Trends in
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Review

Therapeutic opportunities to modulate immune tolerance through the metabolism-chromatin axis

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The ability of the immune system to discriminate external stimuli from self-components – namely immune tolerance – occurs through a coordinated cascade of events involving a dense network of immune cells. Among them, $CD4^+CD25^+$ T regulatory cells are crucial to balance immune homeostasis and function. Growing evidence supports the notion that energy metabolites can dictate T cell fate and function via epigenetic modifications, which affect gene expression without altering the DNA sequence. Moreover, changes in cellular metabolism couple with activation of immune pathways and epigenetic remodeling to finely tune the balance between T cell activation and tolerance. This Review summarizes these aspects and critically evaluates novel possibilities for developing therapeutic strategies to modulate immune tolerance through metabolism via epigenetic drugs.

Highlights

Metabolic and epigenetic events control T cell differentiation and function to ensure immune competence while avoiding immune pathology.

Small metabolic intermediates can switch in parallel energy metabolism and gene expression.

Intracellular metabolism and chromatin modifications converge to finely tune *Foxp3* transcription.

Immune-related disorders display metabolic and epigenetic alterations.

Epigenetic therapies are a novel attractive tool to restore immune tolerance through metabolism.

Self-recognition and immune tolerance

Immune tolerance is the ability of the immune system to distinguish **self** (see Glossary) and safe components from non-self and dangerous signals; an intricate and fascinating process to protect organs and tissues from their autoimmune destruction. This is achieved through several coordinated events occurring in primary lymphoid organs and blood, referred to as central and peripheral tolerance [1]. Central tolerance is maintained in the thymus through a negative selection in which self-reactive thymocytes undergo clonal deletion. This is controlled by the human **autoimmune regulator** (AIRE) transcriptional factor, highly expressed in a subset of **medullary thymic epithelial cells** (mTECs). AIRE promotes the promiscuous expression of tissue-specific antigens; that is, ectopic expression of otherwise tissue-restricted antigens (TRAs) by mTECs, that are presented by thymic antigen-presenting cells (APCs) to developing thymocytes, enabling deletion of self-reactive T cells [2]. However, this process is not flawless and some T cells migrate to the periphery where they can recognize self-components, leading to an autoimmune activation and tissue damage. A subset of self-reactive thymocytes is converted into $CD4^+CD25^+$ Forkhead box p3 $(Foxp3)^+$ T regulatory (Treg) cells that control peripheral tolerance by suppressing self-reactive T effector (Teff) cells that have escaped adverse selection in the thymus [3].

Treg cell generation and peripheral tolerance

Treg cells are a specialized subpopulation of $CD4^+$ T lymphocytes having a critical role in the maintenance of **immune homeostasis**, inhibition of autoimmune diseases and control of anticancer immune responses [3]. Peripheral tolerance is guaranteed through the suppression of proliferation and effector function of target cells via several mechanisms: **inhibitory receptors**, such as cytotoxic T lymphocyte antigen (CTLA)-4, programmed cell death protein (PD)-1, and T cell immunoglobulin and ITIM domains (TIGIT), soluble factors, such as transforming growth factor (TGF)-β, interleukin (IL)-10, IL-35, or through growth factor/nutrient competition [4].

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Effector Treg cells can be divided into functionally distinct subsets based on their suppressive cytokine induction [5]. IL-35-producing Treg cells preferentially localize in the T cell zone to target autoreactive T cells; in contrast, IL-10-producing Treg cells express high levels of multiple **chemokine** receptors responsible for their migration to peripheral tissues to control autoimmunity and suppress inflammation [5]. Additionally, a specialized subset of Treg cells, named T follicular regulatory (Tfr) cells, characterized by the expression of Foxp3, CXCR5, ICOS, PD-1, and Bcl6, has been shown to control humoral and allergic immunity by restraining early B cell responses [6].

Another intriguing immunosuppressive mechanism of Treg cells is related to their concomitant surface expression of the ectoenzyme ATP apyrase (CD39) and ecto-5′-AMP-nucleosidase (CD73), which dampens T cell receptor (TCR) activation and effector function via cyclic-AMP [7]. Treg cells express high levels of nutrient and metabolite receptors such as folate receptor (FR)4, vitamin D receptor (VDR) [8], alanine serine cysteine transporter (ASCT)2, cationic transporter (CAT)1, class B scavenger receptor (CD36), and monocarboxylate transporter (MCT)1 [9]. Also, Treg cells express the α chain of the IL-2 receptor (CD25) together with the metabolic sensor liver kinase (LK)B1, AMP-activated protein kinase (AMPK), and mammalian target of rapamycin (mTOR) [10]. Treg cells display a surface and biochemical phenotype typical of activated and growing cells [11]. This phenotype increases their ability to rapidly sense the inflammatory environment and the metabolic milieu, favoring their expansion and the maintenance of long-lasting suppressive capacity. Interestingly, the metabolic competition (through nutrient deprivation) represents one of the primary mechanisms through which Treg cells inhibit innate and adaptive immune cell activation [12]. In physiological conditions, when Treg cells reach the inflammatory environment, they suppress **T helper** (Th)-2 cell autoimmunity and the associated increased levels of serum **immunoglobulin** (Ig)G1 and IgE as efficiently as Th1 and Th17 activation, even though Th2 responses are the most sensitive to diminished Treg cell number or functionality [13]. In autoimmune disorders such as multiple sclerosis (MS), thyroiditis, rheumatoid arthritis (RA), and type 1 diabetes, have been reported defective Treg cells, due to altered expression of Foxp3 **splicing variants** [14–16]. Treg cells also play a major role in preventing both innate and adaptive antitumor cell recognition [17]. This dichotomous role in control of peripheral tolerance and favor of tumor progression makes them an interesting target and an effective therapeutic tool to restore immune homeostasis in several pathophysiological conditions.

Foxp3 holds on immune tolerance

Epigenetic regulation

The transcription factor Foxp3 represents the master regulator of Treg cell generation and function. It establishes their number and maintains immunological tolerance through a finely tuned process involving transcriptional repression and activation of specific target genes [18]. Indeed, *Foxp3* deficiency leads to multiorgan failure and autoimmunity, known as IPEX syndrome (immune dysregulation polyendocrinopathy enteropathy X-linked) in humans [19] and as scurfy phenotype in mice [20]. Epigenetic regulation of *Foxp3* after TCR stimulation involves, as early events, the DNA demethylation at the **CpG islands** of the promoter and chromatin remodeling, allowing the binding of transcription factors (TFs) to specific enhancers that are conserved noncoding sequences (CNS0–CNS3) in the *Foxp3* locus [21] (Figure 1A). In addition, positive epigenetic regulation such as mono- and trimethylation of the lysine 4 of the histone 3 (H3K4me1-3) as well as H3K9 and H3K27 acetylation anticipates the binding of TFs. SATB homeobox (Satb)1 binds closed chromatin regions at the double-positive (DP) thymocyte stage. Satb1 interacts with mixed leukemia linked (MLL)4 factor and occupies the newly identified CNS0 enhancer region at the *Foxp3* locus. Thanks to MLL4, in these Satb1-bound regions the chromatin becomes gradually open, allowing the recruitment of the Foxp3-promoting TFs, such as Runt-

