Perspective

PD-1/PD-L1 pathway inhibition to restore effector functions in exhausted CD8$^+$ T cells: chances, limitations and potential risks

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Abstract: T cell exhaustion is a well-known mechanism involved in escape of degenerated cells or certain pathogens from CD8$^+$ T cell-mediated immune surveillance, ultimately resulting in tumor development and chronic infections, respectively. Next to activated T cells, exhausted CD8$^+$ T cells typically express high levels of the programmed cell death-1 (PD-1) receptor. While interaction of PD-1 with its ligand programmed death-ligand 1 (PD-L1) on hemotopoietic and non-hemotopoietic cells is important for the re-establishment of homeostasis following immune activation, PD-1/PD-L1 interaction represents a major drawback in certain other disease settings such as cancer or chronic viral infections. Here PD-1 signalling in T cells prevents efficient anti-tumor or anti-viral immune responses. Thus, therapeutic interference with the PD-1/PD-L1 pathway represents a promising approach for releasing exhausted CD8$^+$ T cells from PD-1-dependent suppression and reactivation of effector functions. However, recent reports have highlighted unexpected outcomes of PD-1/PD-L1 pathway inhibition in the context of chronic infections. We provide here a comprehensive overview of the recent discoveries made in the context of PD-1/PD-L1 checkpoint inhibition that are considered relevant with respect to the targeted reactivation of effector functions in exhausted CD8$^+$ T cells. We briefly discuss the impact of PD-1 signalling on the expression of certain transcription factors, on epigenetic modifications affecting chromatin accessibility, on cellular metabolism and the expression of certain cytokine receptors involved in immune homeostasis. These newly uncovered facts should be carefully considered before further development of therapies targeting the PD-1/PD-L1 pathway that are aiming at the restoration of pathogen-specific and anti-tumor CD8$^+$ T cell effector functions in order to prevent adverse side effects.

Keywords: Programmed cell death 1 (PD-1); programmed death-ligand 1 (PD-L1); programmed cell death-1/programmed death-ligand 1 pathway blockade (PD-1/PD-L1) pathway blockade; CD8$^+$ exhausted T cells; chronic infection; cancer

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Programmed cell death-1 (PD-1)/programmed death-ligand 1 (PD-L1) pathway

The PD-1 receptor is expressed at the surface of activated T cells. It has two known ligands, PD-L1 and PD-L2 (B7-DC). Its high affinity ligand PD-L1 is commonly expressed at the surface of hematopoietic cells such as dendritic cells (DCs) and macrophages as well as on non-hematopoietic cells such as epithelial and endothelial cells. In contrast, PD-L2 is mainly expressed on antigen presenting cells (APCs), DCs and macrophages (1,2). Both ligands are usually described as immune checkpoint proteins that act

as co-inhibitory molecules and can terminate the effector phase of T cell-mediated immune responses (3). When PD-L1 binds to PD-1, an inhibitory signal is transmitted into the T cell that reduces cytokine production, suppresses T cell proliferation (1,4) and mainly incapacitates the cytotoxic and memory potential of $CD8^+$ T cells (5-7). The homeostatic PD-1/PD-L1 interaction ensures immune activation for only an appropriate time in order to curtail the likelihood for the development of chronic inflammation. Accordingly, disruption of the PD-1 or PD-L1 gene aggravates T cell responses resulting in the generation of self-reactive T cells, development of lupus like disorder and dilated cardiomyopathy (8,9). However, PD-1/PD-L1 interaction represents a major drawback in certain other disease settings such as cancer or chronic viral infections, where PD-1 signalling in T cells impinges on anti-tumor or anti-viral immune responses. In particular, tumor cells or pathogens causing chronic infections exploit this immune checkpoint pathway of over-expressing PD-1 and PD-L1 as a mechanism to evade cytotoxic immune surveillance (10-12).

