Reducing CD73 Expression by IL1β-Programmed Th17 Cells Improves Immunotherapeutic Control of Tumors

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Abstract

T cells of the T helper (Th)17 subset offer promise in adoptive T-cell therapy for cancer. However, current protocols for ex vivo programming of Th17 cells, which include TGFβ exposure, increase the expression of CD39 and CD73, two cell surface ATP ectonucleotidases that reduce T-cell effector functions and promote immunosuppression. Here, we report that ATP-mediated suppression of IFNγ production by Th17 cells can be overcome by genetic ablation of CD73 or by using IL1β instead of TGFβ to program Th17 cells ex vivo. Th17 cells cultured in IL1β were also highly polyfunctional, expressing high levels of effector molecules and exhibiting superior short-term control of melanoma in mice, despite reduced stem cell-like properties. TGFβ addition at low doses that did not upregulate CD73 expression but induced stemness properties drastically improved the antitumor effects of IL1β-cultured Th17 cells. Effector properties of IL1β-dependent Th17 cells were likely related to their high glycolytic capacity, since ex vivo programming in pyruvate impaired glycolysis and antitumor effects. Overall, we show that including TGFβ in ex vivo cultures used to program Th17 cells blunts their immunotherapeutic potential and demonstrate how this potential can be more fully realized for adoptive T-cell therapy. Cancer Res; 74(21): 6048-59. ©2014 AACR.

Introduction

Adoptive T-cell therapy (ACT), which involves the isolation of antigen-specific T cells, followed by their ex vivo expansion and then infusion into autologous tumor-bearing host, is a promising approach for treating patients with advanced malignancies (1). New strategies to improve adoptive immunotherapy are now emerging; including blocking inhibitory molecules (CD28, 4-1BB, OX-40, ICOS, and VISTA), engaging costimulatory molecules (2, 3), expanding T cells in different cytokines (CD28, 4-1BB, OX-40, ICOS, and VISTA), and generating distinct T cells, followed by their in vivo expansion (4), expressing higher levels of effector molecules and exhibiting superior short-term control of melanoma in mice, despite reduced stem cell-like properties. TGFβ addition at low doses that did not upregulate CD73 expression but induced stemness properties drastically improved the antitumor effects of IL1β-cultured Th17 cells. Effector properties of IL1β-dependent Th17 cells were likely related to their high glycolytic capacity, since ex vivo programming in pyruvate impaired glycolysis and antitumor effects. Overall, we show that including TGFβ in ex vivo cultures used to program Th17 cells blunts their immunotherapeutic potential and demonstrate how this potential can be more fully realized for adoptive T-cell therapy. Cancer Res; 74(21): 6048-59. ©2014 AACR.

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as compared with Th17\(^{\text{TGF}^\beta}\) T cells, which translates into improved in vivo tumor control. We believe this strategy will help us to design conditions for ex vivo expansion that will minimize Tregs property, maximize Th1 features while maintaining Th17 phenotype, and potentiate the long-term antitumor response after ACT.

**Materials and Methods**

**Mice**

C57BL/6, CD73\(^{-/-}\) (B6.129S1-Nrd\(^{260/+}\)J), B6- Thy1.1 (B6. Pl-Thy1\(^{+/+}\)(Cyp), and OT-II (B6.Cg-Tg(Tcrb Tcrb-425Cbn/J) mice were obtained from The Jackson Laboratory. Development of h3T transgenic mouse bearing T-cell receptors (TCR) reactive to the human tyrosinase 368-376 (YMDTMSQV) epitope has been described recently (14). OT-II-GFP-FoxP3 mice coexpressing EGFP and FoxP3 were kind gift from Dr. C. Vasu, Medical University of South Carolina (MUSC, Charleston, SC). Animals were maintained in pathogen-free facilities and procedures approved by the Institutional Animal Care and Use Committee.

**Reagents and cell lines**

Ova (ova\(_{323-339}\); SQAVHAAHAEMAGR) and MART-1 (ELA-GIGILTV) peptides were purchased from GenScript. Penicillin, streptomycin, glucose-free RPMI-1640 medium and after 48 hours cells were fed with IL2 (50 IU/mL). anti-CD28 (5\(\mu\)g/mL) and TGF-\(\beta\) (10\(\mu\)g/mL) were termed as Th0 in this study. In some experiments, T cells polarization was performed either in presence or absence of 50 \(\mu\)mol/L ATP (Sigma). On day 3 of culture, T cells were harvested and either processed for intracellular cytokine analysis, RNA preparation using TRizol (Life Technologies) or used for adoptive cell therapy. For ova-specific generation of different Th17 cells, total splenocytes from OT-II TCR transgenic mice were stimulated with 1 \(\mu\)g/mL ova\(_{23-33}\) peptide in presence of above-mentioned polarizing conditions.

