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Reduced CD73 Expression by IL-1 β Programmed Th17 Cells Improves Tumor Control

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Abstract

T helper (Th)-17 subsets hold promise in adoptive T cell transfer therapy for cancer. However, *ex vivo* programming of Th17 cells in presence of TGF- β increases cell surface expression of ectonucleotidases CD39 and CD73, that in turn increases susceptibility to immunosuppression and reduces effector functions. Our data shows that ATP mediated suppression of IFN- γ production by Th17 cells can be overcome either by genetic ablation of CD73 or by generating TGF- β independent Th17 in presence of IL-1 β . Th17 cells cultured in IL-1 β are also highly polyfunctional, express high level of effector molecules and exhibit better short-term control of B16-F10 murine melanoma, despite reduced stem cell like properties. Adding TGF- β at low dose that does not up regulate CD73 expression, but induces stemness, drastically improves anti-tumor function of IL-1 β cultured Th17 cells. It is likely that effector property of IL-1 β dependent Th17 is due to their high glycolytic capacity, since generating IL-1 β dependent Th17 cells in pyruvate containing media impaired glycolysis and its anti-tumor potential. Thus, our data suggests that due to induction of ectonucleotidase expression by TGF- β , *ex vivo* culture conditions for generating Th17 cells need to be reconsidered for exploiting their full potential in adoptive T cell therapy.

Keywords

Immunotherapy; Th17; CD73; melanoma; glycolysis

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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, VISTA), engaging co-stimulatory molecules (2,3), expanding T cells in different cytokines (IL-2, IL-15, IL-12, IL-21, IL-27) (4) and generating distinct T helper (Th) cell subsets (Th9, Th17) with enhanced *in vivo* persistence (5,6). However, recent studies show that immunosuppressive mechanisms induced by the tumor, such as indoleamine-2, 3-dioxygenase (IDO), PD-L1/B7-H and FoxP3⁺ regulatory T cells (Tregs), might serve as negative feedback mechanisms that follows rather than precedes the infiltration of T cells into the tumor (7). These results underscore the need to understand the T cell derived factors that aid in promoting an immunosuppressive tumor microenvironment, and to use this knowledge in designing cellular therapies that effectively treat patients with advanced malignancies.

There has been a recent resurgence of the CD4⁺ T cell subsets (Th1, Th9, Th17) in tumor immunotherapy (5–7). While studies have shown that Th17 cells do promote tumor growth (8,9), an effective anti-tumor property of Th17 cells can be observed when they co-express key Th1 cytokine IFN- γ (5). These hybrid Th17+Th1 phenotype bearing T cells display enhanced persistence and robust memory response to tumors compared to Th1 cells when infused into mice bearing melanoma (5). This implies that while anti-tumor effector function of hybrid Th17+Th1 cell depends on Th1 cytokine IFN- γ , the other Th17 properties of ‘stemness’, which may contribute to persistence (10,11), or reduced susceptibility to activation induced cell death may be dependent specifically on Th17 programming conditions (12). Given that Th17 cells can also convert into a regulatory Th17⁺FoxP3⁺ phenotype under inflammatory conditions in the tumor microenvironment (13), it is crucial to understand which cytokines are responsible for regulating the pro- vs. anti-tumorogenic properties of Th17 cells. Our data characterizing the hybrid Th17+Th1 cells generated using TGF- β dependent (i.e. Th17^{TGF- β 1} with TGF- β 1/IL-6) or TGF- β independent (i.e. Th17^{IL-1 β} with IL-1 β /IL-6) culture conditions show that Th17^{IL-1 β} cells exhibit a dominant Th1 phenotype with nominal expression of ectonucleotidase CD73, high effector (i.e. T-bet^{hi}, Granzyme B^{hi}, IL-10^{lo}) and glycolytic effector phenotype (i.e. IFN- γ ^{hi}, CD107a^{hi}, HIF-1 α ^{hi}) as compared to Th17^{TGF- β 1} 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 regulatory T cells (Treg) property, maximize Th1 features while maintaining Th17 phenotype- potentiating the long-term anti-tumor response after ACT.

Materials and Methods

Mice

C57BL/6, CD73^{-/-} (B6.129S1-*Nt5e^{tm1Lft}/J*), B6-Thy1.1 (B6.PL-*Thy1^a/CyJ*), OT-II (B6.Cg-Tg (Tcr α Tcr β 425Cbn/J) mice were obtained from Jackson Laboratory (Bar Harbor, ME). Development of h3T transgenic mouse bearing TCR reactive to the human tyrosinase 368–376 (YMDTMSQV) epitope has been described recently (14). OT-II-GFP-FoxP3 mice co-

expressing EGFP and FoxP3 were kind gift from Dr. C. Vasu, MUSC. Animals were maintained in pathogen-free facilities and procedures approved by IACUC.

