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How metabolism bridles cytotoxic CD8+ T cells through epigenetic modifications

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Abstract

In the direct competition for metabolic resources between cancer cells and tumor-infiltrating $CD8^+$ T cells, the latter are bound to lose out. As such, these effector lymphocytes are rendered exhausted or dysfunctional. Emerging insights into the mechanisms of T cell unresponsiveness in the tumor microenvironment (TME) point towards epigenetic mechanisms as being crucial regulatory factors. In this review, we discuss the effects of characteristic components of the TME, i.e. glucose/amino acid dearth and high ROS concentration, on DNA methylation and histone modifications in $CD8^+$ T cells. Subsequently, we take a closer look at the translational potential of epigenetic interventions that aim to improve current immunotherapeutic strategies, including the adoptive cell transfer of TCR- or CAR-engineered T cells.

The role of CD8+ T cells in tumor regression

$CD8^+$ cytotoxic T lymphocytes (CTLs) are favored immune effector cells for targeting human cancers. Indeed, their infiltration in the **tumor microenvironment** (TME) of several tumor types (i.e. breast, colorectal and hepatocellular cancer) correlates with a positive prognosis [1]. Hence, two main immunotherapeutic strategies are founded on cancer cell killing by CTLs with unprecedented results in the treatment of certain malignancies: *(i)* **immune checkpoint inhibitors**, and *(ii)* **adoptive cell transfer** (ACT) [2,3]. Unfortunately, immune intervention does not yet offer a durable response in certain patients and in a variety of tumors. In the worst case, the tumor is completely refractory to treatment. The TME considerably influences therapeutic responses; this has been linked to the ability of the TME to compromise the performance and fate of $CD8^+$ tumor-infiltrating lymphocytes (TILs)

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Resources

I This trial is listed in https://clinicaltrials.gov/ct2/show/NCT01799083

II The clinical trial identifiers listed in Table 1 are found in https://clinicaltrials.gov/ with their respective trial ID numbers (https://clinicaltrials.gov/ct2/show/NCT03913455, etc.).

in favor of immunological tolerance [4]. While the major mechanisms leading to **T cell exhaustion** are still unclear, the harsh TME is now considered to be one of the important contributing factors. Therefore, knowledge on the hallmarks of truly protective $CD8^+$ TILs (resistant to the immunosuppressive milieu), would represent a big leap forward in the field.

The metabolic alteration of the TME is a hallmark of cancer - a concept first introduced by Otto Warburg in the 1920's [5]. Cancer cells redirect their metabolism to maintain an uninterrupted supply of energy and building blocks, safeguarding their own survival and growth. This occurs at the expense of the anti-tumor immune response, whereby CTLs need to survive with nutrient and oxygen dearth. These conditions profoundly shape immune cell fitness, localization, and phenotype [6]. Moreover, the combined occurrence of both hypoxia and glucose deprivation in the TME imposes non-compatible conditions on infiltrating T cells. Namely, whereas hypoxia reduces the use of oxidative phosphorylation (OXPHOS) and enhances glycolysis in CTLs, the reduced glucose availability in the TME cannot support this switch (Figure 1, Key Figure) [7]. Additionally, T cell dysfunction is endorsed by, among others, elevated concentrations of adenosine in the TME [8], macrophage-driven arginine depletion [9], glutamine scarceness [10], and tryptophan deprivation or kynurenine excess [11,12].

The field of immunometabolism is gaining momentum due to the recognition that metabolic remodeling triggers various aberrant immune responses. As such, the interaction between metabolic stress and immune dysfunction in cancer, and the potential to reprogram cell metabolism to bolster immune responses, has become one of the most exciting areas of translational research. In the last couple of years, accumulating evidence suggests that epigenetic remodeling is key in immunometabolic processes. Furthermore, the involvement of epigenetics (Box 1) in defining T cell functioning and fate has increasingly been recognized [13]. Hence, the question arises: can epigenetics be the enticing, missing link between the composition of the TME (embracing an array of metabolic features, cellular components, and cytokine surges), and CTL functioning, ultimately leading to overt clinical responses to immunotherapy? This review first highlights the strong link between the epigenetic machinery and the metabolic features of the TME. In a second part, the article focuses on past and future efforts to translate such findings into the rational reinforcement of current T cell-based immunotherapies.

Epigenetic regulation of T cell exhaustion

As we move towards the point where we might instill T cells with desired, acquired characteristics that are suitable for cell-based therapies (i.e. capacity to infiltrate the tumor site, persistence), targeting their epigenetic programs offers a valuable perspective to improve their efficacy. In fact, the epigenetic signature of $CD8^+$ T cells dictates their differentiation status [14,15]. Stemming from this, the question arises as to whether the exhausted state of T cells in the TME can be traced back to their epigenetic fingerprint (Box 2). The answer to this may open several new opportunities to therapeutically target exhaustion and functional impairment of T cells in the TME, and, conceivably, improve the response to **immune checkpoint blockade**. Even though immune checkpoint inhibitors are now able to temporarily restore T cell effector functions, the chromatin of treated

cells may be left unchanged. However, experiments in murine models of lymphocytic choriomeningitis virus (LCMV) infection have shown that $CD8^+$ T cells rapidly return to an exhausted state upon therapy withdrawal [16,17]. This suggests an irreversible installation of an exhaustion-specific genetic landscape, which can lead to defective tumor control. Moreover, it represents a likely mechanism of tumor resistance, which certainly merits further investigation.

Although T cell receptor (TCR) signaling is a well-recognized contributor to CTL **exhaustion**, general insight into the mechanisms that reinforce exhausted T cells is lacking. Nevertheless, epigenetic remodeling is key. Various murine models of chronic infection or cancer have shown that **TOX**-driven epigenetic changes can turn memory precursor effector cells into progenitor exhausted $CD8^+$ T cells [18,19]. The expression of TOX is in turn driven by chronic TCR stimulation and NFAT activation [20]. Furthermore, analysis of exhausted $CD8^+$ T cell transcriptomes have revealed that the nuclear receptor transcription factor NR4a has an expression pattern similar to that of TOX [21]. Thus, NR4a has been identified as being crucial for the epigenetic and transcriptional program of $CD8^+$ T cell exhaustion [21,22]. While this TCR-NFAT-TOX/NR4A axis contributes to guiding the $CD8^+$ T cell exhaustion program and presents new avenues for the development of anticancer therapies [18,19], its relationship with the metabolic conditions of the TME remains unresolved. Since exhausted T cells exhibit metabolic insufficiency with suppressed mitochondrial respiration and glycolysis, poor metabolic fitness may well reinforce T cell exhaustion [23–26]. In line with this, two research groups recently reported that the soaring amounts of reactive oxygen species (ROS), generated under mitochondrial stress, can drive T cell terminal exhaustion [27,28]. As such, these data preliminarily suggest that the unique metabolic state of exhausted $CD8^+$ T cells might not only be a consequence of its cellular differentiation status, but perhaps also be its cause.

Rewiring of the $CD8^+$ T cell epigenome by the TME

Here, we discuss the major alterations in nutrient availability and utilization underlying certain differences in effector $CD8^+$ T cells vs. exhausted $CD8^+$ T cells, and address how these cells can rewire their epigenome and transcriptome under the metabolic cues of the TME. A solid understanding of such metabolic adaptation by $CD8^+$ T cells may harbor important implications for achieving more effective tumor-targeting strategies.