$CD8^+$ T cell exhaustion and PD-1/PD-L1 pathway inhibition

$CD8^+$ T cell exhaustion is characterized by the dysfunctionality of the cells to mount appropriate immune responses against tumor antigens or persisting pathogens. Generally, exhausted $CD8^+$ T cells exhibit poor effector functions, which is associated with elevated expression of multiple sets of immune inhibitory receptors (13). The well-studied PD-1/PD-L1 receptor-ligand pair interaction inhibits the functional renewal of the $CD8^+$ T cell repertoire in many human chronic diseases (14,15). The extracellular region of PD-1 consists of a single IgV-like domain and its cytoplasmic tail has two motifs, immunoreceptor tyrosine based inhibitory motif (ITIM) and immunoreceptor tyrosine based switch motif (ITSM) domain, respectively. ITIM and ITSM serve as docking sites for SH2 phosphatases that dephosphorylate and thereby inactivate ZAP-70, an integral component of T cell receptor–mediated signalling (16). This results in the inhibition of T cell proliferation and effector functions like cytotoxicity and IFN-γ secretion (17). Recently, it has been demonstrated that the PD-1-dependent Src Homology 2 (SH2) phosphatase preferentially dephosphorylates the cytoplasmic tail of the CD28 costimulatory receptor to potentiate T cell inhibition (18). Thus, blocking of the PD-1/PD-L1 pathway with antagonistic antibodies would substantially improve the functionality of exhausted cytotoxic $CD8^+$ T cells. Such therapeutic intervention was successfully applied to boost $CD8^+$ T cell reactivity to tumors and to invigorate the body's own defensive mechanisms to remain effective against any malignancies. Food and Drug Administration (FDA) approved monoclonal antibody therapies targeting PD-1 (nivolumab and pembrolizumab) and PD-L1 (atezolizumab) revealed promising outcomes in clinical trials. Nivolumab is an anti-PD1 drug developed by Bristol-Myers Squibb, which is approved for metastatic melanoma and squamous non-small cell lung cancer (NSCLC) (19), while the PD-1 antagonist Pembrolizumab developed by Merck, is approved for metastatic melanoma (20). Atezolizumab is a new anti-PD-L1 drug developed by Roche and is approved for the treatment of patients with metastatic NSCLC (21) and metastatic urothelial carcinoma (22). The mechanisms of $CD8^+$ T cell rejuvenation upon anti-PD-1 therapy is shown in *Figure 1*.

Hidden facts behind therapeutic PD-1/PD-L1 pathway inhibition

Despite tremendous collaborative efforts made by academic-industrial partners to develop monoclonal antibodies specifically inhibiting the PD-1/PD-L1 axis, a recent study has revealed some extraordinarily important facts that force us to re-consider the therapeutic concept underlying the targeting of the PD-1 signalling pathway during chronic diseases. Here, an in-depth comparison between αPD-L1 treated and untreated exhausted $CD8^+$ T cells was done using unbiased transcriptional profiling and epigenomics. In addition, αPD-L1 treated and untreated exhausted $CD8^+$ T cells were compared with functionally competent memory or effector $CD8^+$ T cells, wherein αPD-L1 treated exhausted $CD8^+$ T cells lost their recall ability, a hallmark feature of memory $CD8^+$ T cells, and displayed an inflexible epigenome correlating with the repression of immune inhibitory genes like *PDCD1*, *CTLA4* and *IL10* (23).

