glycolysis fulfills bioenergetic need and also provides building blocks for the biosynthesis of macromolecules, such as lipids, a proper balance between uptake and synthesis of which is required for immunogenicity of DCs (48, 49). As functionally plastic cells, macrophages are capable of tightly coordinating their metabolic programs with their functional properties. This allows macrophages to grow, survive, and properly respond to a variety of pathophysiological signals in their changing microenvironments. Within the tumor microenvironment, TAMs are often identified as pro-tumoral M2 type macrophage (50, 51). Mounting evidences have showed that switching the TAM phenotype from M2 to M1 may promote anti-tumor activity, implicating a phenotypic plasticity of TAM (52–57). To mount a rapid inflammatory response, M1 macrophages coordinately engage aerobic glycolysis, pentose phosphate shunt (PPP), glutamine, and arginine catabolism to produce nitric oxide (NO) and reactive oxygen species (ROS) (58–60). However, anti-inflammatory M2 macrophages largely utilize lipid oxidation (61–63) meanwhile shift arginine catabolism from iNOS-mediated production of NO to the production of urea and ornithine (64–68). Similar to macrophage, the polarization of MDSC, a heterogeneous immunosuppressive population in tumor microenvironment, toward a pro-inflammatory phenotype (often referred as M1) is associated with heightened glycolysis meanwhile reduced immunosuppressive function (69). Beyond this, metabolic regulation in NK and neutrophils are largely unknown.

Table 1 | Metabolic profiles of immune cells.

Immune cells	Naïve T cells	Activated T cells	T_{eff} cells	T_{reg}/T memory cells	Activated dendritic cells	M1 macrophages	M2 macrophages (TAM)
Metabolic profile	fatty acid oxidation and some glycolysis	Glycolysis and glutaminolysis	Glycolysis	Fatty acid oxidation	Glycolysis	Glycolysis, pentose phosphate shunt (PPP), glutamine, and arginine catabolism	Lipid oxidation

METABOLIC ANTAGONISM AND SYMBIOSIS IN TUMOR MICROENVIRONMENT

Aerobic glycolysis and glutaminolysis are dominant cancer metabolic routes. Heightened glucose and glutamine consumption often results in the depletion of nutrients (glucose, glutamine, etc.) whereas accumulates metabolic end- or by-products (lactate, proton, etc.) in tumor microenvironment (70, 71). In addition to the above general metabolic features that are required to support the needs of proliferation and other neoplastic features, tumor cells also exhibit diverse metabolic phenotypes that are often due to the adaptation of pre-existing cell/tissue lineage specific metabolic network. It is well documented that in tumor cells, biosynthesis, and catabolism of glycine and serine, as well as catabolism of tryptophan and cysteine, are essential to support tumor cell survival (25, 72–74). Acidic extracellular pH, which is resulted from the accumulation of lactate and CO_2 production, has been demonstrated to be important for cancer progression (75, 76). Recent studies have demonstrated that anti-tumoral immune population, such as CTL and T_{eff} cells, engage robust aerobic glycolysis and glutaminolysis, suggesting a potential metabolic antagonism (competition) for nutrients between tumor and those immune cells. On the contrary, pro-tumoral immune suppressive cells may preferentially utilize metabolic products of tumor to form a potential metabolic symbiosis in tumor microenvironment (**Figure 1**).

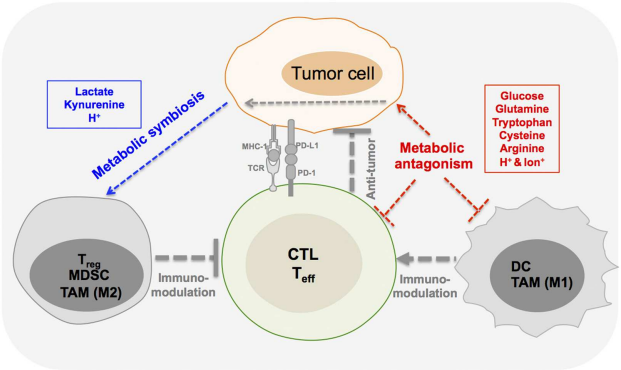

FIGURE 1 | Metabolic interplay in tumor immunity. Metabolic interplay through shuttling of metabolites among different cell compartments in tumor microenvironment serves as a form of intercellular communication and intercellular coordination. T effector cells (T_{eff}), cytotoxic T cells (CTL), dendritic cells (DC), and tumor-associated macrophage (TAM-M1) may form a potential metabolic antagonism (red color marked) with tumor cells. On the contrary, T regulatory cells (T_{reg}), myeloid-derived suppressor cells (MDSC), and TAM-M2 may preferentially utilize metabolic products of tumor to form a potential metabolic symbiosis (blue color marked) in tumor microenvironment.

ANTAGONISM

Glucose and glutamine

The similarity of metabolic programs between tumor and T cells (CTL and T_{eff}) leads to fierce competition for limited source of glucose and glutamine in local environment. The restriction of glucose and glutamine to fast proliferating cells could result in metabolic stress on both tumor and immune cells. Nutrient deprivation elicits signaling responses through AMP-dependent kinase (AMPK), mammalian target of rapamycin (mTOR), transcriptional factor p53, and other unknown signaling modulators to confer metabolic plasticity allowing cancer cells survive under low glucose and low glutamine condition (77–81). Furthermore, lactate and CO_2 produced from glycolysis and glutaminolysis lead to microenvironment acidification, favoring the development of more aggressive and invasive tumor cells (75, 76). Unlike tumor cells, metabolic stresses are less tolerated in non-malignant cells, such as T cells, and are often immune suppressive, partially due to a preferential development of T_{reg} cells following nutrients restriction (41, 42). Several studies also indicated that nutrient starvation perturbs T_{eff} cytokine production, macrophage phagocytic activity, and superoxide production. Therefore, metabolic microenvironment may render tumor cells a selective advantage due to their resistance to apoptosis and rapid adaptation under metabolic stress.

Tryptophan catabolism

The catabolism of the essential amino acid tryptophan has been reported to be a biomarker of tumor tissues in various studies. In tumor cells, the conversion of tryptophan to kynurenine is primary mediated by two dioxygenases, indoleamine-2,3-dioxygenase (IDO) and tryptophan-2,3-dioxygenase (TDO). IDO is expressed in many types of tumor cells and antigen-presenting cells, whereas TDO exists in certain IDO-negative tumor cells, such as malignant gliomas and hepatocellular carcinoma (82–85). The breakdown of tryptophan has been shown to dramatically affect the function of T cells against tumor cells. On one hand, upregulated tryptophan catabolism in tumor tissue resulted in the depletion of tryptophan in the extracellular space, which causes T_{eff} cells anergy and apoptosis and, in turn, suppresses anti-tumor-immune responses. On the other hand, kynurenine and potentially other catabolic metabolites of tryptophan are nature ligands of aryl hydrocarbon receptor (AHR), which plays a broad role in modulating immunity (86, 87). As such, extracellular accumulation of kynurenine elicits an AHR-mediated response to reciprocally enhance function of T_{reg} and suppress function of T_{eff} and immunogenicity of DCs (88–90). Thus, tryptophan depletion and kynurenine accumulation cooperatively create an immunosuppressive microenvironment in tumor (25, 90).

Cysteine and glycine

Physiological levels of ROS play essential roles in various signaling cascades for cell survival and proliferation, whereas excess ROS causes cell injury and tissue damage (91, 92). The thiol group in glutathione (GSH) acts as a reducing agent that can quench the cytotoxic ROS, and thus GSH is considered as an essential cellular antioxidant system to maintain redox homeostasis. Heightened GSH level is observed in numerous types of cancers, and the enriched GSH improves tumor cell survival by protecting them against oxidative stress (73, 74). Tumor cells uptake cysteine and cystine from the local environment and convert them into GSH together with glutamate and glycine, which are often derived from glutamine and glucose. Similarly, T cell proliferation depends on the uptake of exogenous cysteine. T cells lack cystathionase enzyme that converts methionine to cysteine and xc-transporter that imports cystine as an alternative source of

cysteine (93). Thus, the competition between tumor cells and T cells for cysteine and glycine may lead to the suppression of T cell activation and proliferation.

Arginine catabolism

As another form of free radical, NO plays multifaceted roles in cancer initiation, progression, differentiation, and angiogenesis (94–96). In mammals, NO is converted by a family of nitric oxide synthases (NOS) from arginine. It has been reported that arginine depletion retards the growth of some types of tumor, whereas other studies demonstrated that arginine supplementation assists anti-tumor treatment possibly by enhancing immune function (97, 98). As such, arginine has been discovered to stimulate T cell and NK cell activity and promote production of pro-inflammatory cytokines (99, 100). Also, tumor-derived NO may elicit cytotoxic effects on tumor-associated immune cells. However, the intrinsic resistance to NO-mediated cytotoxicity of tumor cells with mutated p53 offers a selective growth advantage of cancer cells over normal cells (101–104).

Proton and sodium ion

It has been known that the acidification of microenvironment caused by the accumulation of lactic acid and CO_2 enhances tumor radioresistance and favors tumor cell migration and invasion (75, 76, 105). Beyond this, acidic environment decreases the activity of NK cells, suppresses T cell proliferation, and impairs cytokine production and cytotoxic activity of T cells. Accumulating evidences also suggest that acidic microenvironment has a profound impact on monocytes differentiation and cytokine release (106–108). As one of the key inorganic ions in our body, cross-membrane transport of sodium ion is intimately coupled with proton and amino acids transport and also profoundly impact on tumor microenvironment (75, 109). Recent studies show that high-sodium chloride conditions induce the development of pathogenic Th17 cells with elevated release of pro-inflammatory cytokines (GM-CSF, TNF-α, and IL-2) and thus promote tissue inflammation, which may either promote or suppress tumor formation. While some of the effects of sodium are mediated through serum/glucocorticoid-regulated kinase 1(SGK1), further investigations are warranted to assess the impact of sodium on proton and amino acids transport. As such, a sodium ion-proton axis may coordinately modulate anti-tumor response (110, 111).

METABOLIC SYMBIOSIS

In contrast to T_{eff} cells, the enriched lactate and the acidic microenvironment do not have obviously suppressive effect on T_{reg} cells, as they have a different energy metabolism that relies on fatty acid oxidation. The lactate accumulated in the microenvironment is generally considered as metabolic "waste." However, numerous studies have indicated the possible function of lactate as a prominent substrate in oxidative metabolism among various types of cells such as muscle cells, neurons, and certain tumor cells (112–114). Although, it has not been demonstrated, the preference of mitochondrial-dependent oxidative metabolism of T_{reg} indicates the possibility that T_{reg} may utilize lactate under nutrient scarcity, which often happens in tumor microenvironment. The concentration of lactate in vertebrate plasma ranges from 1 to 30 mM under physiological and pathological conditions (2). Beyond serving as a potential alternative energy source, early studies suggested that high lactate concentrations (2–30 mM) enhance T_{reg} differentiation through the stimulation of IL-2 production (115, 116). Similarly, increased production of lactate by tumor cells promotes the development of MDSC (117). Also, lactate and acidic environment have a profound impact on secretory profile of TAM, promoting tumor angiogenesis (108, 118, 119). Beyond lactate, the catabolic metabolites of tryptophan, such as kynurenine, promote T_{reg} differentiation and immune suppressive function (89). Thus, tumor-derived lactate and tryptophan catabolic metabolites may form a layer of metabolic symbiosis with various immune cells to favor tumor growth.

CONCLUSION AND PERSPECTIVE

The metabolites that present in tumor microenvironment may also have signaling functions independent of their roles of bioenergetics fuels. This may represent a general feature of the intercellular metabolic crosstalk mediated by metabolites. To fully understand the underlying complexity of intercellular metabolic interplay, new techniques that allow us to quantitative measure metabolites, assess metabolic flux *in situ*, and detect physical interaction between metabolites and cell surface proteins are warranted. The fast moving cancer metabolism field and immunotherapy field have generated tremendous excitement regarding new therapeutic strategies and will likely change the paradigm of therapeutic interventions for cancer. However, the perturbed metabolic landscape of the tumor microenvironment can have a profound impact on anti-tumor immunity. As such, understanding the metabolic interplay between tumor and immune system will guide the development of optimal metabolic interventions on cancer without compromising anti-tumor immunity. Beyond this, intercellular metabolic interplay may also play an essential role in forming a pro-tumoral inflammatory microenvironment.

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Role of T cells in malnutrition and obesity

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Nutritional status is critically important for immune cell function. While obesity is characterized by inflammation that promotes metabolic syndrome including cardiovascular disease and insulin resistance, malnutrition can result in immune cell defects and increased risk of mortality from infectious diseases. T cells play an important role in the immune adaptation to both obesity and malnutrition. T cells in obesity have been shown to have an early and critical role in inducing inflammation, accompanying the accumulation of inflammatory macrophages in obese adipose tissue, which are known to promote insulin resistance. How T cells are recruited to adipose tissue and activated in obesity is a topic of considerable interest. Conversely, T cell number is decreased in malnourished individuals, and T cells in the setting of malnutrition have decreased effector function and proliferative capacity. The adipokine leptin, which is secreted in proportion to adipocyte mass, may have a key role in mediating adipocyte-T cell interactions in both obesity and malnutrition, and has been shown to promote effector T cell function and metabolism while inhibiting regulatory T cell proliferation. Additionally, key molecular signals are involved in T cell metabolic adaptation during nutrient stress; among them, the metabolic regulator AMP kinase and the mammalian target of rapamycin have critical roles in regulating T cell number, function, and metabolism. In summary, understanding how T cell number and function are altered in obesity and malnutrition will lead to better understanding of and treatment for diseases where nutritional status determines clinical outcome.

Keywords: obesity, inflammation, T cells, malnutrition, leptin

NUTRITIONAL STATUS ALTERS T CELL IMMUNITY

Both obesity and malnutrition are major health problems around the globe. The World Health Organization has listed both obesity/overweight and childhood malnutrition/underweight on its top 10 causes of global mortality and disease. In developed countries, there has been an emphasis on the deleterious health effects of obesity. Obesity is associated with life-threatening co-morbidities including cardiovascular disease and type 2 diabetes that shorten lifespan. However, we must remember that for the majority of the world population, malnutrition from chronic food deprivation is the more common nutritional problem. Both obesity and malnutrition are associated with changes in immune cell number and function that alter immunity and have consequences for infection and inflammation. In this review, we will discuss the effects of nutrition on T cell distribution and function and examine the role of T cells in altered immunity in both malnutrition and obesity.

