



Transgene-derived overexpression of miR-17-92 in CD8⁺ T-cells confers enhanced cytotoxic activity



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ARTICLE INFO

Article history:

Received 21 January 2015

Available online 10 February 2015

Keywords:

miRNA-17-92

Antigen-specific CD8⁺ T-cells

IFN- γ

Cytotoxicity

TGFBR2

ABSTRACT

MicroRNAs (miRs) play important roles in regulation of a variety of cell functions, including immune responses. We have previously demonstrated that miR-17-92 expression in T-cells enhances Th1 phenotype and provides a long-term protection against glioblastoma when co-expressed as a transgene in T-cells along with a chimeric antigen receptor. To further elucidate the function of miR-17-92 in tumor antigen-specific CD8⁺ T-cells, we generated transgenic (Tg) mice in which CD8⁺ T-cells overexpress transgene-derived miR-17-92 under the Ick promoter as well as T-cell receptor specific for human gp100₂₅₋₃₃ (Pmel-1) (miR-17-92/Pmel-Tg). CD8⁺ T-cells from miR-17-92/Pmel-Tg mice demonstrated enhanced interferon (IFN)- γ production and cytotoxicity in response to the cognate antigen compared with those from control Pmel-Tg mice without the transgene for miR-17-92. In addition, miR-17-92/Pmel-Tg mouse-derived CD8⁺CD44⁺ T-cells demonstrated increased frequencies of cells with memory phenotypes and IFN- γ production. We also found that miR-17-92/Pmel-Tg-derived CD8⁺ T-cells expressed decreased levels of transforming growth factor (TGF)- β type II receptor (TGFBR2) on their surface, thereby resisting against suppressive effects of TGF- β 1. Our findings suggest that engineering of tumor antigen-specific CD8⁺ T-cells to express miR-17-92 may improve the potency of cancer immunotherapy.

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1. Introduction

Adoptive cell transfer (ACT) of tumor-specific T-cells has shown promise as a potentially curative treatment for cancer [1]. Based on the cytokine-producing profiles and functions, T-cell immune responses are classified into at least four distinct subsets: type-1, type-2, type-17 and regulatory T-cells [2–4]. Although type-1 effector T-cells are important in the development of efficient

antitumor activity [5], tumors secrete numerous type-2 and immunosuppressive cytokines that promote tumor proliferation and immune escape [6–8]. Tumor microenvironment also induces regulatory T-cells, which suppress antitumor immune responses resulting in promotion of tumor growth [9]. Type-17 T-cells play an important role in the clearance of pathogens [10], however, the antitumor effects of type-17 T-cells remains controversial [11]. Thus, the strategic skewing type-1 immunity is critical for the development of more effective cancer immunotherapy.

MicroRNAs (miRNAs) are endogenous small noncoding RNAs that regulate the expression of target genes by binding to the complementary element of target messenger RNAs [12]. miRNAs in the miRNA-17-92 (miR-17-92) cluster are elevated in various tumor types and promote tumor progression [13–16], while transgenic mice with miR-17-92 overexpressed in lymphocytes develop lymphoproliferative disorder and autoimmunity but not cancer [17].

Abbreviations: ACT, Adoptive cell transfer; gp100, glycoprotein 100; IFN- γ , interferon-gamma; miR, microRNA; Tg, transgenic; TGF, transforming growth factor; TGFBR2, transforming growth factor beta type II receptor.

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Furthermore, miR-17-92-overexpressing T-cells increase IFN- γ production [17,18], and we have recently demonstrated that transgene-mediated overexpression of miR-17-92 in T-cells provides long-term protection against gliomas when co-expressed with a chimeric antigen receptor [19]. These findings suggest that miR-17-92 promotes type-1 T-cell skewing and antitumor immunity.

Tumors produce a variety of immunosuppressive factors, such as transforming growth factor (TGF)- β , which may disturb antitumor immune responses [20]. TGF- β type II receptor (TGFBR2) has a high affinity for TGF- β 1 [21], and is a verified target of miR-17-92 in solid cancers [22,23]. miR-17-92 expression in CD34⁺ hematopoietic stem cells inversely correlated with the level of TGFBR2 transcript [24]. We therefore hypothesized that engineering of tumor antigen-specific CD8⁺ T-cells to overexpress miR-17-92 would confer resistance to immunosuppressive effects of TGF- β via decreased TGFBR2 expression.