**Adoptive T-cell protocol**

Mouse melanoma tumor (B16-F10-ova), human melanoma (624-MEL), and T cells lymphoma cells (EL-4) were maintained in vitro in IMDM. EL-4 cells (0.25 \times 10\(^6\)) were injected i.p. into C57BL/6 mice, and on day 12 a total of 1 \times 10\(^6\) Th17 cells (either Th17\(^{\text{TGF}^\beta}\) or Th17\(^{\text{IL1}}\)) were transferred i.p. into the tumor site. Following 48 hours of T-cell transfer, peritoneal ascites fluid was drawn and donor cells were tracked using congenic Thy1.1 marker. B16-F10-ova (0.25 \times 10\(^6\)) and 624-MEL (2.5 \times 10\(^6\)) were injected s.c. into left flank of C57BL/6 or Rag1\(^{-/-}\) C57BL/6 mice or NSG-A2 mice, respectively. Twenty-four hours before adoptive transfer of T cells (CD4\(^+\) V\(\beta\)5\(^+\) ova-specific Th17\(^{\text{TGF}^\beta}\), Th17\(^{\text{IL1}}\), or Th17\(^{\text{IL1+TGF}^\beta}\)) on day seventh, the recipient mice were injected with cyclophosphamide (4 mg/mice). Tumors bearing C57BL/6 or Rag1\(^{-/-}\) C57BL/6 mice were either kept untreated or adoptively transferring with either CD4\(^+\) V\(\beta\)5\(^+\) (1 \times 10\(^6\)) ova-specific Th17\(^{\text{TGF}^\beta}\), Th17\(^{\text{IL1}}\), or Th17\(^{\text{IL1+TGF}^\beta}\) cells (1 \times 10\(^6\) cells/mice) on day 7. For xenograft tumor experiment, 15 days subcutaneously established 624-MEL, in NSG-A2 mice were either kept untreated or treated with either 0.2 \times 10\(^6\) CD4\(^+\) V\(\beta\)12\(^+\) Th17\(^{\text{TGF}^\beta}\) or Th17\(^{\text{IL1+TGF}^\beta}\) cells.

**Activation induced T-cell death**

Differentiated ova-specific Th17 (Th17\(^{\text{TGF}^\beta}\), Th17\(^{\text{IL1}}\), or Th17\(^{\text{IL1+TGF}^\beta}\)) restimulated for 4 hours with either cognate antigen (ova\(_{323-339}\)) or nonspecific antigen (MART-1) loaded irradiated C57BL/6 splenocytes at the 5:1 (T cells:B6 splenocytes) ratio. Apoptosis was measured by Annexin V (BD Biosciences) versus 7AAD staining according to the manufacturer’s protocol, followed by flow cytometry. Data were analyzed with FlowJo software (Tree Star).

**Cytotoxicity assay**

B16-F10-ova (specific target) or EL-4 (nonspecific target) cells labeled with CFSE (carboxyfluorescein diacetate succinimidyl ester; Life Technologies) and cocultured with differentiated Th17\(^{\text{TGF}^\beta}\) and Th17\(^{\text{IL1}}\) cells were used to determine cytotoxic potential, as detailed in Supplementary Methods.

**Flow cytometry and qPCR**

Detailed protocols for staining the cells for surface markers and intracellular cytokines have been described earlier (15), pyruvate or 20 mmol/L pyruvate plus 3-Mercaptopicolinic acid (glucagonogenesis blocker; Sigma) were used for Th17\(^{\text{IL1}}\) polarization. Purified CD4\(^+\) T cells stimulated with plate-bound anti-CD3 (5 \(\mu\)g/mL) and anti-CD28 (5 \(\mu\)g/mL) were termed as Th0 in this study. In some experiments, T cells polarization was performed either in presence or absence of 50 \(\mu\)mol/L ATP (Sigma). On day 3 of culture, T cells were harvested and either processed for intracellular cytokine analysis, RNA preparation using TRizol (Life Technologies) or used for adoptive cell therapy. For ova-specific generation of different Th17 cells, total splenocytes from OT-II TCR transgenic mice were stimulated with 1 \(\mu\)g/mL ova\(_{23-33}\) peptide in presence of above-mentioned polarizing conditions.
and in Supplementary Methods. Detailed methodology for qPCR is provided in Supplementary Methods.

Glucose uptake, oxygen consumption, and glycolytic flux
Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were evaluated as described earlier (16). Glucose uptake was determined by 2NBDG (Cayman Chemical) uptake assay according to the manufacturer's protocol and as described in Supplementary Methods.

Statistical analysis
All data reported are the arithmetic mean from three or five independent experiments performed in triplicate ±SD unless stated otherwise. The unpaired Student t test was used to evaluate the significance of differences observed between groups, accepting P < 0.05 as a threshold of significance. Data analyses were performed using the Prism software (GraphPad).

