The tumor microenvironment disarms CD8\(^+\) T lymphocyte function via a miR-26a-EZH2 axis

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ABSTRACT
One of the most important factors that limit the potency of CD8\(^+\) cytotoxic T lymphocyte (CTL) responses is the tumor microenvironment (TME). Here, we provide evidence that miR-26a is a negative regulator of CTL function in the TME. Specifically, we identified miR-26a as a crucial suppressor gene in CTLs from the TME, as we found that, miR-26a expression was elevated in CTLs to respond to TME secretome stimulation. CTLs from miR-26a-transgenic mice showed impaired IFN\(\gamma\) and granzyme B production in response to their cognate antigen. Conversely, we found that miR-26a inhibition in CTLs could effectively increase the cytotoxicity and suppress tumor growth. Mechanically, we identified EZH2 as a direct target of miR-26a. miR-26a and EZH2 expression were found to be inversely correlated in CTLs, and the inhibition of EZH2 in CTLs impair CTL function. These functional correlations were validated in a cohort of non-small cell lung cancer patients, indicating that the miR-26a-EZH2 axis is clinically relevant. Our findings suggested that miR-26a silencing as a novel strategy to improve the efficacy of CTL-based cancer immunotherapy.

Abbreviations: CAFs, cancer-associated fibroblasts; CM, conditioned media; CTL, cytotoxic T lymphocyte; IDO, indoleamine 2,3-dioxygenase; MDSCs, myeloid-derived suppressor cells; NSCLC, non-small cell lung carcinoma; PBMCs, peripheral blood mononuclear cells; TAMs, tumor-associated macrophages; TGF-\(\beta\), transforming growth factor-\(\beta\); Th1, T helper type 1; TME, tumor microenvironment; Tregs, regulatory T cells; VEGF, vascular endothelial growth factor

Introduction
CD8\(^+\) cytotoxic T lymphocytes (CTLs) play a pivotal role in immune surveillance and antitumor immunity.\(^1,2\) Unsurprisingly, they are also primary targets of tumors for immune evasion.\(^3\) The co-existence of immunogenic tumor cells and antigen-specific CTLs in the same patients unequivocally indicates that the dominant mechanism leading to immune escape is immune suppression, and specifically, suppression of CTLs.\(^4\) For CTLs to execute their antitumor function, major challenges are imposed by the tumor microenvironment (TME).\(^5\) In established tumors, the TME is complexly composed of tumor cells and various infiltrating cells, the extracellular matrix, metabolites, growth factors, cytokines, and chemokines that they secrete and dysregulated vasculature.\(^6\) Recently, reversal of CTL dysfunction by blocking either PD-1 on the T cell surface or PD-L1 displayed by cells in the TME has shown unprecedented efficacy in the treatment of a broad range of human cancers, including melanoma,\(^7,8\) non-small cell lung carcinoma (NSCLC),\(^7\) bladder carcinoma,\(^9\) and renal carcinoma.\(^10\) These successes clearly demonstrate that alleviating immune checkpoint suppression is an effective way to treat cancer. Meanwhile, non-responders in these trials and mediocore responses to anti-PD1 and anti-PD-L1 treatment in ovarian and pancreatic cancer patients\(^9\) strongly suggest that other, perhaps more powerful, immunosuppressive mechanisms operating in the TME need to be surmounted.

Besides PD-L1 and its family members, a range of extracellular immunosuppressive components have been identified in the TME. Within the tumor, various types of stromal cells, including cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs) collaborate with tumor cells to damage the viability and blunt the function of CTLs. Secreted by these cells are growth factors (such as vascular endothelial growth factor, VEGF),\(^11\) cytokines (such as transforming growth factor-\(\beta\), TGF-\(\beta\)),\(^12\) and metabolic enzymes (such as indoleamine 2,3-dioxygenase, IDO),\(^13\) which cumulatively damage the expansion and cytotoxicity of tumor-infiltrating CTLs. While immunosuppressive functions of these secreted molecules are well established, many novel mechanism of action are still emerging\(^14\) or need to be explored. Deciphering these mechanisms could pave new ways to overcome immune suppression or reactivate CTLs, leading to improved immune therapies and prolonged survival of patients.
Educated by these extrinsic signals, CTLs are intrinsically reprogrammed by the TME to a dysfunctional state. This reprogramming is carried out by various signaling pathways to profoundly change the genome-wide transcription factor network and the epigenetic landscape. For example, activation of Smad2/3 and TGF-β in the TME suppresses transcription of the entire panel of cytotoxicity genes in both naïve and full-fledged effector CTLs. Also, activation of STAT3 in CTLs by multiple TME growth factors and cytokines directly antagonize type-I immunity. Epigenetic regulation carried out by DNA methylation and histone modification represents another fundamental regulatory mechanism in the TME to blunt CTL activity. Polycomb group protein EZH2 is one such essential epigenetic regulator, which catalyzes trimethylation of H3K27 and acts primarily as a transcription silencer. Ovarian cancer cells employ the EZH2 machinery to suppress expression of the chemokines CXCL9 and CXCL10, which thereby serves as a prominent mechanism to exclude T helper type 1 (Th1) cells from the tumor. Unexpectedly however, in CTLs, EZH2 was identified as an essential gene activator for promoting effector function. This was first identified in allogeneic CTL responses mediating graft-versus-host disease. Recently, in the TME, it was identified that EZH2 supports CTL-mediated antitumor immunity by silencing repressors of Notch signaling. In addition, the clinical relevance of EZH2 expression in ovarian cancer highlights that it is part of a major pathway targeted by the TME for immunosuppression. However, the relevance of this epigenetic modifier in other cancers remains to be determined.