OBESITY AND INFLAMMATION

Obesity is a growing epidemic in developed countries. For example, over 30% of Americans are currently classified as overweight or obese (1). Unfortunately, the obesity epidemic is accompanied by a myriad of associated health risks. Exacerbating the problem is the absence of safe and effective treatments to reverse obesity. Lifestyle modifications such as diet and exercise are oftentimes ineffective, and at present, bariatric surgery is the most effective treatment for obesity but can be associated with post-surgical complications and side effects.

Obesity is associated with life-threatening co-morbidities such as insulin resistance leading to type 2 diabetes mellitus, as part of the metabolic syndrome (2). Indeed, the prevalence of both obesity and diabetes has increased in parallel during the last 20 years, and is highly associated (3). From these trends, the United States Centers for Disease Control (CDC) predicts that 1 in 3 American adults will have diabetes by the year 2050 (4). As part of the metabolic syndrome, obese individuals are also at risk for hyperlipidemia and hypertension leading to increased cardiovascular and renal disease. Additionally, obesity increases the risk of multiple forms of autoimmunity (5–7), including multiple sclerosis (MS), thyroid autoimmunity, and type 1 diabetes. Obesity is further associated with an increased risk of certain forms of cancer, including esophageal, breast, endometrial, colorectal, kidney, pancreatic, gallbladder, and thyroid cancer (8, 9). And finally, obese individuals have increased susceptibility to infections due to impaired host defense (10). In fact, obese individuals were more susceptible to the pandemic H1N1 influenza outbreak of 2009 (11).

Obesity is associated with both systemic inflammation and an influx of pro-inflammatory immune cells into visceral adipose tissue (VAT) (12). These VAT-localized immune cells secrete inflammatory cytokines, which have been shown to promote insulin resistance (12, 13). It is now clear that T lymphocytes (T cells), in particular, have a critical role in the early stages of this inflammatory process. Both regulatory and inflammatory T cells are found in VAT and influence the recruitment and function of other inflammatory cells into VAT, thereby contributing to changes in

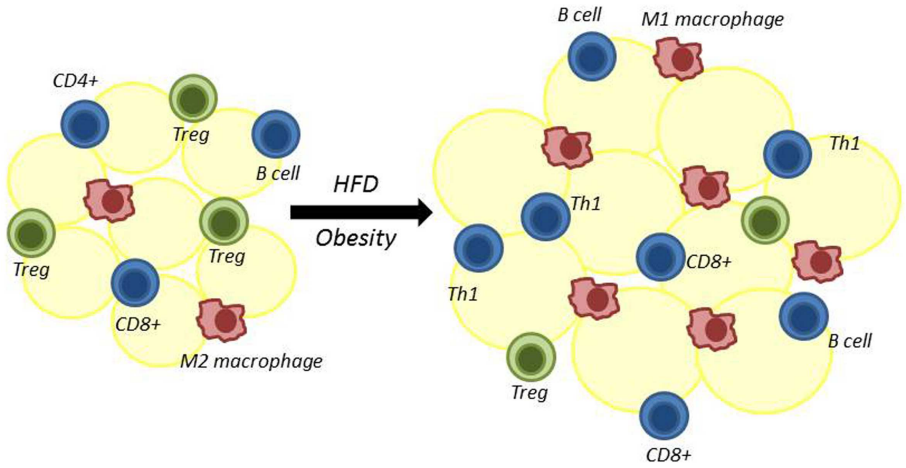

FIGURE 1 | Adipose tissue is remodeled following high-fat diet-induced obesity. Multiple immune cells reside in adipose tissue. High-fat diet-induced obesity results in decreased regulatory T cells (Treg) and alternatively activated M2 macrophages. At the same time, there is an increase in adipose-resident inflammatory macrophages (classically activated M1 macrophages), CD4+ Th1 cells, CD8+ T cells, and B cells. The role of CD4+ Th2 and Th17 cells in adipose tissue in obesity is less certain.

insulin sensitivity in obesity (**Figure 1**). For these reasons, it is critically important to determine how obesity and increased adiposity alter T cell distribution and function to drive inflammation.

EARLY STUDIES ON OBESITY-RELATED INFLAMMATION

In the 1990s, several observations were made that first linked obesity with inflammation. A key early study by Hotamisligil et al. demonstrated that expression of the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) was increased in the adipose tissue of obese animals (14). Moreover, loss of TNF-α in obesity resulted in improved insulin sensitivity and glucose homeostasis (14–16). Not long after these initial findings with TNF-α, other pro-inflammatory cytokines were implicated. In a prospective study in 2001, both C-reactive protein (CRP) and IL-6 were found to be predictive of the development of type 2 diabetes in obese patients (17).

Soon after, macrophages were identified as an inflammatory cell of interest in obesity-associated inflammation. In 2003, it was discovered that many macrophage-specific genes were upregulated in both genetic and diet-induced forms of obesity in animals (18, 19). Around the same time, macrophages were found to accumulate in adipose tissue in obese animals (19). A few years later, macrophages in obese adipose tissue were ascribed an inflammatory phenotype that differs from the phenotype of macrophages in lean adipose tissue (20, 21).

These early studies have led to an understanding of multiple immune cells, chemokines, and cytokines contributing to the network of inflammation leading to insulin resistance and metabolic syndrome in obesity. Since these early studies, multiple pro-inflammatory cells, in addition to macrophages, have been ascribed a role in either promoting inflammation (lymphocytes, mast cells, neutrophils, and NK cells) or regulating inflammation (regulatory T cells) in obesity. Additionally, adipocytes themselves can secrete pro-inflammatory cytokines, many of which overlap with cytokines produced by macrophages, including TNF-α and IL-6, as well as hormones that influence inflammation, such as the pro-inflammatory hormone leptin and the anti-inflammatory hormone adiponectin.

An important question remained, how do inflammatory cytokines and signals promote insulin resistance? Elevated inflammatory signals were found to induce both c-Jun N-terminal kinase (JNK) signaling pathways and activation of the inhibitor of nuclear factor kappa-B (NF-κB) kinase subunit beta (IKKβ), which goes on to promote NF-κB activation. Both JNK and IKKβ/NF-κB signaling pathways were found to decrease insulin action of adipocytes and hepatocytes (22, 23). Multiple studies have now shown that blocking inflammatory cells or cytokine signaling in adipose tissue prevents development of inflammation and subsequent insulin resistance in obesity.

MACROPHAGES IN OBESITY-RELATED INFLAMMATION

Macrophages are present in both lean and obese VAT and, in fact, make up 40–60% of VAT immune cells in obesity. The role of the macrophage in VAT and promoting insulin resistance in obesity is well studied and has been extensively reviewed (24, 25). Interestingly, there is an overlapping biology and function of VAT-localized macrophages and adipocytes, and these two cell types produce many hormones and cytokines in common, including inflammatory cytokines implicated in obesity-associated inflammation and insulin resistance (26). These inflammatory cytokines have paracrine effects on other adipose-localized cells and may be secreted into circulation to exert endocrine effects systemically.

Macrophages are polarized toward either an M1 or M2 phenotype. Macrophages in lean tissue are typically M2 macrophages, also known as "alternatively activated macrophages," which secrete anti-inflammatory cytokines, including IL-10, IL-1 receptor agonist, and arginase-1. Obesity-associated VAT macrophages are largely M1 macrophages, or "classically activated macrophages" that secrete large amounts of inflammatory cytokines including TNF-α, IL-6, IL-12, IL-1β, and monocyte chemotactic protein 1

(MCP-1) (24). These M1 macrophages in obese VAT are either derived from macrophages already present in adipose tissue, which have changed their phenotype in response to obesity, or they are recruited into adipose tissue from circulation. Obesity-associated effects on VAT include the release of chemokines, such MCP-1. MCP-1 binds to the chemokine (C–C motif) receptor 2 (CCR2) on macrophages, and has been described to recruit macrophages into adipose tissue in obesity (27). Many studies have demonstrated the role for classically activated M1 macrophages in promoting insulin resistance, and numerous mouse models have confirmed that disabling the macrophage inflammatory response pathway in obesity is protective against the development of insulin resistance (25, 28).

In addition to macrophages, other innate immune cells appear to participate in regulating inflammation and insulin resistance in obesity. Both pro-inflammatory neutrophils and mast cells are activated in obesity, and mice lacking these innate inflammatory cells are protected against insulin resistance (29). Conversely, eosinophil number is decreased in the VAT of obese mice, and eosinophil-deficient mice have increased inflammation and insulin resistance (29). While innate cells are clearly important to regulate inflammation in obesity, we will not focus on the role of the innate cells in obesity-associated inflammation and insulin resistance here, but rather highlight the role of the lymphocyte.

LYMPHOCYTES IN OBESITY-RELATED INFLAMMATION

In 2009, Nature Medicine published a series of three papers that altogether established a critical role for both inflammatory and regulatory T cells in mediating adipose tissue inflammation and altering insulin sensitivity in obesity (30–32). Together, these studies demonstrated that obesity was associated with T cell changes in VAT that included decreased regulatory T cells (Treg) and Th2 cells and increased inflammatory Th1 and CD8+ T cells. Soon after, other studies confirmed a role for T cells in adipose tissue in promoting systemic inflammation in obesity (33). These reports established a potentially early and critical role for inflammatory lymphocytes (CD8+ T cells, Th1 helper cells, and B cells) and regulatory lymphocytes (Th2 helper cells and Treg) in obesity-associated inflammation leading to insulin resistance. Thus began a new era of investigation into the role of lymphocytes in obesity-associated inflammation. It soon became clear that both the type and the proportion of lymphocytes and lymphocyte subsets change in VAT in obesity. We will discuss findings regarding lymphocyte subsets that either promote or down regulate obesity-induced inflammation here.

PRO-INFLAMMATORY LYMPHOCYTES

CD4+ Th1 cells

VAT from high-fat diet-induced obese mice has higher levels of both CD4+ and CD8+ T cells, as compared to lean controls. T cells from obese VAT produce high levels of the Th1 cytokine interferon gamma (IFN-γ) when stimulated *in vitro* (34). IFN-γ secreted from adipose tissue induces the polarization of macrophages toward the M1 phenotype. IFN-γ also increases the production of other inflammatory cytokines, including TNF-α, from adipose tissue cultured *in vitro*. Obese animals lacking IFN-γ expression produced less adipose TNF-α and MCP-1, had decreased inflammatory cell accumulation in adipose tissue, and had improved insulin sensitivity compared to animals with normal IFN-γ expression (34, 35). Most recently, Khan et al. examined T cell receptor beta (TCRβ) deficient mice, which are protected against obesity-induced macrophage infiltration and insulin resistance, and found that adoptive transfer of Th1 cells into high-fat diet-fed TCRβ-/- mice led to increased muscle and adipose tissue inflammation as well as increased insulin resistance (36).

The T cell-specific T-box transcription factor, T-bet, which is known to induce Th1 development and promote transactivation of the IFN-γ gene, has also been implicated in obesity-associated inflammation. T-bet knockout mice have decreased energy expenditure and increased visceral adiposity compared to wildtype littermates, yet have better insulin sensitivity on both normal chow and high-fat diet (37). While the T-bet knockout mice were expected to have increased insulin sensitivity, the finding that they were more obese, with increased visceral adiposity, was surprising, as obesity and insulin resistance are typically co-associated. Improved insulin sensitivity is perhaps due to decreased expression of Th1 cytokines and increased expression of Th2 cytokines and the Th2 transcription factor GATA binding protein 3 (GATA3) in the T-bet knockout animal (38). Moreover, adoptive transfer of T-bet-deficient CD4+ T cells, but not wildtype CD4+ T cells, into Rag2-/- mice, which lack both B and T lymphocytes, modestly improves insulin sensitivity (37). As Th1 cells typically produce both T-bet transcription factor and IFN-γ cytokine, their role in insulin sensitivity was examined in IFN-γ knockout mice. Loss of T-bet on an IFN-γ knockout mouse conferred no additional improvement to insulin sensitivity (39).

CD4+ Th17 cells

The role of Th17 cells in VAT in obesity is less clear. In 2009, Winer et al. described an association between obesity and IL-17 production in mice (40). IL-17 expression has also been found to be high in obese human beings (41), and peripheral blood samples from human beings with type 2 diabetes mellitus have increased numbers of circulating Th17 cells (42, 43). Obesity can exacerbate autoimmune diseases that have a strong Th17-dependent mechanism, including experimental autoimmune encephalomyelitis (EAE) in mice, MS in human beings, and colitis (44). IL-17 has been found to be produced by T cells in adipose tissue, and IL-17 knockout mice are overweight and have increased obesity following high-fat diet compared to littermate controls, but show improved insulin sensitivity (45). However, IL-17 can be produced by both Th17 cells and gamma delta (γδ) T cells and appears to be secreted from γδ T cells in VAT (45). One group has even reported IL-17 production from neutrophils in obesity (46). Therefore, the role of Th17 cells in inflammation and insulin resistance in obesity remains uncertain.

CD8+ T cells

Obesity is associated with increased CD8+ T cells in adipose tissue (47). In 2009, Nishimura et al. demonstrated that CD8+ T cells preceded macrophages into VAT in obesity (30). Moreover, depletion of CD8+ T cells in diet-induced obesity resulted in decreased accumulation of macrophages into obese VAT as well as improved insulin sensitivity. Conversely, adoptive transfer of CD8+ T cells

into CD8-deficient mice increased infiltration of macrophages into VAT as well as expression of the inflammatory cytokines IL-6 and TNF-α, along with development of insulin resistance following high-fat diet (30). These findings suggest a critical role for CD8+ T cells in the development of inflammation and insulin resistance in obesity. More recently, Jiang et al. confirmed VAT accumulation of CD8+ T cells in obesity and examined the mechanism of CD8+ T cell accumulation in adipose tissue (48). The authors found that VAT CD8+ T cells are activated *in vitro* by Th1 cytokine IFN-γ. Moreover, CD8+ T cells from VAT in obese mice expressed high levels of the integrin CD11a, which was important for infiltration of CD8+ T cells into adipose tissue in obesity (48).