Glossary

Anergy: lack of reaction by the immune system to a particular antigen or allergen.
ATP: an organic compound that provides energy to drive many processes in living cells.
Autoimmune regulator: a transcription factor playing an essential role to promote self-tolerance in the thymus by regulating the expression of a wide array of self-antigens.
Autophagy: an intracellular degradation system wherein damaged organelles, unneeded proteins, as well as pathogenic agents, are digested and the resulting macromolecular content is released back into the cytosol.
β oxidation: the catabolic process by which fatty acid molecules are broken down in the mitochondria to generate acetyl-CoA, which enters the citric acid cycle, and NADH and FADH2, which are coenzymes used in the electron transport chain.
Chemokines: a family of small cytokines or signaling proteins that promotes directional movement of cells.
Chromatin loops: the anchor points providing contacts between regulatory regions and promoters.
CpG islands: short region of DNA with high frequency of the CG sequence, often located around gene promoters.
Epigenetic cofactor: a protein that acts inducing modifications of DNA and gene expression without affecting gene sequence.
Exon: the polynucleotide sequence of the nucleic acid coding information for protein synthesis.
Fatty acid oxidation: the mitochondrial aerobic process of breaking down a fatty acid into acetyl-CoA units (see β oxidation).
Glycolysis: cytoplasmic pathway that breaks down glucose into two three-carbon compounds (pyruvate) and generates energy.
Hypoxia: a condition in which the body or a region of the body is deprived of adequate oxygen supply at the tissue level.
Immune homeostasis: the finely regulated balance of appropriate immune activation and suppression in tissues and organs.
Immunoglobulins: also known as antibodies; glycoproteins produced by B lymphocytes that bind and neutralize particular antigens, such as bacteria or viruses.

related transcription factor (Runx)1 and core-binding factor (Cbf)-β. Genetic deletion studies of single CNS indicated that CNS3 controls *Foxp3* induction in thymic precursors while CNS1 and CNS2 are required for the conversion of T conventional (Tconv) into peripheral Treg (pTreg) and *in vitro* induced Treg (iTreg) cells [21]. Recently, Nair and colleagues found that CNS2 demethylation is maintained by ten to eleven translocation (TET) proteins recruited to the CNS2 locus by IL-2 [22]. Stable demethylation of this sequence enables critical TFs to access the CNS2 enhancer region (Figure 1A). Conversely, the CD28–PKC–NFκB axis represses *Foxp3* CNS2 demethylation in activated Tconv hampering iTreg cell differentiation [23]. Moreover, the binding of the homeobox protein (Hhex) to the *Foxp3* locus during Tconv cell activation completely inhibits the activity of the promoter and CNS1 as well as of several Treg signature genes, such as *Il2ra* and *CTLA4* (Figure 1A); this points up Hhex as a Foxp3-independent Treg cell regulator that inhibits iTreg cell differentiation and function under specific circumstances [24].

Foxp3 splicing variants

In humans, *Foxp3* gene comprises 12 **exons**, with 11 coding and one noncoding exon within the 5′ untranslated region [25]; it encodes for different transcript variants, among which four have been characterized in depth [14,15,26] (Figure 1B). The alternatively spliced isoforms of Foxp3 include the total length (Foxp3FL), those lacking the 105 bp exon-2 of the Foxp3 mRNA (Foxp3Δ2), those lacking the entire 81 bp region encoding the exon-7 (Foxp3Δ7) or those missing both (Foxp3Δ2Δ7) (Figure 1B and Box 1). The splicing variants are expressed at different levels in circulating Treg cells, being Foxp3FL and Foxp3Δ2 abundant, while Foxp3Δ7 variants present only at low proportion [26]. The interaction of Foxp3 with the required cofactor can be impaired when the splicing occurs in the relative binding domain. Indeed, loss of the exon-2, which is part of the repressor domain, hampers the binding of Foxp3 with RAR-related orphan receptor (ROR)γt; the transcription factor required for the development of proinflammatory Th17 cells. In the same way, loss of the exon-7, encoding a part of the leucine-zipper domain, alters homo and hetero-association of Foxp3 and its DNA binding [27] (Figure 1B,C and Box 1). However, how and whether the epigenetic regulation could impact on the transcription of specific *Foxp3* splicing variants is still unknown.

Foxp3 transcriptional activity

Foxp3 orchestrates a genetic program driving the development of Treg cells with strong immunosuppressive phenotype and function through the expression of suppressive molecules (e.g., CD25, CTLA-4, CD39, and IL-10) and the inhibition of proinflammatory cytokines (e.g., IL-2, IFN-γ, and IL-17) [28]. Recently, Kwon *et al.* attractively dissected this process showing that Foxp3 controls its transcriptional function by interacting with two different sets of cofactors (Figure 1C,D and Box 1). When bound to RelA, Foxp3 localizes to the center of the nucleus activating or repressing target genes; in contrast, when complexed with enhancer of zeste homolog (EZH)2 and IKAROS family zinc finger (IKZF)3, Foxp3 is confined to the periphery of the nucleus and has diminished transcriptional activity (Figure 1C,D and Box 1) [29]. Moreover, while the binding to some cofactors – such as RELA and IKZF2 – positively associates with the expression of Foxp3-induced genes (e.g., *CTLA4*), binding to others (e.g., IKZF3 and EZH2) inversely correlates with Foxp3 function [29]. Moreover, there is functional evidence that Foxp3 cofactors facilitate the binding to a given site either through the direct recruitment of Foxp3-containing complexes or by favoring interactions with Foxp3-bound sites (containing Forkhead domain) via **chromatin loop** formation [30]. Indeed, Foxp3 binds to both histone deacetylases (HDACs), such as p300 and TIP60, and histone methyl transferases, such as EZH2 [31], to promote epigenetic stability. Intriguingly, the interplay between RORγt and Foxp3 has been considered the Yin and Yang of the immune system (Figure 1C,D and Box 1). These two factors act reciprocally to keep one another in check to balance immune activation versus tolerance.

Immunometabolism: a branch of biology that studies the interplay between metabolism and immunology in all organisms.
Inhibitory receptors: the receptors able to prevent the cell from receiving proper activation signals.
Medullary thymic epithelial cells: a stromal cell population of the thymus which plays an essential role in the establishment of tolerance.
Oxidative phosphorylation: an electron transfer chain driven by substrate oxidation that is coupled to the synthesis of ATP within the inner membrane of the mitochondria.
Resting cell: a quiescent cell not undergoing mitosis.
Self: antigens of an organism, such as cellular proteins, peptides, ribonucleoprotein complexes and DNA, to which the immune system is tolerant.
Splicing variants: the alternative forms of mRNA derived from a single gene sequence.
T helper: subsets of CD4 T cells that play an important role in adaptive immunity.
Tricarboxylic acid cycle: a series of chemical reactions used in aerobic organisms to generate energy via the oxidation of acetyl-CoA derived from carbohydrates, fatty acids and proteins.
Polyamine pathway: a metabolic pathway in which the amino acids arginine, ornithine and methionine are decarboxylated by ornithine decarboxylase to generate putrescine, spermidine, and spermine.
Tumor microenvironment: the system surrounding a tumor including several types of immune cells, the extracellular matrix, blood vessels and other biochemical and cellular components in direct contact with cancer cells.
Pentose phosphate: branches from glucose 6-phosphate, produces NADPH and ribose 5-phosphate, and shunts carbons back to the glycolytic or gluconeogenic pathway.