In addition, miRNAs represent another layer of post-transcriptional machinery targeted by the TME for immunosuppression. Our group recently identified that TGF-β produced in the TME executes its inhibitory function through at least two pathways: (1) direct transcriptional repression of effector molecules, and (2) indirect post-transcriptional control through miR-23a to diminish expression of master regulator Blimp-1. Ectopic expression of miR-124 in CTLs enhanced effector function in the TME and exerted potent therapeutic efficacy in a murine model of glioblastoma. Also, in the context of ovarian cancer, deprivation of glucose in memory T cells elevated expression of miR-101 and miR-26a, resulting in diminished functionality and survival capacity of CTLs. These studies indicate that miRNAs are common targets for TME reprogramming of CTL function, and that identifying key miRNAs in CTLs would benefit the design of novel immunotherapy strategies.

In our study, we aimed to bridge these knowledge gaps in the context of lung cancer. We chose this context because lung cancer is well known to be a difficult target for immunotherapy, since the lung acts as an immunoprivileged site. We compared the miRNA expression profiles of TILs and peripheral blood mononuclear cells (PBMCs) collected from individual NSCLC patients. Through functional and clinical relevance analyses, as well as gain-of-function and loss-of-function studies, we identified miR-26a-EZH2 as a key axis in CTLs that is targeted by the TME for immunosuppression. Moreover, we evaluated the efficacy of miR-26a as a target for T cell adoptive transfer immunotherapy.

**Results**

**Differential expression of miR-26a correlates with CTL functional suppression in TME**

Because miRNAs represent a layer of post-transcriptional regulation that controls T cell differentiation and effector function, we hypothesized that the TME modulates the expression of miRNAs in order to suppress tumor-infiltrating lymphocyte (TIL)-mediated tumor rejection. To identify key miRNAs that mediate immunosuppression by the TME, we profiled miRNA expression in isolated CTLs from the PBMCs and pleural effusion of advanced NSCLC patients. Pleural effusion CTLs served as surrogates for lung TILs since it has been reported that these CTLs have experienced a similar microenvironment as that of tumors and display similar phenotypes as those of TILs. Indeed, our functional analysis showed that, in comparison to CTLs collected from PBMCs, CTLs from the pleural effusion of NSCLC cancer patients exhibited a deficiency in granzyme B production (Fig. S1). Among the 340 miRNAs assessed, six miRNAs were found to be highly upregulated in patient CTLs sorted from pleural effusion as compared to CTLs sorted from PBMCs (Fig. 1A; miR-140, p = 0.0007; miR-26a, p = 0.0007; miR-21, p = 0.014; miR-194, p = 0.012; miR-378, p = 0.023; let-7f, p = 0.014, Fig. S2). To explore the suppressive potential of these miRNAs, we analyzed the correlation between the expression of each of the six upregulated miRNAs and granzyme B production (a hallmark of CTL function) in our eight pairs of patient samples. Among the candidate miRNAs, miR-26a was the only one whose expression was inversely correlated with granzyme B production, as determined by the mean fluorescence intensity (MFI) (Fig. 1B, p = 0.048).

The correlation between elevated miR-26a expression and functional suppression in CD8+ TILs suggested that miR-26a expression could be upregulated in CTLs by the TME. It has been shown by others that the restricted glucose concentration in the TME upregulates miR-26a. We examined whether other factors within the TME could also have such an impact. To evaluate the role of factors directly produced by the tumor cell, we stimulated CD8+ T cells in the presence of lyophilized supernatant from 3T3 cell or tumor cell conditioned media (CM). By supplying CM in the solid form, we avoided alteration of glucose or serum concentrations in the T cell culture medium. While factors enriched in 3T3 CM failed to impact miR-26a expression, factors secreted by either B16 or LLC tumor cells augmented miR-26a expression in activated CD8+ T cells by 5-fold (Fig. 1C). Taken together, we speculated that multiple factors in the TME coordinately promote miR-26a expression to functionally impair CTLs.