Reagents and cell lines

Ova (ova₃₂₃₋₃₃₉) (ISQAVHAAHAEINEAGR) and MART-1 (ELAGIGILTV) peptide were purchased from GenScript (Piscataway, NJ). Penicillin, streptomycin, glucose free RPMI-1640, IMDM were purchased from Life Technologies, Grand Island, NY. FBS was procured from BioAbChem Inc., Ladson, SC. All the recombinant cytokines except IL-2 (Shenandoah Biotechnology, Warwick, PA) and fluorochrome conjugated anti-mouse CD4 (GK1.5), CD73 (TY/11.8), CD26 (H194-112), CD44 (IM7), CD62L (MEL-14), IFN- γ (XMG1.2), IL-17a (TC11-18H10.1), IL-22 (Poly5164), IL-2 (JES6-5H4), TNF- α (MP6-XT22), CD25 (PC61) and T-bet (4B10) were purchased from BioLegend, San Diego, CA. Fluorochrome conjugated anti-mouse V β 5.1,5.2 (MR9-4), IRF-4 (3E4), CD39 (24DMS1) and ROR γ t (AFKJS-9) were obtained from eBiosciences (San Diego, CA). Anti-human V β 12 was from Thermo Scientific (Rockford, IL). Purified anti-CD3, anti-CD28, anti-IFN- γ , anti-IL-4 were obtained from UCSF monoclonal antibody core, UCSF, CA. Anti-mouse pS6 conjugated with Alexa647 was purchased from Cell Signaling Technology (Danvers, MA). Tumor cells tested for antibody production were obtained from our collaborators as follows: 624-MEL (Dr. Michael Nishimura, Loyola University, Chicago), EL4 (Dr. Zihai Li, MUSC), B16-ova (Dr. Mark Rubinstein, MUSC).

Culture condition

Magnetic-bead sorted CD4⁺ T cells (>95%) from B6 splenocytes or in some cases FACS (MoFlo Astrios, Beckman Coulter, Fullerton, CA) sorted CD4⁺T cells from h3T splenocytes were differentiated into either Th17^{TGF- β 1} (3 ng/ml TGF- β 1, 25 ng/ml IL-6) or Th17^{IL-1 β} (20 ng/ml IL-1 β and 25 ng/ml IL-6) or Th17^{IL-1 β +TGF- β} (20 ng/ml IL-1 β , 25 ng/ml IL-6 and 250 pg/ml TGF- β 1) in presence of plate bound anti-CD3 (5 μ g/ml) and anti-CD28 (5 μ g/ml). Monoclonal antibody (mAb) to IFN- γ (XMG1.2; 10 μ g/ml) and mAb to IL-4 (11B11; 10 μ g/ml) were added to the polarizing media and after 48h cells were fed with IL-2 (50 IU/ml). Complete IMDM (cIMDM) media containing 10% FBS, penicillin, streptomycin was used for T cells differentiation. However, in some cases, glucose and pyruvate free RPMI-1640 media supplemented either with 20mM glucose plus 1mM pyruvate or 20mM pyruvate plus 3-Mercaptopicolinic acid (gluconeogenesis blocker; Sigma, St. Louis, MO) was used for Th17^{IL-1 β} polarization. Purified CD4⁺ T cells stimulated with plate bound anti CD3 (5 μ g/ml) and anti CD28 (5 μ g/ml) were termed as Th0 in this study. In some experiments, T cells polarization was performed either presence or absence of 50 μ M ATP (Sigma, St. Louis, MO). On day 3 of culture, T cells were harvested and either processed for intracellular cytokine analysis, RNA preparation using TriZol (Life technologies, Grand Island, NY) 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 μ g/ml ova₃₂₃₋₃₃₉ 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 (0.25×10^6) were

injected intraperitoneally (i.p.) into C57BL/6 mice, and on day twelve a total of 1×10^6 Th17 cells (either Th17^{TGF- β 1} or Th17^{IL-1 β}) were transferred i.p. into the tumor site. Following 48h of T cell transfer, peritoneal ascites fluid was drawn and donor cells were tracked using congenic Thy1.1 marker. B16-F10-ova (0.25×10^6) and 624-MEL (2.5×10^6) were injected subcutaneously (s.c.) into left flank of C57BL/6 or Rag1^{-/-} C57BL/6 mice or NSG-A2 mice respectively. Twenty-four hour before adoptive transfer of T cells (CD4⁺V β 5⁺ ova specific Th17^{TGF- β 1}, Th17^{IL-1 β} or Th17^{IL-1 β + TGF- β}) 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 β 5⁺ (1×10^6) ova specific Th17^{TGF- β 1}, Th17^{IL-1 β} or Th17^{IL-1 β + TGF- β} cells (1×10^6 cells/mice) on day 7. For xenograft tumor experiment, 15 days s.c. established 624-MEL in NSG-A2 mice were either kept untreated or treated with either 0.2×10^6 CD4⁺V β 12⁺ Th17^{TGF- β 1} or Th17^{IL-1 β + TGF- β} cells.

Activation induced T cell death

Differentiated ova specific Th17 (Th17^{TGF- β 1}, Th17^{IL-1 β} or Th17^{IL-1 β +TGF- β}) re-stimulated for 4h with either cognate antigen (ova₃₂₃₋₃₃₉) or non-specific antigen (MART-1) loaded irradiated C57BL/6 splenocytes at 5:1 (T cells:B6 splenocytes) ratio. Apoptosis was measured by Annexin V (BD Biosciences, San Jose, CA) vs. 7AAD staining according to the manufacturer's protocol, followed by flow cytometry. Data were analyzed with FlowJo software (Tree Star, OR).