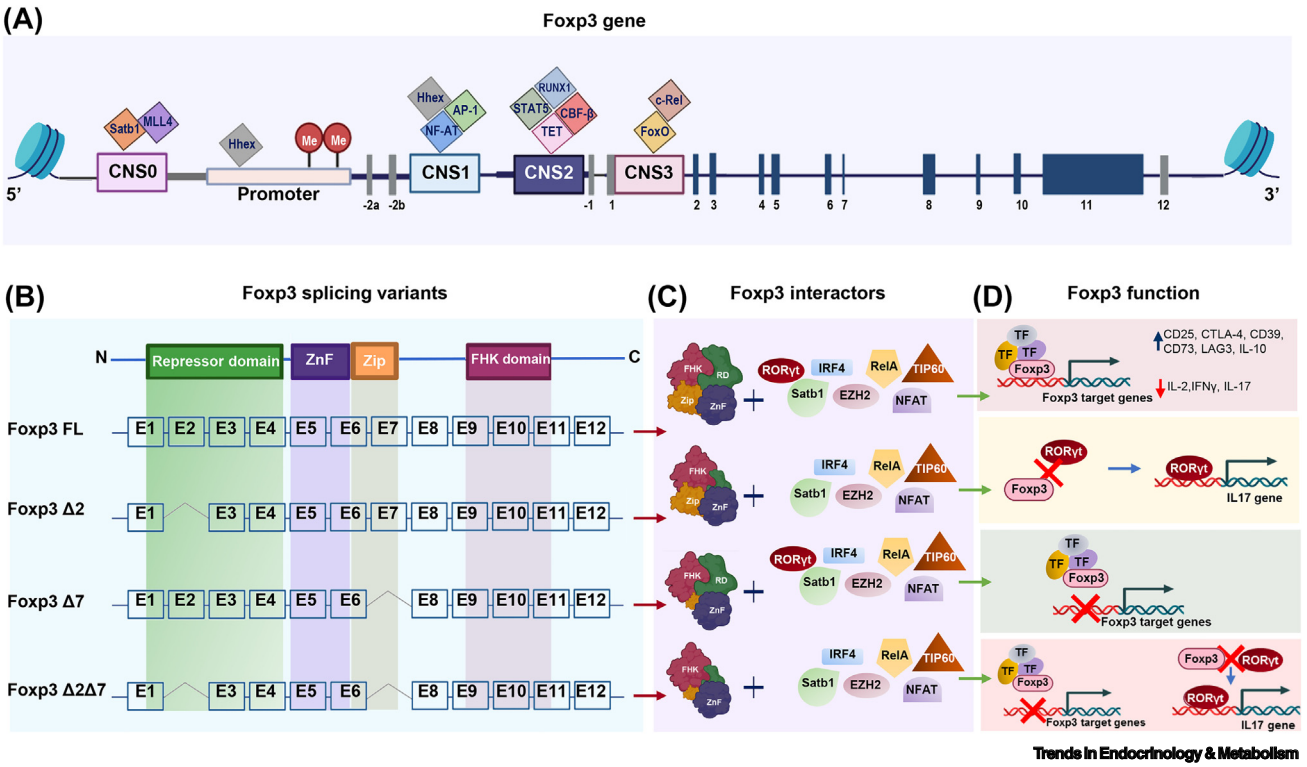

Figure 1. Schematic diagram of the human *Foxp3* gene, mRNA splicing variants, interactors, and their functional activity. (A) The *Foxp3* gene comprises 12 exons and four CNS (CNS0–CNS3) involved in the binding of Foxp3-promoting TFs. (B) Foxp3 encodes different protein domains: N-terminal repressor (RD, green box), zinc finger (ZnF, purple box), leucine zipper (Zip, orange box), and forkhead (FHK, burgundy box). The main Foxp3 mRNA splicing variants are the full-length (Foxp3FL), those lacking exon 2 (Foxp3Δ2), exon 7 (Foxp3Δ7), or both (Foxp3Δ2Δ7). (C, D) Foxp3 protein domains control the interaction with the different TFs and the DNA binding; lack of the repressor domain (RD) prevents the interaction with RORγt, while loss of the Zip domain prevents the DNA binding affecting Foxp3 transcriptional activity. Abbreviations: CNS, conserved noncoding sequence; CBF-β, core-binding factor subunit β; EZH2, enhancer of zeste homolog 2; Fox, Forkhead box; Hhex, homeobox protein; IRF4, interferon-regulatory factor 4; Me, methylation; MLL4, mixed leukemia linked factor 4; RORγt, retinoic acid-related orphan receptor γt; RUNX1, Runt-related transcription factor 1; Satb1, SATB homeobox 1; TET, ten-eleven translocation methyl cytosine dioxygenases; TF, transcription factor. Figures have been created with BioRender.

More in detail, the binding of Foxp3 to RORγt inhibits the relative transcriptional activation of its target genes, among which IL-17, hampering Th17-cell lineage differentiation. In contrast, **hypoxia**-inducible factor (HIF)-1α interacts with Foxp3 through the C-terminal domain and promotes its degradation in a proteasome-dependent manner; when Foxp3 is complexed

Box 1. Foxp3 domain structure

Foxp3 protein belongs to the forkhead (FOX) transcription factor family, which mainly localizes in the nucleus [34]. It contains different protein domains: a proline-rich N-terminal (1–97 aa) responsible for transcriptional activation or repression, a central zinc-finger and a leucine-zipper domain (98–260 aa) involved in its dimerization or association with other factors, and a conserved C-terminal forkhead domain (337–423 aa) responsible for DNA binding. The proline-rich N-terminal domain regulates Foxp3 transcriptional activity through the interaction with several TFs, among which AML1/Runx1, TIP60, and NFAT1 [35]. The high-resolution crystal structure of Foxp3 unveils the mechanism controlling Treg cell development and function. Indeed, the forkhead domain of Foxp3 forms a domain-swapped dimer into a ternary complex which contains the NFAT1 DNA-binding domain and the Foxp3 forkhead domain bound to two DNA molecules. The domain-swapped dimer allows Foxp3 to coordinate the expression of genes connecting two distal regions of DNA and reorganizing the genome architecture; disruption of this domain attenuates Foxp3-mediated suppressor function [36]. Conversely, mouse zinc finger and leucine zipper domain (Foxp3-ZL) form an unusual Foxp3 two-stranded anti-parallel α-helical coiled-coil with twofold symmetry. Deletion of the lysine (K) 251 in this domain hampers the repression of IL-2 production. Moreover, post-translational modification (i.e., acetylation) of human Foxp3 in the K250 and K252 of the coiled-coil region regulates Treg cell function. The crystal structure of the Foxp3 N-terminal region and the full-length Foxp3 are still unavailable due to an intrinsic disorder of the N-terminal part [36].

with HIF-1α, RORγt wins the battle against Foxp3 in favor of Th17 differentiation [32]. This intriguing relationship finds its proof in autoimmune disorders, where decreased frequency of the Foxp3 splicing variants containing exon-2 associates with increased expression of RORγt and HIF-1α, with the subsequent establishment of the Th17-cell lineage differentiation program [33].

Metabolic flexibility as the main immunomodulator

Metabolic flexibility is the ability of an organism to adapt to energetic variations and nutritional changes provided by the microenvironment. This is particularly represented in immune cells, which rapidly adapt to the milieu by modifying their metabolic profile to support immune activation or preserve homeostasis (Figure 2 and Box 2). These reactions mainly rely on the abundance and accessibility of nutrients and on the host metabolic state. Such **immunometabolism** is governed by metabolic intermediates and has a pivotal role in regulating immune cell function [37]. Indeed, the metabolic activity of **resting** T cells is directed towards maintaining housekeeping processes having minimal energetic demands. Therefore, T cells predominantly produce energy via the **tricarboxylic acid** (TCA) cycle coupled with **oxidative phosphorylation** (OXPHOS), while for their rapid expansion preferentially use glucose as primary **ATP** source (Figure 2 and Box 2). This approach can rapidly generate energy by converting glucose into pyruvate and lactate, providing the intermediates necessary for cell division and proliferation. Upon activation, T cells reprogram their