Taken together, we sought to determine whether overexpression of miR-17-92 in tumor antigen-specific CD8⁺ T-cells confers type-1 activation and resistance to TGF- β 1 suppression.

2. Materials and methods

2.1. Mice

C57BL/6 background wild-type (WT), transgenic (Tg) for the human gp100₂₅₋₃₃-specific TCR (Pmel-1), miR-17-92 (C57BL/6-Gt [ROSA]26Sor^{tm3(CAG-MIR17-92,EGFP)Rsky/J}) or Lck-Cre (B6.Cg-Tg[Lck-cre]548Jxm/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6-background mice that express transgene-derived miR-17-92 as well as the human gp100₂₅₋₃₃-specific TCR were created by crossing miR-17-92 Tg mice and Pmel-1 mice, followed by further crossing with C57BL/6 background Lck-Cre mice. These mice overexpress miR-17-92 cluster in hgp100 25-33-specific T cells (miR-17-92/Pmel-TG cells hereafter). All mice were maintained and handled in accordance with the Animal Facility at the University of Pittsburgh per an Institutional Animal Care and Use Committee-approved protocol.

2.2. Reagents

The following antibodies were purchased from BioLegend (San Diego, CA): anti-CD8a (53-6.7), anti-CD44 (IM7) and anti-CD62L (MEL-14). H-2D^b restricted gp100 tetramer was provided from The National Institutes of Health (NIH) Tetramer Core Facility. Goat anti-mouse TGFBR2, goat IgG isotype control and recombinant mouse TGF- β 1 were purchased from R&D Systems (Minneapolis, MN). Rabbit anti-goat IgG was purchased from Abcam (Cambridge, MA). Recombinant human IL-2 was purchased from PeproTech (Rocky Hill, NJ). Human gp100₂₅₋₃₃ (KVPRNQDWL) peptide was synthesized by the University of Pittsburgh Peptide Synthesis Facility.

2.3. Flow cytometry

Cells were stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, PE combined with a cyanine dye (PE-Cy7)-, or allophycocyanin (APC)-conjugated mAbs. The samples were collected and analyzed using BD Accuri C6 flow cytometer and software (BD Biosciences, San Jose, CA).

2.4. RNA isolation and quantification of gene expression

Total RNA was extracted using RNeasy Mini Kit (Qiagen Inc., Valencia, CA), and subjected to RT-PCR analysis using TaqMan

microRNA Reverse Transcription Kit and microRNA Assays (Applied Biosystems). Quantitative real-time PCR analysis was performed by StepOne™ Real-Time PCR Systems and Software (v2.3) (Life Technologies, Grand Island, NY). SNO202 was used as a housekeeping small RNA reference gene and to normalize another microRNA expression level. Relative expression of microRNA compared with control samples was calculated by the ddCt method.

2.5. ELISA

The amount of IFN- γ in the supernatant was measured by BD OptEIA ELISA sets (BD Biosciences, San Jose, CA) according to the manufacturer's instructions.

2.6. CTL analysis

Cytotoxicity was conducted using 6 h ⁵¹Cr-release assay as described previously [25]. In brief, CD8⁺ T-cells were isolated from Pmel-Tg and miR-17-92/Pmel-Tg mice and incubated with GL261 cells loaded with or without human gp100₂₅₋₃₃ peptide before and after priming *in vitro*.

2.7. Cell sorting

CD8⁺ T-cells were purified using CD8a MicroBeads and magnetic cell sorting system (Miltenyi Biotec Inc., San Diego, CA). CD8⁺CD44⁺ and CD8⁺CD44⁻ T-cells were sorted by MoFlo (Beckman Coulter, Inc., Brea, CA) at the Cytometry Facility of the University of Pittsburgh Cancer Institute.