Cytotoxicity assay

B16-F10-ova (specific target) or EL-4 (non-specific target) cells labeled with CFSE (Life technologies, Grand Island, NY) and co-cultured with differentiated Th17^{TGF- β 1} and Th17^{IL-1 β} cells were used to determine cytotoxic potential, as detailed in supplementary methods.

Flow cytometry and q-PCR

Detailed protocols for staining the cells for surface markers and intracellular cytokines have been described earlier (15), and in supplementary methods. Detailed methodology for q-PCR is provided in supplementary method section.

Glucose uptake, oxygen consumption and glycolytic flux

Oxygen consumption rate (OCR), Extracellular acidification rate (ECAR) was evaluated as described earlier (16). Glucose uptake was determined by 2NBDG (Cayman chemical, Ann Arbor, Michigan) uptake assay according to the manufacture'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 $\pm$ SD unless stated otherwise. The unpaired Student's *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, San Diego, CA).

RESULTS

TGF- β induced CD73 expression on Th17 cells increases susceptibility for IFN- γ suppression

CD39 and CD73 are two ectonucleotidases that sequentially cleave ATP to produce adenosine and are expressed at higher levels in TGF- β polarized Th17 (Th17^{TGF- β 1}) cells as compared to the unpolarized Th0 cells (Fig. 1a). Since, ATP is present at high concentration in the tumor microenvironment (17) and the ATP-byproduct adenosine (a potent immune suppressive) subverts anti-tumor immunity (18), we therefore tested whether ATP could affect the functionality of Th17^{TGF- β 1} cells. Flow analysis revealed that ATP (50 μ M) significantly suppressed IFN- γ production without affecting the IL-17 production by Th17^{TGF- β 1} cells subset with CD39⁺CD73⁺ phenotype, as compared to Th0 cells that express minimal CD73 on their cell surface (Fig. 1b). This data implies that CD73 expression could lead to impairment of IFN- γ production by Th17^{TGF- β 1} cells in ATP rich tumor microenvironment. To further evaluate the functional fate of Th17^{TGF- β 1} cells co-expressing CD39 and CD73 in tumor microenvironment, purified CD4⁺ T cells from congenic Thy1.1 mice were differentiated towards Th17^{TGF- β 1} and injected into C57BL/6 (Thy1.2) mice bearing EL-4 ascites tumor. Donor cells retrieved from the tumor site after 48h showed decreased IFN- γ production following re-stimulation (Fig. 1c). Further, to confirm the role of ectonucleotidases CD73 in ATP mediated suppression of IFN- γ production by Th17^{TGF- β 1} 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 to Wt Th17^{TGF- β 1} cells, CD73^{-/-} Th17^{TGF- β 1} cells showed no decrease in IFN- γ secretion when cultured in ATP (Fig. 1d). These data supports a role of CD73 in suppressing IFN- γ production by Th17^{TGF- β 1} cells exposed to ATP.

Next, we tested if the polarization of Th17 cells in absence of TGF- β 1, but in presence of IL-1 β that is reported to have lower cell surface expression of CD39 and CD73 (19,20), would affect the persistence and function of anti-tumor Th17 cells (Fig. 1e). We thus tested the susceptibility of Th17^{IL-1 β} cells to IFN- γ suppression in ATP rich environment. Naïve CD4⁺ T cells were polarized to Th17^{IL-1 β} in presence or absence of 50 μ M ATP and cytokines production was analyzed. We found that Th17^{IL-1 β} cells express less CD39 and CD73 on cell surface, and were resistant to suppression of IFN- γ production in presence of ATP (Fig. 1f). Furthermore, Th17^{IL-1 β} cells significantly retain their IFN- γ production at tumor site when retrieved from tumor site after 48h of cell transfer (Fig. 1g). This data establishes that Th17^{IL-1 β} cells could be better than Th17^{TGF- β 1} cells in retaining their functionality in tumor microenvironment.

Distinct functionality of Th17^{TGF- β 1} and Th17^{IL-1 β} cells

IFN- γ plays a pivotal role in Th17 mediated control of tumor growth (5). Since, both Th17^{IL-1 β} vs. Th17^{TGF- β 1} cells differ in their susceptibility to suppression of IFN- γ production at tumor site, we further characterized these cells. On comparing the effector