Intriguingly, Pauken *et al.* has shown that αPD-L1 treatment induces the expression of interleukin-7 receptor (IL-7R) (CD127) on exhausted T cells during chronic lymphocytic choriomeningitis virus (LCMV) infection. PD-L1 induced IL-7R re-expression on exhausted $CD8^+$ T cells may in turn render them responsive to the homeostatic cytokine IL-7 (23). Until now, the two best characterized features of IL-7 are that it acts as a pivotal factor for (I) the long-term survival of mature effector $CD8^+$ T cells

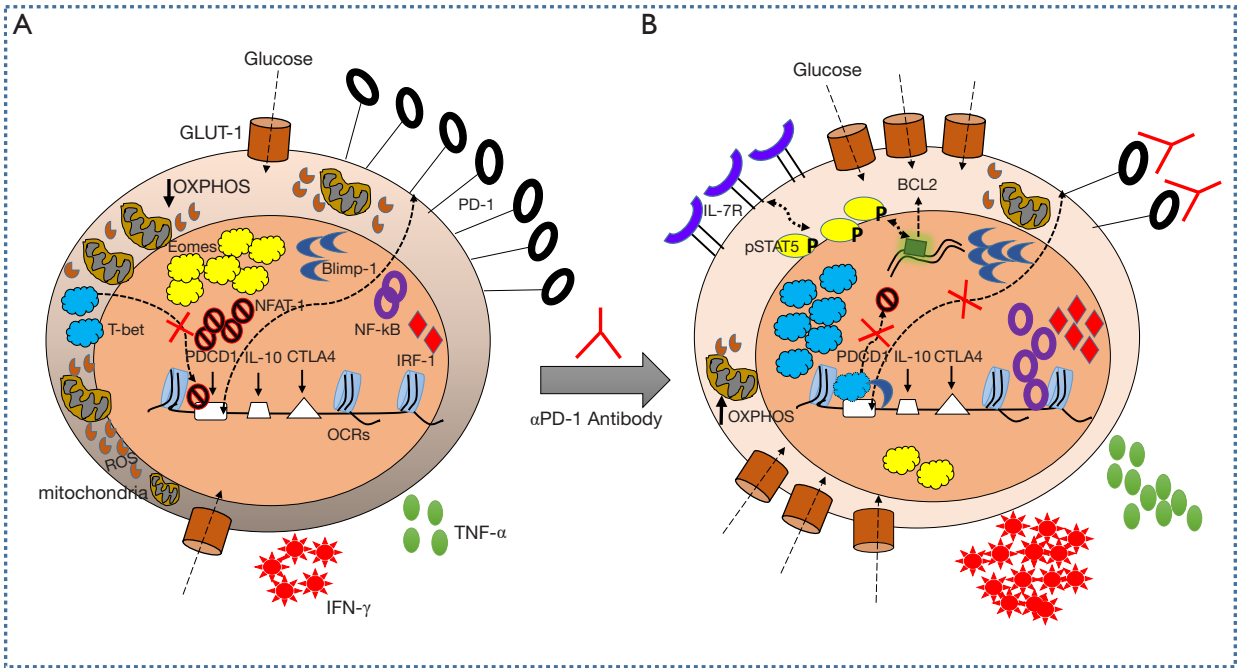

Figure 1 Exhausted CD8+ T cells and programmed cell death 1 (PD-1)/programmed death-ligand 1 (PD-L1) pathway inhibition during chronic disease. (A) Ongoing antigen exposure in the context of chronic infections and cancer induces CD8+ T cell exhaustion which is associated with elevated surface expression of the inhibitory receptor PD-1. Exhausted PD-1^{high}CD8+ T cells exhibit a variety of intracellular alterations occurring at the level of signalling molecules, chromatin accessibility and metabolism. Here, exhausted CD8+ T cells tend to express decreased levels of glucose transporter (GLUT-1) resulting in glucose deprivation. Due to limited glucose availability, the mitochondrial membrane potential changes, eventually causing decreased oxidative phosphorylation (OXPHOS). In addition, mitochondrial mass increases and excessive ROS production occurs. Exhausted PD-1^{high}CD8+ T cells exhibit increased Eomes but decreased T-bet expression, with Eomes being localized in the nucleus and T-bet being localized in the cytoplasm. Expression of effector cell-related TFs such as NF-κB, IRF-1, Blimp-1 are decreased, while the expression of NFAT-1 which is known to induce transcription of the *PDCD1* gene is increased in exhausted PD-1^{high}CD8+ T cells. Transcriptional alterations are further driven by changes in the chromatin structure with OCRs in close proximity to genes encoding for inhibitory molecules including PDCD1, CTLA4 and IL10. Moreover, exhausted PD-1^{high}CD8+ T cells are impaired with respect to the secretion of the effector cytokines IFN-γ and TNF-α; (B) PD-1/PD-L1 pathway inhibition by αPD-1 treatment induces the expression of the cytokine receptor interleukin-7 receptor (IL-7R) in exhausted CD8+ T cells. Responsiveness to IL-7 is associated with increased phosphorylation of signal transducer and activator of transcription 5 (STAT-5) that might induce the expression of the anti-apoptotic factor Bcl-2 and thereby supporting prolonged survival of exhausted CD8+ T cells. Moreover, alteration at the level of transcription factor expression does occur, with increased T-bet and decreased Eomes expression. In the same line the expression of NF-κB, IRF-1 and Blimp-1 