2.8. Statistical analysis

Mean values between two groups were compared using Student's *t*-test. Data are presented as mean \pm SD. *P* < 0.05 was considered significant.

3. Results

3.1. Expression of miR-17-92 conferred activation of type-1 CD8⁺ T-cells

To evaluate the effects of miR-17-92 expression in tumor antigen-specific CD8⁺ T-cells, we generated mice whose CD8⁺ T-cells express transgene-derived hgp100₂₅₋₃₃-specific TCR (Pmel-1) as well as miR-17-92 (miR-17-92/Pmel-Tg). The control cells are obtained from mice transgenic for Pmel-1 and miR-17-92 alleles but not bred with the Lck-cre mice so the transgene miR-17-92 is not expressed. There was no difference between the two groups in terms of frequency of the hgp100₂₅₋₃₃-tetramer-positive CD8⁺ T-cells (Fig. 1A). As expected, miR-17-5p as a miR-17-92 family member was expressed at higher levels in miR-17-92/Pmel-Tg than in control CD8⁺ T-cells (*P* < 0.001; Fig. 1B).

We next determined whether miR-17-92 overexpression in antigen-specific CD8⁺ T-cells would enhance IFN- γ production and cytotoxic activity in response to the cognate antigen, hgp100₂₅₋₃₃. As shown in Fig. 1C, CD8⁺ T-cells isolated from miR-17-92/Pmel-Tg mice produced higher levels of IFN- γ compared with those from control mice when stimulated with hgp100₂₅₋₃₃ peptide (*P* < 0.05). None of those cells expressed detectable levels of IFN- γ without the peptide stimulation (Fig. 1C). miR-17-92/Pmel-Tg mouse-derived CD8⁺ T-cells showed significantly higher levels of antigen-specific cytotoxicity than control CD8⁺ T-cells even without *in vitro* stimulation with the cognate peptide (*P* < 0.05 or *P* < 0.01; Fig. 1D). After culture with hgp100₂₅₋₃₃ peptide and IL-2 for 6 days, both T-cell types exhibited increased cytotoxicity levels against the