Glucose

To fulfill the high bioenergetic and biosynthetic requirements of their effector functions, naïve $CD8^+$ T cells can abruptly switch their metabolic program from oxidation of glucose, lipids, and amino acids, to robust consumption and metabolism of glucose and amino acids [29]. However, in the TME, the effector functions of $CD8^+$ T cells are jeopardized due to glucose deprivation known as the “**Warburg effect**”[30]. Subsequently, a drop in the frequency of nucleo-cytosolic acetyl-CoA pools can be noticed. Indeed, intracellular acetyl-CoA concentrations are heavily dependent on the breakdown of glucose into pyruvate during glycolysis (Figure 1) [31]. This might contribute to explain the unique histone acetylation landscape that differentiates effector $CD8^+$ T cells from exhausted T cells [32].

In line with this, acetate supplementation can promote histone acetylation and chromatin accessibility, thereby restoring IFN-γ production in T cells rendered hyporesponsive by glucose deprivation; this was observed in T cells isolated from both B16 melanoma tumors, and the blood of patients chronically infected with hepatitis C virus [31]. Of note, transitory glucose restriction was recently reported to enhance donor-derived $CD8^+$ T cell tumor infiltration and function in a mouse B16 melanoma model [33]. This not only suggested sustained functional changes induced by nutrient availability, but also refined the general notion that glucose deprivation can harm T cell effector functions. Unfortunately, the authors did not explore the epigenetic aspects of their findings. In addition to histone acetylation, an elegant study shed some light on how lactate, as a byproduct of glycolysis, could be utilized in a new histone modification, histone lysine lactylation, and re-shape the epigenome of murine macrophages and human cancer cells [34]. The latter finding opened various questions on the broader roles of this modification. Indeed, elevated lactate concentrations in the TME can promote immunosuppression [35], and tumor-associated macrophages isolated from B16 melanoma and Lewis lung carcinoma tumors demonstrate a positive correlation between an anti-inflammatory phenotype and histone lactylation [34]. Thus, how are T cells affected by increased lactate amounts in the TME? Is there a connection between histone lysine lactylation in $CD8^+$ TILs and an exhausted phenotype?

Apart from controlling the availability of substrates for epigenetic modifications, glucose metabolism strictly regulates the activity of epigenetic enzymes. For instance, in one study, under conditions of glucose restriction, the expression of methyltransferase EZH2 was restricted by microRNA (miRNA)26a and miRNA101 in mouse CTLs [36]. This led not only to reduced cytokine expression and diminished cytotoxicity [36], but also to general metabolic insufficiency and CTL exhaustion, relative to controls [37]. This influence of glucose availability on $CD8^+$ T cell epigenetics and function is clinically relevant. High infiltration of $EZH2^+$ $CD8^+$ T cells in ovarian cancer patients correlates with a particularly long term survival [36]. Similarly, with nutrient deprivation, methyltransferase G9a can dissociate from the *LC3B, WIPI1,* and *DOR* promoters, whose products are required for the formation of **autophagosomes** [38,39] -- essential cellular structures for replenishing scarce nutrients. Collectively, these studies directly or indirectly highlight the importance of an epigenetic approach to overcome T cell dysfunction in a low-in-glucose TME (Box 3).

Free fatty acids and cholesterol

Exogeneous free fatty acid uptake, lipid metabolism, and the concentration of lipids in the T cell plasma membrane can affect T cell functioning [40,41]. Even if glucose-derived acetyl-CoA is an important source of substrates for histone acetylation, lipid-derived acetyl-CoA obtained through β-oxidation has been suggested to be equally important [42]. However, this finding contradicts earlier claims [43], emphasizing the necessity to investigate the context-dependent role of free fatty acid oxidation in epigenetic regulation.

Cholesterol is of vital importance as it specifically affects TCR clustering and the formation of an **immunological synapse**; pharmacologically inhibiting cholesterol esterification has resulted in an increased plasma membrane cholesterol concentration, boost in CTL function, and tumor control in mouse models of melanoma [44]. One of the main players preserving

cholesterol metabolism equilibrium in human primary $CD8^+$ T cells is the epigenetic regulator RORα: indeed, the RORα/ histone deacetylase (HDAC) complex acts as a transcriptional repressor of ***ACAT1/2*** and ***ABCA1*** [45]. Moreover, since the two-carbon acetate group of acetyl-CoA is used for cholesterol synthesis, alterations in cholesterol metabolism influence cellular acetyl-CoA pools in vitro [42,46], and thus, may affect the epigenetic histone acetylation landscape of $CD8^+$ TILs. Nevertheless, the general scarcity of reports on cholesterol metabolism and epigenetics in cancer does not allow to make general statements to date. Hence, unraveling the epigenetic machinery of TILs might offer an answer to how cholesterol metabolism might support cancer cells while impairing immune cell functions.

Amino acids

Glutamine is a major fuel required for maintaining the tricarboxylic acid (TCA) cycle and a key source for lipid synthesis in both cancer cells and CTLs [47]. Consequently, the disrupted glutamine metabolism in $CD8^+$ TILs will result in an imbalance of metabolic intermediates from the TCA cycle, such as α-ketoglutarate (α-KG). This in turn, influences the epigenome of $CD8^+$ T cells (Figure 1) [47]. For instance, the presence of α-KG is crucial for the activity of histone and DNA demethylation enzymes such as Jumonji N/C terminal domains (**JmjCs**) and ten-eleven translocation (**TETs**) enzymes. Additionally, α-KG is oxidized within the TCA cycle and converted into succinyl-CoA. Then, succinyl-CoA can provide the necessary substrate for the histone modification known as histone succinylation [48,49], while also inhibiting TET- and JmJC-mediated demethylations [50]. This whole process has been deemed necessary for the maintenance of anti-tumor effector T cell functions[50]. Of note, JHU083 (developed in [10])-- a prodrug of the glutamine antagonist 6-diazo-5-oxo-L-norleucine-- reduced tumor growth and improved survival when injected subcutaneously in murine MC38 and CT26 colon cancer, EL-4 lymphoma, and B16 melanoma mouse models. It also demonstrated efficacy in boosting $CD8^+$ T cells by enhancing their acetate metabolism to fuel the dysregulated TCA cycle [10], which might be interesting for future testing.

$CD8^+$ T cells predominantly uptake exogenous methionine to maintain their cellular S-adenosyl-methionine (**SAM**) pools [51], the universal methyl donor for DNA, RNA and protein methyltransferases (Figure 1). As such, T cell activation seems to go hand in hand with methionine uptake and the generation of SAM, sustaining histone and RNA methylation [52,53]. However, due to the upregulation of methionine transporter SLC43A2 on both human and murine cancer cells, $CD8^+$ TILs are known to undergo methionine deprivation, resulting in a decrease of histone methylation and cytokine production, relative to controls [52]. This illustrates again how activated T cells may share many metabolic similarities with cancer cells, and how the competition for metabolites deriving thereof can impair the anti-tumor effector functions of $CD8^+$ TILs through epigenetic mechanisms. We should, however, be careful with ‘wearing $CD8^+$ T cell blinders’. Although supplementation seems like a plausible solution, mice placed on a methionine-restricted diet have demonstrated slow tumor growth in two patient-derived xenograft mouse models of colorectal cancer with RAS mutation [54].