cytokines secretion ability between by Th17^{TGF-β1} and Th17^{IL-1β} cells *in vitro*, we observed that about ~1.5 fold higher proportion of Th17^{IL-1β} cells (12%) secreted IFN-γ than Th17^{TGF-β1} cells (8%) (Fig. 2a). Similarly, Th17^{IL-1β} cells also showed higher percent of TNF-α (80% vs. 65%) and IL-22 (6.5% vs. 0.5%) as compared to Th17^{TGF-β1} cells. The increased secretion of Th1 effector cytokines (IL-2, IFN-γ, TNF-α) and reduction in IL-17 by Th17^{IL-1β} cells correlated to the higher expression level of transcription factor T-bet, IRF-4, and reduced level of ROR-γ, respectively (Fig. 2b). Interestingly, evaluation of the cell surface markers showed that Th17^{IL-1β} cells express high CD62L⁺CD44⁺ central memory phenotype (88%) as compared to Th17^{TGF-β1} cells (50%) (Fig. 2ci), and display a prominent active phenotype as elucidated by CD25 expression (Fig. 2cii). Th17^{IL-1β} cells also showed high surface expression of CD26 (Fig. 2ciii), which has been shown to be associated with high IL-22, GM-CSF and IL-23R (21). Th17^{IL-1β} cells also exhibit higher *IL-23R* (that stabilizes Th17 phenotype) (22), *GzmB* (encodes for cytolytic molecule granzyme B), *CSF2* (encodes for GM-CSF), cytokines *IL-22*, and *IL-3* (Fig. 2di). It has been recently shown that IL-23 enhances/stabilizes Th17 phenotype, and similar to GM-CSF that also increases effector function leading to pathogenic phenotype in experimental autoimmune encephalomyelitis (EAE) model (23,24). Importantly, the expression of cytokine IL-10, that could suppress T cell response, was decreased in Th17^{IL-1β} cells as compared to Th17^{TGF-β1} cells. Further, a q-PCR array based analysis also revealed that transcription factors regulating TCR signaling, Erk1/2, Notch and EGF pathway were highly expressed in Th17^{IL-1β} cells as compared to Th17^{TGF-β1} cells (Fig. 2dii). In addition, a signal transduction array analysis also revealed that Th17^{IL-1β} cells engage in multiple signaling pathway than Th17^{TGF-β1} cells (Fig. 2diii), which may be responsible for their overall enhanced effector capability (25).

Enhanced anti-tumor function of Th17^{IL-1β} vs. Th17^{TGF-β1} cells

Since Th17^{IL-1β} 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-β1} and Th17^{IL-1β} phenotype, first we tested their ability to lyse murine melanoma cell, *in vitro*. Our data shows that Th17^{IL-1β} cells can lyse the tumor cells directly and are more cytolytic than Th17^{TGF-β1} cells (Fig. 3a and supplementary Fig. 1a). Next, adoptive transfer of 1×10^6 ova specific Th17^{TGF-β1} and Th17^{IL-1β} 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^{IL-1β} vs. Th17^{TGF-β1} cells (Fig. 3b). At the experimental end-point, a cytokine analysis of adoptively transferred donor cells retrieved from various sites (tumor and non-tumor) showed that the IFN-γ secretion was dramatically reduced in Th17^{TGF-β1} cells retrieved from tumor site (Fig. 3c, lower left panel), as compared to Th17^{IL-1β} cells that maintained their IFN-γ secretion at tumor site (Fig. 3c, lower right panel). Importantly, donor cells retrieved from the lymph node, spleen and blood did not show significant decrease in IFN-γ secretion upon restimulation. This data establishes that Th17^{TGF-β1} cells with high CD73 expression are more prone to loosing effector cytokine IFN-γ in tumor micro-environment as compared to Th17^{IL-1β} cells. In addition, it is possible that higher expression of CD25 on Th17^{IL-1β} cells resulted in their increased homeostatic proliferation as compared to Th17^{TGF-β1} cells (26), and thereby improved anti-tumor effect. Next, to determine whether the anti-tumor potential

of Th17^{IL-1 β} cells are independent of any endogenous T cells, we transferred 1×10^6 of either OT-II Th17^{TGF- β 1} or Th17^{IL-1 β} cells to Rag^{-/-} C57BL/6 mice bearing B16-F10-ova melanoma. We obtained similar tumor regression by Th17^{IL-1 β} cells in tumor bearing Rag^{-/-} C57BL/6 mice as we observed for immunocompetent mice suggesting that Th17^{IL-1 β} cells could control the tumor growth independent of CD8 T cells (data not shown).

To further address the issue of Th17 plasticity, i.e. the ability to convert to FoxP3⁺ cells, which is considered a key reason for the failure of Th17 cells in tumor-immunotherapy, we tested if there is a differential susceptibility for conversion to FoxP3⁺Th17 cells between the Th17^{TGF- β 1} and Th17^{IL-1 β} cells. Using CD4⁺ T cells from GFP-FoxP3 developed on OT-II background for programming to Th17^{TGF- β 1} and Th17^{IL-1 β} cells, we noted an increased percent of FoxP3⁺GFP⁺ cells in conventional Th17^{TGF- β 1} 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 shows that conversion to iTreg phenotype is decreased in Th17^{IL-1 β} cells as compared to Th17^{TGF- β 1} ones, potentially due to higher Gfi-1 expression (Fig. 3d, right panel). These sets of data establish that Th17^{IL-1 β} cells possess better anti-tumor properties and are less 'plastic' than conventional Th17^{TGF- β 1} cells.