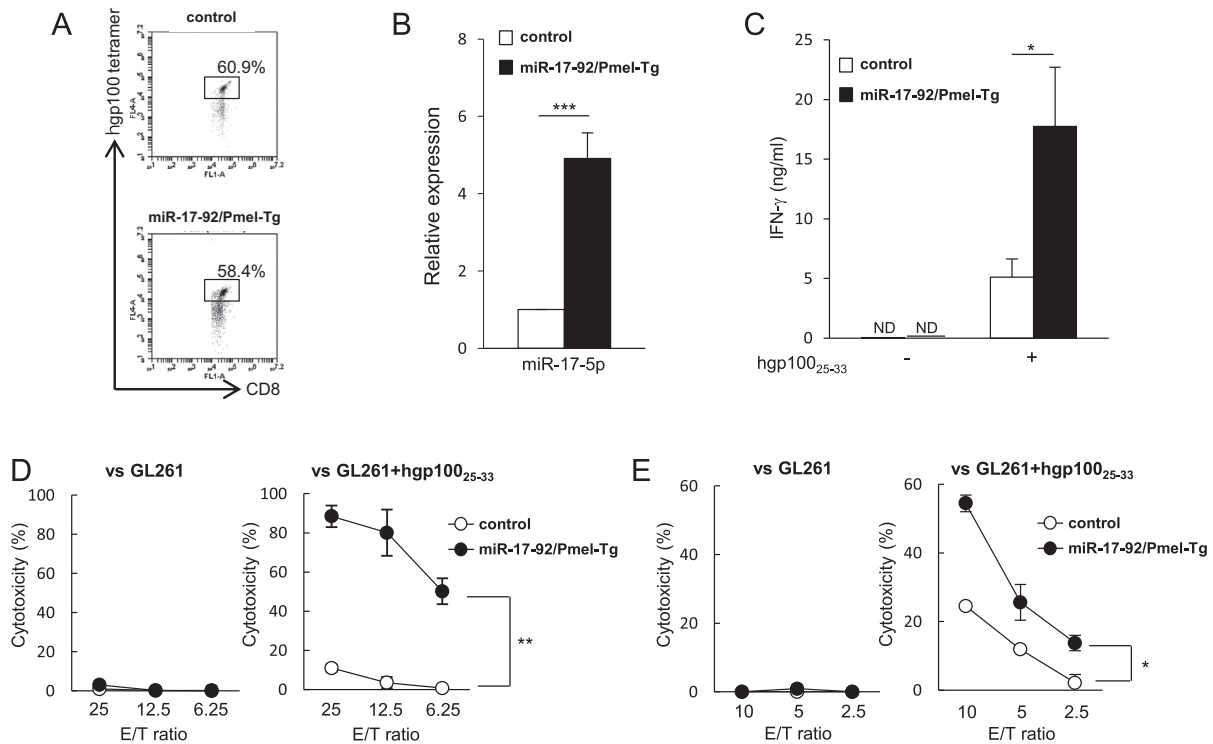


Fig. 1. Transgene-mediated overexpression of miR-17-92 in CD8⁺ T-cells enhanced IFN- γ production and cytotoxicity. CD8⁺ T-cells were isolated from control and miR-17-92/Pmel-Tg mice. (A) Frequencies of gp100 tetramer⁺ cells were analyzed by flow cytometry. Flow data are representative of three independent experiments. (B) Expression levels of miR-17-5p were measured by qRT-PCR. (n = 3, ***P < 0.001, t-test) (C) CD8⁺ T-cells were stimulated with hgp100₂₅₋₃₃ (0.125 μ g/ml) in the presence of feeder cells for 46 h and evaluated for IFN- γ production by ELISA. (*P < 0.05, t-test) (D) Antigen-specific cytotoxicity of CD8⁺ T-cells without *in vitro* stimulation was evaluated against GL261 glioma cells loaded with or without hgp100₂₅₋₃₃ peptide by a 6-h ⁵¹Cr-release assay. (**P < 0.01, t-test) (E) CD8⁺ T-cells were cultured with IL-2 (100 IU/ml) and hgp100₂₅₋₃₃ peptide (0.125 μ g/ml) in the presence of feeder cells. After 6 days, these cells were harvested and evaluated for antigen-specific cytotoxic activities. (*P < 0.05, t-test) Graphs are presented as mean \pm SD from at least two independent experiments.

cognate antigen, while miR-17-92/Pmel-Tg CD8⁺ T-cells showed a higher cytotoxic activity than control CD8⁺ T-cells (Fig. 1E). The transgene-derived overexpression of miR-17-92 in CD8⁺ T-cell enhanced the antigen-specific cytotoxic activity even without *in vitro* stimulation.

3.2. Transgene-derived miR-17-92 overexpression in CD8⁺ T-cells increased the frequency of CD8⁺CD44⁺ memory T-cells that produce IFN- γ

CD8⁺ T-cells can be divided into three subsets: naïve, central memory, and effector memory cells using CD44 and CD62L cell-surface marker [26], and CD8⁺CD44⁺ memory-phenotype CD8⁺ T-cells exist even in mice which did not receive apparent stimulations with the cognate antigen [27–29]. To find whether transgene-mediated overexpression of miR-17-92 influences the frequency of naïve and memory CD8⁺ T-cells, we evaluated expression of CD44 and CD62L on CD8⁺ T-cells in splenocytes. Non-stimulated CD8⁺ T-cells from miR-17-92/Pmel-Tg mice demonstrated significantly increased proportions of both CD44⁺CD62L⁺ central and CD44⁺CD62L[−] effector memory cells compared to those from control mice (P < 0.001; Fig. 2A and B). Next, we isolated CD8⁺, CD8⁺CD44[−] naïve and CD8⁺CD44⁺ memory T-cells from miR-17-92/Pmel-Tg and control mice, and compared IFN- γ production levels following stimulation with hgp100₂₅₋₃₃ peptide-pulsed GL261 glioma cells for 24 h (Fig. 2C). Consistent with data in Fig. 1C, miR-17-92/Pmel-Tg mouse-derived CD8⁺ T-cells produced higher levels of IFN- γ than control CD8⁺ T-cells (P < 0.01). We observed a strikingly high level of IFN- γ in miR-17-92/Pmel-Tg