Results
TGFβ-induced CD73 expression on Th17 cells increases susceptibility for IFNγ suppression
CD39 and CD73 are two ectonucleotidases that sequentially cleave ATP to produce adenosine and are expressed at higher levels in TGFβ-polarized Th17 (Th17TGFβ1) cells as compared with the unpolarized Th0 cells (Fig. 1A). Because ATP is present at high concentration in the tumor microenvironment (17) and the ATP-byproduct adenosine (a potent immune suppressive) subverts antitumor immunity (18), we therefore tested whether ATP could affect the functionality of Th17TGFβ1 cells. Flow analysis revealed that ATP (50 μmol/L) significantly suppressed IFNγ production without affecting the IL17 production by Th17TGFβ1 cells subset with CD39+CD73+ phenotype, as compared with Th0 cells that express minimal CD73 on their cell surface (Fig. 1B). These data imply that CD73 expression could lead to impairment of IFNγ production by Th17TGFβ1 cells.

Figure 1. TGFβ1–induced CD73 expression on Th17 cells increases susceptibility for IFNγ suppression. A, flow-cytometric analysis of ectonucleotidases (CD39 and CD73) expression by unpolarized (Th0) or TGFβ1–mediated Th17 (Th17TGFβ1) cells. Data are representative of five independent experiments. B–D, intracellular IFNγ and IL17 production in presence or absence of ATP (50 μmol/L) by Th17TGFβ1 or unpolarized (Th0) cells (B); Thy1.1+Th17TGFβ1 cells retrieved from the tumor site of C57BL/6 (Thy1.2+) mice (n = 4) bearing EL-4 ascetic tumor following 48 hours of T cells transfer (C); and Th17TGFβ1–polarized cells from either wt or CD73−/− C57BL/6 mice (D). Cumulative data from three different experiments are represented in bar diagram alongside the dot-plot for the percentage of cells producing IFNγ in presence or absence of ATP (50 μmol/L). E, flow-cytometric analysis (right) of CD39 and CD73 expression by Th0, Th17TGFβ1, and Th17C57 cells. F and G, intracellular IFNγ and IL17 secretion in presence or absence of ATP by Th17C57 cells (F) and Thy1.1+Th17C57 cells retrieved from the tumor site of C57BL/6 (Thy1.2+) mice (n = 4) bearing EL-4 ascetic tumor following 48 hours of T cells transfer (G). Results are representative of three (E) and five (F and G) independent experiments; ***P < 0.0001.
cells in ATP-rich tumor microenvironment. To further evaluate the functional fate of Th17^TGF\(_\beta1\) cells coexpressing CD39 and CD73 in tumor microenvironment, purified CD4^+ T cells from congenic Thy1.1 mice were differentiated toward Th17^TGF\(_\beta1\) and injected into C57BL/6 (Thy1.2) mice bearing EL-4 ascites tumor. Donor cells retrieved from the tumor site after 48 hours showed decreased IFN\(\gamma\) production following restimulation (Fig. 1C). Furthermore, to confirm the role of ectonucleotidases CD73 in ATP-mediated suppression of IFN\(\gamma\) production by Th17^TGF\(_\beta1\) cells, CD4^+ T cells were purified from wild-type (Wt) or CD73^−/− mice (CD73 converts AMP to adenosine) and then differentiated to Th17 phenotype in presence or absence of ATP. In contrast with Wt Th17^TGF\(_\beta1\) cells, CD73^−/− Th17^TGF\(_\beta1\) cells showed no decrease in IFN\(\gamma\) secretion when cultured in ATP (Fig. 1D). These data support a role of CD73 in suppressing IFN\(\gamma\) production by Th17^TGF\(_\beta1\) cells exposed to ATP.

Next, we tested whether the polarization of Th17 cells in absence of TGF\(_\beta1\), but in presence of IL1, that is reported to have lower cell surface expression of CD39 and CD73 (19, 20), would affect the persistence and function of antitumor Th17 cells (Fig. 1E). We thus tested the susceptibility of Th17^IL1^ cells to IFN\(\gamma\) suppression in ATP-rich environment. Naïve CD4^+ T cells were polarized to Th17^IL1^ in presence or absence of 50 \(\mu\)M/L ATP, and cytokines production was analyzed. We found that Th17^IL1^ cells express less CD39 and CD73 on cell surface, and were resistant to suppression of IFN\(\gamma\) production in presence of ATP (Fig. 1F). Furthermore, Th17^IL1^ cells significantly retain their IFN\(\gamma\) production at the tumor site when retrieved from the tumor site after 48 hours of cell transfer (Fig. 1G). These data establish that Th17^IL1^ cells could be better than Th17^TGF\(_\beta1\) cells in retaining their functionality in tumor microenvironment.