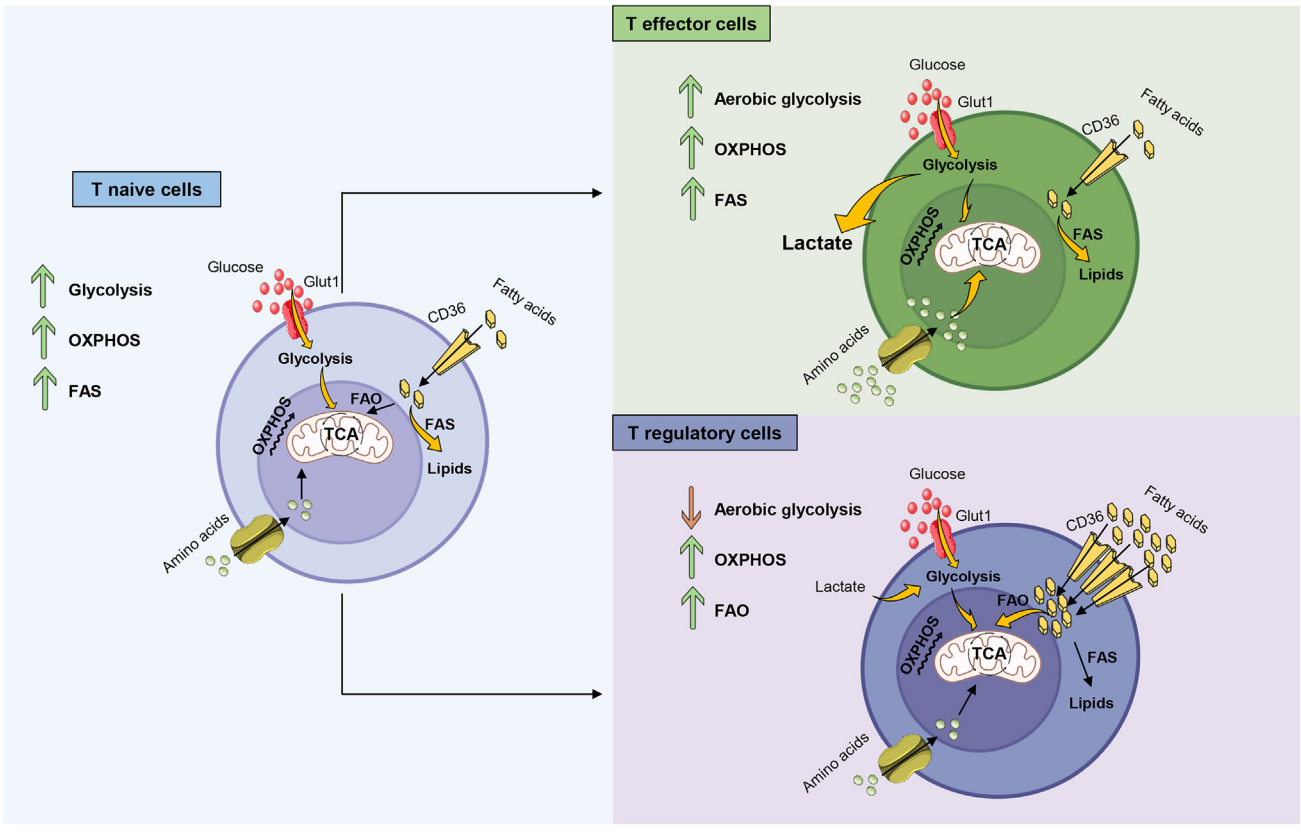

Figure 2. Metabolic pathways supporting T cell differentiation and function. Different metabolic pathways sustain T effector (green box) and regulatory (violet box) cells. Naïve T cells (light blue box) engage glycolysis, OXPHOS and FAS but switch to different metabolic pathways to support their differentiation. T effector cells engage aerobic glycolysis, OXPHOS, and FAS; conversely, the suppressive function of Treg cells is sustained by OXPHOS and FAO. Abbreviations: FAO, fatty acid oxidation; FAS, fatty acid synthesis; OXPHOS, oxidative phosphorylation; TCA, tricarboxylic acid cycle. Figures have been created with BioRender.

Box 2. Metabolic network

Metabolism is a dense network of enzymatic reactions that produce interchangeable metabolic intermediates. The conversion of glucose to glucose-6-phosphate (G6P) by hexokinase (HK) or glucokinase (GCK) is the first limiting step of glycolysis. G6P can proceed into glycolysis producing pyruvate, or can be shuttled towards the **pentose phosphate** (PPP) pathway to synthetize nucleotides or NADPH, essential for the anabolic pathways. Pyruvate can be either converted to lactate, by lactate dehydrogenase (LDH), or to acetyl-CoA, entering into the TCA cycle. Of interest, pyruvate can also be re-formed from citrate, malate, and oxaloacetate through cytosol-mitochondrial shuttles [48]. Moreover, alanine, serine, threonine, glycine, cysteine, and tryptophan can be converted to pyruvate as well. Additionally, FAO and glutaminolysis can feed the TCA cycle through their products, acetyl-CoA and α-KG, respectively. This subsequently provides metabolic intermediates for OXPHOS and FAS but also cofactors required for the epigenetic enzymes [49]. Subsequently, the TCA cycle fuels OXPHOS and FAS. One of the keystones in energy metabolism is represented by NAD^+ that influences many critical cellular functions, including metabolic pathways (glycolysis and TCA), DNA repair, chromatin remodeling, cellular senescence, and immune cell function [50,51]. NAD^+ derives directly from tryptophan, an essential amino acid, or is recycled via the nicotinamide (NAM) rescue pathway, a fundamental step to restore NAD^+ levels [52]. High levels of NADH can inhibit both glycolysis and the TCA cycle [53].

metabolism to sustain functional activity: Teff cells engage aerobic **glycolysis** and OXPHOS, while Treg cells mainly use **fatty acid oxidation** (FAO) and OXPHOS to support their suppressive function [38] (Figure 2 and Box 2).

Metabolic configurations handle T cell fate

Typically, growth signals promote glycolysis through phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and mTOR [38,39]. In this context, AMPK senses low ATP concentrations and increases catabolic processes, such as glycolysis, FAO, and **autophagy,** while dampening anabolic processes. Glycolysis, mediated by the glucose transporter Glut1, promotes effector T cell proliferation and function; conversely, it supports Treg cell proliferation and migration but inhibits their suppressive function [38–40] (Figure 2 and Box 2). Increased glucokinase activity leads to a reduced number of circulating Treg cells due to enhanced migratory activity, that still preserve their suppressive function, thus confirming the critical role for glycolysis in promoting Treg cell proliferation/migration [41]. FAO represents a multiphase process in which CD36 (encoding a scavenger receptor responsible for long-chain fatty acid and oxidized low-density lipoprotein uptake) imports fatty acids inside the cells, that are oxidized (**β oxidation**) to produce acetyl-CoA, entering the TCA cycle [39] (Figure 3 and Box 2). High levels of AMPK and CD36 in Treg cells sustain FAO and ATP generation increasing the uptake of long-chain fatty acids and their transport to the mitochondria (Figure 3 and Box 2) [39].