**Forced miR-26a expression diminishes the induction of cytotoxicity in primed naive CD8+ T cells**

To examine the function of miR-26a during the initial priming of naive CD8+ T cells, we generated a transgenic mouse strain that constitutively expresses miR-26a under the control of Ubiquitin promoter (Mir26a-Tg). Flow cytometry analysis of lymphocyte populations in the thymus, spleen, and lymph nodes indicated that T cells developed normally in Mir26a-Tg mice (Fig. S3). Based on qPCR quantification, miR-26a was moderately overexpressed in naive CD8+ T cells collected from the spleens of
Mir26a-Tg mice (3-fold on average) (Fig. 2A). However, in activated Mir26a-Tg CD8⁺ T cells, the expression of effector molecules was significantly inhibited. In comparison to WT controls, fewer Mir26a-Tg CD8⁺ T cells were able to produce granzyme B (87.2% vs. 34.5%) and less granzyme B was produced per cell (MFI decreased by 1.6-fold on average) (Fig. 2B). At the mRNA level, both granzyme B (3.75-fold, Fig. 2C) and IFNγ (5.11-fold, Fig. 2D) expression were severely inhibited. Consequently, when the overall cytotoxicity against EL4 lymphoma cells was examined in vitro, we observed that the killing capacity of Mir26a-Tg CD8⁺ T cells was significantly reduced at various effector:target ratios (Fig. 2E). Therefore, we concluded that miR-26a is an intrinsic inhibitor of CTL function.

**EZH2 is a bona fide target of miR-26a**

To assess the mechanism of miR-26a-mediated suppression, we employed miRecord (http://c1.accurascience.com/miRecords/) to search for potential miR-26a target genes. Among hundreds of predicted targets, four genes (Ezh2, Mcl-1, Pten, and Bach2) were highly conserved for miR-26a seed region pairing (Fig. 3A) and reported to be involved in CTL function. We took a targeting decoy approach to validate the impact of miR-26a on candidate gene expression. A miR-26a decoy was designed as a tandem of eight different miR-26a binding elements attached to the 3′-end of a GFP gene. Also, in the same retroviral construct, infrared RFP protein was inserted as a marker under the control of the viral LTR promoter to identify virally-transduced cells (Fig. S4A). In decoy-transduced CD8⁺ T cells (miR-26a decoy), miR-26a transcripts were efficiently sequestered by exogenous decoy-GFP mRNA, diminishing miR-26a expression (Figs. S4B and C), and releasing the suppression of endogenous target expression. We observed that, in comparison to mock-transduced cells, expression of Mcl-1, Pten, and Bach2 in miR-26a decoy CD8⁺ T cells was intact (Fig. S5). However, there was a 3-fold increase in EZH2 mRNA expression.

*Figure 1. miR-26a expression is induced by the TME and negatively correlates with CTL function. (A) The difference in miRNA expression between CD3⁺ CD8⁺ cells is shown for CTLs sorted from paired pleural effusion and PBMC samples. The statistical significance of the differences was measured using an unpaired Student’s t-test (n = 8). (B) The correlation between granzyme B⁺ production and candidate miRNA expression was tested (n = 8). R represents the Pearson product-moment correlation coefficient, and 2-tailed p-values are shown. (C) miR-26a expression in activated CTLs treated with lyophilized CM from B16 or LLC cell cultures. Unconditioned medium or lyophilized CM from 3T3 cell cultures were used as controls (n = 4). The statistical significance of the difference was measured using an unpaired Student’s t-test, ‘’p < 0.001’, ns means no significant difference.*
in miR-26a

CD8+ T cells (Fig. 3B), as well as a 2.9-fold increase in EZH2 protein expression (Fig. 3B). To further investigate whether EZH2 could be a direct target of miR-26a in T cells, we constructed luciferase reporters containing the full-length Ezh2 3′/C19-UTR or a 3′/C19-UTR with a predicted miR-26a targeting site mutation. Each of these luciferase reporters were co-transfected into Jurkat T cells with a mock vector, a miR-26a overexpression vector or a miR-26a decoy vector. Using the GFP-mock vector-transfected cells as a control, miR-26a overexpression suppressed and miR-26a decoy reciprocally promoted luciferase reporter expression (Fig. 3C). Moreover, miR-26a-mediated suppression of luciferase expression was released when its predicted binding site was mutated in the Ezh2 3UTR, which strongly suggested that EZH2 mRNA is a direct target of miR-26a (Fig. 3D).