enhanced along with a decreased expression of NFAT-1. Both, T-bet as well as Blimp-1, compete with NFAT-1 for PDCD1 binding sites and thereby repress the expression of PD-1. At the same time, GLUT-1 expression is increased leading to a normalization in glucose uptake by the cells, which in turn decreases mitochondrial mass, improves OXPHOS and energy production in αPD-1-treated exhausted CD8+ T cells. Together, these mechanisms partially reactivate CD8+ T cell effector functions resulting in enhanced secretion of IFN-γ and TNF-α. On the other hand, αPD-1-treatment of exhausted CD8+ T does not induce any obvious modification with respect to chromatin accessibility/OCRs in proximity to genes encoding for the inhibitory molecules PDCD1, CTLA4 and IL10.

by inducing upregulation of the anti-apoptotic marker Bcl-2 (24) and (II) the generation of a memory T cell phenotype (25). Apart, IL-7 regulates multiple functions during chronic LCMV infection by (I) augmenting the co-production of effector cytokines such as IL-17, IL-6, IFN-γ; (II) inducing the secretion of the cytoprotective cytokine IL-22; (III) repressing the suppressor of cytokine signalling (SOCS3) and, importantly; (IV) down-regulating PD-1

expression to reinvigorate the effector functions of CD8$^+$ T cells. According to data reported by Pauken *et al.* (23), the course of chronic LCMV infection following monotherapy with IL-7 alone was not positively affected, although the homeostatic proliferation of memory CD8$^+$ T cells was indeed IL-7 dependent. In contrast, combined αPD-L1 and IL-7 therapy was shown to have additive effects resulting in the expansion of LCMV-specific CD8$^+$ T cells co-producing IFN-γ and tumor necrosis factor-alpha (TNF-α). Apparently, IL-7 acts in concert with IL-15 to maintain *Bcl-2* expression, which is involved in the phosphorylation of signal transducer and activator of transcription 5 (STAT-5) and thus supports the generation of long-lived effector CD8$^+$ T cells (26). Although, an increased STAT-5 phosphorylation is observed in αPD-L1 treated exhausted CD8$^+$ T cells upon additional IL-7 stimulation, Pauken *et al.* have noticed that the expression of the IL-15 receptor CD122 did not differ between αPD-L1 treated and untreated exhausted CD8$^+$ T cells in the chronically LCMV infected host. This imbalance in the expression pattern of homeostatic cytokine receptors could possibly account for only a partially improved survival of αPD-L1 treated exhausted CD8$^+$ T cells, even in an antigen-free environment (23). These data clearly pinpoint drastic changes that are occurring possibly at both transcriptional and epigenetic levels in exhausted CD8$^+$ T cells and that are not sufficiently altered upon αPD-L1 treatment.