Differential amino acid concentrations and byproducts in the local environment can influence the engagement of specific metabolic pathways impacting T cell fate. A well-characterized mechanism is represented by the induction of the mTOR complex I kinase (mTORC1) [42]. When glucose availability is reduced, key intermediates, such as pyruvate and citrate, can arise from the glutamine-fueled TCA cycle [43] (Figure 3 and Box 2). T cells grown in glutamine-free media or in the absence of glutamine transporter (Slc1a5) fail to adequately engage mTORC1, highlighting the critical role that glutamine plays in the signaling cascade after TCR stimulation [44]. Additionally, the Rag GTPase–mTORC1 interaction, either through GATOR- or ADP-ribosylation factor (ARF)1-dependent mechanism, is induced by leucine, methionine, and glutamine and sustains mTOR signaling. Amino acid deprivation in activated T cells leads to a potent mTORC1 inhibition, promoting T cell survival and **anergy** [44]. As proinflammatory Teff, Treg cells display increased expression of the amino acid sensors Sestrin1, CASTOR1, CASTOR2, and components of the GATOR1 and GATOR2 complexes, enhancing mTORC1 activity [44]. However, hyperactivation of the mTORC1 pathway has been shown to inhibit *Foxp3* induction during the generation of pTreg and iTreg cells and to constrain Treg cell proliferation *in vitro* [45]. Recent findings show that also innate cells can adapt to environmental inflammatory signals through a metabolic shift from glycolysis and glutaminolysis to TCA, OXPHOS, FAS, and higher cellular oxygen-consumption

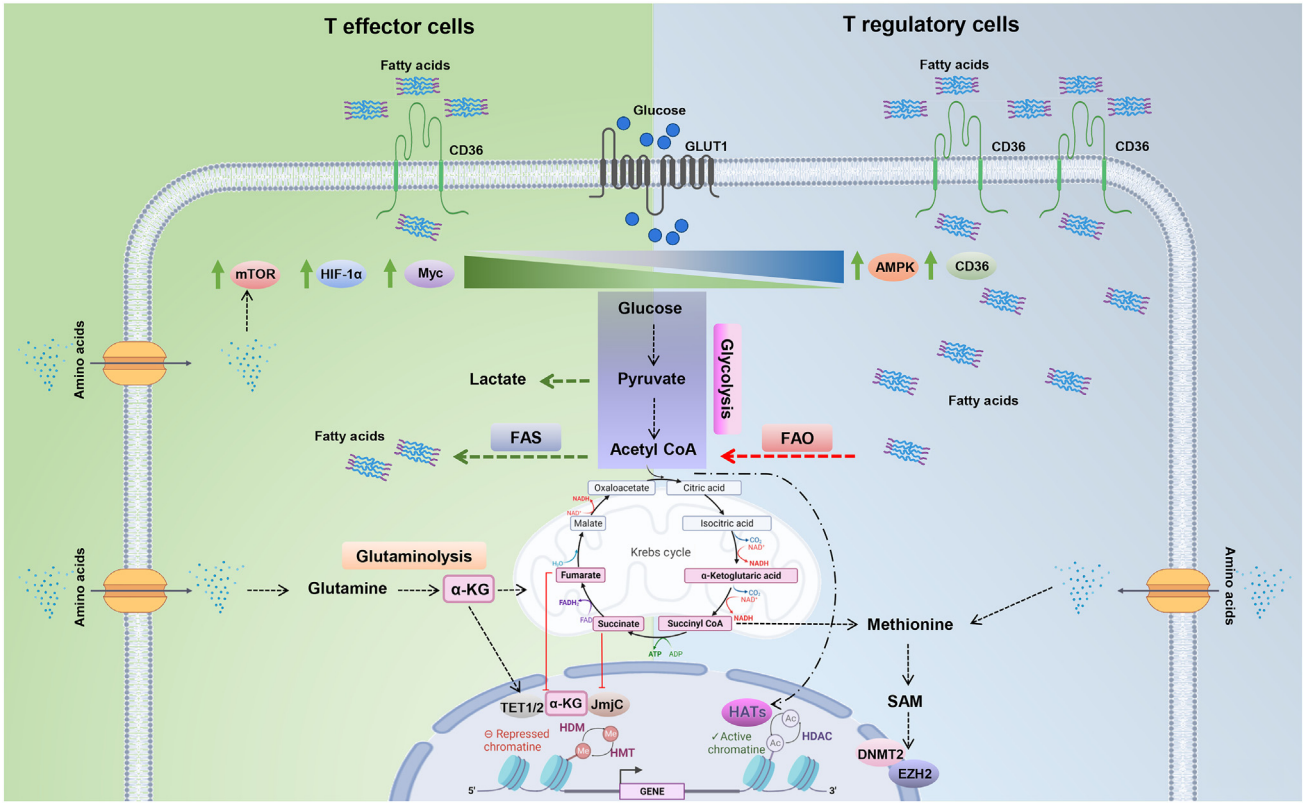

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Figure 3. Metabolic configurations and epigenetic modifications in T effector and regulatory cells. In T effector cells (green box) high levels of mTOR, Myc, and HIF-1α support aerobic glycolysis and FAS. Conversely, T regulatory cells (light blue box) show high levels of CD36 and AMPK that sustain fatty acid metabolism in the OXPHOS through FAO. In T effector cells, OXPHOS can be fueled also by glutaminolysis. The TCA-derived metabolic intermediates can act as epigenetic regulators. Indeed, SAM derived from methionine is a methyl donor for DNA and histones methyltransferases, such as DNMT and EZH2; α-KG regulates the activity of multiple 2-OGDO families, including TET1/2 and JmjC demethylases. Other TCA metabolites, such as succinate and fumarate, inhibit the activity of the 2-OGDO family, competing with α-KG. Again, acetyl-CoA is an acetyl donor for HATs that catalyzes the acetylation of lysine residues. Abbreviations: 2-OGDO, 2-oxoglutarate-dependent dioxygenases Ac, acetylation; α-KG, α-ketoglutarate; AMPK, AMP-activated protein kinase; DNMT, DNA methyl transferase; EZH2, enhancer of zeste homolog 2; FAO, fatty acid oxidation; FAS, fatty acid synthesis; HATs, histone acetyltransferases; HIF-1α, hypoxia inducible factor 1 subunit alpha; JmjC, jumonji C domain-containing demethylases; Me, methylation; Myc, myc proto-oncogene; BHLH transcription factor; mTOR, mammalian target of rapamycin; OXPHOS, oxidative phosphorylation; SAM, S-adenosyl methionine; TET, ten-eleven translocation methyl cytosine dioxygenases. Figures have been created with BioRender.

rate (OCR), generating 'trained' cells displaying a memory phenotype [46,47]. In summary, abundance and accessibility of nutrients have a direct impact on the metabolic configuration of the activity of immune cells.

Chromatin-modifying enzymes as metabolic sensors

Energy metabolism and gene regulation share small intermediates that connect these fundamental biological processes [54]. The engagement of the specific metabolic pathway in T cells produces key metabolites such as lactate, succinate, S-adenosylmethionine (SAM), alfa-ketoglutarate (α-KG) and acetyl-CoA, which can regulate the activity of many chromatin-modifying enzymes, thus working like cofactors or inhibitors (Figure 3 and Box 3). Although many of them can cross the nuclear membrane, some metabolic reactions can also occur directly in the nucleus; the result is a modification of DNA structure and gene expression. In this context α-KG, derived from amino acid and protein synthesis, TCA and nitrogen transport, acts as an **epigenetic cofactor**, regulating the activity of multiple 2-oxoglutarate-dependent dioxygenases (2-OGDO) family, including TET1/2 and the Jumonji C (JmjC)-containing domain histone demethylases KDM4A, KDM4D, and