We also investigated the causal connections between miR-26a, EZH2, and EZH2-mediated transcriptional regulation. We observed that in wild-type (WT) CD8+ T cells, during naive to effector differentiation, EZH2 expression was highly induced. However, in Mir26a-Tg cells, this induction was dramatically suppressed at both the mRNA level (Fig. 3E) and protein level (Fig. 3F). Consequently, the reduction in EZH2 expression in activated Mir26a-Tg CD8+ T cells led to a global decrease in H3K27 tri-methylation (Fig. 3F), which strongly suggested that excessive miR-26a expression is sufficient to perturb the epigenetic landscape during effector CTL differentiation. Reciprocally, we observed that, by redirecting miR-26a suppression to its decoy, we significantly enhanced expression of CTL effector molecules, such as IFNγ and granzyme B (Fig. 3G). Most strikingly, as quantified by qPCR, the expression of EZH2 strongly correlated with the expression of IFNγ (R = 0.83, p = 0.01), granzyme B (R = 0.83, p = 0.01) and the upstream master transcriptional regulator for these two genes, Blimp-1 (R = 0.83, p = 0.0028) (Fig. 3H). This strongly suggested that miR-26a-mediated control of EZH2 expression is sufficient to determine CTL functional maturation. To validate this hypothesis, we administered GSK343, a selective and cell-permeable inhibitor of EZH2, to activated mock- or miR-26a decoy-transduced CD8+ T cells. Measured by intracellular cytokine staining and flow cytometry, GSK343 was capable of suppressing IFNγ production in mock-CTLs at the low nanomolar range (Fig. S6). Importantly, miR-26a decoy-induced CTL hyperactivation (based on IFNγ production) could be completely quenched with the same concentration of GSK343.

Figure 2. miR-26a reduces CTL cytotoxicity against tumor cells in vitro. (A) Relative gene expression values for miR-26a in CTLs from Mir26a-Tg mice. CTLs from WT mice were used as a control (n = 5). (B) MACS-purified naive CD8+ T cells were activated using anti-CD3 and anti-CD28 antibodies for 2 d, and flow cytometry was used to assess CTL effector molecule expression. Both the expression percentage and MFI are shown (n = 5). (C, D) qPCR analysis of granzyme B and IFNγ expression was also performed in CTLs from Mir26a-Tg mice and WT mice (n = 5). (E) CTLs from WT or Mir26a-Tg mice were incubated with target cells (EL4 cells) for 6 h. The ratios of target cells: effector cells were 1:1, 1:5, 1:10, and 1:20. Error bars in the curve represent the SD among four mice. Statistically-significant differences were measured using an unpaired Student’s t-test (A, C, D, and E) and a paired Student’s t-test (B), ”p < 0.05, ””p < 0.01, ”””p < 0.001.
which indicated that augmented EZH2 function is necessary for miR-26a-induced cytotoxicity enhancement. Taking all evidence together, we concluded that EZH2 is a direct and functionally-relevant target of miR-26a during CTL differentiation.

In a mouse tumor model, the miR-26a-EZH2 axis is exploited by the TME for immunosuppression

We showed above that miR-26a expression is elevated in TILs and that the miR-26a-EZH2 axis is an intrinsic pathway that epigenetically regulates CTL function. We speculated that miR-26a is exploited by the TME to target EZH2 in TILs for immune suppression. We tested this hypothesis first in a mouse tumor model. We treated activated CD8+ T cells with lyophilized secreted factors from B16 melanoma cells. Corresponding to the elevated miR-26a expression we previously observed (Fig. 1C), we found a 2-fold reduction of EZH2 expression (Fig. 4A), and H3K27Me3 level was also significantly reduction (Fig. 4B). We also isolated CD8+ TILs from established mouse melanoma tumors and measured their EZH2 mRNA expression. Using splenic CD8+ T cells sorted from the same mice as a control, we found that EZH2 expression in TILs was significantly reduced (Fig. 4C). This indicated that EZH2 is targeted by the TME in mouse CD8+ TILs.

The human lung cancer microenvironment exploits the miR-26-EZH2 axis to suppress CTL function

We also collected and combined pleural effusion samples from five NSCLC patients to test whether human CD8+ T cell function could be suppressed through miR-26a. Cell-free fluid containing soluble factors in the TME was solidified by lyophilization. CD8+ T cells isolated from the PBMCs of healthy donors were primed in vitro in the presence of mixed, solidified pleural effusion samples at various concentrations. After CD8+ T cells were activated in normal media for 3 d, miR-26a expression was reduced by 56.6% and EZH2 expression was induced by 7.79-fold. However, in the presence of soluble factors enriched from pleural effusion, even at the volume ratio of 1:8, miR-26a expression was significantly enhanced and EZH2 expression was diminished (Figs. 5A and B). This suggested...
Figure 4. In a mouse tumor model, the miR-26a-EZH2 axis is exploited by the TME for immunosuppression. (A) EZH2 expression in activated CD8⁺ T cells treated with lyophilized CM from B16 melanoma cells or unconditioned medium for 24 h (n = 6). (B) H3K27Me3 expression levels were tested in naive CD8⁺ T cells, activated CD8⁺ T cells treated with B16 CM or unconditioned medium for 24 h by FACS (n = 5). (C) EZH2 expression in CTLs isolated from spleen and tumor tissue of melanoma tumor-bearing mice (n = 6). Splenic CD8⁺ T cells sorted from the same mice as a control. Statistically significant differences were determined using an unpaired Student’s t-test, *p < 0.05, ***p < 0.001.