mouse-derived CD8⁺CD44⁺ T-cells compared with corresponding cells from control mice (P < 0.001). Similarly, miR-17-92/Pmel-Tg CD8⁺CD44⁺ memory T-cells produced a higher levels of IFN- γ than control CD8⁺ T-cells when exposed to Quad-GL261 glioma cells which endogenously express transgene-derived major histocompatibility (MHC) class I-restricted hgp100₂₅₋₃₃ [30] (P < 0.001; Fig. 2D). These results indicate that overexpression of miR-17-92 in CD8⁺ T-cells *in vivo* enhanced not only the frequency of CD8⁺CD44⁺ memory T-cells but also IFN- γ secretion levels by memory cells.

3.3. miR-17-92/Pmel-Tg-derived CD8⁺ T-cells are resistant to TGF- β 1-mediated suppression

TGF- β type II receptor (TGFB2) is a verified target of miR-17-92 in solid cancers [22,23], and miR-17-92 expression in CD34⁺ hematopoietic stem cells inversely correlates with the level of TGFB2 transcript [24]. Hence, we investigated whether overexpression of miR-17-92 in CD8⁺ T-cells would lead to down-regulation of TGFB2, thereby rendering CD8⁺ T-cells more resistant to the suppressive effects of TGF- β 1. As shown in Fig. 3A, while CD8⁺ T-cells from control mice expressed detectable levels of TGFB2, ones from miR-17-92/Pmel-Tg mice were negative for TGFB2. Furthermore, presence of recombinant TGF- β 1 significantly suppressed hgp100₂₅₋₃₃ peptide-induced IFN- γ production levels in control CD8⁺ T-cells but not significantly in miR-17-92/Pmel-Tg CD8⁺ T-cells (Fig. 3B). These data suggest that miR-17-92 confers resistance of CD8⁺ T-cells against TGF- β 1 via down-regulation of TGFB2 expression.