Box 3. Metabolic–epigenetic connection

The abundance of metabolites can modulate gene expression by regulating epigenetic modifications [59]. Acetylation and methylation are affected respectively by the availability of the acetyl donor acetyl-CoA and the methyl donor SAM, synthetized through the folate and methionine cycles. Histone and DNA methylation require SAM as the high-energy methyl donor [60]. In addition to SAM, other metabolites are also are essential cofactors for chromatin-modifying enzymes. This is the case of the 2-OGDO superfamily, including the JmjC-demethylases and the DNA oxygenases TET1 and 2, which use α-KG and molecular oxygen for their catalytic functions [61]. Indeed, 2-OGDOs can function as oxygen sensors; however, their affinity for oxygen and α-KG can vary among specific enzymes, suggesting that their activity can be differentially affected by the availability of the aforementioned metabolites. The acetylation of histones is a key component of transcriptional regulation and associates with gene activation. The pool of acetyl-CoA arises from citrate via the enzymatic action of ATP citrate lyase (ACLY) [62]. ACLY is also activated by the PI3K/Akt/mTOR axis to maintain acetyl-CoA in the nucleus during starvation; moreover, the levels of acetyl-CoA and histone acetylation are also affected by the activity of AMPK. It is worth mentioning that several metabolic enzymes are also regulated by acetylation. Another link between metabolism and epigenetic regulation is represented by NAD^+ which, besides its role in histone deacetylation, is also used by poly-(ADP-ribose) polymerases (PARP) acting close to DNA and histones [54]. It has been reported that PARP-1 activity can reduce the availability of NAD^+ for other enzymes, thus affecting the expression of histone deacetylase sirtuin 1 (SIRT1)-target genes. Evidence has been reported that the sirtuins can adapt to the metabolic state; moreover, NAD^+ levels increase upon caloric restriction, thereby offering an alternative means of sirtuin activation [63].

KDM4DL. Succinate and fumarate, other TCA-metabolites, can act as competitors of α-KG, inhibiting the activity of the 2-OGDO family (Figure 3 and Box 3). This makes α-KG a promising candidate as a key metabolic sensor regulating the whole organism differentiation program [54]. Other glycolytic intermediates involved in the epigenetic regulation are pyruvate dehydrogenase (PDH), ATP-citrate lyase (ACL), and acetyl-CoA synthetase (ACSS2), which provide acetyl-CoA to support histone acetylation [54] (Figure 3 and Box 3). Moreover, the M2 tumor-specific isoform of pyruvate kinase (PKM2), which catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate during glycolysis, has a different impact on T cell fate [55]. More in detail, when in its dimeric form, PKM2 can enter the nucleus increasing the expression of glycolytic enzymes through the Myc-HIF-1α axis [56,57]. Conversely, PKM2 tetramerization promotes Treg cell generation and reduces the development of proinflammatory Th17 and Th1 cells, resulting in inhibition of autoimmunity *in vivo* [58]. In summary, chromatin-modifying enzymes represent critical intracellular sensors, adapting gene expression to the cell metabolic state (Box 3).

Epigenetic and metabolic control of immune tolerance: the Foxp3 hub

The equilibrium between the generation of effector and regulatory $CD4^+$ T cells is a critical step to ensure immune competence while avoiding immune pathology and autoimmunity; this is achieved through the coordinated control of metabolic and epigenetic events during naïve T cell activation [64]. It is well known that the metabolic switch provides ATP and a pool of catabolic intermediates that can supply anabolic pathways to sustain T cell proliferation, differentiation and function [65] (Figure 4). However, what is now emerging is the novel concept that this 'metabolic choice' is not just an adaptation to the increased energy demand but a deliberate strategy to regulate gene transcription to control T cell fate. One of the main opportunities is represented by the molecular events occurring at the *Foxp3* locus – hence defined the Foxp3 Hub – where metabolic processes and epigenetic modifications converge to dictate the choice between activation and tolerance through the control of *Foxp3* transcription. A pioneer work from Merkenschlager and colleagues showed that suboptimal TCR stimulation together with inhibition of PI3K, protein kinase B (Akt), or mTOR conferred Foxp3 expression and Treg-like gene profile to $CD4^+$ T cells [66]. From that time, other studies clarified that the main inhibitory effect of the PI3K/Akt/mTOR pathway on Treg cell induction is the inactivation of the Foxo1–3 TFs, essential for *Foxp3* transcription [67] (Figure 4). Intriguingly, when naïve T cells are cultured under Th17-polarizing conditions, OXPHOS promotes the expression of basic leucine zipper ATF-like transcription factor (BATF), the Th17-pioneer transcription factor, and stimulates TCR and mTOR signaling. Conversely, when naïve