Figure 5. The human lung cancer microenvironment exploits the miR-26-EZH2 axis to suppress CTL function. (A, B) qPCR analysis of miR-26a and EZH2 expression in naive and day-4 stimulated healthy donor CTL cultures, in the presence of solidified pleural effusion from lung cancer patients at a pleural effusion: medium ratio of 1:1, 1:4, and 1:8. Dots represent fold change relative to the naive sample (n = 5). (C) qPCR analysis of EZH2 expression in CD3⁺CD8⁺ CTLs from paired PBMC and pleural effusion samples (n = 8). (D) FACS analysis of H3K27Me3 level in CD3⁺CD8⁺ CTLs from paired PBMC and pleural effusion samples (n = 4). (E) The relationship between miR-26a and EZH2 expression was assessed using a Pearson correlation array (n = 8 pairs). (F) The correlation between granzyme B percentage and EZH2 expression was tested (n = 8 pairs). (G) The correlation between IFNγ and EZH2 expression was tested (n = 8 pairs). R indicates the Pearson product-moment correlation coefficient, and two-tailed p-values are shown. Statistically-significant differences were determined using an unpaired Student’s t-test (A, B) and a paired Student’s t-test (C), *p < 0.05, **p < 0.01, ***p < 0.001.
that soluble factors in NSCLC pleural effusion can effectively capitalize on the miR-26a-EZH2 axis.

We further examined the clinical relevance of the miR-26a-EZH2 regulatory axis in NSCLC patients. In the original screening cohort of eight patients (Fig. 1A), EZH2 mRNA expression in CTLs from patient pleural effusion was significantly reduced in comparison to PBMC CTLs collected from the same patients (Fig. 5C). Accordingly, H3K27Me3 level was found reduced in CTLs from patient pleural effusion (Fig. 5D). To further validate this miRNA and target relationship, we analyzed the correlation between miR-26a and EZH2 expression in another cohort of nine patients. In this new sample set, miR-26a expression was also elevated in TILs, while EZH2 mRNA levels were reduced. More importantly, when all nine pairs of samples were combined for Pearson analysis, we found an inverse correlation between miR-26a and EZH2 expression (R = 0.482, p = 0.04, Fig. 5E). To further evaluate the functional relevance of miR-26a-controlled EZH2 expression in NSCLC patients within the same cohort, we analyzed the correlation between EZH2 and granzyme B/IFN-γ expression. In general, in comparison to PBMCs, expression of granzyme B protein or IFN-γ mRNA was highly suppressed in TILs. Meanwhile, EZH2 expression was positively associated with both granzyme B (R = 0.573, p = 0.012, Fig. 5F) and IFN-γ (R = 0.686, p = 0.002, Fig. 5G) production in CD8+ T cells. Taken together, we concluded that the miR-26a-EZH2 axis responds to the NSCLC microenvironment with high sensitivity and functional relevance.

**miR-26aDecoy is sufficient to rescue TME-suppressed CTL function in vitro**

We set out to assess the efficacy of targeting miR-26a in the context of tumor cell-mediated immunosuppression. pMel-1 transgenic CD8+ T cells were primed in vitro with their cognate antigen (gp10025–33 peptide) and transduced with retrovirus expressing mock or miR-26aDecoy. Two days after initial activation, CD8+ T cells were deprived of antigen, or treated with CM (B16 cells derived) for an additional 48 h. As expected, even under optimal priming conditions, miR-26aDecoy was capable of enhancing IFN-γ and granzyme B protein production by CTLs, based on both population percentage and per-cell production. More importantly, while the presence of CM severely impaired the functional maturation of mock-transduced CTLs, this impairment was efficiently rescued in miR-26aDecoy-transduced CTLs (Figs. 6A and B). Consequently, based on in vitro cytotoxicity assays with gp10025–33 peptide-pulsed EL4 lymphoma cells, the tumor-specific killing capacity of miR-26aDecoy-pMel-1 cells was significantly improved (Fig. 6C). Accordingly, the efficacy of miR-26aDecoy strongly suggested that miR-26a is a key modulator in CTLs during targeting by tumor cells for immunosuppression.

**miR-26aDecoy improves the efficacy of CTL adoptive transfer therapy**

To further validate the role of miR-26a in TME-mediated immunosuppression and the efficacy of engineering CTLs by targeting miR-26a in adoptive transfer therapy, we intratumorally injected mock- or miR-26aDecoy-transduced pMel-1 cells for B16 melanoma treatment. Transferring 2 × 10^5 mock-transduced pMel-1 cells induced moderate suppression of tumor growth; however, transferring the same number of miR-26aDecoy CTLs substantially impeded tumor progression (Fig. 7A). We assessed the effector function of transferred CTLs in the TME by flow cytometry analysis of sorted pMel-1 T cells from tumors on day 5 post-transfer. Again, miR-26aDecoy transduction led to a higher percentage of functional CTLs (IFN-γ+, granzyme B+, Fig. 7B). Also, the enhancement of CTL function was evident at the single-cell level, based on the MFI of IFN-γ (245.9 vs. 427.8) or granzyme B (239.2 vs. 443.4) protein (Fig. 7C).