B cells

In addition to increased CD4+ and CD8+ T cells, B cells also accumulate in VAT in diet-induced obesity (49). Diet-induced obese mice that lacked B cells, following treatment with B cell depleting CD20 antibody, were protected against insulin resistance, despite weight gain on a high-fat diet (50). Treatment of these B cell-deficient mice with IgG from wildtype obese mice resulted in restoration of insulin resistance (50). These findings, therefore, establish a role for both B cells and IgG antibodies in driving insulin resistance in obesity. In recent work from a separate group, cytokine production was compared in obesity from B cell null mice and wildtype controls. Obese B cell null mice had decreased inflammatory cytokines, including IL-6 and IFN-γ, increased anti-inflammatory IL-10 levels, and protection against insulin resistance compared to obese wildtype mice (51). Absence of B cells in obesity was also associated with an increased number of regulatory T cells (Treg) in VAT, as compared to obese wildtype mice. Additionally, human B cells from type 2 diabetics, but not from non-diabetic controls, were able to activate T cells *in vitro* (51). These results suggest a possible mechanism in which B cells precede T cells, and possibly macrophages, into VAT and regulate T cell differentiation, inflammation, and activation in obesity.

LYMPHOCYTES WITH AN ANTI-INFLAMMATORY ROLE IN OBESITY

Regulatory T cells (Treg) have been found to have a key role in the regulation of inflammation and the development of insulin resistance in obesity. Obese human beings have decreased circulating Treg cells (52), and genetic mouse models of obesity and diet-induced obese mice show decreased Treg numbers in VAT (31). When Treg cells were depleted acutely, investigators observed increased transcription of inflammatory genes in VAT along with increased insulin levels and decreased insulin receptor signaling (31). This suggests that Treg play an important role to suppress obesity-related inflammation. Indeed, expansion of the Treg compartment in high-fat diet-fed mice was associated with increased levels of IL-10, statistically significant lower blood glucose levels, and trends toward lower insulin resistance and glucose tolerance (31).

In 2012, Cipolletta et al. described peroxisome proliferator-activated receptor gamma (PPAR-γ) expression as a critical player in Treg accumulation in VAT and in insulin sensitivity (53). PPAR-γ expression was increased in VAT Treg as compared to peripheral lymphoid Treg cells, and establishment of VAT Tregs from naïve CD4+ T cells depended on both PPAR-γ and Foxp3 expression. Indeed, conditional knockout of PPAR-γ in Treg resulted in decreased Treg number in VAT but did not affect Treg number in peripheral lymphoid tissue (53). PPAR-γ is well known to influence adipocyte differentiation and is considered anti-inflammatory. Members of the class of drugs called thiazolidinediones are PPAR agonists, and have been used in the treatment of type 2 diabetes mellitus for many years, although their use over the past five years has been limited due to cardiovascular side effects and outcome. The authors of this study found that PPAR-γ expression was required for action of the thiazolidinedione drug pioglitazone to restore insulin sensitivity in obesity, as pioglitazone enhanced the accumulation of VAT Treg and improved insulin sensitivity in obese mice (53). These findings suggest a novel mechanism by which PPAR agonists can promote insulin sensitivity through the regulation of T cell differentiation and function, as opposed to modulation of PPAR expression in adipocytes. Moreover, these findings highlight Treg as an interesting potential drug target of obesity-related inflammation and type 2 diabetes mellitus.

More recently, Han et al. described a key role for insulin in regulating Treg function (54). The authors found that insulin receptors are expressed on Treg, and that insulin signaling directly influenced Treg function by decreasing IL-10 production via activation of the AKT/mammalian target of rapamycin (mTOR) signaling pathway (54). Insulin was also able to reduce Treg suppression of TNF-α production by macrophages. When Treg were isolated from obese or lean VAT in mice, Treg from obese VAT produced less IL-10 and more IFN-γ than Treg from lean animals (54). These findings suggest that increased insulin levels in obesity can promote inflammation by directly reducing Treg suppression and thereby driving the chronic inflammation of obesity.

MIGRATION AND ACTIVATION OF T CELLS IN VAT IN OBESITY

While the presence of inflammatory T cells in adipose tissue is now well established, the mechanism by which T cells are recruited into obese VAT and how they are activated has been less clear. Recent publications have started to address these questions. The chemokine receptor CXCR3 has recently been found to have a critical role in T cell recruitment into VAT in obesity. CXCR3 knockout mice on high-fat diet had fewer VAT T cells than wildtype mice on high-fat diet (55). Obese CXCR3 knockout mice also developed less insulin resistance following 8 weeks of high-fat diet compared to wildtype obese mice, although this protection was lost by 16 weeks. The diet-induced obese CXCR3 knockout mice also expressed lower levels of mRNA for several pro-inflammatory genes including MCP-1 in adipose tissue, and higher levels of anti-inflammatory genes, including Foxp3 and IL-10 (55). A separate group also examined CXCR3 knockouts on high-fat diet for 20 weeks. They observed similar weight gain in CXCR3-/- and wildtype mice, but observed decreased fasting glucose and improved glucose tolerance in CXCR3 knockouts, along with decreased infiltration of immature myeloid cells into VAT (56). Other chemokine systems may also play a role in this process. Expression of the chemoattractant RANTES is induced in adipocytes in obesity along with its chemokine receptor CCR5 (57, 58). CCR5 also has a critical role in obesity-associated inflammation as CCR5 knockout

mice on high-fat diet demonstrate a shift from pro-inflammatory M1 to M2 macrophages in VAT, and are protected against insulin resistance in obesity (58).

Once T cells are recruited to adipose tissue in obesity, they require activation to maintain a pro-inflammatory state. There is evidence for both macrophages and B cells (51, 59), as well as adipocytes (60), in acting as antigen-presenting cells to stimulate T cell activation and inflammation of VAT in obesity. Adipocytes certainly secrete hormones and cytokines that are well known to promote inflammatory T cell activation, including leptin, resistin, TNF-α, and IL-6. Additionally, adipocyte-derived lipids have been shown to modulate T cell function (60). However, in addition to secreting cytokines, hormones, and lipids that can influence T cell inflammation, adipocytes may also serve as antigen-presenting cells by expressing MHC class II and co-stimulatory membrane receptors on their surface, thereby activating CD4+ T cells (61).

TARGETING T CELL INFLAMMATION IN THE TREATMENT OF INSULIN RESISTANCE

Understanding the role of lymphocyte inflammation in promoting insulin resistance in obesity opens up new possibilities for treatment of type 2 diabetes. Weight loss has been shown to both decrease inflammatory cytokine production and improve insulin sensitivity (62). Even exercise alone has been shown to reduce inflammatory cells in VAT in obesity (63). However, as stated above, weight loss through lifestyle modifications of diet and exercise is challenging for many and oftentimes unsuccessful. In addition, new evidence supports the idea that weight loss may be harder to achieve in an inflammatory state. In a recent report published in The Journal of Clinical Endocrinology and Metabolism in 2014, the presence of increased inflammation, as measured by inflammatory cytokine levels, prior to bariatric surgery, led to decreased body mass index reduction following weight-loss surgery (64).

Subsequently, the idea of immunotherapy in the treatment of type 2 diabetes had gained considerable interest. Thus far, there have been a handful of targets of inflammation that have shown therapeutic promise for the treatment of diabetes. The first is TNF-α blockade, which has shown some mixed success in improving insulin resistance in diabetic patients (65–67). The second target of interest is NF-κB activation blockade. Early-animals studies in 2001 showed that salicylates could reverse obesity-associated insulin resistance in both genetically obese and diet-induced obese mice (68, 69), which resulted in decreased NF-κB activation. In a more recent series of studies, salicylates were found to be potentially useful for the treatment of high-fat diet-induced insulin resistance in diabetes in human subjects (70), yet results are mixed (71), and blockade of NF-κB is very downstream in the VAT inflammatory pathway in obesity, so inflammatory cell numbers are not typically affected (13). Finally, there is some evidence that selective blockade of IL-1 receptor activation may prevent insulin resistance in obesity-associated inflammation (72). A more recent study investigated the effect of T cell co-stimulation blockade on obesity-associated insulin resistance, and found that both CD40L antibody and cytotoxic T lymphocyte antigen 4 (CTLA-4) immunoglobulin (Ig)-treated diet-induced obese mice had reduced numbers of VAT macrophages and CD8+ T cells, as compared to obese mice treated with control antibody (73). However, only the CD40L antibody-treated, and not the CTLA-4 Ig-treated mice, had decreased weight gain and subsequent improvement in insulin sensitivity. Altogether, modulation of obesity-associated inflammation is of considerable interest at present, and may offer novel therapeutic targets for type 2 diabetes in obesity.

MALNUTRITON AND IMMUNODEFICIENCY

Like obesity, malnutrition is a serious global health issue affecting many. In 2011, it was estimated that over 52 million children were severely malnourished (74). This has important health implications, as children that are malnourished have suppressed immunity and higher mortality due to infections (75–77). Childhood malnutrition is also associated with impaired cognitive development, as well as persistent defects in learning and memory (78, 79). Young children and infants are particularly at risk, as 45% of deaths in children under age five years are due to undernutrition (74). Malnutrition specifically accounts for 2.6 million childhood deaths annually due to infections including diarrheal illness and pneumonia (80, 81).

In addition to chronic malnutrition in developing countries, malnutrition can also affect immune function in other clinical settings in which nutritional status influences outcome. Such scenarios include patients with cancer- and AIDS-related cachexia, critically ill ICU patients, and low birth weight neonates with inadequate adipose stores. In fact, it is estimated that 60–80% of patients with advanced cancer are cachexic, and weight loss prior to chemotherapy greatly increases mortality in cancer patients (82, 83). Additionally, the U.S. National Cancer Institute has estimated that up to 40% of cancer deaths result from infections related to malnutrition. Malnutrition-induced immune suppression is, therefore, a major cause of morbidity and mortality in multiple susceptible patient populations. Despite an abundance of epidemiological evidence linking malnutrition and immunodeficiency, little is known about the impact of malnutrition on specific immune cell populations. Here, we describe recent findings on the role of malnutrition on lymphocyte number and function.

MALNUTRITION AND T CELLS

Malnutrition has been linked to immune dysfunction in a variety of settings, including starvation and cachexia in both human beings and mice (77, 80–82). As T cells are a vital component of the adaptive immune system, several studies have specifically examined the effect of malnutrition on T cell number and function. Mice fasted for 48 h had drastically decreased thymocyte and splenocyte counts compared to fed-control mice (84–86). Within the spleen, total T cell and CD4+ T cell numbers from fasted mice were decreased by 40–50% compared to control animals (85, 86). Additionally, mice fed a protein-deficient diet had atrophic spleens and decreased T cell numbers compared to control mice (87, 88). Decreased T cell numbers observed in fasted mice are mimicked in malnourished human beings. Malnourished children had decreased CD4+ and CD8+ T cell numbers in whole-blood samples compared to T cell numbers from well-nourished children (89).

In addition to drastically reducing T cell numbers, malnutrition also affects T cell cytokine production. Protein energy malnutrition impaired the ability of rat lymphocytes to proliferate and produce the Th1-associated cytokine IFN-γ (90). Similarly, T cells from mice that were fasted for 48 h and then activated *in vitro* had greatly decreased production of the Th1 cytokines IL-2 and IFN-γ, compared to T cells from fed control animals (86). This decrease in cytokine production observed in malnourished mice was also observed in human studies, as malnourished children had decreased levels of cytokines important for Th1 differentiation (IL-12, IL-18, and IL-21) as well as decreased Th1 cytokines IFN-γ and IL-2 (91). In a separate study, the same group showed that malnourished children had increased expression of Th2 cytokines IL-4 and IL-10 (92). Altogether, these findings suggest that malnutrition shifts the balance of pro-inflammatory Th1 versus anti-inflammatory Th2 cytokines, and may offer an explanation as to how malnutrition predisposes to infection.

Upon activation, effector T cells undergo metabolic reprogramming that results in transition from an oxidative state to a highly glycolytic phenotype. Accompanying this metabolic switch is an increase in expression of the ubiquitous glucose transporter, Glut1, which leads to increased glucose uptake and subsequent glycolysis (93, 94). This shift from oxidative to glycolytic metabolism is critical to maintain T cell function, as decreased glucose availability inhibits T cell cytokine production and proliferation (93, 95). We have found that acute malnutrition inhibits activation-induced T cell glucose metabolism (86). Rescue of this metabolic defect with T cell-specific overexpression of a Glut1 transgene normalizes glucose uptake to levels in T cells from fed controls. Additionally, Glut1 overexpression returns inflammatory cytokine production back to levels seen in T cells isolated and activated from fed mice, suggesting that rescue of T cell glucose metabolism reverses the T cell functional defects seen in malnutrition (86). This leads to the intriguing possibility that decreased circulating glucose levels in fasting may directly affect T cell metabolism and, therefore, T cell function. Indeed, anti-CD3 antibody treatment to activate T cells *in vivo* led to an increase in T cell glucose uptake and a marked decrease in circulating glucose levels, resulting in hypoglycemia in mice (96). Hypoglycemia following anti-CD3 antibody treatment did not occur in Rag1-/- mice that lack lymphocytes (96). This suggests that T cells utilize a substantial amount of glucose during activation and may be affected by altered circulating glucose levels due to starvation or malnutrition. More work is needed to gain a complete understanding of the relationship between malnutrition, T cell metabolism, and function.