**Distinct functionality of Th17^TGF\(_\beta1\) and Th17^IL1^ cells**

IFN\(\gamma\) plays a pivotal role in Th17-mediated control of tumor growth (5). Because both Th17^IL1^ versus Th17^TGF\(_\beta1\) cells differ in their susceptibility to suppression of IFN\(\gamma\) production at the tumor site, we further characterized these cells. On comparing the effector cytokines secretion ability by Th17^TGF\(_\beta1\) and Th17^IL1^ cells in vitro, we observed that about approximately 1.5-fold higher proportion of Th17^IL1^ cells (12%) secreted IFN\(\gamma\) than Th17^TGF\(_\beta1\) cells (8%; Fig. 2A). Similarly, Th17^IL1^ cells also showed the higher percentage of TNF\(\alpha\) (80% vs. 65%) and IL22 (6.5% vs. 0.5%) as compared with Th17^TGF\(_\beta1\) cells. The increased secretion of Th1 effector cytokines (IL2, IFN\(\gamma\), and TNF\(\alpha\)) and reduction in IL17 by Th17^IL1^ cells correlated with the higher expression level of transcription factor T-bet, ROR\(\gamma\), and reduced level of ROR\(\alpha\), respectively (Fig. 2B). Interestingly, evaluation of the cell surface markers showed that Th17^IL1^ cells express high CD62L. CD4^+ central memory phenotype (88%) as compared with Th17^TGF\(_\beta1\) cells (50%; Fig. 2C, i), and display a prominent active phenotype as elucidated by CD25 expression (Fig. 2C, ii). Th17^IL1^ cells also showed high surface expression of CD26 (Fig. 2C, iii), which has been shown to be associated with high IL22, GM-CSF, and IL23R (21). Th17^IL1^ cells also exhibit higher IL23R (that stabilizes Th17 phenotype; ref. 22), Gzmb (encodes for cytolytic molecule granzyme B), Csf2 (encodes for GM-CSF), cytokines IL22, and IL3 (Fig. 2D, i). It has been recently shown that IL23 enhances/stabilizes Th17 phenotype, and similar to GM-CSF that also increases effector function, leading to pathogenic phenotype in the experimental autoimmune encephalomyelitis (EAE) model (23, 24). Importantly, the expression of cytokine IL10, that could suppress T-cell response, was decreased in Th17^IL1^ cells as compared with Th17^TGF\(_\beta1\) cells. Furthermore, a qPCR array–based analysis also revealed that transcription factors regulating TCR signaling, Erk1/2, Notch, and EGF pathway were highly expressed in Th17^IL1^ cells as compared with Th17^TGF\(_\beta1\) cells (Fig. 2D, ii). In addition, a signal transduction array analysis also revealed that Th17^IL1^ cells engage in multiple signaling pathway than Th17^TGF\(_\beta1\) cells (Fig. 2D, iii), which may be responsible for their overall enhanced effector capability (25).

**Enhanced antitumor function of Th17^IL1^ versus Th17^TGF\(_\beta1\) cells**

Because Th17^IL1^ cells are polyfunctional and produce an array of various cytokines/effector molecules, we next investigated their potential to control tumor growth. Using CD4^+ T cells from OT-II mice that were programmed to Th17^TGF\(_\beta1\) and Th17^IL1^ phenotype, first we tested their ability to lyse murine melanoma cell, in vitro. Our data show that Th17^IL1^ cells can lyse the tumor cells directly and are more cytolytic than Th17^TGF\(_\beta1\) cells (Fig. 3A and Supplementary Fig. S1A). Next, adoptive transfer of \(1 \times 10^6\) ova–specific Th17^TGF\(_\beta1\) and Th17^IL1^ cells (i.v.) to immunocompetent C57BL/6 mice bearing established B16-F10-ova melanoma showed a marked delay in tumor growth in the group receiving Th17^IL1^ versus Th17^TGF\(_\beta1\) cells (Fig. 3B). At the experimental endpoint, a cytokine analysis of adoptively transferred donor cells retrieved from various sites (tumor and nontumor) showed that the IFN\(\gamma\) secretion was dramatically reduced in Th17^TGF\(_\beta1\) cells retrieved from the tumor site (Fig. 3C, lower left), as compared with Th17^IL1^ cells that maintained their IFN\(\gamma\) secretion at the tumor site (Fig. 3C, bottom right). Importantly, donor cells retrieved from the lymph node, spleen, and blood did not show significant decrease in IFN\(\gamma\) secretion upon restimulation. These data establish that Th17^TGF\(_\beta1\) cells with high CD73 expression are more prone to loosing effector cytokine IFN\(\gamma\) in tumor microenvironment as compared with Th17^IL1^ cells. In addition, it is possible that higher expression of CD25 on Th17^IL1^ cells resulted in their increased homeostatic proliferation as compared with Th17^TGF\(_\beta1\) cells (26), and thereby improved antitumor effect. Next, to determine whether the antitumor potential of Th17^IL1^ cells are independent of any endogenous T cells, we transferred \(1 \times 10^6\) of either OT-II Th17^TGF\(_\beta1\) or Th17^IL1^ cells to Rag^−/− C57BL/6 mice bearing B16-F10-ova melanoma. We obtained similar tumor regression by Th17^IL1^ cells in tumor-bearing Rag^−/− C57BL/6 mice as we observed for immunocompetent mice, suggesting that Th17^IL1^ cells could control the tumor growth independent of CD8^+ T cells (data not shown).