Transcription factors and PD-1 expression

Antigen-experienced cytotoxic CD8$^+$ T cells are known to express the transcription factor eomesodermin (Eomes) (27) in addition to the T-box transcription factor (T-bet). Eomes$^+$ CD8$^+$ T cells exhibit characteristic features of memory cells (28), whereas T-bet$^+$ CD8$^+$ T cells are competent to execute effector functions (29). These transcription factors play reciprocal roles in fine-tuning the balance between terminally differentiated effector cells and self-renewing memory CD8$^+$ T cells (30). During chronic viral infections, Eomes^{hi} CD8$^+$ T cells outnumber T-bet^{hi} CD8$^+$ T cells in bone marrow, liver, gut, lung, skin, brain, blood, with varying proportions (31) and most importantly, the majority of exhausted CD8$^+$ T cells is generally described to be T-bet^{low} and Eomes^{high}, despite some minor proportion of the exhausted CD8$^+$ T cells exhibit a T-bet^{high} Eomes^{low} phenotype (32). Such Eomes-expressing CD8$^+$ T cells display high expression of several inhibitory receptors, including PD-1, which is associated with a severe state of exhaustion (33,34). Furthermore, Eomes^{high} virus-specific CD8$^+$ T cells show reduced co-production of TNF-α and IFN-γ (32). Since T-bet expression was shown to directly repress the expression of PD-1, a substantial increase in Eomes^{high} T-bet^{low} antigen-specific CD8$^+$ T cells loses its capability to suppress PD-1 expression of (35). In line with this, the transcription factor T-bet was shown to bind to the regulatory elements located upstream of the PDCD1 gene, which represents as well a binding site for the transcription factor nuclear factor of activated T cells (NFAT). NFAT generally enhances PDCD1 expression which explains the antagonism between T-bet and NFAT in terms of PD-1 expression (36). However, during the state of chronic infection direct T-bet-PDCD1 interaction could not be demonstrated, which may mainly be due to the overall low expression level of T-bet in exhausted CD8$^+$ T cells. Reciprocal effects of Eomes and T-bet on PD-1 expression could, at least in part, also be explained by the localization of the transcription factors with high levels of Eomes in exhausted CD8$^+$ T cells being associated with its nuclear localization and low levels of T-bet being associated with its cytoplasmic localization. In line with this, it has been reported that in effector CD8$^+$ T cells 85% of T-bet is located in the nucleus (37), suggesting a role of T-bet in effector functions. This is further corroborated by reports stating that T-bet^{high}Eomes^{low}PD-1^{int} exhausted CD8$^+$ T cells are more susceptible to functional rescue by PD-1 blockade than T-bet^{low}Eomes^{high}PD-1^{high}exhausted CD8$^+$ T cells (23).

Despite having the potential for memory cell differentiation, these Eomes^{high} exhausted CD8$^+$ T cells fail to develop a recall response upon secondary antigenic challenge. This could be in a part explained by elevated expression of PD-1. On the other hand, it might be possible that Eomes^{high} CD8$^+$ T cells are paralyzed since they lack help from CD4$^+$ T cells, which also express high levels of PD-1 during chronic infection (38,39).

Epigenetic changes in exhausted T cells

Chronic antigenic stimulation is associated with unique changes in the chromatin accessibility in exhausted CD8$^+$ T cells. During chronic LCMV infection, an enrichment of open chromatin regions (OCRs), which is generally associated with transcriptional activation, was observed in the vicinity of immune-inhibitory genes such as PDCD1, IL10, and CTLA4 in exhausted T cells. αPD-L1 treatment did not affect these modifications, indicating a relatively