MALNUTRITION AND INFECTION

Increasing evidence suggests that malnutrition can lead to more severe viral infections and can affect vaccine responses in children (76, 97). Although most epidemiological studies examined general nutrient deficiencies, many of the animal studies specifically assess the effect of protein energy malnutrition (PEM) on viral immunity, using low-protein or protein-deficient diets in mouse models (87, 88, 98, 99). In a respiratory infection model, mice fed a low-protein diet required a 1000-fold lower viral titer for 50% lethality than normal fed controls (87). Similarly, malnourished mice inoculated with *Mycobacterium tuberculosis* had 2–3 logs more bacilli in the lungs than mice receiving a full protein diet (100). PEM also increased the susceptibility to influenza infection due to impaired viral clearance and decreased lymphocyte numbers (88). Importantly, supplementation with protein enhanced viral clearance and decreased mortality from influenza (88).

Furthermore, PEM impaired the homeostatic proliferation of mouse memory CD8+ T cells in response to lymphocytic choriomeningitis virus (LCMV) (98). A separate group showed a decreased number of viral-specific CD8+ T cells upon protein malnutrition during LCMV infection (99). The low-protein diet impaired the recall response and maintenance of memory CD8+ T cells; this could be rescued by protein supplementation, suggesting that dietary protein is critical for maintaining a functional pool of memory T cells (98). This defect in CD8+ T cell function during malnutrition also occurs in human beings. Malnourished children hospitalized with bacterial infections were shown to have a lower fraction of memory T cells than well-nourished infected controls (101), as well as decreased total CD4+ and CD8+ T cell numbers (89). Together, these data suggest that malnutrition decreases CD8+ T cell number and function and predisposes to infection.

MALNUTRITION AND AUTOIMMUNITY

As malnutrition reduces T cell number and function, there is increasing evidence that calorie-restriction and fasting protect against autoimmune disease. One study examined BXSB mice, which spontaneously develops an autoimmune disease similar to systemic lupus erythematosus (SLE) (102). A 40% calorie-restriction inhibited the onset of autoimmunity and increased the lifespan of mice compared to those on a non-restricted diet (103). The calorie-restricted mice also had decreased IL-2 production and lymphocyte proliferation compared to controls (103). Piccio et al. examined a 40% calorie-restriction in the context of two EAE models. In both EAE models, calorie-restriction increased survival and decreased disease scores during EAE progression (104). Additionally, calorie-restriction significantly decreased central nervous system inflammation and demyelination as well as plasma levels of the pro-inflammatory cytokine IL-6 (104). A separate group showed that mice fasted for 48 h had decreased EAE disease scores as well as decreased IFN-γ production (105). Similar findings were also shown in calorie-restricted rats (106). Interestingly, mice fed *ad libitum* every other day (intermittent feeding) also developed less severe EAE compared to control animals fed *ad libitum* (107). Together, these data suggest that calorie-restriction and fasting prevent autoimmunity, likely by decreasing T cell responses and inflammatory cytokine production.

ROLE OF LEPTIN IN MEDIATING NUTRITIONAL EFFECTS ON T CELLS

Leptin is an adipokine secreted in proportion to adipocyte mass. In addition to its well-described role in regulating appetite, energy expenditure, and body weight, leptin is also a pro-inflammatory cytokine. Leptin has direct and indirect effects on T cell number and function, promoting Th1 and Th17 cell number and cytokine production while inhibiting Th2 cytokine production and Treg proliferation (108). The effects of leptin on T cell number and function have been extensively reviewed and will not be discussed

in depth here, yet special consideration must be given to the role of leptin in mediating T cell changes in malnutrition or obesity.

Acute starvation causes a drastic reduction in leptin levels in both mice and human beings; indeed, many studies utilize fasting as a way to model hypoleptinemia in mice (84–86). Low levels of leptin are associated with high rates of death from infectious diseases (109, 110). In a recent study published in the Journal of Clinical Endocrinology and Metabolism, the hormonal and metabolic status of malnourished Ugandan children was examined. In this study, low leptin levels were found to be the single most important biomarker to predict mortality during inpatient treatment of malnutrition (111).

Additionally, leptin-deficient *ob*-/- mice, despite their genetic obesity, have many features similar to those seen in malnutrition, including decreased body temperature, infertility, and low metabolic rate (112). *Ob*-/- mice also have immune defects similar to those seen in malnourished human beings and animals, including decreased thymocyte and splenocyte numbers, increased susceptibility to infection, and protection against certain forms of autoimmunity (84). Both the metabolic and immune defects seen in *ob*-/- mice can be reversed by treatment with recombinant leptin.

A role for leptin in reversing malnutrition-induced immunosuppression has been examined by our group and others. Although fasting leads to decreased T cell number and function, these defects can be reversed by treatment with recombinant leptin during the period of starvation (85, 86). Either leptin administration to fasting mice *in vivo* or leptin treatment of T cells isolated from fasted animals *in vitro* was sufficient to rescue inflammatory cytokine production in activated T cells from fasted mice (86). Importantly, leptin also rescued the T cell metabolic defects seen in fasting. Leptin treatment led to increased Glut1 expression and increased glucose uptake and glycolysis in fasted animals (86). Leptin is, therefore, a critical regulator of T cell glucose metabolism to fuel T cell activation. In other studies, leptin replacement was found to reverse starvation-induced immunosuppression, as measured by a delayed-type hypersensitivity (DTH) response (113), and prevented starvation-induced protection against EAE (105). Together, these data suggest that leptin is an important modulator of nutritional effects on T cell metabolism and function (**Figure 2**).

Leptin itself or the regulatory pathways that control leptin expression or action may be important new targets to promote immunity (114). In a small clinical study of malnourished children hospitalized for infection in Mexico, treatment of peripheral blood T cells with leptin *in vitro* increased T cell activation and inflammatory IL-2 and IFN-γ cytokine production, while decreasing IL-4 and IL-10 production (115). Additionally, leptin has been used as a mucosal vaccine adjuvant for *Rhodococcus equi* bacterial infections in mice, and leptin signaling was also associated with higher *Helicobacter pylori* antibody titers following vaccination (116, 117). Understanding the pathways by which leptin regulates T cell metabolism and function may lead to new ways to augment immunity in select clinical disorders associated with undernutrition.

Conversely, the fact that leptin is a pro-inflammatory cytokine secreted by adipocytes, in proportion to adipocyte mass, points to a role for leptin in obesity-associated inflammation and insulin resistance, and many have speculated on the role for leptin in promoting inflammation in obesity. This is complicated, however, by the fact that both the leptin-deficient and leptin receptor-deficient *ob*-/- and *db*-/- mice, respectively, still develop insulin resistance in the setting of genetic obesity (118). As leptin acts on every cell in the immune system as well as stromal cells that influence immune cell development, the direct effect of leptin on lymphocyte and macrophage inflammation in obesity remains unclear.

NUTRIENT-SENSING T CELL SIGNALING

Key molecular signals are involved in T cell metabolic adaptation during nutrient stress; among them, the metabolic regulator AMP-activated protein kinase (AMPK) and its upstream kinase, liver kinase B1 (LKB1), have critical roles in regulating

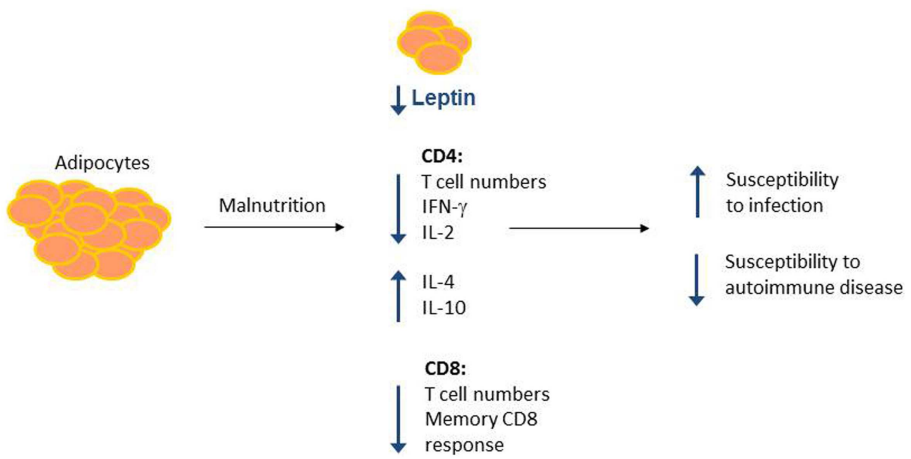

FIGURE 2 | Malnutrition leads to changes in adipocyte-secreted hormones and T cell number and function. Malnutrition leads to decreased adipocyte mass, which results in decreased circulating leptin. Concurrently, malnourished individuals show changes in CD4+ and CD8+ T cell number and function, leading to increased susceptibility to infection and protection against certain forms of autoimmunity. Leptin may mediate a subset of these malnutrition-related changes to T cell number and function, as leptin is known to alter CD4+ T cell number and cytokine production.

T cell number, function, and metabolism (119). AMPK is a well-described metabolic regulator that responds to energy stress and depletion of ATP reserves. AMPK is maximally active when bound to AMP and phosphorylated by an upstream kinase, typically LKB1. In select tissues, AMPK may also be phosphorylated by alternative kinases, including calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2), which is responsive to changes in intracellular Ca2+ concentration (120). In fact, in T cells, activation of AMPK via phosphorylation by CaMKK2 occurs following TCR activation, presumably in anticipation of the increased energy demands of activation (120). AMPK activation leads to increased energy-producing/catabolic pathways while inhibiting energy-consuming/anabolic pathways. Interestingly, the diabetic drug metformin activates AMPK, which leads to improved insulin sensitivity and increased glucose uptake in metabolic cells.

We and others have found that LKB1 is a central regulator of T cell number, activation, and metabolism (121–123). T cell knockout of LKB1 resulted in blocked thymocyte development and decreased numbers of both CD4+ and CD8+ T cells. LKB1-knockout T cells had defects in both viability and proliferation, as well as increased expression of inflammatory cytokines IFN-γ and IL-17 and increased glucose metabolism (121). AMPK-knockout reproduced only a portion of the defects seen in the LKB1-knockout mouse. Loss of T cell AMPK did not alter thymocyte development or T cell number, but did result in increased expression of inflammatory cytokines as well as increased glucose metabolism. Increased mTOR complex 1 (mTORC1) signaling in both AMPK- and LKB1-knockouts, contributed to the phenotype (121).

The nutrient-sensing mTOR pathway is also important for cellular nutrient/energy sensing and drives inflammatory T cell differentiation and function. T cell specific knockout of mTOR kinase to delete both mTORC1 and mTORC2 suppresses effector T cell generation but allows Treg differentiation (124). The specific contribution of mTORC1 activity to Treg function appears to be complex. Deletion of mTORC1 activity has been shown to prevent Treg suppressive function in some settings, and not affect Treg in others (125, 126). AMPK has also been shown to promote Treg cell function while inhibiting signaling pathways, including mTORC1, which promote effector T cell differentiation, particularly Th1 and Th17 (127). The role of the nutrient-sensing mTOR and AMPK pathways on T cells in malnutrition, therefore, may be to act as metabolic checkpoints so that inflammatory T cell expansion is limited when nutrient availability is low. In the context of obesity, adipokines such as leptin may promote mTOR activity to promote effector T cell generation and contribute to inflammation (85).

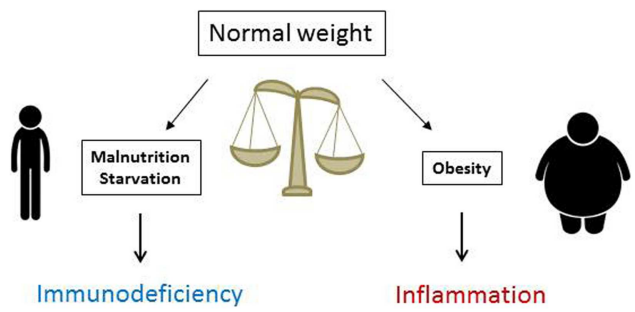

FIGURE 3 | Nutritional status is critical for normal immune function. Malnutrition reduces immunity and increases markedly the risks and mortality from severe infections, whereas obesity heightens immune reactivity and predisposes to systemic inflammation that fosters the development of inflammatory disorders and insulin resistance leading to type 2 diabetes mellitus.

CONCLUSION

In summary, T cell number, function, and metabolism are significantly affected by nutritional status. Whereas malnutrition lowers T cell number and metabolism and increases susceptibility to infection, obesity increases inflammatory T cell numbers in VAT and promotes systemic inflammation (**Figure 3**). Understanding how T cells are altered in obesity and malnutrition will lead to better understanding of and treatment for diseases where nutritional status determines clinical outcome. Targeting the inflammatory T cell response in obesity offers novel therapeutic options for insulin resistance and type 2 diabetes, whereas augmenting T cell response in malnutrition may promote immunity in clinical scenarios where malnutrition leads to poor outcome. Such scenarios could include augmenting vaccine response in malnourished children from underdeveloped countries and protecting chronically ill patients with cachexia against infection. An understanding of nutritional effects on T cells is therefore critically important for public and global health.

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Metabolic control of B cells: more questions than answers

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It is now accepted that metabolic disorders such as type 2 diabetes (T2D) and obesity are characterized by alterations in immune cells. Indeed, immune cells have been shown to play a critical role in fueling the inflammation that accompanies increased fat deposition and insulin resistance. Both the innate (macrophages) and the adaptive arm (CD4 and CD8 T cells, in particular) significantly contribute to the chronic inflammatory state that characterizes these conditions. B cells, however, have until recently remained significantly understudied. This is not due to the fact that they do not play as significant a role, as more recent publications appear to show. Here, we briefly review the most recent literature and highlight the remaining outstanding questions.

A CROSS TALK BETWEEN B CELLS AND ADIPOCYTES?

B lymphocytes or B cells are the arm of the adaptive immune system responsible for the generation of antibodies. In addition, they produce cytokines that support the activation of other immune cells (1). Whether they interact and influence the physiology or pathophysiology of other non-immune cells, such as adipocytes in obesity, is only now beginning to emerge.

Adipocytes themselves secrete cytokines also secreted by immune cells, such as TNFα, IL-6, and IL-8 (2). However, adipocytes secrete cytokines at significantly lower levels than the immune cells infiltrating adipose tissue in obesity (2). Adipocytes also secrete adipocyte-specific cytokines known as adipokines, these include leptin, adiponectin, resistin, which are also altered in obesity (3). The receptors for both leptin and adiponectin have been found on B cells; however, their role is still not understood. There is some evidence that leptin induces secretion of IL-10, IL-6, and TNFα in human B cells (4). However, how adipokines affect B cells and whether this plays a role in obesity has not been sufficiently addressed yet.