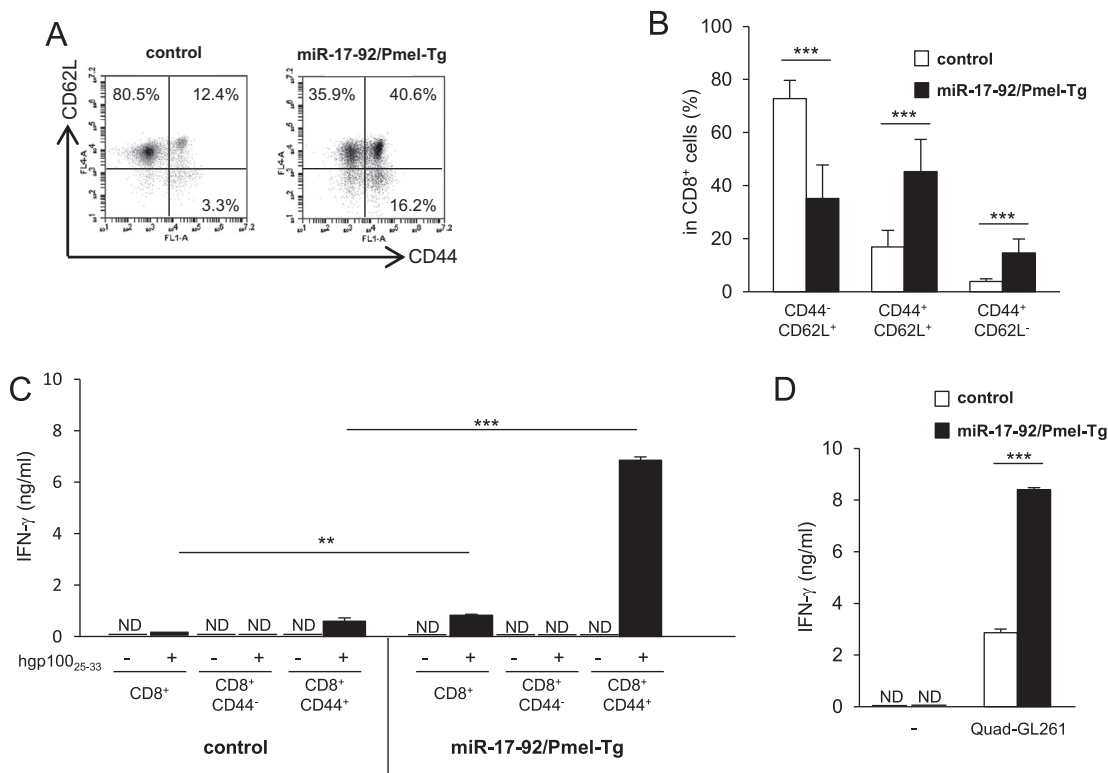


Fig. 2. CD8⁺ T-cells from miR-17-92/Pmel-Tg mice demonstrated increased the frequency and IFN-γ production of CD8⁺CD44⁺ T-cells. Spleens were harvested from control and miR-17-92/Pmel-Tg mice. (A) Representative flow data for CD44 and CD62L expression on CD8⁺ T-cells. (B) Frequencies of CD44⁻CD62L⁺ (naïve), CD44⁺CD62L⁺ (central memory) and CD44⁺CD62L⁻ (effector memory) cells on CD8⁺ T-cells. Data are presented as mean ± SD. (n = 6 in each strain; ***P < 0.001, t-test) (C) CD8⁺, CD8⁺CD44⁻ and CD8⁺CD44⁺ T-cells were isolated from the spleens of control and miR-17-92/Pmel-Tg mice. These cells were cocultured with hgp100₂₅₋₃₃ peptide-pulsed or non-pulsed GL261 cells for 24 h and evaluated for IFN-γ production by ELISA. (ND, not detected; **P < 0.01, ***P < 0.001, t-test) (D) CD8⁺CD44⁺ T-cells from control and miR-17-92/Pmel-Tg mice were cocultured with Quad-GL261 cells for 24 h and evaluated for IFN-γ production by ELISA. (ND, not detected; ***P < 0.001, t-test) Bars and error bars indicate the mean and SD, respectively, from three independent experiments.

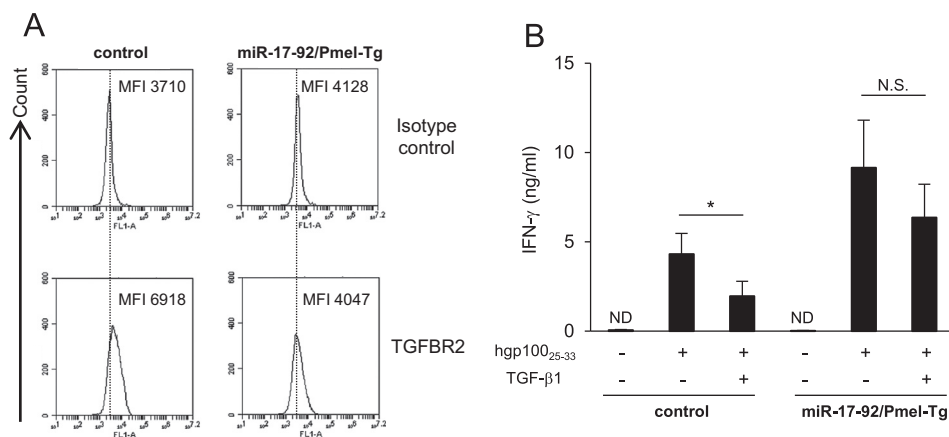


Fig. 3. miR-17-92 expression in CD8⁺ T-cells provided resistance to suppressive effect of TGF-β1. CD8⁺ T-cells were isolated from control and miR-17-92/Pmel-Tg mice. (A) Cell surface expression level of TGFBR2 was evaluated by flow cytometry. Flow data are representative of two independent experiments. (B) CD8⁺ T-cells were cultured with hgp100₂₅₋₃₃ (0.125 μg/ml) and feeder cells in the presence or absence of TGF-β1 (4 ng/ml) for 46 h. IFN-γ secretion was determined by ELISA. Bars and error bars indicate the mean and SD, respectively, from three independent experiments. (ND, not detected; N.S., no significant; *P < 0.05, t-test).