stable epigenetic imprinting in exhausted CD8+ T cells. Moreover, almost 6,000 OCRs modifications were uncovered to be unique for the exhausted T cell phenotype when compared to effector or memory T cells implying that exhausted T cells may develop into a distinct T cell lineage due to their rigid epigenome. Upon PD-1 pathway blockade, almost 650 OCRs modification were induced. More careful exploration revealed that αPD-L1 treated exhausted T cells exhibit considerable changes in a set of genes located close to OCRs containing effector cell-related transcription factors-binding motifs, wherein the activity of NF-κB, IRF-1 and Blimp-1 was augmented. At the same time, a reduced expression of the genes located within OCRs encoding for T cell exhaustion-promoting transcription factors such as *NFAT*, *NFAT-AP-1*, *IRF-4* and *BATF* was observed upon αPD-L1 treatment of exhausted CD8+ T cells. Thus, the notion behind the PD-L1 blockade implicates rewired transcriptional activity to re-establish the expression of effector genes within non-reprogrammable PD-1hi dysfunctional CD8+ T cells (23).

Blimp-1, one of the effector molecules that was induced in exhausted CD8+ T cells upon PD-L1 blockade, was reported to act directly on the *PDCD1* gene locus by supporting the formation of a closed chromatin structure and thereby preventing direct binding of NFATc1 (40). Moreover, Blimp-1 supports the expansion of perforin+ granzyme+ cytolytic CD8+ effector T cells, whereas the formation of memory precursor cells is hindered. Here, Blimp-1 binds to the promoter region of Id3 to repress its expression in effector CD8+ T cells. The reduced expression of Id3 triggers CD8+ effector T cell death and thereby limits memory cell development (41). This could explain the mechanism underlying the defective recall response by memory T cells during re-infection upon αPD-L1 blockade. Likewise, NF-κB is considered to be a critical factor for the survival of proliferating CD8+ T cells (42). Furthermore, PD-L1 blockade augments the activity of IRF-1 in exhausted CD8+ T cells. Likewise, the therapy might also modulate PD-L1 reverse signalling activities inside APCs. It has been reported that the expression of IRF-1 as well as NF-κB in APCS are considered to be pivotal for the antigen processing machinery that is needed for the proper expression of MHC-I molecules on APCs, thereby rendering them susceptible to CD8+ T cell mediated cytotoxicity (43). In particular, IRF-1 deficient mice exhibit impaired expression of *TAP* and *LMP-2* genes. Since these molecules are crucial for MHC-I-dependent antigen presentation, IRF-1 deficiency contributes to the reduction of CD8+ T cell numbers (44). Though IRF-1 is a regulator of type I interferon expression during viral infections, it is also a well-characterized factor known to suppress the tumor activity as evidenced by an early onset tumorigenesis, increased tumor burden and multiplicity, and striking changes in the tumor spectrum in mice lacking the *IRF-1* gene (45). In addition, tumor-derived lysosomes containing IRF-1 also enhance the recruitment of CD8+ T cells to the tumor microenvironment and mediate antitumor immune responses (46). Thus, such shift in the potential effector-related transcriptional factor motif enrichments in the chromatin could act as a predictive marker for the ability of infection or cancer-associated diseases to respond to the PD-L1 blockade therapy.

Metabolic reprogramming and PD-1/PD-L1 pathway inhibition

During chronic disease, the functional exhaustion of antigen-specific CD8+ T cells is reported to be directly linked to metabolic dysfunction. In fact, the early developmental stage of exhausted CD8+ T cells is mainly due to metabolic alterations. The transition of activated to exhausted CD8+ T cells is linked to reduced expression of glucose transporter-1 (GLUT-1) that is needed for glucose uptake into the cells and therefore serves as a limiting factor for glucose utilization (47,48). Moreover, PD-1 expressing CD8+ exhausted T cells also display dysregulated mitochondrial metabolism, which includes increased mitochondrial mass with depolarized mitochondria and increased production of ROS. It is well-known that a proper mitochondrial membrane potential is needed for the production of ATP during the process of oxidative phosphorylation (OXPHOS). During the early state of T cell exhaustion, mitochondrial dysfunction results in reduced OXPHOS and eventually reduced energy production. Mitochondria depolarization and dysfunction is mainly driven by the mammalian target of rapamycin (mTOR) signalling pathway that induces anabolic mitochondrial programming under glucose deprived conditions (48).