To further address the issue of Th17 plasticity, that is, the ability to convert to FoxP3^+ cells, which is considered a key reason for the failure of Th17 cells in tumor immunotherapy, we tested whether there is a differential susceptibility for...
Figure 2. Distinct functionality of Th17TGFβ1 and Th17IL1β cells. A, naïve CD4+ T cells from C57BL/6 mice were differentiated toward either Th17TGFβ1 or Th17IL1β and intracellular production of various cytokines was analyzed. The percentage of cells producing different cytokines is also represented in pie-diagram (50,000 cells/group were analyzed to draw pie-diagram). B, qPCR analysis (top) and flow-cytometric analysis (bottom) of various Th17 signature transcription factors expression by Th17TGFβ1 and Th17IL1β cells. C and D, flow-cytometric analysis of CD62L versus CD44 expression (C, i) and CD25 expression Th17TGFβ1 and Th17IL1β cells at day 3 of polarization (C, ii). D, i, qPCR analysis of expression of key effector genes in Th17TGFβ1 and Th17IL1β cells after 3 days of polarization. Data represent three independent experiments; **, P < 0.005; ***, P < 0.0001. Transcription factors array (D, ii) and signal transduction array (D, iii) were performed using the 84-Gene qPCR-Based Array Kit (SABiosciences) as per the manufacturer’s recommendation. Fold upregulation (blue) or downregulation (red) of Th17IL1β over Th17TGFβ1.
conversion to FoxP3+Th17 cells between the Th17_{GFP} and Th17_{IL1} cells. Using CD4+ T cells from GFP-FoxP3 developed on OT-II background for programming to Th17_{GFP} and Th17_{IL1} cells, we noted an increased percentage of FoxP3+ GFP+ cells in conventional Th17_{GFP} cultures (Fig. 3D). In accordance with the recent study that established the inhibitory role of transcription repressor growth factor independent 1 (Gfi-1) in nTreg generation, our data show that conversion to iTreg phenotype is decreased in Th17_{IL1} cells as compared with Th17_{GFP} ones, potentially due to higher Gfi-1 expression (Fig. 3D, right). These sets of data establish that Th17_{IL1} cells possess better antitumor properties and are less "plastic" than conventional Th17_{GFP} cells.

**Improved antitumor ability of Th17_{IL1} cells correlates with increased glycolysis**

Recent evidences suggest that T-cell energy metabolism is not merely a cellular phenomenon, rather it can determine the cytokine productions and functional outcome of T cells (27, 28). Because IFNγ secretion has also been shown to be dependent on glucose consumption (29, 30), we reasoned that Th17_{IL1} cells could be more glycolytic. A comparison of glucose consumption reveals that Th17_{IL1} cells were more glycolytic than Th17_{GFP} cells (Fig. 4A). To further address this issue, ECAR, an indicator of glycolysis, and OCR, an indicator of oxidative phosphorylation (OXPHOS), were measured in Th17_{IL1} and Th17_{GFP} cells using Seahorse bioanalyzer. Our data show that Th17_{IL1} cells had higher ECAR value as compared with Th17_{GFP} cells, indicating their high glycolytic capacity (Fig. 4B, i). Furthermore, the basal OCR was less in Th17_{IL1} cells as compared with Th17_{GFP} cells (Fig. 4B, ii), leading to a higher OCR/ECAR ratio in Th17_{GFP} cells (Fig. 4B, iii). To further ascertain the metabolic difference between Th17_{IL1} and Th17_{GFP} cells, we analyzed mRNA expression of various glycolysis-associated genes using qPCR. Our data show that the mRNA expression levels of all glycolytic genes evaluated were several fold higher in Th17_{IL1} cells as compared with Th17_{GFP} cells (Fig. 4C). The increased expression at mRNA level also translated to increased expression of glycolytic proteins, as represented by hexokinase II (HKII) expression (Fig. 4D). The increased glycolysis in Th17_{IL1} cells also correlated with increased activation of the mTOR pathway, as determined by phosphorylation of S6 (Fig. 4E). These data support that higher effector function and antitumor control displayed by
Shift from glycolysis dampens Th17IL1β cells effector functions