Potassium

Potassium is released from necrotic cancer cells into the TME, causing profound suppression of $CD8^+$ TIL functioning [55]. Indeed, the concentrations of K^+ in the interstitial fluid of mouse and human tumor tissues can be 5–10 times higher than in (normal) serum [55]. This results in an upsurge of intracellular K^+ in $CD8^+$ T cells, eventually blocking TCR-mediated activation of the Akt/mTOR signaling pathway, in a PP2A phosphatase-dependent manner (based on genetic disruption of PP2A via overexpression of a dominant-negative isoform (PP2A_DN) or via short-hairpin-mediated RNA interference of the PP2A subunit *Ppp2r2d*) [55]. Furthermore, altered transmembrane potassium concentration and membrane potential has led to functional caloric restriction in mouse $CD8^+$ T cells, characterized by, among others, enhanced mitochondrial metabolism [56]. Consequently, nucleo-cytosolic acetyl-CoA concentrations were reduced, favoring its use in mitochondria for oxidative phosphorylation. The latter impacted histone acetylation at effector- and exhaustion- relevant loci, i.e. reduced H3K9 acetylation of *Ifng, Pdcd1, Cd244, Havrc2,* and *Klrg1,* (suppressing effector CTL programs). At the same time, the potassium-mediated starvation response also restricted the availability of methionine intermediates, curtailing methylation of histone marks that typically quell **stemness**-associated programs [56]. Of note, apart from the availability of metabolites, other components of the TME, including ion concentrations, are able to alter the metabolism of $CD8^+$ TILs. Hence, new research projects should consider all the so-far unexplored components of the tumor interstitial fluid.

Hypoxia

The TME is characterized by areas of oxygen scarcity, directly impacting the activity of enzymes and substrates involved in epigenetic regulation. Hence, as hypoxia reduces the use of OXPHOS and enhances glycolysis in CTLs, a broad impact on the epigenetic level can be expected (Figure 1) [7]. For instance, low oxygen concentrations promote glutamine import, glutaminolysis, and the synthesis of α-KG [57]. However, tumor hypoxia subsequently induces the conversion of α-KG into 2-hydroxyglutarate (2-HG)-- a competitive inhibitor of α-KG-dependent enzymes, resulting in increased methylation of DNA and histone repressive marks, relative to controls [58]. In $CD8^+$ T cells specifically, 2-HG has been shown to accumulate through HIF-1α-mediated LDHA expression inside the cells; and, the excess supply of 2-HG has led to altered DNA methylation patterns, particularly due to the inhibition of H3K27me2/3 demethylase KDM6A and TET enzymes [59]. Moreover, hypoxia can induce histone and DNA methylation in a HIF- and 2-HG-independent manner; indeed, certain histone demethylases, such as KDM5A and KDM6A, are oxygen sensitive [60,61]. Similarly, under pathophysiological oxygen concentrations found in tumors, TET enzyme activity has been reported to be reduced, relative to controls [62]. However, given that most work on the influence of hypoxia on immune cells has so far been conducted using *in vitro* systems, more work is needed to determine how these data can be translated to the *in vivo* setting in the context of the TME.

Oxidative stress

On the one hand, the TME is characterized by high concentrations of ROS that cause direct cellular damage, but also serve as signaling molecules [63]. The effect of ROS on epigenetic T cell regulation has recently been studied *ex vivo* and in LCMV infection *in vivo* mouse models [64]. Phosphatase of activated cells 1 (PAC1) was selectively upregulated in exhausted $CD8^+$ TILs upon ROS exposure [64]. It accumulated on chromatin and recruited a HDAC1–HDAC2 complex, eliciting chromatin-closing enzymatic activity [64]. This is of translational importance, considering that multiple studies using PAC1 knockout mice have demonstrated attenuated cancer progression [65,66]; indeed, PAC1 expression is aligned with the expression of various inhibitory receptors [64], and its expression has also been associated with poor prognosis for certain cancer patients (e.g. colon cancer and ovarian carcinoma) [64,67]. Nitric oxide (NO) can also be present in the TME, produced, among others, by cancer cells themselves, as well as by NO synthase-positive tumor-infiltrating myeloid cells [68]. In a EG7 murine lymphoma model, it was reported that this NO might be essential for the anti-tumor activity of CTLs [69]. Possibly, this might be the result of an epigenetic modification, given that NO is capable of directly inhibiting the catalytic activity of the histone demethylase KDM3A [70]; however, this remains to be directly tested.

On the other hand, ROS are also generated as a byproduct of numerous enzymatic reactions in T cells themselves, playing an important role in cellular physiology [71]. This homeostasis can be disrupted in different malignancies. For example, glucose, glutamine, or pyruvate starvation, induce superoxide production. Since pyruvate functions as an antioxidant [72], a low concentration of the latter might contribute to enhanced ROS amounts in CTLs, warranting further investigation. In line with this, $CD8^+$ TILs in renal cell carcinoma display reduced glucose uptake concomitant with hyperpolarized, fragmented mitochondria producing large amounts of ROS [25]. Enhanced concentrations of mitochondrial superoxide result in a decrease in total DNA methylation [71]. This might be caused by a disrupted methionine cycle, in view of its tight regulation with SAM's metabolism; however, this remains hypothetical (Figure 1) [71]. Consequently, due to disrupted metabolic homeostasis, $CD8^+$ T cell activation is flawed and CTLs are unable to appropriately perform their anti-tumor functions [25]. Of note, recently, one study demonstrated that antioxidants could reactivate gene expression of loci known to be inaccessible in exhausted T cells [27].

Finally, the reduction of nicotinamide adenine dinucleotide $(NAD)^+$ to NADH is a limiting reaction for the glycolysis pathway-- a factor highly demanded in many other redox reactions, i.e. TCA cycle and fatty acid oxidation. Sirtuins (SIRT1-7) are NAD^+-dependent class-III HDACs. In line with this, cytotoxic $CD8^+$ T cells with enhanced glycolytic capacity exhibit decreased expression of SIRT1, promoting both glycolysis and secretion of granzyme B [73].

Oncometabolites

An important oncometabolite is 2-Hydroxyglutarate (2-HG). In cancer cells, mutations in the isocitrate dehydrogenase (IDH) enzyme result in the loss of its ability to convert isocitrate to α-KG. Instead, IDH acquires the ability to catalyze the NADPH-dependent

reduction of α-KG to 2-HG [74,75]. Tumor cell-derived 2-HG is taken up by activated $CD4^+$ and $CD8^+$ T cells, where it interferes with α-KG-dependent demethylases (Figure 1) [76]. The latter can impair T cell tumor infiltration, suppress early TCR signaling events, and hamper T cell anti-tumor immunity (e.g. mouse and human gliomas) [77,78]. These data add to our understanding of how tumors can progress despite the infiltration of T cells that harbor the ability to destroy it.

Therapeutically tackling epigenetics in onco-immunology

During the last couple of years, epigenetic therapy has emerged as a promising strategy to combat malignancy, either on its own, or in combination with other treatments [81]. Hence, epigenetic modifiers could be used to harness the adaptive (antigen)-specific immune response against certain cancers. Two main strategies can be envisioned *(i)* targeting (tumor-infiltrating) $CD8^+$ T cells *in vivo* (Table 1) and *(ii)* endorsing the (*ex vivo*) generation of superior anti-tumor T cell grafts for ACT.

Improving *in vivo* $CD8^+$ T cell responses

An important goal of cancer immunotherapy is to avert the exhausted phenotype of $CD8^+$ TILs while promoting an effector state. Since a *de novo* DNA-methylation program controls the formation of fully exhausted T cells, the use of DNA methyltransferases (DNMT)-inhibiting cytosine nucleoside analogs, such as **decitabine**, could block the establishment of an exhaustion-associated epigenetic signature [16]. In a phase I/II clinical trial enrolling patients (n=100) with solid tumors or B cell lymphoma, treatment with decitabine promoted **Th1 polarization** and CTL cytolytic activity (Figure 2) (NCT01799083)[II] [82]. This study supports the feasibility of using epigenetic modulators as candidate anti-tumor therapeutics targeting $CD8^+$ T cells in the TME.