B CELLS IN OBESE ADIPOSE TISSUE AND T2D: SIMILARITIES AND DIFFERENCES BETWEEN MOUSE AND MAN

Recently, a number of studies have revealed B cells contribute to the progression and severity of obesity and T2D.

During weight gain, the earliest infiltrators into adipose tissue are IFNγ-secreting CD8+ T cells, then followed by M1-polarized, inflammatory macrophages, which secrete IL-6, IL-12, and TNFα (5). Conversely, anti-inflammatory mediators such as CD4+ T regulatory cells and M2-polarized macrophages are reduced in obese adipose tissue (6). The activation of pro-inflammatory immune cells during obesity is likely caused by the release of free fatty acids (FFAs), high-glucose levels, reactive oxidative species, and hypoxia (7). Once established, this inflammatory environment is thought to contribute to the development of metabolic disease symptoms, such as insulin resistance, caused by a disruption in normal metabolic signaling pathways.

In the adipose tissue of obese humans, B cells, together with IgG and IgM deposition, have been observed in crown-like structures (8), areas defined by accumulation of macrophages around dying adipocyte cells (9). Similarly, in wild-type mice, B cells are rarely observed within lean adipose tissue but begin to infiltrate after 4 weeks of mice being fed a high-fat diet (HFD) (10). Mice lacking both B and T cells do not show any differences in weight gain or induction of glucose intolerance on a HFD. Interestingly, however, obese adipose tissue has a greater macrophage infiltration in the absence of B and T cells, suggesting that lymphocytes may influence the recruitment of macrophages in obesity (10).

In humans, T2D is strongly linked to obesity (11), and many murine models of obesity have been used to study induction of T2D symptoms (12). Cytokine production by B cells is altered in both T2D patients and obese mice. In T2D patients, B cells constitutively secrete the pro-inflammatory chemoattractant IL-8, while also secreting lower levels of IL-10, TNFα, and IL-6 than non-diabetic controls (13). Similarly, LPS-stimulated B cells from obese mice secrete higher amounts of macrophage inflammatory protein-2 alpha (MIP-2), the murine ortholog of human IL-8, as well as lower levels of IL-10 (14). Thus, the pro-inflammatory phenotype seen in human T2D patients is recapitulated in obese mice. Not all cytokines behave in the same way, though, secretion of IL-6 and TNFα is increased in murine B cells from obese mice (14), yet decreased in human T2D patients (13). These differences might be species-specific or highlight the limitations of using obese murine models to model T2D. Overall, murine B cells will have a more pronounced inflammatory phenotype compared to the human counterpart in T2D.

LESSONS FROM B CELL-DEFICIENT MICE

In order to define whether B cells are critical players in obesity and metabolic disease, different research groups used B cell-deficient mice, such as the μMT line, in which disruption of the B cell receptor μ chain, required for differentiation and survival of mature B cells, results in

loss of B cells (15). Overall, μMT mice on a HFD developed obesity similarly to wild-type mice. Crucially, however, they did not present the glucose intolerance normally induced by HFD (14, 16). μMT mice had resting serum glucose and insulin levels closer to that of lean mice, as well as improved glucose and insulin tolerance test responses. This implies a strong role for B cells in promoting the development of T2D during obesity; nonetheless, the mechanism by which B cell exert this effect is still controversial.

One proposed mechanism is that B cells influence the development of T2D by promoting a pro-inflammatory phenotype in macrophages and T cells. μMT mice had fewer macrophages within the adipose tissue and increased expression of anti-inflammatory M2 markers, such as Arg 1 and Ym1 (14). Furthermore, B cell-deficient mice showed reduced serum levels of inflammatory cytokines such as TNFα, IL-6, and MCP-1, as well as reduced mRNA expression of IFNγ and CD8 within the adipose tissue (14). Thus, B cells appear to be involved in inducing a pro-inflammatory phenotype in both macrophages and T cells during obesity. Interestingly, μMT mouse adipocytes also secreted less leptin; however, the authors did not investigate if this is a direct or indirect effect of B cells (14).

How exactly B cells influence macrophages and T cells has been in part addressed by another study, which also highlighted the importance of antibody secretion by B cells. Winer et al. (16) observed IgG antibodies within adipose tissue of obese mice and B cell infiltration of visceral adipose tissue (VAT) early in obesity development (16). Furthermore, the proportion of B cells within VAT that were class-switched (IgG+) increased over time when mice were fed a HFD. Splenic B cells from obese mice secreted more IgG and less IgM, which suggests that B cells are activated and switch to a memory phenotype during obesity. In the serum and adipose tissue, the IgG2c subclass, in particular, was significantly increased. Deposits of IgM and IgG were also reported in crown-like structures within VAT (16), similar to that observed in human (8). Winer et al. (16) proposed the obesity-induced antibody response to be pathogenic and to directly contribute to development of T2D via macrophages. Macrophages express Fc receptors that bind antibodies, the binding of which can be activating or inhibitory depending on the class of Fc receptor (17). Within the adipose tissue, μMT mice had fewer infiltrating M1-macrophages (16). Injection of purified IgG antibodies from obese mice in μMT mice increased the M1-macrophage infiltrate into VAT, as well as increasing macrophage TNFα secretion in culture (16). Most importantly, the symptoms of T2D were worsened in obese mice injected with purified IgG from obese mice, but not with IgG from lean mice (16). This strongly suggests the presence of pathogenic autoantibodies. How exactly the pathogenic IgG affects the phenotype of macrophages remains to be elucidated. It is important to note, however, that two additional publications reported lack of pathogenic IgGs (14) and no evidence of IgG secretion or class-switching of B cells (18), indicating that the involvement of antibodies in obesity may be model-dependent. Further studies with additional models are required to clarify this important point.

On a different level, Winer at al. (16) also observed less IFNγ+ CD8+ cells within obese adipose tissue in μMT. They proposed that, as well as influencing macrophages via immunoglobulin secretion; B cells also prime both CD4+ and CD8+ T cells via MHCI/II interaction. By specifically removing MHCI or MHCII from B cells, glucose tolerance and insulin sensitivity improved TNFα levels within the adipose tissue decreased and IFNγ secretion by CD8+ cells was down regulated (16). Importantly, the authors were then able to lessen the symptoms of obesity-dependent insulin resistance by depleting B cells with an anti-CD20 antibody. Although the depletion of B cells did not affect weight, the levels of IgM and IgG in the blood as well as the levels of TNFα and TNFα+ macrophages were reduced. As the depletion occurred after obesity was established, it suggests that B cells are required for both inducing and also maintaining the pro-inflammatory phenotype in macrophages and T cells that contributes to insulin resistance. It also indicates that targeting B cells therapeutically could improve the management of T2D.

B CELLS ARE ALSO INVOLVED IN ADIPOSE TISSUE HOMEOSTASIS

In contrast to the pro-inflammatory role of B cells in promoting metabolic disease reported by others, Nishimura et al. (18) provided evidence of a protective role for a subset of B cells, regulatory B cells, also known as B10 or IL-10-producing B cells. IL-10 is an immunosuppressive cytokine strongly associated with reducing autoimmune disease and inflammation (19). Regulatory B cells or Bregs are a subset of B cells, which secrete IL-10 and TGF-β (19), mediating suppressive effects on other immune cells. In their research, Nishimura et al. (18) showed that murine B cells isolated from adipose tissue produce high levels of IL-10, even in the absence of any *in vitro* stimulation. In contrast, B cells isolated from the spleen and lymph nodes required stimulation in order to have detectable levels of IL-10.

To prove the hypothesis that Bregs influence adipose inflammation, B cell specific IL-10 knockout (KO) mice were established using bone marrow chimeras. To do this bone marrow cells comprising 10% IL-10 KO cells and 90% B cell KO cells were transferred into wild-type mice, such that all resulting B cells were IL-10 deficient (18). The infiltration of macrophages in adipose tissue increased, as well as the expression of pro-inflammatory markers such as CD44 and IFNγ (18). From these findings, it seems that there is a population of regulatory B cells within the adipose tissue that maintains homeostasis by suppressing pro-inflammatory responses, thus the production of IL-10 by B cells is important in counterbalancing insulin resistance.

The authors hypothesized that resident B cells within adipose tissue are dependent on local factors secreted by adipocytes, such as FFAs. Indeed, FFA supported adipose B cell survival and IL-10 production in culture (18). The binding of FFA to immune cells has previously been suggested to be via binding to toll-like receptor 4 (TLR4) (20). However, if FFA secretion helps support Bregs within adipose tissue, it does not explain their reduction in obesity. It is possible that the secretion of additional factors by obese adipocytes skew the Breg phenotype; however, this is not addressed in the paper.

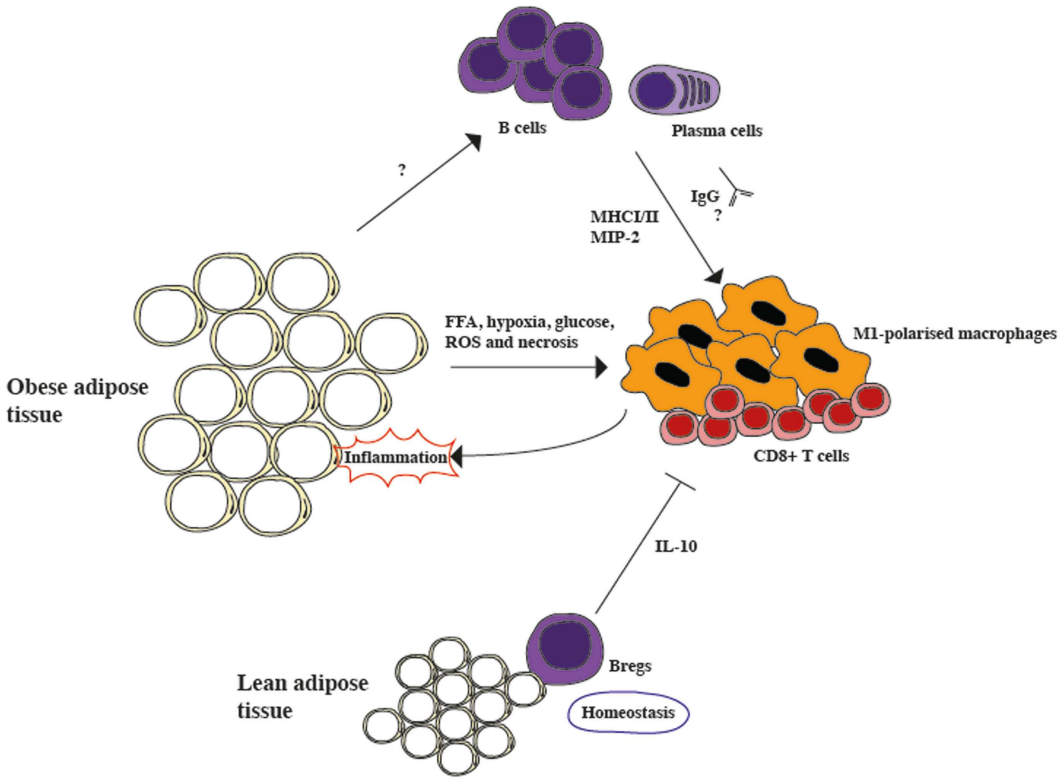

FIGURE 1 | The role of B cells in modulating inflammation in adipose tissue. Lean adipose tissue contains resident Bregs, which constitutively secrete IL-10 and suppress inflammation during homeostasis. However, in obese adipose tissue the secretion of free fatty acids (FFA), hypoxia, high glucose levels, reactive oxygen species (ROS), and necrosis activates pro-inflammatory CD8+ cells and M1-polarized macrophages, generating inflammation. B cells are also activated during obesity, although the mechanism is still unclear. During obesity, B cells class-switch and secrete pathogenic IgGs, as well as the macrophage-recruiting chemokine, macrophage inflammatory protein-2 alpha (MIP-2). Furthermore, they also activate T cells via MHCI/II interactions. Overall, it seems that the protective role of Bregs is overcome by the pro-inflammatory role of activated B cells.

CONCLUDING REMARKS

The idea that Bregs counterbalance the pro-inflammatory processes that characterize adipose tissue in obesity does not necessarily contrast with previous studies showing that the complete absence of B cells ameliorates inflammation and insulin resistance in obese mice. It is possible to speculate that Bregs are present in high numbers within the lean adipose tissue, possibly to avoid the development of inflammation in normal conditions. In support of this view, it has been reported that in humans IL-10 RNA levels in adipose tissue are inversely correlated with body mass index, whether this is due to Bregs or not remains to be established (18). Once homeostasis is lost, however, as observed in the adipose tissue of mice on HFD, the effect of activated B cells that prime T cells and generate immunoglobulins that affect macrophages, fueling the inflammatory response, overwhelms the protective effect of IL-10 producing Bregs (summarized in **Figure 1**). Experiments to determine how B cell subsets change during obesity progression and if individuals with lower levels of Bregs are more prone to insulin resistance would be required to further clarify the role of Bregs in obesity. Nonetheless, the observation that once obesity is established, insulin resistance can be ameliorated by B cell depletion via an anti-CD20 treatment offers an interesting therapeutic opportunity and warrants further investigation into B cell function in obesity.

FURTHER QUESTIONS FOR B CELLS AND METABOLISM

In order to have a better understanding of how B cells affect all body-metabolism, it will also be necessary to gain more information on what metabolic pathways regulate B cells, their differentiation, cytokine production, and antibody secretion. Whereas glucose metabolism and the pentose phosphate pathways are necessary to support B cell clonal expansion (21), we still know little about the differentiation to antibody-secreting cells and cytokine production. Fatty acid biosynthesis is up regulated in antibody-secreting cells, in order to sustain the expansion of the endoplasmic reticulum to support antibody production (22). But what effect will an increase in free fatty acid uptake have on the B cell phenotype? Further research efforts are required to get a more comprehensive picture, which will ultimately help understanding the role of B cells in metabolic disorders and how to intervene therapeutically.