Improved anti-tumor ability of Th17^{IL-1 β} cells correlates to 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). Since IFN- γ secretion has also been shown to be dependent on glucose consumption (29,30), we reasoned that Th17^{IL-1 β} cells could be more glycolytic. A comparison of glucose consumption reveals that Th17^{IL-1 β} cells were more glycolytic than Th17^{TGF- β 1} cells (Fig. 4a). To further address this issue, extracellular acidification rate (ECAR), an indicator of glycolysis, and oxygen consumption rate (OCR), an indicator of oxidative phosphorylation (OXPHOS), was measured in Th17^{IL-1 β} and Th17^{TGF- β 1} cells using seahorse bioanalyzer. Our data shows that Th17^{IL-1 β} cells had higher ECAR value as compare to Th17^{TGF- β 1} cells indicating their high glycolytic capacity (Fig. 4bi). Further, the basal OCR was less in Th17^{IL-1 β} cells as compared to Th17^{TGF- β 1} cells (Fig. 4bii), leading to a higher OCR/ECAR ratio in Th17^{TGF- β 1} cells (Fig. 4biii). To further ascertain the metabolic difference between Th17^{IL-1 β} and Th17^{TGF- β 1} cells, we analyzed mRNA expression of various glycolysis-associated genes using q-PCR. Our data shows that the mRNA expression levels of all glycolytic genes evaluated were several fold higher in Th17^{IL-1 β} cells as compared to Th17^{TGF- β 1} cells (Fig. 4c). The increased expression at mRNA level also translated to increased expression of glycolytic proteins, as represented by HKII expression (Fig. 4d). The increased glycolysis in Th17^{IL-1 β} cells also correlated with increased activation of mTOR pathway, as determined by phosphorylation of S6 (Fig. 4e). This data supports that higher effector function and anti-tumor control displayed by Th17^{IL-1 β} cells as compared to Th17^{TGF- β 1} cells could be attributed to higher glycolytic commitment.

Shift from glycolysis dampens Th17^{IL-1β} cells effector functions

Next, we tested if the increase in glycolytic commitment observed in Th17^{IL-1β} cells is key to increased expression of effector molecules that are responsible for better anti-tumor effector response. For this purpose, the Th17^{IL-1β} cells were polarized in complete media containing glucose that supports glycolysis, or media with no glucose but pyruvate with gluconeogenesis blocker (that supports oxidative phosphorylation). We observed that cells grew at same rate (Fig. 5ai) and also had similar activation profile (Supplementary Fig. 1b) in pyruvate containing (without glucose) media as compared to complete media. On analyzing the Th17^{IL-1β} cells polarized in different media for signature cytokines production after stimulation with PMA/Ionomycin, we found that blocking glycolytic pathway by culturing in pyruvate containing media markedly inhibited the IFN-γ and IL-17 production implying that these cytokines production dependent on glucose. However, TNF-α secretion remained unaffected by pyruvate (Fig. 5aii). It has been documented that IFN-γ and TNF-α have a complimentary relationship, and it is likely that similar to the differences in secretion pattern between IFN-γ and TNF-α ON/OFF cycle (31,32), different metabolic requirements exist for these cytokines to compensate for effector function under changing microenvironment. To evaluate whether inhibiting glycolytic pathway could also affect the cytolytic capacity Th17^{IL-1β} cells, mRNA expression GzmB was analyzed after polarization of cells in pyruvate containing media. Expression analysis using q-PCR revealed that key effector molecules (GzmB, GM-CSF, T-bet, IL-22, IL-3) was significantly down regulated in Th17^{IL-1β} cells cultured in presence of only pyruvate containing media as compared to complete media (Fig. 5b). Moreover, we also found that antigen specific killing of tumor cells B16-F10-ova were greatly reduced when Th17^{IL-1β} cells were polarized in pyruvate containing media (Fig. 5c and supplementary Fig. 1c). However, no correlation between glycolysis and CD39/CD73 expression by Th17^{IL-1β} cells was established, since culturing Th17^{IL-1β} cells in pyruvate containing media (glucose free) did not affect the CD39/CD73 expression (Supplementary Fig. 2a). Next to further confirm that glycolysis is necessary to mount proper anti-tumor response by Th17 cells, Rag^{-/-} C57BL/6 mice with seven days established B16-F10-ova tumor were treated by adoptively transferring 1×10⁶ ova specific Th17^{IL-1β} cells polarized either in normal or only pyruvate containing media. Anti-tumor potential of Th17^{IL-1β} cells were 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 Th17^{IL-1β} cells polarized in normal media markedly delayed tumor growth. These data together strongly suggest that anti-tumor potential of Th17 cells is highly dependent on their metabolic commitment, and higher glycolysis may have contributed to improved anti-tumor response observed with Th17^{IL-1β} cells.

Lack of stemness in Th17^{IL-1β} cells could be restored by very low dose of TGF-β

Despite superior anti-tumor activity of adoptively transferred Th17^{IL-1β} cells *in vivo*, we noticed that the Th17^{IL-1β} recipient mice could not control tumor growth after 30–35 days. The inability to control tumor growth long-term was also correlated to the poor persistence of these Th17^{IL-1β} cells *in vivo* (Supplementary Fig. 2b). Since, long-term persistence of conventional Th17^{TGF-β1} cells is attributed to ‘stemness’ signature (10,11), we analyzed various stemness-associated genes in Th17^{IL-1β} vs. Th17^{TGF-β1} cells. Real time-PCR analysis revealed that expression of various stem cell associated genes (*β-catenin*, *Bcl6*,