Next, we tested whether the increase in glycolytic commitment observed in Th17IL1β cells is key to increased expression of effector molecules that are responsible for better antitumor effector response. For this purpose, the Th17IL1β cells were polarized in complete media containing glucose that supports glycolysis, or media with no glucose but pyruvate with glucose transport blocker (that supports oxidative phosphorylation). We observed that cells grew at same rate (Fig. 5A, i) and also had similar activation profile (Supplementary Fig. S1B) in pyruvate-containing (without glucose) media as compared with complete media. On analyzing the Th17IL1β cells polarized in different media for signature cytokines production after stimulation with PMA/Ionomycin, we found that blocking the glycolytic pathway could also affect the cytolytic capacity of Th17IL1β cells, mRNA expression of GzmB was analyzed after polarization of cells in pyruvate-containing media. Expression analysis using qPCR revealed that key effector molecules (GzmB, GM-CSF, T-bet, IL22, and IL3) were significantly down-regulated in Th17IL1β cells cultured in presence of only pyruvate-containing media as compared with complete media (Fig. 5B). Moreover, we also found that antigen-specific killing of tumor cells B16-F10-ova was greatly reduced when Th17IL1β cells were polarized in pyruvate-containing media (Fig. 5C and Supplementary Fig. S1C). However, no correlation between glycolysis and CD39/CD73 expression by Th17IL1β cells was established, because culturing Th17IL1β cells in pyruvate-containing media (glucose free) did not affect the CD39/CD73 expression (Supplementary Fig. S2A). Next, to further confirm that glycolysis is necessary to mount proper antitumor response by Th17 cells, Rag1-/- C57BL/6 mice with 7 days established B16-F10-ova tumor were treated by adoptively transferring 1 × 10⁶ ova–specific Th17IL1β cells polarized either in normal or only pyruvate-containing media. Antitumor
potential of Th17IL1b cells was severely impaired when cells were polarized in pyruvate-containing media as determined by the rapid progression of tumor growth in treated mice (Fig. 5D). However, mice treated with Th17IL1b cells polarized in normal media markedly delayed tumor growth. These data together strongly suggest that antitumor potential of Th17 cells is highly dependent on their metabolic commitment, and higher glycolysis may have contributed to improved antitumor response observed with Th17IL1b cells.

Lack of stemness in Th17IL1b cells could be restored by very low dose of TGFβ

Despite superior antitumor activity of adoptively transferred Th17IL1b cells in vivo, we noticed that the Th17IL1b-recipient mice could not control tumor growth after 30 to 35 days. The inability to control tumor growth long-term was also correlated with the poor persistence of these Th17IL1b cells in vivo (Supplementary Fig. S2B). Because long-term persistence of conventional Th17TGFβ1 cells is attributed to "stemness" signature (10, 11), we analyzed various stemness-associated genes in Th17IL1b versus Th17TGFβ1 cells. Real-time PCR analysis revealed that expression of various stem cell–associated genes (β-catenin, Bcl6, Tcf7, and Lef1) was significantly lower in Th17IL1b cells as compared with Th17TGFβ1 cells (Fig. 6A). We also found greater induction of AICD in Th17IL1b cells as compared with Th17TGFβ1 cells following antigen restimulation (data not shown). These data together indicate that Th17IL1b cells exert the characteristics of terminally differentiated population with profound antitumor potential, however, lack stemness features, and thus persist for a short period of time in tumor-bearing host. Because TGFβ has been shown to induce expression of various stem cell–associated genes (33), we argued that it may be responsible for the stemness signature reported in conventionally programmed Th17TGFβ1 cells (10, 11). We thus titrated for a minimum dose of TGFβ that could impart stemness without inducing the expression of

Figure 5. Shift from glycolysis dampens Th17IL1b cells effector functions. A, i, schematic diagram of the culture conditions used to generate the Th17IL1b cells. ii, intracellular staining of various cytokines. B, qPCR analysis of the expression of key effector genes. C, cytolysis of B16-F10-ova cells was evaluated using Th17IL1b cells polarized either in complete media (green bars) or in 20 mmol/L pyruvate (no glucose)-containing media (brown bars). D, C57BL/6 Rag1−/− mice (n = 5 mice/group) were inoculated (s.c.) with 106 B16-F10-ova, and after 7 days, mice were either kept untreated as control or adoptively transferred with either 1 × 106 OT-II cells or Th17IL1b cells effector (b cells. ii, CFSE dilution analysis of OT-II cells transferred with either 1 × 106 b cells polarized in pyruvate-containing media as determined by the rapid progression of tumor growth in treated mice (green bars) or in 20 mmol/L pyruvate (no glucose)-containing media. Tumor growth was measured using digital calipers every fourth day. Data, mean tumor size at each time point in one of the two experiments with similar results; *, P < 0.05; **, P < 0.005; and ***, P < 0.0001.