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Metabolic syndrome and the immunological affair with the blood–brain barrier

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Epidemiological studies reveal an increased incidence of obesity worldwide, which is associated with increased prevalence and severity of cognitive disorders. The blood–brain barrier (BBB) represents the interface between the peripheral circulation and the brain, and plays a fundamental role in the cross-talk between these two compartments. The homeostatic function of the BBB is the protection of the brain from peripheral insult/inflammation. Alterations in the function of the BBB lead to pathologies of the central nervous system. Recently, metabolic imbalance has been shown to be an important risk factor associated with the decline of BBB integrity and function. This has direct etiological consequences on a variety of cerebrovascular and neurodegenerative pathologies with great impact to society. Priority areas for future preclinical research include strategies to improve clinicians' ability to diagnose, prevent, and manage BBB abnormalities. In sharp contrast with epidemiological studies and clinical needs, little is known about the mechanisms that link metabolic syndrome to BBB functionality and cognitive disorders. Our view is that immune responses caused by metabolic stress might play a major role in this conundrum.

Keywords: blood–brain barrier, metabolism, immune response, leukocytes, diet

INTRODUCTION

Central nervous system (CNS) homeostasis is a prerequisite for the proper communication and function of neuronal cells. The endothelial blood–brain barrier (BBB) and the epithelial blood–cerebrospinal fluid barrier (BCSFB) tightly seal off the CNS from the continuously changing milieu within the bloodstream (1) by globally controlling immune cell trafficking. The BBB is a complex three-dimensional structure composed of specialized endothelial cells that are reinforced by pericytes, astrocytes end feet, and extracellular matrix (2). The extreme tightness of the BBB is due to specialized endothelial junctional complexes: the adherens junctions (AJs) and the tight junctions (TJs). TJs formation between endothelial cells is regulated by astrocytes, astrocyte-derived soluble factors as well as metalloproteases (glia limitans) and pericytes (so called neurovascular unit-NVU), which are important modulators of the BBB permeability (3). Endothelial cells also express transmembrane molecules called integrins, which are heteromeric molecules involved in anchoring the cellular structure to the subendothelial basal lamina/extracellular matrix (4). Integrins interact with so called "focal adhesion" molecules, which are tightly connected to the actin cytoskeleton and together with TJs and AJs are key in regulating paracellular permeability (5). We have recently reported that another important component for barrier tightness is the anti-inflammatory protein Annexin A1 (ANXA1), which has a dual role in stabilizing actin and TJ formation, and protecting the brain by promoting resolution of inflammation (6), a molecule that we propose to have great potential in correcting BBB leakage.

Since BBB damage appears to be present in many neurological disorders, it is not surprising to see much effort focused on the development of therapies targeting the barrier (7). As BBB dysfunction can either be a causative phenomenon or a propagative/exacerbating event in the course of diseases, limiting its impairment could potentially reduce the severity of pathology and facilitate recovery (5).

Equally, inflammatory mediators are the primary cause of the negative effects at the barrier level, and most of the attempts have been focused at halting the inflammatory reaction. In particular glucocorticoids like dexamethasone have shown improvement of the physical and transport properties of the BBB (8), but their usefulness, for instance, in patients suffering from multiple sclerosis (MS) decreases with time (9).

THE IMMUNOLOGY OF BBB

The immune-surveillance of the CNS is essential to keep under control the entry of potential mediators of infection into the brain parenchyma (10). Resident microglia are the primary guardians of the brain parenchyma; however, a small number of T and B cells, macrophages, and neutrophils from the periphery lie within close proximity, patrolling the specialized CNS compartments (11). Lymphocyte recruitment across the BBB into CNS, although very low in healthy individuals, is responsible to maintain CNS immune-surveillance. Immune cells gain access to the CNS via three routes: (a) non-fenestrated vascularized stroma of the blood–cerebrospinal fluid (CSF) barrier that is surrounded by the epithelial cells of the choroid plexus, (b) the perivascular space,

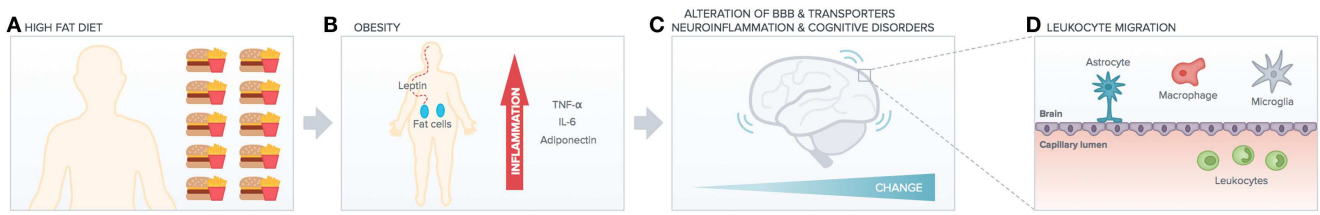

FIGURE 1 | High-fat diet impact on peripheral versus central function. High-fat diet **(A)** induces obesity **(B)** increasing peripheral chronic inflammation (cytokines release). This has deleterious effects on brain functionality (e.g., alteration of BBB transporters, neuroinflammation, and cognitive disorders such as AD). Peripheral inflammation and increased BBB leakage **(C)** induce leukocytes migration into the brain **(D)**, which exacerbates neuroinflammation and neurodegenerative diseases.

where the deep arteries are continuous with the subarachnoid space, and (c) post-capillary venules that enter the parenchyma directly. Extravasation migration across the vascular wall and the glial limitans propagates entry into the brain parenchyma. Within CSF of healthy individuals, the majority of immune cells (~80%) include T cells predominantly activated central memory T cells (CD4+/CD45RA−/CD27/CD69+) (12). They traffic within the CSF until encountering an antigen-presenting cell (APC) (13). In the experimental autoimmune encephalomyelitis (EAE), effector T cells entering the CNS become activated after short contacts with leptomeningeal phagocytes at the onset of disease. During established disease, the activation process is extended to the depth of the CNS parenchyma, where cells form contacts with microglia and recruited phagocytes, suggesting that they become able to infiltrate the brain parenchyma (14). Upon activation, T cells upregulate integrins and adhesion molecules, which enable leukocyte rolling and arrest at the vessel wall. Multiple integrins, cytokines, and adhesion molecules expressed on circulating and CNS-resident cells are responsible for the initial events of the immune cascade (12), which leads to leukocytes extravasation in the brain parenchyma. It has recently been shown that intravenously transferred effector T cells gain the capacity to enter the CNS after residing transiently within the lung tissues. Inside the lung tissues, they move along the airways to bronchus-associated lymphoid tissues and lung-draining mediastinal lymph nodes before entering the blood circulation from where they reach the CNS (15). On their way, T cells reprogram their gene-expression profile, characterized by downregulation of their activation status and upregulation of cellular locomotion molecules together with chemokine and adhesion receptors. The adhesion receptors include ninjurin (16), which participates in T cell intravascular crawling on cerebral blood vessels. In addition, alternative routes can be used by T cells to gain access to the CNS. In (EAE) Th17 pathogenic T cells enter the CNS via the choroid plexus, a route controlled by the CCR6–CCL20 axis (17). Furthermore, an elegant study by Arima et al. (18) clearly shows that CD4+ pathogenic T cell access the CNS trough lumbar spinal blood vessel cord being regulated by CCL20. Such data are very important in the context of neurological disease with a clear immune component.

METABOLIC STRESS AND BBB DISRUPTION

It is nowadays clear that metabolic syndrome (MetS), a cluster of risk factors for cardiovascular disease, diabetes, and stroke, is becoming endemic. Epidemiological studies show that lifestyle (absence of physical exercise) and misbalanced diet (based on the so-called "junk food") are major causes of MetS. Obesity-related disorders have risen by nearly 90% in the last decade (19). It is therefore of paramount importance to educate young and elderly people to a proper lifestyle. However, since socio-economical pressure accounts for the difficulty in meeting these targets, at the moment, early diagnosis and treatment are possible venues to halt and prevent the escalation of MetS. For instance, type 2 diabetes and Alzheimer disease (AD) have been recently pinpointed as likely linked to aging (20), clearly indicating a possible path for the identification of drugs able to control both pathologies (21).

Despite this emerging importance of the environment in triggering adaptive immunity of the CNS, a potential role for metabolic stress, an important risk factor for pro-inflammatory immune imbalance (22) and cognitive imbalance, has not been investigated so far (**Figure 1**). For instance, the link between metabolic stress and BBB functions is far from clear, aging factors may account for alteration in tightness, and chronic peripheral inflammation may also be accountable but no clear studies yet indicate the molecular and cellular mechanisms underlying such link. One molecule that we know is downregulated with aging is Annexin A1 (23), but no studies have related yet such molecule to diet or even metabolic syndrome. Very recently, the commensal gut flora has been shown to be essential in triggering immune responses in the brain (24), implying that the gut-associated-lymphoid-tissue (GALT) is a potential site for priming brain-targeted immune responses.

DIET AND CEREBROVASCULAR FUNCTION

The escalating incidence of obesity worldwide has mounted large interest in studying the pathological consequences of increased fat and cholesterol intake (25) that heavily contribute to the progression of MetS. MetS develops as a consequence of low-grade chronic inflammation due to high-fat consumption and increases the risk of deleterious outcomes such as cognitive impairment (25), stroke (26), and neurodegenerative conditions such as Alzheimer's disease (AD) (27) and MS (28).

Although the BBB is exposed to both the peripheral and central environments, the effects of obesity and MetS are bleak. In addition, age and excessive energy intake/obesity are reported as risk factors for cerebrovascular disease. A recent study suggested that reduction in neurofactors (named brain-derived neurotrophic

factor and basic fibroblast growth factor) as well as inflammatory pathways (the antioxidant enzyme heme oxygenase-1) with aging is responsible for the poor outcome post-infarction in aging (26). Moreover, epidemiological studies on the effect of western diet on learning and memory are ascribed to BBB degradation (29).

CEREBRAL METABOLISM AND BBB TRANSPORTERS

Being one of the most metabolically active organs in the body, the brain does not store excess energy and derives almost all of its energetic needs from the aerobic oxidation of glucose. Therefore, it requires continuous supply of glucose and oxygen to meet its energy demands. Glucose can enter the brain from the blood through two different routes: (a) the sodium-dependent glucose transporters (SGLTs) and (b) the sodium-independent glucose transporters (GLUTs). GLUT-1 is expressed in brain capillary endothelial cells (30, 31) and safeguards glucose transport across the BBB. Glucose transport and transporters are also affected by systemic autoimmune-inflammation, for example, GLUT-1 is up-regulated by interleukin-1β (IL-1β) at endothelial level although its effects at neuronal level are deleterious (32). Therefore, one could argue that since metabolism is associated with a degree of on-going inflammation, the alteration of glucose transport and transporters at the BBB could impact on brain networking resulting in learning deficit in young obese individuals and in cognitive impairment in aged subjects. Moreover, another important BBB transporter member of the ATP-binding cassette protein family, the P-glycoprotein (which transports various molecules in and out the brain parenchyma) appears to be modulated both in its activity and expression by inflammatory events occurring at the level of the BBB endothelium (33). Consequently, as P-glycoprotein activity is important for the passage of therapeutic molecules through the BBB, understanding its regulation during inflammation would aid in the development of drugs (32, 33).

Another important molecule with different functions in the CNS compared to the peripheral system is insulin. Brain endothelial cells (BEC) contain saturable transporter pores (34) that translocate insulin from the blood to the brain. Produced most exclusively by the pancreas, insulin crosses the BBB affecting feeding and cognition (35). Similar to insulin, leptin (secreted by adipocytes) crosses the BBB through a saturable transport mechanism possibly independent of insulin (36) regulating appetite and energy balance (37). Moreover, leptin produced by adipocytes and lymphocytes (metabolic inflammation) has pro-inflammatory activity possibly contributing to CNS inflammation (38).

BBB FUNCTION AND PATHOLOGY

The composition and structural organization of the BBB is key to maintaining a constant and optimal cerebral environment for neuronal function through a combination of barriers and selective transport systems that tightly regulate the passage of essential and unwanted molecules (39). Nowadays, the BBB is not considered as a static, passive structure that serves as an impediment to molecular access into the CNS (40). In contrast, it modulates and actively filters molecules and blood born cells into the CNS, and functions as a highly specialized transport, metabolic, physical, and immunological barrier. Consequently, alterations in BBB integrity and function are associated with many pathologies of the CNS (41), namely, MS, hypoxia and ischemia, edema, Parkinson's disease (PD) and AD, epilepsy, glaucoma, and lysosomal storage diseases (3). Barrier dysfunction can range from mild and transient TJ opening to chronic barrier breakdown and changes in transport systems and enzymes (5). Breakdown of the BBB allows immune cell infiltration to aid clearing debris and repair injuries. However, in several cases, it results in damage to the CNS, causing neuronal dysfunction, injury, and degeneration (41). Hence, it is not clear if changes in BBB physiology should be considered as one of the causes of the disease, part of the pathophysiological process, a consequence of the disease, or a combination of all. Nonetheless, recent evidence supports the hypothesis of endothelial dysfunction as a link between vascular disorders and neurological impairment, exacerbating the development of CNS disorders (40, 42).

METABOLIC IMBALANCE AND COGNITIVE DECLINE

A link between nutrition and mental health has been recently established. Many eating-related peptides and regulatory proteins produced by peripheral tissues and with receptors in the brain have been found to cross the BBB. Thus, the fact that BBB results permeable to factors that are important for the brain functionality can be seen under the umbrella of BBB physiological conduits toward the control of signaling between the peripheral and central system. Consequently, dysfunction of BBB and its transporters can result in disease. Resistance to leptin caused by its decreased transport across the BBB in obesity is an example (37). Although rare, patients with Glut-1 deficiency (caused by genetic mutations) can have severe learning difficulties that may be corrected through the diet (43).

Impairment of insulin transport due to change of BBB component such as pericytes is also at the origin of pathological manifestation in diabetes and hyperglycemia. Resistance to insulin may occur also in the brain (so called diabetes mellitus type III) and it may or not be linked to peripheral resistance, as for ADs (35).