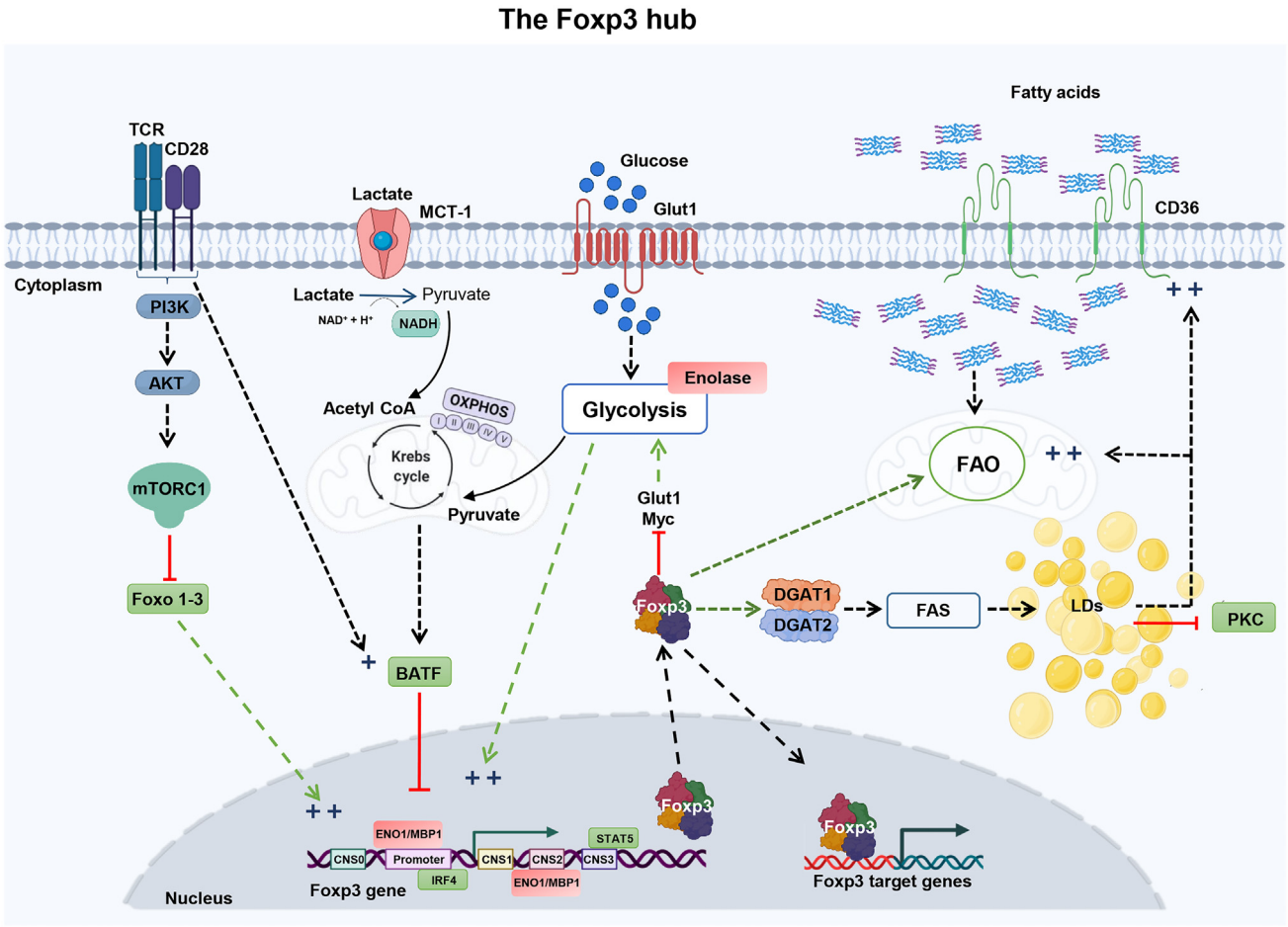

Figure 4. Metabolic pathways and epigenetic events controlling *Foxp3* gene expression. Strong TCR engagement stimulates the PI3K/AKT/mTORC1 pathway which inhibits Foxo1–3. Pyruvate entering the mitochondria is converted into acetyl-CoA, which drives the Krebs cycle and sustains energy production via OXPHOS; this promotes BATF expression which affects *Foxp3* transcription. Inhibition of glycolysis hampers *Foxp3* expression through the binding of ENO1/MBP1 to the promoter and CNS2 region. Lipids enter the cell through the CD36 transporter and then traffic to the mitochondria for FAO. This pathway generates acetyl-CoA that can be used for protein acetylation and FAS. Lipids can also be stored in cytosolic LDs. In parallel, Foxp3 acts as a master metabolic regulator inhibiting glycolysis (through the reduction of Glut1 and Myc expression) and promoting FAO and LD storage (stimulating DGAT-1/2 synthesis). Abbreviations: BATF, basic leucine zipper ATF-like transcription factor; CNS, conserved non-coding sequence; DGAT, diacylglycerol O-acyltransferase; ENO1/MBP1, enolase-1/Myc-binding protein-1; FAO, fatty acid oxidation; FAS, fatty acid synthesis; Glut1, glucose transporter-1; LD, lipid droplets; Myc, myc proto-oncogene BHLH transcription factor; mTORC1, mechanistic target of rapamycin complex 1; OXPHOS, oxidative phosphorylation; PI3K, phosphoinositide 3-kinases; PKC, protein kinase C; TCR, T cell receptor; Treg, T regulatory cells. Figures have been created with BioRender.

T cells are activated under OXPHOS-inhibited Th17-polarizing conditions, they alternatively express *Foxp3* and become suppressive Treg cells due to the absence of BATF, allowing the recruitment of interferon regulatory factor (IRF)-4 and signal transducer and activator of transcription (STAT)-5 on *Foxp3* gene [68]. Intriguingly, strong TCR signals also drive activation of BATF and IRF4, which increases chromatin accessibility for the Th17-promoting TFs, hampering the spontaneous induction of *Foxp3* during naïve T cell activation [44] (Figure 4). On the contrary, glycolytic engagement during suboptimal TCR stimulation of Tconv cells is a key metabolic factor for *Foxp3* induction; indeed, inhibition of glycolysis leads to the accumulation of enolase-1/Myc-binding protein-1 (ENO1/MBP1) on *Foxp3* promoter and CNS2 region, hampering its transcription and iTreg cell differentiation [14] (Figure 4).

Notwithstanding its well-known role as master gene of Treg cell function, what is now emerging is that Foxp3 represents a master metabolic regulator that directly modulates the intracellular metabolism to preserve Treg cell suppressive function. More in detail, Foxp3 inhibits glycolysis – hampering their function – through the direct repression of Glut1 and c-Myc [69], thus promoting OXPHOS for NAD^+ regeneration. As result, $Foxp3^+$ Treg cells increase the production of NAD^+ (OXPHOS) which favors lactate/NAD^+ to pyruvate/NADH conversion through LDH and fosters immune tolerance by impairing protective anticancer immune responses [70] (Figure 4). Not by chance, when compared with Tconv cells, Treg cells have a greater uptake of long-chain fatty acids due to the higher CD36 expression [71]; also, Foxp3 promotes the induction of diacylglycerol o-acyltransferase 1 (DGAT)-1 and -2, necessary for the synthesis of triglycerides. Recently, a unique crossregulation feedback has been described in Treg cells, where Foxp3 reprograms the metabolic state through lipid droplet (LD) loading, which in turn imparts stability to Foxp3 itself. Indeed, LDs are essential for fuel storage, reduce lipotoxicity and limit PKC activity, well known to hamper *Foxp3* induction. Inhibition of DGAT1 results in higher PKC activity and impaired Treg cell generation in response to TGFβ [72]. Intriguingly, through LD storage, Foxp3 could mimic low intracellular availability of fatty acids, which continuously stimulates CD36 expression, promoting fatty acid import and FAO (Figure 4). In sum, through the control of LD formation and the inhibition of glycolysis, Foxp3 manages the intracellular metabolic program to sustain its expression and promote Treg cell suppressive function. Whether this could represent a common mechanism regulating also Tfr needs further investigations.

Epigenetic and metabolic manipulation to restore immune tolerance

The intrinsic reason for T cell plasticity is twofold: it may be part of a mechanism to shut down an active immune response once the pathogen has been cleared, but also a strategy to tailor a reaction from a typical T cell progenitor, based on the challenge type. Cell exhaustion or chronic overstimulation result in transcriptional or epigenetic defects leading to *Foxp3* loss of function and consequent impaired suppressive capacity, introducing the concept of 'ex-Treg' cells [73]. Several studies demonstrate that the balance between IL-2 and IL-6 regulates the development of Th17 from $Foxp3^+$ T cells implicating ex-Treg cells in susceptibility to MS and RA [73,74]. Because of the reciprocal generation of Treg and Th17 cells, it has been shown that the **polyamine pathway** promotes autoimmune pathogenicity restricting the Treg cell program in Th17 cells (Table 1). As increased polyamine levels have been reported in autoimmune disorders and aberrant polyamine metabolism contributes to autoantigen stabilization, inhibiting the polyamine pathway may be an innovative strategy to treat Th17-related diseases [75]. In addition, mitochondrial respiration promotes Th17 cell lineage specification over immunosuppressive Treg cell fate in experimental autoimmune encephalomyelitis (EAE), a mouse model of MS. RNA sequencing of Th17 cells from oligomycin-treated mice demonstrated that disease amelioration associates with transcriptional repression of the Th17 genes, including *Tgfb-3*, *Il23r*, and *STAT4*. This underlines a fundamental opportunity to constrain the transcriptional program of Th17 cells through the inhibition of ATP-synthase-mediated mitochondrial respiration during autoimmunity [68] (Table 1). Moreover, the work by Mariño *et al.* provides the first evidence that a short chain fatty acid (SCFA)-enriched diet prevents autoimmune diabetes by triggering the release of high amounts of gut acetate or butyrate. The first increases the acetylation at the *Foxp3* locus in Treg cells, while butyrate expands the $Foxp3^+$ Treg cell subset, limiting the autoimmune damage [76].

Intratumoral T cells need to adjust their metabolic profile to survive in the nutrient-scanty **tumor microenvironment** (TME). Sequencing of Treg cells that infiltrate lymph nodes and B16 melanomas revealed reduced expression of *Ikzf2* (Helios), *Il2ra* (CD25) upon glucose uptake, suggesting that despite glucose-avid Treg cells are still $Foxp3^+$, they harbor a weaker Treg cell signature. To face this issue, Treg cells incorporate lactate-derived carbon into PEP to provide upstream glycolytic

Table 1. Epigenetic and metabolic targets to restore immune tolerance[a]