Intriguingly, it has been reported that the metabolic activity differs between PD-1hi and PD-1Int exhausted CD8+ T cells. Of note, a PD-1hi subset of exhausted T cells exhibits decreased glucose uptake, a reduced glycolytic and OXPHOS metabolism and higher mitochondrial mass with increased mTOR activity. This clearly implies a distinct role of PD-1 in the metabolic programing of exhausted T cells. The mechanistic link between PD-1 and metabolic modulation in

$CD8^+$ exhausted T cells is mainly due to the repression of the co-activator of peroxisomes proliferator- activator receptor gamma (PGC-1a), a key transcriptional regulator of genes controlling mitochondrial biogenesis and energy metabolism. Thus, metabolic reprogramming is equally important for the effector function of exhausted T cells (48).

PD-1/PD-L1 pathway inhibition: perspectives

Targeting PD-1/PD-L1 pathway represents an ideal tool to reactivate exhausted $CD8^+$ T cells. PD-L1 does not only represent a ligand for PD-1 but also binds to CD80 on T cells, thereby inducing the transmission of negative signals to the T cells, resulting in reduced T cell proliferation and impaired IL-2 and IFN-γ production. Moreover, co-targeting of other inhibitory elements such as CTLA-4 or IL-10 cytokine, that are unaffected during the PD-1/PD-L1 pathway inhibition should be considered for effective reactivation of exhausted $CD8^+$ T cells. In addition, treatment with an agonistic αCD28 antibody together with PD-L1 blockade might represent an attractive therapeutic approach for effective rejuvenation of exhausted T cells (49).

Reactivation of the expression of the transcription factors IRF-1 and NF-κB that was observed in exhausted $CD8^+$ T cells upon PD-L1 blockade might possibly result in paradoxical outcomes. Despite IRF-1 is generally considered to be essential for antiviral defence inhibition of IRF-1 expression was shown to result in massive expansion of IFN-γ^+ granzyme B^+ antigen-specific effector $CD8^+$ T cells. Cell intrinsic as well as cell extrinsic properties of IRF-1 were identified to contribute to shaping $CD8^+$ T cell responses, including the IRF-1-dependent inhibiting of $CD8^+$ T cell proliferation I addition to reducing the frequencies of regulatory T cells that under normal conditions restrain $CD8^+$ T cell responses (50). Furthermore, IRF-1 and NF-κB retain the capability to induce PD-L1 expression on tumor cells (51,52). Apart, transcription factors such as IRF-4 and BATF, which were not re-expressed in exhausted $CD8^+$ T cells following PD-L1 blockade, could also contribute to the overall maintainence of the effector function in $CD8^+$ T cells, by increasing the IFN-γ production, the frequencies of antigen-specific $CD8^+$ T cells and reducing the rate of apoptosis of $CD8^+$ T cells during LCMV infection (53). PD-L1 blockade restores IRF-2 transcription factor expression that is best known for its ability to promote pro-oncogenic properties. Intriguingly, IRF-2 has been recently described to also possess a tumor-suppressive role, since silencing of the *IRF-2* gene directly impairs the function of p53 (54). Hence, a careful consideration of the so far known consequences of αPD-L1 blockade with respect to the activation and repression of transcription factors, epigenetic modifications and metabolic reprogramming of exhausted $CD8^+$ T cells is required to achieve a safe and beneficial reactivation of exhausted $CD8^+$ T cells capable of conferring efficient effector function against persisting pathogens and tumors. In this context, co-targeting of the PD-1/PD-L1 pathway together with molecules of the *IRF* gene family or molecules related to metabolic pathways such as PGC-1a may have implications for the design of future therapeutics.

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