4. Discussion

In this study, we demonstrate that engineering of tumor antigen-specific CD8⁺ T-cells to overexpress miR-17-92 improves type-1 effector functions, such as enhanced IFN-γ production and cytotoxicity, in an antigen-specific manner and provides resistance to immunosuppressive effect of TGF-β1.

We have been dedicated to the development of effective immunotherapeutic strategies for central nervous system (CNS) tumors, such as gliomas. Gliomas account for approximately 30% of all primary central nervous system tumors and 80% of malignant brain tumors [31]. The current standard treatment for patients with malignant gliomas includes surgery, followed by chemotherapy and radiation therapy [32]. Despite recent advances in cancer

therapy, prognosis is dismal. Previously, we have reported that CD49d (α_4 integrin), which forms $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrin heterodimers at the cell surface [33], was preferentially expressed on type-1 CD8⁺ compared with type-2 CD8⁺ T-cells [34], and VLA-4 (the heterodimer of α_4 and β_1 integrins) expression on CTLs plays a significant role in the efficient CNS tumor infiltration of antigen-specific CTLs [35]. Although CD49d can form heterodimers with both β_1 and β_7 integrins, $\alpha_4\beta_7$ complexes were not expressed by CD8⁺ T-cells [34], suggesting that CD49d is a suitable surrogate for VLA-4. We also found miR-17-92/Pmel-Tg CD8⁺ T-cells without *in vitro* stimulation expressed elevated levels of CD49d (data not shown) in addition to IFN- γ production (Fig. 1C) and cytotoxicity (Fig. 1D), demonstrating that miR-17-92 expression in tumor-antigen-specific CD8⁺ T-cells have the potential to improve not only antitumor immunity but also their infiltration into CNS tumor sites.

One of major barriers for successful ACT therapy is an immunosuppressive tumor microenvironment [36]. Malignant gliomas are heavily infiltrated by immature myeloid cells (i.e., tumor-associated macrophages), which play a role in promoting tumor growth and suppressing type-1 immunity by releasing the immunosuppressive factors such as IL-10 and TGF- β [37]. In the normal physiological state, TGF- β actively maintains T-cell homeostasis and regulates T-cell function [38]. However, in the tumor microenvironment, TGF- β creates an immunosuppressive milieu that inhibits antitumor immunity by suppressing or altering activation, maturation and differentiation of both innate and adaptive immune cells [39,40]. miR-17-92 expression in antigen-specific CD8⁺ T-cells reduced the expression of TGFBR2 and conferred resistance to the immunosuppressive effects of TGF- β 1 (Fig. 3). Moreover, IL-10 suppressed IFN- γ production from control CD8⁺ T-cells, but not miR-17-92/Pmel-Tg CD8⁺ T-cells, when stimulated with hgp100₂₅₋₃₃ peptide (data not shown). Taken together, these data suggest that engineering of tumor antigen-specific CD8⁺ T-cells to express miR-17-92 may protect them from immunosuppressive effects in the tumor microenvironment.

Non-stimulated CD8⁺ T-cells from miR-17-92/Pmel-Tg mice demonstrated significantly increased proportions of CD44⁺ memory cells compared to those from control mice (Fig. 2A and B). Under lymphopenic conditions, naïve CD8⁺ T-cells undergo homeostatic proliferation and acquire phenotypic and functional characteristics of memory T-cells in the absence of stimulation with cognate antigens, but require recognition of MHC-bound self-peptides [27–29]. Given that gp100 is expressed in normal melanocytes [41], miR-17-92/Pmel-Tg CD8⁺ T-cells may interact with the endogenous gp100 peptide-MHC complex and convert to CD44⁺ memory-type T-cells.