Alternatively, the reversal of the exhaustion status of $CD8^+$ TILs can be envisioned. By focusing on DNA and histone methylation, targeting the phosphorylated **LSD1 pathway** might be promising, since it is enriched in exhausted T cells. LSD1 forms nuclear complexes with Eomesodermin (**EOMES**) in dysfunctional $CD8^+$ T cells of immunotherapy-resistant melanoma and breast cancer patients [83]. It demethylates and acetylates key lysine residues within the nuclear localization sequence motif or DNA-binding motif of EOMES to restrict nuclear translocation (Figure 2) [83]. This finding is of particular interest, considering it is, to our knowledge, the first report to tackle the so far considered “immunotherapy-resistant” terminally exhausted T cell population (Box 2), and harness this population back into the anti-tumor immune response [16,17].

Regarding histone acetylation, a first exciting target is the serine/arginine-rich splicing factor 2 (SRSF2)-- a regulator of the expression of multiple immune checkpoint molecules. SRSF2 controls their transcription by modifying the H3K27ac status of the relevant gene promoters in human renal cell carcinoma $CD8^+$ T cells [84]. A second strategy includes reinvigorating **Bhlhe40**-deficient $CD8^+$ T cells [85]. Bhlhe40 is a stress-responsive transcription factor, essential for maintaining effector gene acetylation via its support of TCA-cycle activity and OXPHOS. Bhlhe40 expression in $CD8^+$ TILs was suppressed by local **PD-1** signaling in a B16 mouse melanoma model, hampering $CD8^+$ T cell fitness and polyfunctionality

[85]. Upon administration of Tubastatin A, a HDAC6/8 inhibitor, or the supplementation of acetate, effector functions and persistence of $CD8^+$ TILs could be enhanced (Figure 2) [85].

Finally, some noncoding RNAs have been implicated in modulating the exhaustion state of $CD8^+$ T cells [86]. For instance, miRNA-155 can restrict $CD8^+$ T cell functional exhaustion by promoting the expression of **Phf19** via phosphorylated AKT. This leads, in turn, to enhanced **PRC2** functionality, and ultimately restrains T cell senescence by inhibiting key transcription factors driving terminal differentiation and exhaustion [87]. This suggests that targeting these miRNAs might be considered as a putative therapeutic strategy for certain cancers, for example, by investigating miRNA mimetics or miRNA antagonists. Collectively, with a better understanding of the epigenetic states and the molecular pathways that drive $CD8^+$ T cell exhaustion in tumors, epigenetic drugs could have important roles by synergizing with other anti-cancer therapies or in reversing acquired tumor resistance.

Combination therapy

Synergistic effects of epigenetic drugs and established immunotherapeutics are foreseeable. To further improve the success rate of immune checkpoint blockade, different clinical trials are currently ongoing combining epigenetic modifiers and checkpoint inhibitors. This has been the result of encouraging data observed in murine tumor models where several combinations have been tested: bromodomain and extraterminal domain (BET) inhibitor + anti-PD-1 monoclonal antibodies (mAbs) (Ab) [88,89], DNMT inhibitor + anti-CTLA-4 mAbs [90], DNMT inhibitor+ anti-PD-(L)1 mAbs [16,91–93], HDAC inhibitor + antiPD-1 mAbs [94–96] and EZH2 inhibitor + anti-CTLA-4 mAbs [97]. Agonistic mAbs targeting costimulatory molecules constitute an alternative to checkpoint blockade [98]. **Urelumab**, a fully human IgG4k mAb agonist of CD137/4–1BB, regulates DNA methylation, poising $CD8^+$ T cells to respond more robustly upon antigen rechallenge [99].

Epigenetic drugs have also been studied in combination with adoptive T cell therapy, improving the activity and expansion of adoptively transferred $CD8^+$ T cells. In a B16 melanoma model, the addition of HDAC inhibitor **dacinostat** to T cell vaccination therapy promoted its therapeutic action through *(i)* effects on target cancer cells, *(ii)* a decrease in competing endogenous lymphocytes and *(iii)* an upgrade of adoptively transferred lymphocyte functional activity [100]. Similar results were obtained with panobinostat (Figure 2) [101].

A more indirect approach is also possible. For example, the combination of two epigenetic modifiers; **ricolinostat**, a HDAC6 inhibitor, and **JQ1**, a specific inhibitor of BET proteins, shows potential for the treatment of non-small cell lung cancer. This combination promotes T cell-mediated anti-tumor immunity and, as such, immune-mediated tumor growth arrest. Ricolinostat improves the functioning of antigen-presenting cells, leading to an enhanced activation of $CD8^+$ TILs and secretion of effector cytokine IFN-γ. At the same time, the suppressive functions of **regulatory T cells** are reduced by JQ1, increasing the $CD8^+$ T cells / regulatory T cell ratio [102]. In line with this, the combination of HDAC6 and BET inhibition shows potential in a multiple myeloma model. It efficiently reduces c-MYC expression and counteracts the BET inhibition-mediated HDAC6 upregulation, associated with cancer progression and drug resistance [103].

Currently, although the rationale and data seem promising, it is still too soon to make a ruling on the translatability of these findings to humans and, as such, on their clinical significance. Considering the vast number of ongoing clinical trials with epigenetic drugs, in combination with other (immuno)therapeutics or alone, the upcoming years are expected to inform on the subject. Here, special attention will be required in selecting patients who might gain the greatest benefit. Might there be a role for epigenetic biomarkers? Further research should address this possibility.

Adoptive cell transfer improvement

One of the current challenges of **ACT** is the improvement of persistence and durability response. The *in vitro* expansion of $CD8^+$ T cells to attain the large numbers required for vaccination, inevitably goes hand in hand with T cell differentiation and loss of proliferative potential [104,105]. To obtain the essential proportion of **stem cell memory T cells** and central memory T cells in a graft, epigenetic modifiers can be employed. Bromodomain inhibitor JQ1 has been shown to maintain these T cell subsets with desired proliferative capacity. In an acute lymphoblastic leukemia (ALL) xenograft mouse model with Nalm-6 pre-BALL human cells, JQ1-treated T cells showed greater cell persistence and superior anti-tumor effects [105]. Data should, however, be treated carefully, since they contradict a more recent study, demonstrating reduced efficacy in a murine B16 melanoma model [106]. Treatment duration and inhibitor concentration could underlie this discrepancy. Timing, choice, and concentration of the epigenetic modifier may prove to be decisive. An alternate approach to reduce T cell exhaustion, improve effector functions and persistence, is the use of DNMT inhibitors during $CD8^+$ T cell expansion [16,107]. The addition of decitabine during **chimeric antigen receptor** (CAR) T manufacturing has improved the tumor-homing ability and anti-tumor potential of the graft, in both Nalm-6 ALL and Raji non-Hodgkin's lymphoma mouse models [108]. One case described a chronic lymphocytic leukemia (CLL) patient who achieved a complete response, with no evidence of CLL in his bone marrow, due to the expansion of one T cell clone with a CAR lentiviral integration site in the *TET2* gene [109]. As such, the disruption of the *TET2* gene and the resulting changes in the epigenetic landscape gave rise to a T cell clone with a significant long-life span, underpinning clinical efficacy of CAR T cell therapy in the concerning patient [109]. Further research on the effects of knocking out the *TET2* gene and the effects on CAR T cell survival is therefore needed.

In view of the earlier discussed approaches involving DNMT and TET2 inhibition, we should briefly touch upon the current provocative nature of the topic. Loss-of-function mutations in *DNMT* and *TET* occur frequently in patients with, among others, ALL and acute myeloid leukemia, and are associated with poor prognosis [110,111]. Recent insights coming from clonal hematopoiesis studies suggest that null mutations in these genes allow for preservation of a stem-like state in hematopoietic cells [110,112–115]. Hence, although transformation to malignancy requires additional mutations, and as such, the *DNMT* and *TET* mutations do not drive malignancy *per se*, therapeutic approaches that center on modifying the enzymatic activity of these epigenetic modifiers should be carefully monitored for long-term effects.