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Figure 6. Low dose of TGFβ induces stem cell–like phenotype in Th17IL1b cells. A, qPCR analysis for expression of key memory and stemness–associated genes in Th17TGFβ1 and Th17IL1b cells. Cumulative data from three independent experiments are presented. B, flow–cytometric analysis for CD39 and CD73 expression on CD4–gated T cells after 3 days of culture in presence of various concentration of TGFβ. C, qPCR analysis of key glycolysis regulating genes (left), and memory/stemness–associated genes (right) in either Th17TGFβ1, Th17IL1b cells, or Th17IL1b cells cultured in presence of 250 pg/mL TGFβ (i.e., Th17IL1b–TGFβ cells). D, OT-II CD4+ T cells were polarized toward different Th17 types and restimulated with either cognate antigen (ova323-339) or nonspecific antigen (MART-1) for 4 hours. Cell death was determined by evaluating Annexin V versus 7AAD by flow cytometry (left) as detailed in the Supplementary Methods. Bar diagram (right), the percentage of Annexin V– and 7AAD–positive cells from three different experiments. E, C57BL/6 Rag1−/− mice (n = 4–5 mice/group) were inoculated (i.c.) with 0.25 × 10^6 B16-F10-ova murine melanoma cells and after 7 days, mice were either kept untreated or treated with either 1 × 10^5 ova–specific Th17TGFβ1 or Th17IL1b+TGFβ1 (Vβ15 CD4+) cells. Tumor growth was measured using digital calipers every 3 day. Data, mean tumor size at each time point from three experiments with similar results. F, NSG-A2 mice (n = 5 mice/group) were inoculated with 2.5 × 10^6 HLA-A2/C2 human melanoma 624-MEL cells and after 15 days, mice were either kept untreated or treated with h3T mouse–derived 0.2 × 10^6 human tyrosinase epitope–reactive Th17TGFβ1 or Th17IL1b+TGFβ1 cells. Tumor growth was measured using digital calipers every 3 day. Data, mean tumor size at each time point; *, P < 0.05; **, P < 0.005; and ***, P < 0.0001.

ectonucleotidase CD73. Our dose titration data showed that 250 pg/mL of TGFβ minimally upregulates CD73 expression (Fig. 6B). Th17IL1b cells differentiated in presence of 250 pg/mL of TGFβ (referred to as Th17IL1b+TGFβ1) also markedly increased the mRNA transcripts of various stemness genes as well as the genes associated with T-cell memory (Fig. 6C). However,
comparative analysis of glycolytic pathway molecules and stemness gene signature between three different Th17 populations revealed that Th17\(^{IL1b^{-}}\) cells exhibited intermediate glycolysis (Th17\(^{IL1b^{-}}\) > Th17\(^{IL1b^{+}-TGFb^{+}}\) > Th17\(^{TGFb^{+}}\)), and stemness (Th17\(^{TGFb^{+}}\) > Th17\(^{IL1b^{+}-TGFb^{+}}\) > Th17\(^{IL1b^{-}}\)) gene signature (Fig. 6C, left and right). In addition, a TCR restimulation–induced AICD was decreased in Th17\(^{IL1b^{-}-TGFb^{+}}\) cells as indicated by the lower percentage of cells with Annexin V and 7AAD positivity (Fig. 6D). These data also confirm a recent observation that T cells differentiated in presence of glycolysis inhibitor 2-deoxy glucose programs for better antitumor control and persistence (34). Importantly, the Th17\(^{IL1b^{-}-TGFb^{+}}\) cells also showed significant improvement in the ability to control both B16 murine melanoma (Fig. 6E), and 624-MEL human melanoma (Fig. 6F). The tumors in mice treated with the Th17\(^{IL1b^{-}-TGFb^{+}}\) group did not reach half the tumor endpoint (<100 mm\(^3\)) until sacrificed on day 70, indicating the long-term persistence of Th17\(^{IL1b^{-}-TGFb^{+}}\) cells in vivo (Supplementary Fig. S2C). Thus, Th17 cells generated ex vivo with IL1b and low concentrations of TGFb program antitumor T cells optimally for metabolic commitment and persistence, which in turn affect the ability to control tumor growth long-term.