However, additional consideration has been put forward suggesting that prolonged increased high-fat intake may be linked to inflammatory and aging-related neurodegenerative diseases including MS, PD, and AD (44). The development of various neurodegenerative disorders have been associated with BBB damage and increased permeability. Thus, it is thought that obesity can potentially activate the onset of vascular disorders that affect BBB permeability later in life (29, 45).

Moreover, BBB modifications are often characterized by the disturbance of endothelial glial interactions (2). In addition, decreased number of TJ proteins, in particular Claudin-5 and Claudin-12, is found within the BBB and choroid plexus, increasing the permeability of the barriers and enabling the entry of toxic molecules (29, 46). During BBB disruption, agrin (a large proteoglycan) is lost from the abluminal surface of the BEC. This is thought to contribute to BBB damage in AD. Amyloid-B accumulation, a key characteristic of AD is first seen in the neighboring hood of blood vessels with toxicity on endothelium and astrocytes, before causing extensive neuronal loss and CNS homeostatic imbalance (47).

Population studies are now more and more aligned to the concept that MetS has a negative impact on learning and cognition. Multiple factors and etiology spanning from impaired vascular reactivity to autoimmune-inflammation and oxidative stress are potential factors affecting brain functionality as exhaustively reported in Ref. (48). Insulin resistance and diabetes are indeed strongly associated with deficiency in cerebrovascular functionality. Indeed, it has recently been reported that insulin-signaling dysfunction in AD may be treated with administration of intranasal insulin, which has been reported to improve mood and behavior in diabetic patients (21). Such treatment has great potential because of the beneficial effects not only on the functionality of peripheral organs but also of the brain. Metformin together with amylin and leptin analogs also deserve better investigation of their potential beneficial effects in CNS pathologies that arise as consequence of metabolic syndrome (21).

METABOLISM AND ADAPTIVE IMMUNITY IN THE PERIPHERAL–CENTRAL AXIS

Although different metabolic needs and activity certainly follow changes in signaling and proliferation rate, recent evidence suggests that the regulation of T cell metabolism is tightly linked to T cell function and differentiation. T lymphocytes are finely regulated by signals that, once delivered through T cell receptor (TCR) and cytokine receptors, induce the activation of different intracellular metabolic pathways (49). Signals deriving from growth factors and cytokines such as IL-2 or IL-7, together with ligation of co-stimulatory molecules, lead to an increase in glucose uptake and glycolysis through induction of phosphoinositide-3-kinase (PI3K)-dependent activation of Akt (50, 51). T cell activation is also accompanied by an increased rate of protein synthesis, which supports cell growth and effector functions. Downstream of TCR and CD28, Akt controls the activation of the mammalian Target of Rapamycin, mTOR, which is the main regulator of protein synthesis in T cells (52, 53). The importance of the mTOR pathway for T cell activation is testified by the evidence that rapamycin, a selective inhibitor of mTOR, induces a condition of immunosuppression, through the induction of a cell cycle arrest in proliferating T lymphocytes. It has recently been shown that changes in nutritional status of the host can directly affect survival and proliferation of pro-inflammatory CD4+ T cells. Of note, dietary restriction causes metabolic and physiological changes that have beneficial effects in different pathological conditions, such as obesity, insulin resistance, inflammation, oxidative stress, and autoimmune diseases (54–56). The overall increase of obesity has focused the attention on the biology of adipose tissue, so far considered only a "passive" energy storage site. It is well accepted now that the adipose tissue can also produce hormones and cytokines, named "adipokines," that bridge metabolism and immune homeostasis, such as leptin, IL-1β, IL-6, IL-8, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), leukemia-inhibiting factor (LIF), and many chemokines (57, 58). Leptin is produced by the adipose tissue in proportion to the body fat mass; its role is to regulate body weight through the inhibition of food intake and stimulation of energy expenditure but evidence has indicated that leptin is also one of the main regulators converging signals from the environment (food availability) to effector (Teff)/regulatory (Treg) T cell proliferation both *in vitro* and *in vivo* (59, 60). The evidence that adipose tissue has an important role on the control of central functions, such as immunity and metabolism, is providing novel insights into the pathogenesis of metabolic and inflammatory disorders. Leptin secreted by adipocytes sustains Th1 responses by promoting Teff cell proliferation and pro-inflammatory cytokine production and by constraining Treg cells expansion: this balance between Teff and Treg cells leads to immune tolerance on one side and to protection from infections on the other (59, 60). Recent evidence suggests that leptin acts as an endogenous "sensing" factor, linking the environment (availability of nutrients) to circulating Treg number. Since nutritional deprivation increases the susceptibility to infection and associates with the amelioration of clinical manifestations of inflammation and autoimmunity, it will be important to address how this condition relates to the influence of leptin on both Teff and Tregs. In MS, leptin secretion is increased in serum and CSF of naive-to-treatment subjects and this directly correlates with the secretion of IFN-γ in the CSF. Leptin levels inversely correlate with the percentage of circulating Treg cells, a subset able to dampen the autoimmune response mediated by myelin-specific Teff cells present in MS subjects, thus reinforcing the link between the number of Treg cells and leptin secretion *in vivo* (61). The findings that different intracellular metabolic pathways have an impact in the control of self-immune tolerance and the study of how metabolic dysregulation in overweight and obesity could alter immune tolerance are now topics of intensive investigation. Recent evidence suggests that metabolic and autoimmune diseases, characterized by chronic inflammation and altered self-immune tolerance, are more common in affluent countries; the reasons for such phenomena are still not completely understood, but the "metabolic disturbances" induced by nutritional overload, observed in more developed countries, seem to play the main role.

CONCLUSION

Despite the overwhelming indirect evidence correlating MetS and inflammation to brain cognition, direct causative effects of metabolic imbalance in pathological alteration of the BBB remain to be established. Specifically, the metabolic pathways that affect the cellular component of the BBB, as well as those regulating T cell function and access to the CNS, are yet to be defined. The identification of molecules selectively altered by MetS will provide new targets for corrections/interventions in all those neurodegenerative disorders with a clear metabolic imbalance and autoimmune pro-inflammatory component as well as biomarkers of early-stage BBB malfunction with the aim of preventing disease onset and progression.

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Metabolic reprograming of mononuclear phagocytes in progressive multiple sclerosis

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Multiple sclerosis (MS) is an inflammatory and demyelinating disease of the central nervous system (CNS). Accumulation of brain damage in progressive MS is partly the result of mononuclear phagocytes (MPs) attacking myelin sheaths in the CNS. Although there is no cure yet for MS, significant advances have been made in the development of disease modifying agents. Unfortunately, most of these drugs fail to reverse established neurological deficits and can have adverse effects. Recent evidence suggests that MPs polarization is accompanied by profound metabolic changes, whereby pro-inflammatory MPs (M1) switch toward glycolysis, whereas anti-inflammatory MPs (M2) become more oxidative. It is therefore possible that reprograming MPs metabolism could affect their function and repress immune cell activation. This *mini review* describes the metabolic changes underpinning macrophages polarization and anticipates how metabolic re-education of MPs could be used for the treatment of MS.

Key points:

- Inflammation in progressive MS is mediated primarily by MPs.
- Cell metabolism regulates the function of MPs.
- DMAs can re-educate the metabolism of MPs to promote healing.

Keywords: immune metabolism, macrophages, microglia, Warburg effect, multiple sclerosis, EAE, mitochondria

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) in which perivascular infiltration of self-reactive T lymphocytes leads to demyelination (both primary and secondary) and axonal damage. Inflammation is an early and transient event in MS and remyelination occurs afterwards (1). The early stages of the disease are characterized by episodes of neurological dysfunction that usually recover. Over time, the pathological features of MS become dominated by widespread microglial activation associated with extensive and chronic neurodegeneration, which associates with progressive accumulation of disability (2).

Current immune modulatory treatments are effective at reducing T-cell-mediated damage early in disease (3). However, most of these therapeutic strategies have failed to work in patients with progressive MS, where uncontrolled activation of mononuclear phagocytes (MPs) takes place in the chronically inflamed CNS (4–6).

Mononuclear phagocytes, such as microglia and macrophages, are present in all tissues where they have a range of homeostatic functions including the removal of apoptotic cells and cell debris (7). Although functionally similar, microglia and macrophages are ontogenetically distinct populations (8–11). Microglia, the primary MPs in the CNS, are derived from the yolk-sac blood islands and migrate to the neuroepithelium during early development (7, 12, 13). Microglia interact with neural progenitor cells to regulate both structural and functional responses in the CNS during development, homeostasis, and disease (14, 15). Macrophages are derived from hematopoietic stem cells in the bone marrow that differentiate into peripheral blood monocytes (16). Macrophages are critical for innate immune defense and also control organ homeostasis in a tissue-specific manner. In non-parenchymal areas of the CNS, macrophages and microglia survey for tissue injury and infection (17).

Mononuclear phagocytes are phenotypically classified as classically activated (M1-like; pro-inflammatory) or alternatively activated (M2-like; anti-inflammatory) cells. This paradigm should not be over-interpreted, as it is not a rigid classification. M1-like MPs produce neurotoxic molecules, pro-inflammatory cytokines, and chemokines and present self-antigens to attract cytotoxic $CD8^+$ T cells (18, 19), whereas M2-like MPs are regenerative cells that secrete growth and neurotropic factors (20, 21). MPs polarization is governed by *intrinsic* (22) and *extrinsic* factors, and even differentiated macrophages can be reprogramed when transferred into a new microenvironment (23).

Accumulation and activation of MPs in the CNS is thought to be a crucial step in the pathological cascade of MS, which frequently culminates in irreversible injury to myelin and axons (24). Therefore, MS therapies that steer MPs toward a reparative, rather than pro-inflammatory, phenotype are now emerging as ideal approaches to promote tissue healing without disrupting MPs functions.

This *mini review* describes the metabolic changes underpinning macrophages polarization and anticipates how metabolic re-education of MPs could be used for the treatment of MS.

PROGRESSIVE MS AND MPs

A balanced response between the M1- and M2-like phenotype is necessary for tissue homeostasis, and in MS and its animal model experimental autoimmune encephalomyelitis (EAE), this balance is disturbed. The fact that the MS *per se* exists in the relapsing/remitting type points to the M1/M2 dynamics as potentially relevant for this disease (25–27).

By expressing pattern recognition receptors, including toll-like receptors (TLRs) and NOD-like receptors (NLRs), MPs can sense both danger-associated molecular patterns and pathogen-associated molecular patterns from damaged tissue and microbes, respectively (28). The trigger for activation of MPs in the CNS is unknown, but is thought to be a combination of genetic susceptibility and environmental factors. M1-like MP polarization results in the release of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α and interleukin (IL)-1β; chemokines, such as monocyte chemoattractant proteins and reactive oxygen species (ROS), through increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity. All these factors contribute to demyelination, gliosis, and axonal loss, thus leading to irreversible tissue damage (29). Pro-inflammatory cytokines indirectly damage neurons and oligodendrocytes (ODCs) through sensitization of axons to glutamate excitotoxicity (30, 31). Chemokines promote the recruitment of innate immune cells and T cells to the site of ROS production in the CNS, causing mitochondrial dysfunction of neuronal cells and an increased energy demand due to inefficient nerve conductance, which can result in axonal damage and neuronal death (32, 33).

The role and function of microglia in progressive MS still remains a matter of debate, especially considering the intrinsic plastic nature of these cells (34–36). Classifying the different phenotypes of microglia *in vivo* (i.e., applying to microglia the *old* M1-like vs. M2-like classification of macrophages) has proven challenging. Unlike macrophages, microglial cells are not professional antigen-presenting cells, but they quickly increase the expression of MHC class-I and -II complexes in response to injuries and/or local inflammation. Specifically during brain inflammation, T cells crossing the blood–brain barrier, directly interact with microglia to recognize antigens, and ultimately mediate their skew toward M1-like activation (37). Activated microglia release the Th1-like pro-inflammatory cytokine interferon (IFN)-γ, which might induce their own polarization via IFNGR, trough an autocrine loop (38). This potentially vicious cycle typical of progressive MS, in which microglia contribute to the self-propagation of neuroinflammation, is likely to be determined also by a failure in the M2-like responses in a microenvironment dominated by Th1/Th17 cytokines (39).

In the context of brain repair, the activation of microglia is also necessary for clearing debris and, more importantly, to support the remyelination of damaged axons (40–42). In Cuprizone-fed mice, an animal model of demyelination/remyelination, microglia sustain remyelination with a durable effect involving (i) the phagocytosis of myelin debris and apoptotic cells during demyelination and (ii) the expression of a repertoire of cytokines and chemokines that include insulin-like growth factor (IFG)-1, platelet-derived growth factor (PDGF)-α, and transforming growth factor (TGF)-β, which ultimately mediate the recruitment of oligodendrocyte precursor cells (OPCs) and their differentiation into mature functional ODCs (43). Indeed, remyelination of damaged axons is a process that can be driven by M2-like MPs (21). In mice with lysolecithin-induced focal demyelination of the corpus callosum, a switch from M1-like to M2-like phenotype is described for both microglia and peripheral macrophages. This M2-like polarization takes place as early as remyelination begins, and it is indispensable to promote OPC differentiation. Interestingly, M2-like MPs also produce neurotropic and growth factors, such as the TGF-β superfamily member activin-A, a key signaling intermediate for ODC function, thus contributing to the remyelination process (21). During progressive MS, this remyelination-supportive microglia phenotype may be impaired, thus preventing proper repair. Interestingly, the same failure of M2-like microglia seems to occur also in other neurodegenerative diseases characterized by chronic MP activation in the CNS, such as Alzheimer's disease (44, 45).

METABOLIC REPROGRAMING OF MPs IN MS

Already in the 1980s, macrophages were known to undergo profound metabolic changes upon activation (46). More recent evidence corroborated these findings. While quiescent macrophages predominantly use mitochondrial respiration to generate energy, lipopolysaccharide (LPS)-activated macrophages switch their metabolism from oxidative phosphorylation to glycolysis (47, 48) (**Figure 1**). This metabolic switch, also known in cancer biology as the *Warburg effect* (49), is a key feature of the M1 macrophages. By contrast, mouse IL-4-stimulated MPs activate mitochondrial metabolism, fatty acid uptake, and fatty acid oxidation (50). These observations indicate that the switch between glycolytic or oxidative metabolism could play a role in macrophage polarization (**Figure 1**). However, the relevance of some of these metabolic features in human macrophages is still debated and more work is required to clarify the differences between mouse and human models.