The generation of a universal off-the-shelf CAR T cell product is another hurdle to be tackled to enhance the feasibility and diffusion of this treatment. To attenuate the development of graft-versus-host disease, epigenetic modification could yet again be a solution. DOT1L, a H3 lysine-79 specific methyltransferase, plays different roles in cancer as well as T cell differentiation in both mice and humans [116–118]. DOT1L has been shown to repress allogeneic T cell responses, while retaining potent anti-tumor activity [119]. Mechanistically, the inhibition of DOT1L reduces miRNA-181a expression, which is followed by an upregulation of the genes encoding phosphatases DUSP2 and DUSP6. In turn, DUSP6-mediated ERK dephosphorylation selectively ameliorates low-avidity T cell responses through the modulation of TCR sensitivity (Figure 2) [119]. Of note, HDAC11 associates with the *Eomes* and *Tbet* gene promoter regions in resting cells, inhibiting $CD8^+$ T cell effector functions, and disassociates upon activation. Hence, T cells from HDAC11 knockout mice are hyperresponsive, mediating sturdy anti-tumor activity as well as more forceful graft-versus-host disease [120]. Including the data above, targeting T cell epigenetics holds great potential for cancer therapies. Still, caution is advised concerning acute or long-term toxicity. To cope with (un)known toxicities, in case of the *ex vivo* treatment of T cells with epigenetic drugs, a safety switch could be included in, for example, the CAR design; namely, CAR constructs that also express a suicide gene [121–123] or co-express a cell-surface elimination marker [124,125]. Both methods can result in the irreversible depletion of administered cells, and, as such, cope with their toxicity.

Concluding remarks

A compelling body of evidence endorses the potential of immunotherapy in oncology; however, ample, and robust cross-disciplinary work remains to be done to maximize its clinical efficacy for different malignancies. We find interesting that epigenetic mechanisms affect all aspects of the cancer-immunity cycle. As such, this review highlights the concept that epigenetic therapies might represent novel immunotherapies themselves, harnessing once again, the adaptive $CD8^+$ T cell response in the fight against malignancy. Therefore, delving into $CD8^+$ T cell epigenetics and its regulating role in T cell biology and function, can ideally result in the discovery of targeted pharmacological or genetic interventions, supporting current treatment strategies against cancer (Outstanding Questions Box). An important challenge will be to distinguish between the dysregulation of driver genes and those which result from changes in driver genes. Hence, further research should strive to elucidate the exact roles of epigenetic modifications in $CD8^+$ T cell differentiation, effector functions, and exhaustion. In the context of cancer, we should try to address whether the epigenetic profiles of T cells influence T cell trafficking and homing into tumors, and once arrived in the TME, how the immunosuppressive imprinting might be reversed. Finally, since it has become clear that cancer treatments should progress from one-size-fits-all to personalized therapy, epigenetic biomarkers and epigenetic signatures could especially be of interest.

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Glossary

ABCA1
This protein functions as a cholesterol efflux pump in the cellular lipid removal pathway

ACAT1–2
Cholesterol esterification enzymes that convert free cholesterol to cholesteryl esters for storage

Adoptive cell transfer (ACT)
CTL vaccination, with the goal of recognizing, targeting, and destroying tumor cells. With the ACT of T cell receptor (TCR)- or chimeric antigen receptor (CAR)-engineered T cells, expanded from readily available blood $CD8^+$ T cells, it is now theoretically possible to target any tumor

Autophagosomes
double-membrane sequestering vesicles contributing to the recycling of cytosolic components and organelles when nutrients are scarce

BET proteins
epigenetic readers that control gene transcription by binding to acetylated lysine residues on histones

Bhlhe40
also known as Bhlhb2, belongs to a family of basic helix-loop-helix transcriptional factors. Under cellular stress conditions (such as hypoxia), it translocates to the nucleus where it promotes gene transcription by binding to E-box elements

Chimeric antigen receptor (CAR) T cells
T cells modified with artificial receptor proteins consisting of (i) an ectodomain binding directly a tumor-specific molecule on the cell surface, (ii) an extracellular hinge/spacer and a transmembrane domain spanning the membrane, and (iii) an endodomain providing T cell signaling

Dacinostat
potent pan-HDAC inhibitor

Decitabine
DNA methyltransferases-inhibiting cytosine nucleoside analog

Eomesodermin (Eomes)
T-box transcription factor, implicated in $CD8^+$ T cell exhaustion

EZH2
Histone methyltransferase catalyzing the methylation of H3K27, resulting in chromatin compaction and repression of transcription

G9a
Histone methyltransferase for repressive H3K9me2 marks

Glycolysis
Cytoplasmatic ATP-yielding catabolism of glucose

HIF-1α
The hypoxia signaling pathway is primarily governed by hypoxia inducible transcription factor-1α

Immune checkpoint inhibitors
antibodies directed against checkpoint inhibitory receptors such as cytotoxic T lymphocytes antigen-4 (CTLA-4), programmed death-1 (PD-1) and programmed death-ligand 1 (PD-L1), with the goal of breaking immune tolerance and stimulate $CD8^+$ T cell effector responses

Immunological synapse
stable cell-cell junction between a leukocyte (e.g. T cell) and an antigen-presenting cell, allowing (contact-dependent) communication between two immune cells

Jumonji N/C terminal domains (JmjCs) enzymes
important family of histone lysine demethylases (KDMs)

JQ1
aspecific inhibitor of the BET protein family; notable for its high affinity for bromodomains

LSD1
H3K4 and H3K9 demethylase, also targeting non-histone proteins (i.e. DNMT1, STAT3)

Oncometabolites
Conventional products of metabolism aberrantly accumulating in cancer cells, possessing pro-oncogenic capabilities

Phf19
PHD finger protein 19; a key component of PRC2

PRC2
multisubunit protein complex; a methyltransferase regulating gene expression by catalyzing trimethylation of histone H3 on lysine 27 (H3K27me3), leading to transcription repression

Oxidative phosphorylation (OXPHOS)
Mitochondrion-based oxidation of metabolites, generating ATP through complexes I–V

Regulatory T cells
subset of $CD4^+$ T cells, essential for maintaining peripheral tolerance via the secretion of negative regulatory (anti-inflammatory) cytokines, i.e. IL-10 and TGF-β

Ricolinostat
selective inhibitor of HDAC class 6, also inhibiting HDAC class 1–3 at high concentrations

RORα
member of the orphan nuclear receptor (ONR) family. It binds to hormone response elements upstream of several genes, boosting their expression

S-adenosyl-methionine (SAM)
synthesized in the methionine pathway; universal methyl donor for DNA, RNA, and protein methyltransferases

Stemness
capacity of a cell to perpetuate its lineage, having both the ability for self-renewal and differentiation

Stem cell memory T cell
long-lived memory T cells gifted with stem-like properties (self-renewal and multipotency)

TCA (tricarboxylic acid) cycle
Krebs cycle or citric acid cycle; stepwise oxidation of acetyl-CoA (fatty acid oxidation), glutamate (glutaminolyse) or pyruvate (glycolysis), producing NADH and FADH, which in turn fuel the electron transport chain

T cell exhaustion
describes the functionally impaired differentiation state of T cells induced by persistent antigen stimulation

Tumor microenvironment (TME)
composed of immune cells, extracellular matrix, fibroblasts, proteins, and blood vessels that surround and feed the tumor. The inherent inflammation within the TME and its nourishment by a failing vascular network enable tumor progression, metastasis, and resistance to therapies