Drug	Epigenetic target	Disease	FDA/EMA Approval	Trial no.	Refs
Drugs already in use to treat immune-related disorders and their specific epigenetic targets					
ACY-241	HDACi	Unresectable NSCLC	NO	NCT02635061	[89]
Entinostat	HDACi	Metastatic unresectable HER2-negative breast cancer	NO	NCT02453620	[90]
Mocetinostat	HDACi	Advanced solid tumors and NSCLC	NO	NCT02805660	[91]
Panobinostat	HDACi	Unresectable stage III/IV melanoma	NO	NCT02032810	[90]
Vorinostat	HDACi	Hormone therapy-resistant breast cancer	NO	NCT02395627	[90]
Vorinostat	HDACi	Lymphoma/leukemia	YES (T cell Lymphoma)	—	[90]
Abexinostat	HDACi	Sarcoma	NO	NCT01027910	[92]
Azacytidine	DNMTi	AML	NO	NCT02397720	[93]
Azacytidine	DNMTi	MDS	NO	NCT02599649	[94]
Azacytidine	DNMTi	SLE, SSc, SS	NO	—	[95–97]
Tricostatin A	Pan HDACi	SLE, T1D	NO	—	[98,99]
Givinostat	Pan HDACi	RA	NO	NCT00570661	[100]
Tazemetostat	EZH2	B cell lymphoma and advanced solid tumors	YES	—	[101]
PF 06821497	EZH2	SCLC, CRPC, FL, DLBCL	NO	NCT03460977	[102]
Molibresib	BET proteins	ER^+ breast cancer	NO	NCT02964507	[103]
RO6870810	BET proteins	B cell lymphoma	NO	NCT03255096	[104]
BMS-986158	BET proteins	Advanced solid and hematological cancers	NO	NCT02419417	[105]
ET-1101	BET proteins	AML, MDS, NHL	NO	NCT02543879	[106]
Tranylcypromine	KDM1A	AML, MDS	YES	—	[107]
INCB059872	KDM1A	Advanced malignancies	NO	NCT02712905	[108]
Metabolic intermediates/ pathways	Epigenetic target	Disease	FDA/EMA Approval	Trial no.	Refs
Novel promising metabolic intermediates/pathways to control epigenetic targets and restore immune tolerance					
Polyamines pathway	DHPS, chromatin remodeling	EAE and colitis	NO	—	[75]
OXPHOS	BATF, ATP synthase, histone acetylation	EAE	NO	—	[68]
Lactate transporter (MCT)	Acetylation, PKM2	Melanoma, CRC, CID	NO	—	[77]
CD36	Unknown	CRC, breast cancer	NO	—	[79]
NAD^+	Sirtuins, PARP	EAE	NO	—	[109]

[a]Abbreviations: AML, acute myeloid leukemia; BET, bromodomain and extra-terminal domain; BATF, basic leucine zipper ATF-like transcription factor; CID, chronic inflammatory disorders; CRC, colorectal cancer; CRPC, castration-resistant prostate cancer; DHPS, deoxyhypusine synthase; DLBCL, diffuse large B-cell lymphoma; DNMTi, DNA methyltransferase inhibitor; EAE, experimental autoimmune encephalomyelitis; EMA, European Medicines Agency; ER, estrogen receptor; EZH2, enhancer of zeste homolog 2; FDA, Food and Drug Administration; FL, follicular lymphoma; HDACi, histone deacetylase inhibitor; HER2, human epidermal growth factor receptor 2; KDM1A, lysine demethylase 1A; MCTi, monocarboxylate transporters inhibitor; MDS, myelodysplastic syndromes; NHL, non-Hodgkin's lymphoma; NSCLC, non-small-cell lung cancer; PARP, poly (ADP-ribose) polymerase; PKM2, pyruvate kinase M2; RA, rheumatoid arthritis; SCLC, small cell lung cancer; SS, Sjogren's syndrome; SSc, systemic sclerosis; SLE, systemic lupus erythematosus; T1D, type 1 diabetes.

intermediates essential for proliferation; this offers the opportunity to decrease their need for glucose, thus preserving *Foxp3* induction and suppressive function. Treg cell-specific deletion of the lactate transporter or inhibition of tumor acidity not only decrease tumor growth but also break this metabolic symbiosis to hamper the Treg cell barrier to cancer immunity [77] (Table 1). A recent study also underlined the mechanism by which CTLA-4 blockade interferes with Treg cell function in low-

glucose conditions [78]. Moreover, increased lipid metabolism in intratumoral Treg cells, which boosts their suppressive function, is a joint event in human and mouse cancer, occurring through the CD36 upregulation. CD36 supports mitochondrial fitness and biogenesis via a peroxisome proliferator-activated receptor (PPAR)-β-dependent mechanism by modulating NAD^+ levels that allow Treg cells to adapt to a lactic-acid-enriched TME. Genetic ablation of *Cd36* selectively abolishes the suppressive activity of intratumoral Treg cells and suppresses tumor growth without disrupting immune homeostasis; this underpins CD36 blockade as a novel immunotherapeutic intervention to preserve systemic Treg cells homeostasis in cancer patients [79] (Table 1).

Epigenetic- and immune-therapy combination represents a novel exciting tool to overcome the resistance to immune checkpoint inhibitor (ICI) and increase the duration of their therapeutic response in cancer [80]. Epigenetic therapy refers to the use of drugs (epigenetic drugs) that affect the activity of specific epigenetic enzymes. In this context, DNMT and HDAC inhibitors are known to promote immune-related signaling against cancer [81] (Table 1). Moreover, alternative drugs with immunostimulatory effects include EZH2 inhibitors, KDM1A (LSD1), and the bromodomain and extra-terminal (BET) family. EZH2 methylates lysines 27 and 37 on histone H3 (H3K27 and H3K37), repressing gene transcription. Goswami *et al*. showed that EZH2 inhibitors alter Treg cell function and enhance $CD8^+$ cytotoxic activity, sensitizing mouse bladder cancer to the ICIs [82] (Table 1). LSD1 is the enzyme responsible for the demethylation of H3K4 and H3K9, functioning as a transcriptional co-regulator in a context-dependent manner [83,84]; its expression inversely correlates with $CD8^+$ T cell infiltration in several cancers making this compound a possible therapeutic target [85] (Table 1). BETs comprise several epigenetic readers, namely BRD2, BRD3, BRD4, and BRDT, that promote the opening of the chromatin structures (active transcription), recognizing acetylated lysines [86]. Preclinical studies showed that BET inhibitors increase the percentage of tumor-infiltrating Th1 cells, leading to improved survival compared to those seen with ICIs alone [87] (Table 1). All these data underline the role of metabolism in the epigenetic control of immune cell fate and function; this will aid in developing novel metabolic approaches to regulate immunosuppression and tolerance.

Concluding remarks

Metabolism is critical in orchestrating immune responses, providing energy sources and building blocks needed for macromolecule biosynthesis. What is now emerging is that the metabolic state is revealed to the genome through the nuclear availability of metabolites representing cofactors for chromatin-modifying enzymes. Indeed, the activity of DNA/histone writers and erasers is regulated by metabolic intermediates; in parallel, intracellular metabolism is epigenetically regulated to integrate external stimuli with the metabolic needs [88]. In this context, a key role is exerted by Foxp3, which is finely regulated by intracellular metabolism and in parallel reprograms the metabolic state to control T cell activation and tolerance. The novel concept of chromatin as a key energy-consumer, converging metabolic status and extracellular nutritional signals to specific epigenetic outputs, represents an intriguing opportunity to tune immune homeostasis (see Outstanding questions). As epigenetic and metabolic alterations are both distinctive of chronic inflammation and immune-related disorders, this metabolism-chromatin axis may offer novel therapeutic targets to restore immune tolerance.

Outstanding questions

How do metabolic pathways couple nutrient availability with the epigenetic regulation of T cell responses?

May the spatiotemporal distribution of the metabolite pool represent another essential aspect in the control of chromatin modifications and gene expression?

Does Foxp3 hold on suppressive function by directly controlling Treg cell intracellular metabolism?

Is it possible to identify druggable metabolic pathways or metabolites to target the epigenetic modifications while preserving the core intracellular metabolism?

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Declaration of interests

No interests are declared.

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