Furthermore, to demonstrate that EZH2 is the key target of miR-26a in the CTLs from the TME in a similar experimental setting, we pre-treated miR-26aDecoy CTLs with GSK343 for 2 d before intratumoral injection. We found that inhibition of EZH2 largely abolished the advantage of miR-26aDecoy CTLs in IFN-γ and granzyme B production (Figs. 7D and E). Taken together, we concluded that the miR-26a-EZH2 axis is a crucial pathway for CTL functional suppression by the TME, and that targeting miR-26a in CTLs is a promising strategy to improve the efficacy of CTL-based adoptive transfer therapy.

**Discussion**

It has been well established that the TME secretome, produced by tumor cells, stromal cells such as CAFs and infiltrating immune cells, is a major component of the immunosuppression machinery. Immunosuppressive cytokine TGF-β activates SMAD signaling in both naive and full-fledged effector CTLs to repress their expression of key cytotoxic mediators, including granzyme B and IFN-γ, resulting in CTL dysfunction and impaired tumor rejection.29,30 Inflammatory cytokines, such as IL-6 and IL-10, interfere with multiple steps and pathways in the generation of an effective immune response.31 The TME secretome also includes enzymes and their tolerogenic metabolites, such as IDO,14 and nitrogen-reactive species generated by iNOS, which abrogate lingering lymphocyte activity in most solid tumors.32 In this study, we identified miR-26a as a potent suppressor of CTL function that responds to the secretome within the TME. This secretome rapidly elevates miR-26a expression in CTLs, which in turn is closely associated with impaired CTL function in vitro and in clinical samples. The upregulation of miR-26a expression was recently reported by Zhao et al.19 with primary ovarian cancer cell-conditioned medium. In their study, altered miR-26a expression was attributed to dysregulated glucose consumption by cultured tumor cells.19 However, in our case, we successfully enhanced miR-26a expression using lyophilized secretome from both mouse tumor cell lines and pleural effusion from NSCLC patients. Therefore, we propose that the TME secretome is also a major factor that promotes miR-26a expression in CTLs.

miR-26a was previously found to be immunosuppressive by facilitating the expansion of regulatory T cells (Tregs). By this mechanism, the overexpression of miR-26a prolongs skin allograft survival and attenuates renal ischemia-reperfusion injury.33,34 Recently, Zhao et al. reported that miR-26a is also intrinsically suppressive in CTLs: transfection of CTLs with miR-26a mimics decreases the polyfunctionality of CTLs and
enriches T cell apoptosis in vitro. While our data (from both gain- and loss-of-functions approaches) clearly demonstrated that miR-26a is indispensable for the cytotoxicity of CTLs, we did not observe that altered miR-26a expression could affect CTL survival (data not shown). Nevertheless, as shown by Zhao et al., we also identified EZH2 as the direct and functionally-relevant target of miR-26a. EZH2 is a methyltransferase for H3K27 that generates H3K27me3 in the genome. Therefore, EZH2 acts primarily as a gene silencer. Loss of EZH2 in T cells specifically impairs their differentiation into interferon (IFN)-γ-producing effector cells. In the context of clinical tumor immunity, it has been reported that EZH2 enforces the cytotoxicity of CTLs against ovarian tumors by silencing the Notch repressors NUMB and FBXW7. We speculated that this could be a general mechanism employed by CTLs in our NSCLC context. Alternatively, it has been reported that methylation of STAT3 by EZH2 is required for optimal activation of STAT3 and subsequent activation of its target genes. Since (1) STAT3 activation is critical for OX40 expression and cytokine-stimulated granzyme B transcription, and (2) STAT3-deficient CTLs fail to form virus-specific memory, it is possible EZH2 can also enforce the cytotoxicity of CTLs through epigenetic-independent mechanisms.

In summary, we have demonstrated that miR-26a-mediated EZH2 suppression in response to the TME secretome plays a crucial role in disarming the antitumor function of CTLs. Most importantly, we have shown that silencing miR-26a could be a novel strategy for enhancing CTL-mediated cancer immunotherapy.

Materials and methods

Patient samples

NSCLC patient samples for miRNA qPCR, EZH2 qPCR and IFNy/granzyme B flow cytometry analyses were collected at the Institute of Cancer, Xinqiao Hospital, Third Military Medical
University of Chongqing, China, and processed as previously described. Briefly, eight paired pleural effusion and PBMC samples were collected from newly diagnosed NSCLC patients without any anticancer therapy. The patient details are shown in Table S1. All of the samples were isolated using density centrifugation with human lymphocyte separation medium (TBD, China) according to the manufacturer’s instructions. Prior to cell lysis, CD8^-CD3^+ T cells were purified using fluorescence-activated cell sorting (FACS). The study received the approval of the Institutional Review Board of Xinqiao Hospital and informed consent was obtained from all individuals.