Ten-eleven translocation (TET) enzymes
Active DNA demethylation is carried out by enzymes belonging to the TET family; TET1, TET2 and TET3. They mediate the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC)

Th1 polarization
Differentiation of $CD4^+$ T cells into Th1 cells, characterized by the secretion of IFN-γ and TNF-β, promoting cell-mediated immune responses

TOX
member of the HMG (high mobility group) transcription factors

Urelumab
fully human IgG4k mAb agonist of CD137/4–1BB

Warburg effect
aerobic glycolysis; describes the preference of tumor cells for aerobic glycolysis (followed by lactic acid fermentation) over OXPHOS, even in the presence of abundant oxygen

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Text Box 1 -

Basic epigenetic modifications at a glance

As extensively outlined in multiple reviews [126–129], epigenetics refers to changes made “on top of” genetics; i.e. modifications made to DNA or chromatin that do not interfere with the DNA sequence itself, and include DNA methylation, the post-translational modification of histones (PTMs) and the effects of noncoding RNAs. This creates an opportunity for cells with identical underlying genomes to exhibit different phenotypes in response to environmental cues. It should, however, be noted that classifying noncoding RNAs under the heading of epigenetics is debated. Some researchers also advocate to define processes regulated by noncoding RNAs as non-genetic (rather than epigenetic) modifications.

DNA methylation

DNA methylation is a covalent modification catalyzed by a family of DNA methyltransferases (DNMTs); DNMT1, DNMT3a and DNMT3b. DNMTs add a methyl group to the 5-position of the cytosine ring, converting it to 5-methylcytosine (5mC). While the primary effect of DNA methylation is making the nucleosome relatively inaccessible (inhibiting transcription), the opposite effect can also be observed through binding of repressors, leading to gene activation. Active DNA demethylation is carried out by enzymes belonging to the ten-eleven translocation (TET) family; TET1, TET2 and TET3. They start with the conversion of 5mC to 5-hydroxymethylcytosine (5hmC).

Post-translational modification of histones

Acetylation, methylation, phosphorylation, and ubiquitination are well-known PTMs, but far from the only ones described. PTMs are catalyzed by histone-modifying enzymes, which add (writers), recognize (readers) or remove (erasers) PTMs. The resultant modification status of histones affects chromatin compaction and accessibility in two main ways: *(i)* neutralization of the charge of amino acids and *(ii)* recruitment of regulatory proteins. By way of illustration, histone acetylation defuses the positive charge of lysine residues, opening the chromatin structure. This is managed by histone acetyltransferases (HATs) and undone by histone deacetylases (HDACs).

Noncoding RNAs

The heterogeneous family of the noncoding RNAs consists of functional RNA molecules which are not further translated into proteins, including long noncoding RNA (lncRNA), piwi-interacting RNAs (piRNAs), microRNA (miRNA), ribosomal RNA (rRNA), small interfering RNAs (siRNAs) and transfer RNA (tRNA). Since noncoding RNAs represent up to 60% of the transcriptional output in human cells, it may come as no surprise that they play an import role in regulating cellular processes, including chromatin remodeling, transcription and PTMs.

Text Box 2 –

Exhausted T cell differentiation states

Exhausted T cells represent a range of subsets linked in a hierarchical developmental pathway [130]; (i) progenitor 1 exhausted T cells ($Ly108^{+}CD69^{+}TCF1^{high}TOX^{high}$) are quiescent and reside in lymphoid tissues, (ii) progenitor 2 exhausted T cells ($Ly108^{+}CD69^{-}TCF1^{int}TOX^{high}$) are highly proliferative and migrate into the circulation, (iii) intermediate exhausted T cells ($Ly108^{-}CD69^{-}TCF1^{-}TOX^{int}T\text{-}bet^{high}$) are mildly cytotoxic and are found in the circulation and blood-accessible organs, and (iv) terminally exhausted T cells ($Ly108CD69^{+}TCF1^{-}TOX^{high}T\text{-}bet^{low}Eomes^{high}$), which are resident. These four phenotypes were found in exhausted $CD8^{+}$ T cells of LCMV-clone-13-infected mice, in mouse B16 tumors and among TILs from human melanoma [130]. Hence, progenitor exhausted and terminally exhausted $CD8^{+}$ TILs have distinct epigenetic states and functioning [131,132]. For example, only the former respond to PD-1 blockade and can be re-invigorated by the administration of checkpoint inhibitors. [132,133]. This knowledge has been a major advance in our understanding of T cell exhaustion; rather than being a homogenous $CD8^{+}$ T cell phenotype, exhausted T cells are demarcated into different compartments in analogy with, for example, memory $CD8^{+}$ T cell subsets. Therefore, an imminent question to be answered is how to enhance the rejuvenation of all these exhausted $CD8^{+}$ T cells.

Box 3.

Indirectly targeting epigenetic signatures by rewiring cellular metabolism

An underexploited research avenue is the potential of metabolic interventions to reprogram the epigenome of exhausted T cells. As clearly emerges from the data discussed in this review, the T cell epigenome is heavily influenced by nutrient availability and the overall condition of the TME. This goes hand in hand with $CD8^+$ T cell fitness. Also, the specific metabolic environment of the tumor may underlie the differentiation towards an exhausted phenotype in TILs. Hence, breaking the immune suppressive barrier of the TME by reprogramming the intrinsic metabolism of tumor-reactive T cells and preventing a pro-tumor epigenetic landscape to be established, is an emerging and promising area of cancer immunotherapy. Although still in its initial phase, in vivo epigenetic remodeling through a CRISPR-associated Cas9 system could be interesting in this context [134–136]. However, different questions will need to be answered first: which metabolic alterations in cancer cells/$CD8^+$ T cells are able to sustain T cell fitness in the immunosuppressive TME? Which delivery method can be used *in vivo*? What can be said about tissue distribution and off-target effects? How do we prevent the unintended modification of other cells and tissues, potentially leading to unwanted side-effects?

Outstanding Questions Box

- What are the epigenetic hallmarks of the truly protective $CD8^+$ T cells in cancer patients? Namely, which epigenetic modifications render cytotoxic T cells resistant to the immunosuppressive milieu of the tumor micro-environment (TME)?
- How do the intracellular concentrations and fluxes of different metabolites impact the epigenetic signature of $CD8^+$ T cells? Are the types of post-translational modifications, a direct reflection of the metabolite content of the cell?
- Can we reprogram the epigenome of exhausted $CD8^+$ tumor-infiltrating lymphocytes (TILs) by altering the metabolic composition of the TME?
- Is there a direct link between nutrient intake and the microbiome of a patient on the one hand, and the epigenetic modifications observed in his/her $CD8^+$ TILs on the other?
- Can we extrapolate any knowledge from the epigenetic profile of $CD8^+$ TILs to treatment success in solid tumor patients? What might be inferred for assessing epigenetically-based biomarkers?

Highlights Box

- The tumor microenvironment forms a metabolic barrier against cytotoxic $CD8^+$ T cells, hampering current immunotherapeutic strategies.
- It is increasingly recognized that epigenetics bridge cellular metabolism with gene expression.
- Tackling epigenetic modifications or cell metabolism to (i) target the epigenetic signature of (tumor-infiltrating) $CD8^+$ T cells *in vivo* or (ii) potentiate the *ex vivo* generation of anti-tumor T cell grafts for adoptive cell transfer, has gained much interest as a promising strategy to combat malignancy.