Tcf7, *Left1*) was significantly lower in Th17^{IL-1 β} cells as compared to Th17^{TGF- β 1} cells (Fig. 6a). We also found greater induction of AICD in Th17^{IL-1 β} cells as compared to Th17^{TGF- β 1} cells following antigen restimulation (*data not shown*). These data together indicate that Th17^{IL-1 β} cells exert the characteristics of terminally differentiated population with profound anti-tumor potential, however, lack stemness features and thus persist for a short period of time in tumor bearing host. Since TGF- β 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 Th17^{TGF- β 1} cells (10,11). We thus titrated for a minimum dose of TGF- β that could impart stemness without inducing the expression of ectonucleotidase CD73. Our dose titration data showed that 250 pg/ml of TGF- β minimally up-regulates CD73 expression (Fig. 6b). Th17^{IL-1 β} cells differentiated in presence of 250 pg/ml of TGF- β (referred to as Th17^{IL-1 β +TGF- β}) 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^{IL-1 β +TGF- β} cells exhibited intermediate glycolysis (Th17^{IL-1 β} > Th17^{IL-1 β +TGF- β} > Th17^{TGF- β 1}), and stemness (Th17^{TGF- β 1} > Th17^{IL-1 β +TGF- β} > Th17^{IL-1 β}) gene signature (Fig. 6c, left and right panels). In addition, a TCR restimulation induced AICD was decreased in Th17^{IL-1 β +TGF- β} cells as indicated by lower percentage of cells with Annexin V and 7AAD positivity (Fig. 6d). This data also confirms a recent observation that T cells differentiated in presence of glycolysis inhibitor 2-deoxy glucose programs for better anti-tumor control and persistence (34). Importantly, the Th17^{IL-1 β +TGF- β} 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 Th17^{IL-1 β +TGF- β} group did not reach half the tumor end-point (< 100 mm²) until sacrificed on day 70 - indicating the long-term persistence of Th17^{IL-1 β +TGF- β} cells *in vivo* (Supplementary Fig. 2c). Thus, Th17 cells generated *ex vivo* with IL-1 β and low concentrations of TGF- β program anti-tumor 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 exonucleotidase CD73 expression induced immunosuppression (Supplementary Fig. 3). We believe that this study will uncover important aspects that need to be considered when generating potent tumor specific T cells, in order to improve future T cell cancer immunotherapy trials.

DISCUSSION

Cellular cancer therapies based on stimulating the patient's immune system represent an important treatment modality, but much remains to be understood to optimize their use. Several biological mechanisms may account for their failure to achieve efficient immune protection (35). One important confounding factor is immunosuppression by the tumor microenvironment (35,36). In addition, recent studies have shown that after T cells infiltrate to the tumor, there is a heightened immunosuppression in the host due to effector T cells

themselves (7). As cancer immunotherapy develops, it is thus particularly important to understand the impact of ACT treatments in tumor immunosuppression and immunity.

Recent development in T cell mediated therapeutic approach to control tumor growth has led to the development of various protocols to *ex vivo* program not only Th1/Tc1 cells, but other subsets as Th9/Tc9 and Th17/Tc17 for tumor control (5,6,37). While Th17 cells have shown promise in controlling tumor growth (5), the stable phenotype of these cells has been called in question due to their ability to convert to FoxP3 expressing regulatory Th17 cells (13). Studies have shown that TGF- β cultured Th17 cells express ectonucleotidase CD73 and exhibit the ability to suppress the effector T cells (19,38). Additionally, the TGF- β mediated expression of ectonucleotidase on Th17 cells is also found in commensal bacteria rich intestinal lamina propria with high concentration of luminal ATP, that may be responsible for their maintenance and pathogenicity (39). However, a recent study has addressed that the cytokine requirements for Th17 cell polarization *in vivo* depends on the site of priming, and revealed key differences by which the systemic, mucosal, and cutaneous immune systems guide Th17 cell lineage commitment with IL-1 β in an irreplaceable role (40). Given these issues of Th17 plasticity and conversion to regulatory phenotype, along with the variability of cytokine environment in regulating Th17 generation, we addressed as to how a stable Th17 cell with ability to control the tumor growth long-term could be programmed *ex vivo*. Our data substantiates the potential contribution of ectonucleotidase CD73 expression in self-suppression of the effector Th17 cells generated using conventional method with TGF- β , and proposes the strategies to program long-lived effector Th17 cells by combining inflammatory cytokine IL-1 β along with a low dose of TGF- β (that does not induce CD73, but up regulates 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 the immunosuppression by their sequential action of converting ATP to adenosine (41). While 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 CD73-KO mice (44). Similarly, the role of adenosine generation catalyzed by CD39/CD73 expressing Treg's 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 shows that the expression of CD73 on T cell itself could also lead to its increased susceptibility to suppression and loss of effector function. Since TGF- β is the key contributor of CD73 expression, we evaluated herein the T cell subsets that are programmed in TGF- β , i.e. Th17 cells. *Ex vivo* generated Th17 cells that are programmed with TGF- β 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 TGF- β or in absence of TGF- β (but with IL-1 β). The improved ability of IL-1 β programmed Th17 cells to secrete higher level of IFN- γ , express enhanced level effector molecules, and control tumor could be due to the direct effect of IL-1 β 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 IL-1 β treatment increases the expression of pathogenic genes that results in increased incidence of disease (20,48). The increased expression of IL-23R and other effector molecules that resulted in increased pathogenesis in autoimmune model may have been responsible for rendering tumor epitope specific Th17^{IL-1 β} more efficacious than Th17^{TGF- β} cells in targeting ‘self’ but tumor associated epitope and controlling tumor growth. Our data also shows that IL-1 β cultured Th17 cells exhibit increased level of glucose transported Glut-1 that correlates to increased glucose consumption, which fuels the metabolic need of the rapidly dividing glycolytically active effector Th17^{IL-1 β} cells. Our data also suggests that the expression of CD39/CD73 on Th17^{IL-1 β} cells does not inversely correlates to 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 2a, *left panel*). Contrary to the earlier studies that show CD73 expression correlates closely with HIF-1 α expression either in intestinal epithelia or gastric carcinoma (49,50), *ex vivo* programming of T cells with IL-1 β (that promotes HIF-1 α) in absence of TGF- β inhibited the expression of CD39/73 (Supplementary Fig 2a, *right panel*). Importantly, while these glycolytically active Th17^{IL-1 β} cells do exhibit increased ability to control tumors short-term, they do not persist well in the host. The decreased persistence of Th17^{IL-1 β} cells correlates to decreased expression of ‘stemness’ signature that has been otherwise shown to be a key feature of Th17 cells (10,11). Our data shows that it may be the presence of TGF- β in the culture conditions that renders the ‘stemness’ to Th17^{TGF- β} cells, and thus Th17^{IL-1 β} cells do not exhibit stemness. However, addition of low concentration of TGF- β (250 pg/ml) as opposed to the normally used 3–5 ng/ml does not results in up-regulation of CD73, but increases stemness in the Th17^{IL-1 β} 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^{IL-1 β}) or exhibit high stemness but lower glycolysis (Th17^{TGF- β}).