**Mice**

Eight- to ten-week-old female pMel-1^+^ TCR-transgenic, Thy1.1^+^ (pMel-1) mice were purchased from the Jackson Laboratory. C57BL/6 mice were purchased from the Chinese Academy of Medical Sciences (Beijing, China). miR-26a transgenic (Mir26a-Tg) mice were established in our own lab. Briefly, a miR-26a expression construct was generated by inserting a pre-miR-26a sequence into the FUGW lentiviral vector downstream of EGFP. Lentivirus was generated by calcium phosphate transfection and concentrated by centrifugation. The concentrated lentivirus suspension (1 × 10^9 PFU) was then injected into single-cell embryos from C57BL/6 mice. miR-26a expression in CD8^-^ T cells from Mir26a-Tg mice was validated by qPCR. All of the above mice were housed under specific pathogen-free conditions. These experimental procedures received the approval of the Institutional Animal Care and Use Committee.

**Cell lines and cell culture**

B16 cells (melanoma cell line), LLC cells (Lewis lung carcinoma cells), EL4 cells (thymoma cells), and 3T3 cells (fibroblast cells) were obtained from the American Type Culture Collection (ATCC). These cell lines were tested and authenticated using short tandem repeat profiling in September 2015. The cells...
were cultured in RPMI 1640 medium (HyClone, CA, USA) supplemented with 10% heat-inactivated FBS (HyClone, CA, USA), 100 units/mL penicillin/streptomycin and 2 mM fresh L-glutamine, and were incubated in a 37°C incubator with 5% CO₂. To collect the conditioned medium (CM), cells were seeded at a density of 5 × 10⁶ cells/mL in RPMI 1640 medium without FBS or other additives. CM was collected 24 h later, filtered through a 0.2 µm filter and solidified by lyophilization. Pleural effusion samples were collected and combined from five NSCLC patients. Cell-free effusion samples were collected and combined from 5 NSCLC patients. Cell-free fluid containing soluble factors in the pleural effusion was solidified by lyophilization, which dissolved with the same volume of RPMI 1640 medium before using.

Naïve CD8⁺ T cells were isolated from the splenocytes of pMel-1 mice, C57BL/6 mice or Mir26a-Tg mice as previously described. The Dynal Mouse CD8⁺ Negative Isolation Kit (Invitrogen, Carlsbad, CA) was used to isolate naïve CD8⁺ T cells according to the manufacturer’s instructions. Cells were cultured in complete medium consisting of RPMI-1640 (HyClone, CA, USA) supplemented with 10% FBS (HyClone, CA, USA), 2 mM L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids, 0.1% HEPES, 1% penicillin, 1% streptomycin, and 0.1% β-mercaptoethanol (HyClone, CA, USA). To activate CD8⁺ T cells, the cells were seeded into tissue culture plates with plate-bound anti-CD3 (5 µg/mL, Biolegend, CA, USA) and anti-CD28 (5 µg/mL, Biolegend, CA, USA) antibodies, or in the presence of 5 µM gp10025–33 peptide with antigen-presenting cells (for pMel-1 CD8⁺ T cell activation). In EZH2 inhibition experiments, pMel-1 CTLs were pretreated with 1–10 nM of the EZH2 inhibitor GS-343 (Selleck Chemicals, TX, USA) or an ethanol vehicle control for 48 h in vitro before flow cytometry analysis or intratumoral injection.

Retroviral transduction and transfection

A miR-26a decoy vector was built on the MSCV retroviral plasmid with iRFP or puromycin resistance as a selection marker. Each decoy contained eight repeats of sequences that were complementary to the miR-26a seed sequence. The full-length decoy sequence was constructed by annealing eight primers via equal molar mixing, and 95°C heating and slow cooling to room temperature. The primer designs are listed in Table S2. For retroviral transfection, naïve CD8⁺ T cells were isolated and activated as previously mentioned. On day 1, 50 units/mL of recombinant murine IL-2 (Sigma, USA) was added, and cells were incubated at 37°C with 7% CO₂ for 6 h. Then, cells were spin-infected with retroviral supernatant at 2500 rpm for 90 min at 37°C. Two days later, 1 µg/mL of puromycin (Sigma, USA) was added to enrich infected cells (for vector with puromycin resistance marker). At the indicated time point, ficoll was used to remove dead cells, and CTLs from day 4–6 post-stimulation were used for subsequent experiments.

qPCR expression analysis

Total RNA was isolated using the RNAqueous Microkit or the mirVana miRNA Isolation Kit (Ambion, TX, USA), and cDNA was synthesized using the qScript Flex cDNA Synthesis Kit (Quanta Biosciences, MD, USA) according to the manufacturers’ instructions. qPCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) to quantify the relative expression of mRNA and miRNAs. Primer sequences for real-time PCR are listed in Table S3. The miR-26a expression level was quantified using a qRT-PCR primer set (Ribobio, China). In the miRNA expression profiling array, a total of 340 miRNAs was analyzed in paired pleural effusion and PBMC CD8⁺ T cell samples (n = 8). miRNA expression was normalized by geometric mean-based global normalization using the RealtimeStatMiner (Integromics) analysis software.