HYPOXIA CONTROLS THE M1 POLARIZATION OF MPs

The switch toward aerobic glycolysis in activated macrophages is orchestrated – at least in part – by the transcription factor hypoxia-inducible factor (HIF) 1. HIFs are heterodimeric proteins composed of a constitutively expressed beta subunit and an oxygen-dependent alpha subunit (HIF-1α). In the presence of oxygen, the oxoglutarate-dependent prolyl hydroxylases (PHDs) hydroxylate HIF-1α, producing succinate and carbon dioxide. Once hydroxylated, HIF-1α is targeted for proteasomal degradation. When oxygen becomes limiting, PHDs are inhibited, leading

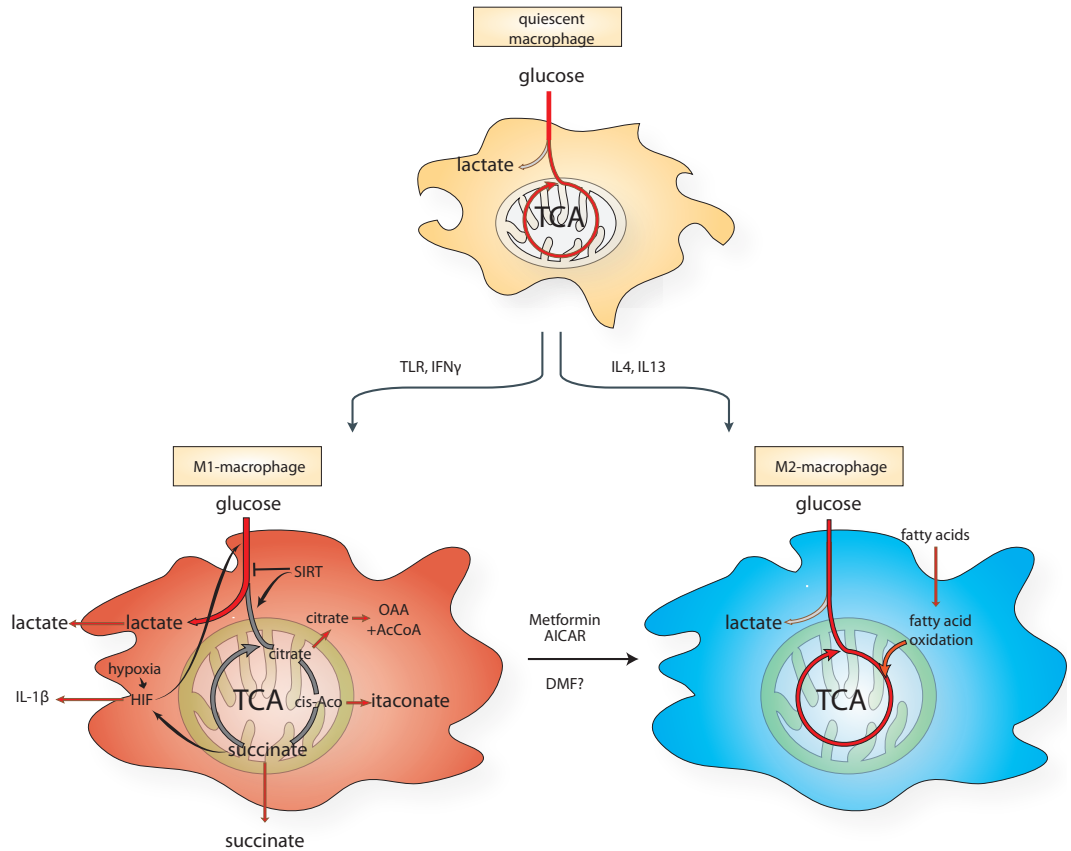

FIGURE 1 | Schematic diagram of the metabolic reprograming of macrophages undergoing M1- vs. M2-like polarization. AICAR, aminoimidazole carboxamide ribonucleotide; cis-aco, cis-aconitase; DMF, dimethyl fumarate; HIF, hypoxia-inducible factor; IL, interleukin; IFN-γ, interferon-γ; TCA, citric acid cycle; TLR, Toll-like receptor.

to HIF-1α stabilization and to the activation of its transcriptional program, which drives the expression of numerous glycolytic enzymes and of the pro-inflammatory cytokine IL-1β (47, 51). Sites of inflammation in MS are often hypoxic and acidic (33, 52, 53) and, accordingly, tissue from MS lesions exhibits hypoxic markers, including increased expression of glucose transporters (GLUTs) and monocarboxylate transporters (MCT), compared with healthy patients (54), most likely due to increased glycolysis of activated MPs. The increased avidity for glucose by MS lesions can be exploited for diagnostic purposes, since these lesions uptake substantial amounts of the glucose analog [^{18}F]-fluorodeoxyglucose, which can be visualized by positron emission tomography during neuroimaging exams (55). In line with these findings, tissue hypoxia and the corresponding increased expression of HIF-1α develops rapidly in response to inflammation in white and gray matter of animals subject to EAE. Levels of hypoxia correlate with neurological defects and the reintroduction of oxygen restores function in EAE mice within an hour of treatment, lasting up to 1 week (56). Interestingly, MPs and microglia from MS patients show different levels of HIF-1α, with MPs increasing HIF-1α expression compared to microglia (11, 23, 57). These observations suggest that MPs, rather than microglia, could be actively involved in the glycolytic switch observed in MS inflammation sites.

MITOCHONDRIAL METABOLITES AND REGULATION OF MPs FUNCTION

Changes in mitochondrial metabolism have important implications for activated macrophages, beyond cellular energetics. It has been recently shown that succinate, which accumulates in LPS-activated macrophages, impairs the enzymatic activity of PHDs by product-inhibition, leading to HIF-1α stabilization even in the presence of oxygen, a phenomenon known as *pseudohypoxia* (47). Importantly, manipulating succinate levels in macrophages *in vitro* can both stabilize HIF-1α as well as drive IL-1β expression (47). Furthermore, the inhibition of succinate dehydrogenase (SDH), the enzyme that converts succinate to fumarate, with diethylbutylmalonate increases intracellular succinate in macrophages and exacerbates the production of LPS-induced IL-1β (47). Interestingly, SDH is less active in the microglia of rats with EAE (58), underlining a possible deregulation of the enzyme in this experimental model of MS.

As well as acting intracellularly, succinate can also be released in the extracellular milieu, where it has been shown to act as a hormone-like molecule. High concentrations of succinate have

been detected in the plasma of patients with peritonitis, in the urine and plasma of diabetic and metabolic disease rodent models (59, 60), and in the synovial fluid of patients with rheumatoid arthritis (61). Interestingly, succinate accumulation is induced as a response to ischemia in several tissues including the brain (62), thus suggesting that several factors could contribute to increase succinate levels in the microenvironment of MS lesions. Succinate has been shown to signal via the G-protein coupled succinate receptor 1 (SUCNR1), a protein highly expressed on a variety of tissues, including the spleen (63) and in immune cells (64). The activation of SUCNR1 by succinate synergizes with TLRs on dendritic cells and is required for enhanced antigen-presenting function of these cells (64). Therefore, blocking succinate receptor on MPs using the highly specific and potent SUCNR1 antagonist (65) could prove to be effective for the treatment of progressive MS.

Two other mitochondrial TCA metabolites, itaconic acid (ITA) and citrate have been shown to be involved in macrophage inflammatory pathways (**Figure 1**). ITA is induced and secreted by macrophages upon LPS and IFN-γ stimulation (66) and it inhibits the growth of bacteria that express isocitrate lyase, such as *Salmonella enterica* and *Mycobacterium tuberculosis*. Immunoresponsive gene 1 (Irg1) protein is the enzyme responsible for the production of ITA in mammalian cells. Gene silencing of *Irg1* in macrophages caused a substantial reduction in antimicrobial activity during bacterial infections (67).

Citrate is another important mediator of LPS-induced signaling in macrophages. Although produced exclusively in the mitochondria, citrate can be exported into the cytosol by the mitochondrial citrate carrier (CIC), and converted to oxaloacetate and acetyl CoA by the enzyme ATP-citrate lyase (ACLY). Interestingly, upon LPS, CIC expression levels increase and its inhibition (68) or the silencing of ACLY (69) was shown to block LPS-induced nitric oxide (NO), ROS, and prostaglandin production in macrophages, consistent with a role of cytosolic citrate as important precursor for these molecules.

In summary, the evidence reported above suggests that the mitochondrial dysfunction observed in MS lesions can lead to imbalance of several mitochondrial metabolites that, beyond being mere intermediates in energy metabolism, can directly influence the immunological function of different cell types involved in MS inflammation. Further understanding of the regulation of these metabolites will be important for the identification of targets to modulate MP metabolism.

METABOLIC RE-EDUCATION OF MPs IN MS

Given the relevance of metabolism in the activation and polarization of MPs, it has been proposed that an M1-to-M2 transition can be achieved by altering cell metabolism. For instance, it has been proposed that activating the key metabolic regulator AMP-activated kinase (AMPk) in MPs would enhance an M2-like phenotype by pushing oxidative metabolism. Indeed, metformin and 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR), well-established AMPk activators can attenuate progression of chronic EAE in mice by inhibiting macrophage infiltration into the CNS (70) and modulating the endothelial–macrophage interaction (71). Interestingly, AMPk-null mice have more severe EAE through an increase in macrophage infiltration to the spinal cord (72).

Another recently proposed metabolic strategy to polarize MPs to an M2-phenotype is the modulation of sirtuins, a family of seven NAD-dependent lysine deacetylases involved in a plethora of cellular processes, including metabolic homeostasis (73–75). Among the sirtuins, SIRT1, SIRT3, and SIRT6 play a key role in the regulation of cellular metabolism. For instance, SIRT3 regulates the enzymatic activity of SDH (76) and both SIRT1 and SIRT6 coordinate a switch from glycolysis to fatty acid oxidation in macrophages (77). Moreover, nicotinamide phosphoribosyltransferase (NAMPT), an important enzyme for NAD^+ biosynthesis and sirtuins function, is required for the inhibition of prolonged macrophage activation via TLR4, indicating that sirtuins can act as anti-inflammatory factors during physiological response to pathogens (78). Therefore, sirtuins activity in MPs could favor an anti-inflammatory M2-like phenotype, by re-educating intermediary metabolism of these cells.

In conclusion, the manipulation of metabolic pathways is a tempting strategy to regulate MPs function in MS. However, using small molecules to regulate ubiquitous enzyme and metabolite levels may be cumbersome as metabolic pathways are crucial for normal cell function and energy production. Therefore, more selective strategies to target MP metabolism are required to regulate inflammation without impacting on metabolism of other tissue.

DIMETHYL FUMARATE AS REGULATOR OF MONONUCLEAR PHAGOCYTE METABOLISM IN MS

A current therapy for relapsing MS is oral dimethyl fumarate (DMF; *Tecfidera*), a methyl ester of fumaric acid that is rapidly hydrolyzed to its active metabolite monomethyl fumarate (MMF), and shown to have a significant effect on relapse rate and time to progression in phase III clinical trials of MS (79, 80). Fumaric acid has been long licensed for the treatment of psoriasis (81), and progressive multifocal leukoencephalopathy, a rare potentially fatal neurologic disease caused by reactivation of JC virus infection, has been reported in rare cases (82, 83).

The mechanisms of action of DMF are still under investigation. DMF interacts with immune cells in the circulation and promotes a shift in cytokine production from a Th1-like (pro-inflammatory) to Th2-like (anti-inflammatory) pattern. Despite being approved for T-cell-mediated relapsing MS only, DMF has been shown to affect MPs *in vivo* in animal disease models. During the acute phase of EAE, Mac-3-positive microglia and macrophages are significantly reduced in DMF-treated mice (84). *In vitro* studies show that DMF can shift MPs from an M1-like to an M2-like phenotype. Evidence for the anti-inflammatory properties of DMF are shown in human PBMCs treated with either IFN-γ or LPS where the expression of the chemokines CXCL8, CXCL9, and CXCL10 are dose-dependently inhibited by DMF (85). In addition, application of MMF to MPs results in increased expression of the anti-inflammatory cytokines IL-4, IL-5, IL-10, and IL-1RA (86). In human macrophages, DMF and MMF block NF-κB activity by inhibiting its nuclear translocation and DNA binding in response to TNF-α and also reduce TNF-α (87). Furthermore, DMF and MMF suppress CCL2-induced chemotaxis of human MPs (87).

These data suggest that this block in chemotaxis would result in decreased infiltration of MPs into the CNS across endothelial surfaces. It has also been proposed that DMF may play a role in CNS oxidative stress by activating the nuclear factor (erythroid-derived 2)-related factor-2 (Nrf2), a transcription factor with antioxidant properties (88, 89). LPS-induced NO, TNF-α, IL-1β, and IL-6 expression in microglia cells is reduced by pre-treatment with DMF, possibly through activation of Nrf2 pathway (90). However, this effect has not been demonstrated *in vivo* in EAE mice (91). In addition, fumaric acid esters induce up-regulation of superoxide in monocytes, which is indicative of a pro-inflammatory response (92). Although the exact mechanisms of action of DMF are not fully understood and still controversial, we postulate that, since the active form of DMF is fumarate, a TCA cycle metabolite, this molecule may act also by altering the metabolism of MPs and favoring an M2-phenotype. However, more data is required to validate this hypothesis.

SUMMARY

The link between metabolism and inflammation has become a hot topic over the past 5 years. The metabolic state of MPs is now thought to affect their *inflammatory status*. Understanding the changes in metabolism that occur in inflammatory and autoimmune diseases is crucial to interpret disease pathogenesis and identify novel therapies for progressive MS. Here, we provided evidence to show that targeting specific metabolic processes in MPs to regulate their inflammatory state might be used as an MS therapy.

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