Overall, our data suggest that modifying ex vivo culture conditions to generate an effective hybrid Th17+Th1 cells for adoptive immunotherapy would benefit from strategies that target to increase effector signature, glycolytic potential, persistence, and concomitantly reducing exonuclease CD73 expression catalyzed by CD39/CD73 KO mice (44). Similarly, the role of adenosine generation discussed as to how a stable Th17 cell with ability to control the tumor growth long-term could be programmed ex vivo. Our data substantiate the potential contribution of ectonucleotidase CD73 expression in self-suppression of the effector Th17 cells generated using the conventional method with TGFb, and propose the strategies to program long-lived effector Th17 cells by combining inflammatory cytokine IL1b along with a low dose of TGFb (that does not induce CD73, but upregulates stemness genes). This, we believe could be an important step forward to generate robust hybrid Th17+Th1 effector cells that could be readily translated to clinics when treating patients with melanoma or other cancers. Expression of ectonucleotidases CD39 and CD73 on tumor cells has been shown to contribute to the immunosuppression by their sequential action of converting ATP to adenosine (41). Although blocking CD73 expression on tumor cells has shown to improve tumor control (42, 43), the engraftment of tumor and its metastases was also reported to be lower in the CD39-KO mice (44). Similarly, the role of adenosine generation catalyzed by CD39/CD73–expressing Tregs in immunosuppression is also established (45). A recent study has also shown that combining anti-CD73 treatment with anti–CTLA-4 and anti-PD1 antibody (negative regulators of T-cell activation) results in improved tumor control (42). These studies imply that expression of either host-derived CD73 on tumors or its expression on the adaptive T-cell subsets may be a key contributor in tumor progression. Our data now show that the expression of CD73 on T cell itself could also lead to its increased susceptibility to suppression and loss of effector function. Because TGFb is the key contributor of CD73 expression, we evaluated herein the T-cell subsets that are programmed in TGFb, that is, Th17 cells. Ex vivo-generated Th17 cells that are programmed with TGFb have been shown to be better than Th1 cells at controlling tumors (5), paradoxically they also express CD73 (19). We thus compared the differences in Th17 cells that were programmed either in presence of TGFb or in absence of TGFb (but with IL1b). The improved ability of IL1b–programmed Th17 cells to secrete higher level of IFN\(\gamma\), express enhanced level effector molecules, and control tumor could be due to the direct effect of IL1b on both CD4 and CD8 T cells that leads to activation of multiple pathways, as reported previously (46, 47). Studies using autoimmune EAE models have shown that IL1b treatment increases the expression of pathogenic genes that results in increased incidence of disease (20, 48). The increased expression of IL23R and other effector molecules that resulted in increased pathogenesis in the autoimmune model may have been responsible for rendering tumor epitope–specific Th17\(^{IL1b^{-}}\) more efficacious than...
Th17<sup>TGF<beta></sup> cells in targeting "self" but tumor-associated epitope and controlling tumor growth. Our data also show that IL1<beta>-cultured Th17 cells exhibit increased level of glucose transporter Glut1-1 that correlates to increased glucose consumption, which fuels the metabolic need of the rapidly dividing glycolytically active effector Th17<sup>IL1<beta></sup> cells. Our data also suggest that the expression of CD39/CD73 on Th17<sup>IL1<beta></sup> cells does not inversely correlate with the level of glycolysis in T cells, as we did not notice any increase in expression of these ectonucleotidases with the decrease in glycolysis (Supplementary Fig. S2A, right). Contrary to the earlier studies that show CD73 expression correlates closely with HIF1α expression either in intestinal epithelia or gastric carcinoma (49, 50); CD73 expression correlates closely with HIF1α expression in the tumor microenvironment (Supplementary Fig. S2A, left). Importantly, although these glycolytically active Th17<sup>IL1<beta></sup> cells do exhibit increased ability to control tumors in the short term, they do not persist well in the host. The decreased persistence of Th17<sup>IL1<beta></sup> cells correlates with decreased expression of "stemness" signature that has been otherwise shown to be a key feature of Th17 cells (10, 11). Our data show that it may be the presence of TGFβ1 in the culture conditions that renders the "stemness" to Th17<sup>TGF<beta></sup> cells, and thus Th17<sup>TGF<beta></sup> cells do not exhibit stemness. However, addition of low concentration of TGFβ (250 pg/ml) as opposed to the normally used 3 to 5 ng/ml does not result in upregulation of CD73, but increases stemness in the Th17<sup>TGF<beta></sup> cells. The detailed analysis also shows that even at 250 pg/ml, the level of glycolysis also drops, and these cells with intermediate level of glycolysis and stemness are better than the cells that are either highly glycolytic with low stemness (Th17<sup>IL1<beta></sup>) or exhibit high stemness but lower glycolysis (Th17<sup>TGF<beta></sup>). Finally, our approach to use the alternative strategy for generating antitumor helper T cells by combining Th17<sup>TGF<beta></sup> culture conditions with low-dose TGFβ could have translational potential owing to long-term persistence and substantial improvement in tumor control. The difference in antitumor effector phenotype based on metabolic commitment also enforces a key role of cellular energy requirements in regulating antitumor function. Overall, this study may significantly forward our understanding of the factors that control long-term and stable antitumor T-cell functions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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CD73 Expression Dampens Antitumor Th17 Response


Reducing CD73 Expression by IL1β-Programmed Th17 Cells Improves Immunotherapeutic Control of Tumors

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