Flow cytometry

Cells were harvested at the indicated end points and assayed using flow cytometry. For analysis of intracellular cytokines, CD8⁺ T cells were re-stimulated with PDBu (0.9 nM, Sigma-Aldrich, Germany), ionomycin (0.5 µg/mL, Sigma-Aldrich, Germany), brefeldin A (0.5 µg/mL, eBioscience, San Diego, CA), and monensin A (2 mM, eBioscience, San Diego, CA). After 4 h, cell suspensions were stained with the Live/Dead Violet Viability Kit (Invitrogen, Carlsbad, CA) to remove dead cells and surface stained for CD8⁺, Thy1.1 or Thy1.2 using anti-mouse flow cytometry antibodies (Biolegend, CA, USA). For intracellular staining, cells were fixed in 2% paraformaldehyde in the dark for 20 min, permeabilized in 0.1% saponin (Sigma, USA) in the dark for 10 min and stained with anti-IFN-γ-PE and anti-granzyme B-APC (Biolegend, CA, USA). Labeled cells were analyzed on a FACSCanto II flow cytometry system (BD biosciences, USA), and data were analyzed using FlowJo software. Counting beads (Invitrogen, Carlsbad, CA) were added to samples before flow cytometry to count absolute cell number.

Western blotting

CD8⁺ T cells were isolated and stimulated as previously described. Cell lysis and western blotting were performed according to the manufacturer’s instructions (KeyGen, China). We used antibodies against EZH2 (1:500, Cell Signaling Technology, Beverly, MA), H3K27Me3 (1:500, Cell Signaling Technology, Beverly, MA), β-actin (1:400, Boster, China), and Histone H3 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA).

In vitro cytotoxicity assays

pMel-1 CD8⁺ T cells were stimulated with gp10025–33 and transduced with mock or miR-26a decoy vector. EL4 cells were loaded with 10 µM of gp10025–33 overnight, then labeled with Cell Tracker Orange (Invitrogen, Carlsbad, CA) (loaded cells: 5 µM, unloading cells: 0.5 µM). Peptide-loaded and unloaded cells were used as target or control cells, respectively, by mixing them at a 1:1 ratio before co-culture with CTLs at the indicated ratio in a humidified 37°C incubator. For CTLs that were derived from WT and Mir26a-Tg mice, CD8⁺ T cells were stimulated with anti-CD3 and anti-CD28 antibodies (Biolegend, CA, USA) for 48 h and used as effector cells. EL4 cells used directly as target cells were labeled with 5 µM CFSE (Invitrogen, Carlsbad, CA). Six hours later, cells were harvested and stained with anti-CD8⁺–FITC (Biolegend, CA, USA) and
the Live/Dead Violet Viability Kit (Invitrogen, Carlsbad, CA). Counting beads (Invitrogen, Carlsbad, CA) were added into the samples before flow cytometry.

Adoptive cell transfers

To study in vivo antitumor effects, mock or miR-26a decoy pMel-1 CTLs were purified, and 2 x 10^6 cells were injected 6 d before subcutaneous injection of 2 x 10^5 B16/F10 cells into the right lateral flank of C57BL/6 recipient mice. Mice were regularly monitored, and tumor volumes were calculated using the following formula: \( V = \frac{4}{3} \times (\text{longest radius} \times \text{shortest radius})^3 \). At the indicated end points, mice were sacrificed and tumors were harvested. Tumor cell suspensions were prepared by digestion using the Papain dissociation system and were then analyzed for CTL effector molecule expression by flow cytometry.

3-UTR reporter luciferase assays

Binding sites of miR-26a were predicted using miRecords (http://mirecords.biolead.org/). Mouse EZH2 (containing a WT or mutant 3’-UTR) was cloned using PCR and ligated into the pmirGLO dual-luciferase reporter vector, downstream of firefly luciferase. The miR-26a decoy vector, miR-26a overexpression vector or mock vector was co-transfected into Jurkat T cells with each dual-luciferase reporter vector. After 48 h, the luciferase activity was measured using the dual-luciferase reporter assay system (Promega, Madison, USA). The data were presented as the ratio between the firefly and renilla fluorescence activities.

Statistics

All of the statistical analyses were performed using GraphPad Prism 5.0 software. Two-tailed unpaired or paired Student’s t-tests were used to compare two groups. Pearson product-moment correlation coefficients were used to assess the correlation between the expression levels of miR-26a and other mRNA transcripts in CD8^+ T cells. Statistical significance was indicated as *p < 0.05, **p < 0.01, and ***p < 0.001. All of the experiments were independently repeated at least three times.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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