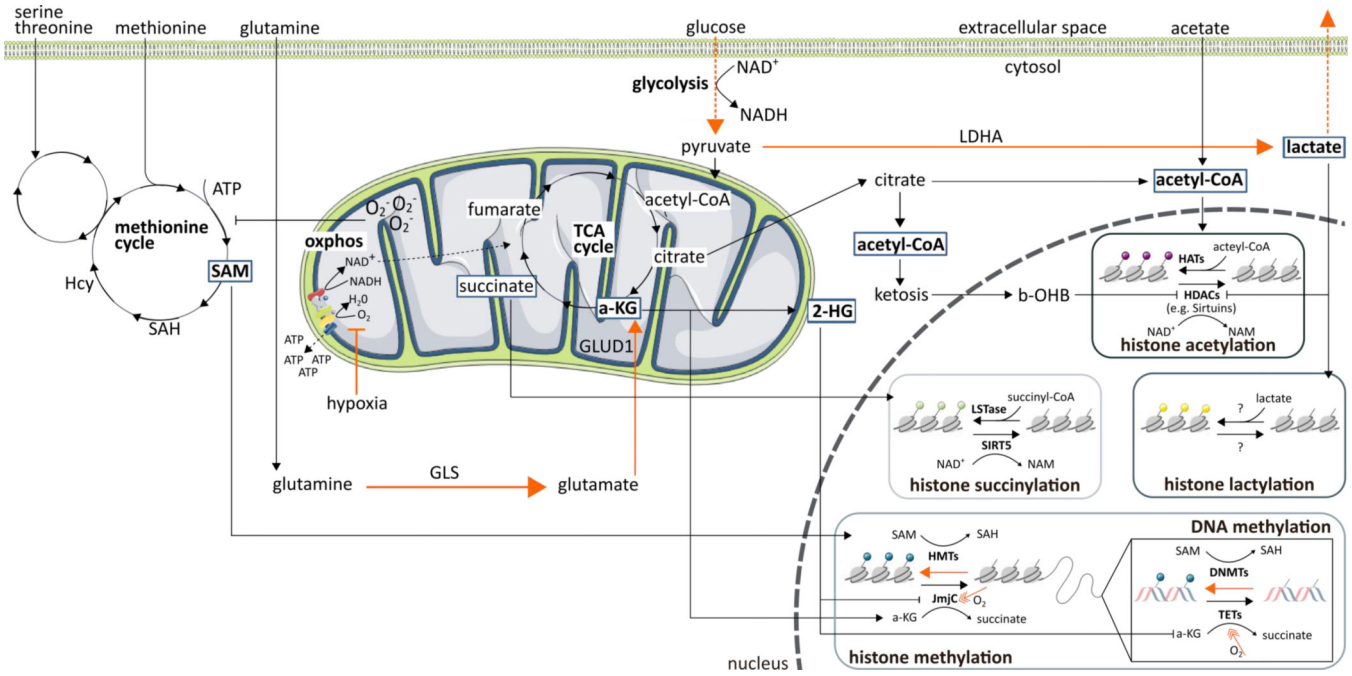

Figure 1. Relationship between immunometabolism, oxygen, and epigenetics.

Numerous metabolic intermediates as well as oxygen availability affect the cellular epigenome. The effects of hypoxia are indicated in orange.

Abbreviations: 2-HG, 2-hydroxyglutarate; a-KG, α-ketoglutarate; ATP, adenosine triphosphate;; b-OHB, β-hydroxybutyric acid; DNMT, DNA methyltransferases; GLS, glutaminase; GLUD1, glutamate dehydrogenase 1; HAT, histone acetyl transferase; Hcy, homocysteine; HDAC, histone deacetylases; HMT, histone methyltransferases; JmjC, Jumonji N/C terminal domains; LDHA, lactate dehydrogenase A; LSTase, lysine succyniltransferase; NAD^+/NADH, nicotinamide adenine dinucleotide; NAM, nicotinamide; O_2^-, superoxide; oxphos, oxidative phosphorylation; SAH, S-adenosylhomocysteine; SAM, S-adenosyl-methionine; SIRT, sirtuin; TCA, tricarboxylic acid; TET, ten-eleven translocation methylcytosine dioxygenases

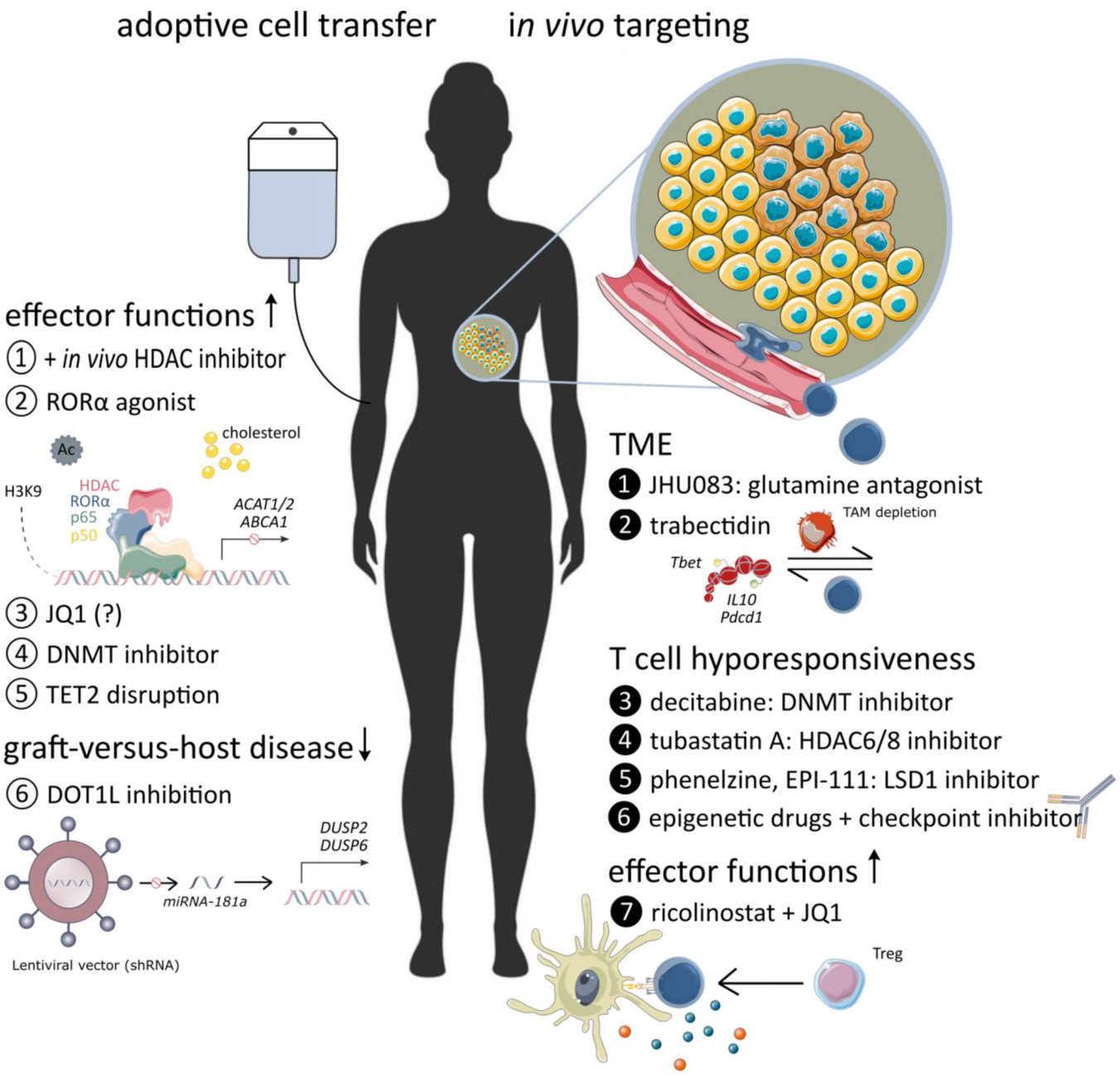

Figure 2. Explored therapeutic interventions based on epigenetic modifications

The diagram depicts the generation of superior anti-tumor $CD8^+$ T cell grafts for adoptive cell transfer (ACT) (left), and targeting (tumor-infiltrating) $CD8^+$ T cells *in vivo* (right). The remaining challenges with ACT, both at the level of efficacy and at the level of toxicity, might potentially be solved with epigenetic drugs (epidrugs). *In vivo*, epidrugs might also be at the center of reinvigorating the adaptive immune response, overcoming tumor immune escape, and resistance to therapy.