Finally, our approach to use the alternative strategy for generating anti-tumor helper T cells by combining Th17^{IL-1 β} culture conditions with low dose TGF- β could have translational potential owing to long-term persistence and substantial improvement in tumor control. The difference in anti-tumor effector phenotype based on metabolic commitment also enforces a key role of cellular energy requirements in regulating anti-tumor function. Overall, this study may significantly forward our understanding of the factors that control long-term and stable anti-tumor T cell functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. TGF- β induced CD73 expression on Th17 cells increases susceptibility for IFN- γ suppression

(a) Flow cytometric analysis of ectonucleotidases (CD39 and CD73) expression by unpolarized (Th0) or TGF- β 1 mediated Th17 (Th17^{TGF- β 1}) cells. Data are representative of 5 independent experiments. (b–d) Intracellular IFN- γ and IL-17 production in: (b) presence or absence of ATP (50 μ M) by Th17^{TGF- β 1} or unpolarized (Th0) cells, (c) Thy1.1⁺ Th17^{TGF- β 1} cells retrieved from the tumor site of C57BL/6 (Thy1.2⁺) mice (n=4) bearing EL-4 ascitic tumor following 48 h of T cells transfer, (d) Th17^{TGF- β 1} polarized cells from either wt. or CD73^{-/-} C57BL/6 mice. Cumulative data from 3 different experiments is represented in bar diagram alongside dot-plot for the percentage of cells producing IFN- γ in presence or absence of ATP (50 μ M). (e) Flow cytometric analysis (right panel) of CD39 and CD73 expression by Th0, Th17^{TGF- β 1} and Th17^{IL-1 β} cells. (f–g) intracellular IFN- γ and IL-17 secretion in: (f) presence or absence of ATP by Th17^{IL-1 β} cells, (g) Thy1.1⁺ Th17^{IL-1 β} cells retrieved from the tumor site of C57BL/6 (Thy1.2⁺) mice (n=4) bearing EL-4 ascitic tumor following 48 h of T cells transfer. Results are representative of 3 (e) and 5 (f–g) independent experiments. *** p <0.0001.

Figure 2. Distinct functionality of Th17^{TGF- β 1} and Th17^{IL-1 β} cells

(a) Naïve CD4⁺ T cells from C57BL/6 mice were differentiated towards either Th17^{TGF- β 1} or Th17^{IL-1 β} and intracellular production of various cytokines was analyzed. Percentage of cells producing different cytokines is also represented in pi-diagram (50,000 cells/group were analyzed to draw pi-diagram). (b) q-PCR analysis (*upper panel*) and flow cytometric analysis (*lower panel*) of various Th17 signature transcription factors expression by Th17^{TGF- β 1} and Th17^{IL-1 β} cells. (c–d) Flow cytometric analysis of: (ci) CD62L vs. CD44 expression, and (cii) CD25 expression Th17^{TGF- β 1} and Th17^{IL-1 β} cells at day 3 of polarization. (e) q-PCR analysis of expression of key effector genes in Th17^{TGF- β 1} and Th17^{IL-1 β} cells after 3 days of polarization. Transcription factors array (dii) and signal transduction array (diii), was performed using the 84-gene q-PCR based array kit (SABiosciences, Valencia, CA, USA) as per manufacturer's recommendation. Fold up-regulation (blue) or down-regulation (red) of Th17^{IL-1 β} over Th17^{TGF- β 1}.