We hypothesized that expression of miR-17-92 in CD8⁺ T-cells would have a lower threshold for TCR activation upon antigen-stimulation based on our own finding that the majority of miR-17-92/Pmel-Tg CD8⁺ T-cells are already primed upon isolation from host mice and a previous study showing that CD4⁺ T-cells with higher miR-17-92 expression have a lower threshold for activation with anti-CD3 antibody [17]. To test this, we used mouse (m)gp100₂₅₋₃₃ peptide instead of human (h)gp 100₂₅₋₃₃ to stimulate miR-17-92/Pmel-Tg-derived CD8⁺ T-cells because mgp100₂₅₋₃₃ peptide is identical to the naturally existing peptide in mice, and binds to H-2D^b with 100-fold lower efficiency than hgp100₂₅₋₃₃ peptide [42], and thus we thought mgp100₂₅₋₃₃ would be more relevant to cancer immunotherapy where endogenous auto-antigens are targeted. Although stimulation with mgp100₂₅₋₃₃ induced higher levels of IFN- γ from miR-17-92/Pmel-Tg CD8⁺ T-cells than from the control Pmel-Tg CD8⁺ T-cells, the threshold of mgp100₂₅₋₃₃ peptide concentration required for detectable IFN- γ production was not different in the two T-cell types (data not

shown). Another possibility of enhanced IFN- γ production and CTL activity in miR-17-92/Pmel-Tg CD8⁺ T-cells is that overexpression of miR-17-92 may enhance T-cell activation by influencing proximal TCR signals and/or by increasing the expression levels of any of these phosphorylated intermediates such as p56lck, ZAP-70 and linker for activation of T cells (LAT) [43]. Furthermore, miR-17-92 targets hypoxia-inducible factor (HIF)-1 α in lung cancer cells [44]. Since HIF-1 α negatively regulates T-cell functions [45–47] and T-cell-targeted disruption of HIF-1 α leads to increased IFN- γ production and improved effector functions [48–51], miR-17-92 expression in activated T-cells may promote type-1 function in part through down-regulation of HIF-1 α .

miR-17-92/Pmel-Tg CD8⁺ T-cells showed cytotoxicity against hgp100₂₅₋₃₃ peptide-pulsed, but not non-pulsed GL261 cells (Fig. 1D). In this regard, in contrary to our observations, Prins and colleagues have demonstrated that syngeneic T-cells primed with hgp100₂₅₋₃₃ peptide can mediate cytotoxic effects on GL261 cells [52]. A possible reason for these inconsistent observations is that they used JAM cytotoxicity assays using ³H-thymidine labeled GL261 cells, whereas we performed ⁵¹Cr-release assays. The JAM assay has been demonstrated to be a more sensitive measurement of cell death than ⁵¹Cr-release assay because the JAM assay depends on DNA fragmentation which precedes loss of membrane integrity in most apoptotic cells [53].

Our findings in this study demonstrate that genetic engineering of tumor antigen-specific CD8⁺ T-cells with miR-17-92 confers both skewing type-1 immunity and resistance to suppressive effects in the tumor microenvironment. We also discovered that miR-17-92 expression in T-cells provided long-term protection against gliomas when co-transduced with the anti-EGFRvIII chimeric antigen receptor [19]. Although further investigations are required to determine the mechanisms, adoptive transfer of tumor antigen-specific CD8⁺ T-cells engineered to express miR-17-92 may have a potential to be a useful treatment strategy for patients with gliomas.

Conflicts of interest

Hideho Okada and Gary Kohanbash are two of inventors on Pitt Ref. No. 01933: Title: Th1-Associated MicroRNAs and Their Use For Tumor Immunotherapy: Application No. 12/818,016, filed Country: United States of America. Because of this, interpretation of data are made by all authors but not solely by Dr. Okada and/or Kohanbash.

Acknowledgments

Grant Supports from: The National Institutes of Health (NIH) (2R01 NS055140) and Musella Foundation for Brain Tumor Research and Information. This project used University of Pittsburgh Cancer Institute (UPCI) shared resources (Animal Facility and Cytometry Facility) that are supported in part by NIH P30CA047904.

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