Note. Data on the effects of JQ1 are not unequivocal, (designated by '?')

Abbreviations: DNMT, DNA methyltransferases; HDAC, histone deacetylase; miRNA, microRNA; TET, ten-eleven translocation

Table 1.

The use of epigenetic drugs in the clinic (current clinical trials)[a,b]

Class	Drug	Tumor type	Clinical phase	Rationale (CD8 T cells)	Setting	Clinical trial identifier
DNMT inhibitor	Guadecitabine	Non-small-cell lung cancer	II	DNMT inhibitors induce the expression of MHC class I molecules, increase the presentation level of tumor-associated antigens and the expression of chemokines that recruit CD8 T cells, promote Th1 polarization and CTL cytolytic activity [88,135].	+ carboplatin	NCT03913455
		refractory germ cell tumor	I		+ cisplatin	NCT02429466
		Acute myeloid leukemia	II		+ cladribine + idarubicin	NCT02096055
		Leukemia	II		After DLI	NCT02684162
		Leukemia	II		After HSCT	NCT03454984
		Leukemia	III		-	NCT02907359
		Leukemia rollover study	II		-	NCT03603964
		Central chondrosarcoma	II		+ **belinostat**	NCT04340843
		Metastatic colorectal cancer	I	Aim of the combination: recruitment of $CD45RO^+$ T cells into the tumor	+ allogeneic colon cancer cell vaccine (GVAX) + yclophosphamide	NCT01966289
		Leukemia	II	DNMT inhibitor + anti-CTLA4 [88], DNMT inhibitor+ anti-PD(L)1 [89–91]	+ atezolizumab	NCT02935361
		Urothelial carcinoma	II		+ atezolizumab	NCT03179943
		Kidney cancer	II		+ durvalumab	NCT03308396
		Advanced Liver, pancreatic, bile duct or gallbladder cancer	I		+ durvalumab	NCT03257761
		Lung cancer	I		+ pembrolizumab + **mocetinostat**	NCT03220477
		Non-small-cell lung cancer Prostate cancer	I		+ pembrolizumab	NCT02998567
		Fallopian tube, ovarian or primary peritoneal carcinoma	II		+ pembrolizumab	NCT02901899
		Lung cancer, melanoma	II		+ ipilimumab + nivolumab	NCT04250246

Class	Drug	Tumor type	Clinical phase	Rationale (CD8 T cells)	Setting	Clinical trial identifier
		Metastatic colorectal cancer	II		+ nivolumab	NCT03576963
EZH2 inhibitor	CPI-1205	B-cell lymphoma	I	EZH2 HMT inhibitors may reverse the suppression of CXCL9 and CXCL10 expression and subsequently improve effector T cell infiltration in the TME [136,137]. EZH2 is activated or overexpressed by several tumors, resulting in the silencing of cancer suppressor genes and diminished antigen presentation [138–140].	-	NCT02395601
		Metastatic castration resistant prostate Cancer	I		+ enzalutamide or abiraterone/ prednisone	NCT03480646
		Advanced solid tumors	II		+ ipilimumab	NCT03525795
	Tazemetostat	B-cell non-hodgkin's lymphoma	II		-	NCT03456726
		follicular lymphoma	III		-	NCT04224493
		Mesothelioma	II		-	NCT02860286
		Metastatic prostate cancer	I		+ enzalutamide or biraterone/ prednisone	NCT04179864
		Advanced sarcoma	III		+ doxorubicin HCl	NCT04204941
		INI1-negative tumors or relapsed / refractory synovial sarcoma	II			NCT02601950NCT02601937
		Advanced solid tumors	II		-	NCT03213665
		Cancer rollover study	II		-	NCT02875548
		Lymphoma	I	EZH2 serves as a molecular switch controlling tumor immune escape. The combination with checkpoint blockade may result in synergistic effects [95,141]	+ atezolimumab	NCT02220842
		Non-small-cell lung cancer	II		+ atezolimumab	NCT03337698
		Urothelial carcinoma, metastatic bladder urothelial carcinoma	II		+ pembrolizumab	NCT03854474
	SHR2554	lymphoid neoplasms	I		-	NCT03603951
		luminal advanced breast cancer	II		+ other treatments	NCT04355858
		solid tumors or B-cell lymphomas	II	The combination of EZH2 inhibitors and PD-L1/TGF-β blockade may enhance the efficiency of immunotherapy	+ anti-PD-L1/TGF-β antibody SHR1701	NCT04407741
BET inhibitor	BMS-986158	Pediatric cancer	I	BET inhibitors increase the presence of active cytotoxic $CD8^+$ T cells in the TME [142,143].	-	NCT03936465
		Advanced tumors	II		+ nivolumab	NCT02419417
	CC-95775	Non-Hodgkin lymphoma	I		-	NCT04089527

Class	Drug	Tumor type	Clinical phase	Rationale (CD8 T cells)	Setting	Clinical trial identifier
	CPI-061	Leukemia	II		Ruxolitinib	NCT02158858
	Molibresib	Advanced and refractory solid tumors and lymphomas	I		+ **Entinostat**	NCT03925428
	MK-8628	Leukemia	I		-	NCT02698189
	ZEN-3694	Metastatic castrationresistant prostate cancer	II		+ enzalutamide, embrolizumab	NCT04471974 NCT02711956
HDACI inhibitor	Entinostat	hormone receptor-positive breast cancer	III	HDAC inhibitors have been shown to induce cancer cell apoptosis and to inhibit angiogenesis. HDAC3: negative regulator of $CD8^+$ T cell cytotoxicity program. HDAC3 inhibition leads to the enhanced expression of granzyme B and the improved eradication of B16 melanoma target cells [144]. Note. HDAC inhibitors can synergize with checkpoint inhibitors [92–94]	+ other treatments	NCT02115282 NCT03538171
HDACIII inhibitor	Nicotinamide	Non-small-cell lung cancer	III		+ gefitinib or erlotinib	NCT02416739
HDACi	Abexinostat	Renal cell carcinoma	III		+ pazopanib	NCT03592472
	Chidamide	Leukemia	III		+ other treatments	NCT03564704NCT03553238 NCT04231448 NCT04038411NCT04040491
	Panobinostat	leukemia	III		+ donor lymphocyte infusions	NCT04326764
		Myelofibrosis rollover study	III		+ ruxolitinib	NCT02386800
	Vorinostat	Cutaneous T-cell lymphoma	III		or mogamulizumab	NCT01728805
		High grade glioma	III		Bevacizumab, temozolomide	NCT01236560
	Valproic acid	High grade gliomas	III		+ temozolomide (or temozolomide + chloroquine)	NCT03243461

Note 1. The DNMT inhibitors azacytidine and decitabine are the most successful epigenetic drugs to date. Due to the overabundance of ongoing clinical trials with these drugs, only the novel hypomethylating agent guadecitabine is mentioned in this table.

Note 2. Considering the plethora of clinical trials ongoing with HDAD inhibitors, only the phase III clinical trials are mentioned