Figure 3. Enhanced anti-tumor function of Th17^{IL-1β} vs. Th17^{TGF-β1} cells

(a). B16-F10-ova cells labeled with CFSE were co-cultured at 1:5 ratio with either Th17^{TGF-β1} or Th17^{IL-1β} for 6h and decrease in the number of cells expressing CFSE was analyzed by using flow cytometry. (b) wild type (WT) C57BL/6 mice (n=5 mice/group) were inoculated (s.c.) with 0.25×10^6 B16-F10-ova murine melanoma cells and treated with cyclophosphamide (CTX) (4mg/mouse) after 7 days. CTX treated mice either kept untreated as control or adoptively transferred one day later with either 1×10^6 ova specific Th17^{TGF-β1} or Th17^{IL-1β} cells. Tumor growth was measured using digital calipers every fourth day. Data in figure demonstrate mean tumor size at each time point from one of the two experiments with similar results. (c) Intracellular cytokine production of ova specific donor Th17^{TGF-β1} and Th17^{IL-1β} cells after retrieving from either lymph nodes, spleen, peripheral blood or tumor site of 21 days tumor bearing mice (n=4). Cytokines production of donor cells was compared with non-transferred cells. Data represent two independent experiments. (d) Naïve CD4⁺ T cells from OT-II GFP-FoxP3 mice were polarized to Th17^{TGF-β1} and Th17^{IL-1β} type in presence of ova and percentage of cells expressing GFP (indicative of FoxP3 expressing cells) were analyzed after 3 days of polarization using flow cytometer (*left panel*). q-PCR analysis (*right panel*) of Gfi-1 expression by Th17^{TGF-β1} and Th17^{IL-1β} cells. Data represent 3 independent experiments. ** $p < 0.005$ and *** $p < 0.0001$.

Figure 4. Th17^{TGF-β1} and Th17^{IL-1β} cells are metabolically different

Differences in glycolysis between Th17^{TGF-β1} and Th17^{IL-1β} cells was observed using: (*a*) Glucose uptake using fluorescent glucose (2-NBDG), (*bi*) Basal Extracellular Acidification Rate (ECAR), (*bii*) Basal Oxygen Consumption rate (OCR), (*biii*) Basal OCR/ECAR ratio, (*c*) q-PCR analysis of the expression of key genes associated with glycolysis, (*d*) Upper panel shows western blot for hexokinase II (HKII); lower panel: blot quantification of HKII, and (*e*) flow cytometric analysis of phosphorylation of S6 (pS6) ribosomal protein. Results in (*a*, *b*) are representative of 4 and (*c*, *d*) are representative of 3 independent experiments with similar results.

Figure 5. Shift from glycolysis dampens Th17^{IL-1β} cells effector functions

(ai) Schematic diagram of the culture conditions used to generate the Th17^{IL-1β} cells. (aii) Intracellular staining of various cytokines, (b) q-PCR analysis of the expression of key effector genes, and (c) cytolysis of B16-F10-ova cells was evaluated using Th17^{IL-1β} cells polarized either in complete media (green bars) or in 20mM pyruvate (no glucose) containing media (brown bars). (d) C57BL/6 Rag1^{-/-} mice (n=5 mice/group) were inoculated (s.c.) with 0.25×10^6 B16-F10-ova, and after seven days mice were either kept untreated as control or adoptively transferred with either 1×10^6 ova specific Th17^{IL-1β} OT-II cells polarized either in complete media or 20mM pyruvate (no glucose) containing media. Tumor growth was measured using digital calipers every fourth day. Data in figure demonstrate mean tumor size at each time point in one of the two experiments with similar results. * $p < 0.05$, ** $p < 0.005$ and *** $p < 0.0001$.

Figure 6. Low dose of TGF-β induces stem cell-like phenotype in Th17^{IL-1β} cells
 (a) q-PCR analysis for expression of key memory and stemness associated genes in Th17^{TGF-β1} and Th17^{IL-1β} cells. Cumulative data from 3 independent experiments is presented. (b) Flow cytometric analysis for CD39 and CD73 expression on CD4 gated T cells after three days of culture in presence of various concentration of TGF-β. (c) q-PCR analysis of key glycolysis regulating genes (left panel), and memory/stemness associated genes (right panel) in either Th17^{TGF-β1}, Th17^{IL-1β} cells or Th17^{IL-1β} cells cultured in presence of 250pg/ml TGF-β (i.e. Th17^{IL-1β}+TGF-β cells). (d) OT-II CD4⁺ T cells were polarized towards different Th17 types and re-stimulated with either cognate antigen (ova₃₂₃₋₃₃₉) or non-specific antigen (MART-1) for 4h. Cell death was determined by evaluating Annexin V vs. 7AAD by flow cytometry (left panel) as detailed in the supplementary method. Bar diagram (right panel) representing 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 (s.c.) with 0.25 × 10⁶ B16-F10-ova murine melanoma cells and after seven days mice either kept untreated as control or adoptively transferred with either 1 × 10⁶ ova specific Th17^{IL-1β} or Th17^{IL-1β}+TGF-β OT-II (Vβ5⁺CD4⁺) cells. Tumor growth was measured using digital calipers every fourth day. Data in figure demonstrate mean tumor size at each time point in one of the three experiments with similar results. (f) NSG-A2 mice (n=5 mice/group) were inoculated with 2.5 × 10⁶ HLA-A2⁺ human

melanoma 624-MEL cells and after fifteen days mice were either kept untreated or treated with h3T mouse derived either 0.2×10^6 human tyrosinase epitope reactive Th17^{TGF- β 1} or Th17^{IL-1 β +TGF- β} cells. Tumor growth was measured using digital calipers every three day. Data in figure demonstrate mean tumor size at each time point. * $p < 0.05$, ** $p < 0.005$ and *** $p < 0.0001$.