MicroRNA-17-92 Controls T-Cell Responses in Graft-versus-Host Disease and Leukemia Relapse in Mice

Yongxia Wu¹,², Jessica Heinrichs²,³, David Bastian², Jianing Fu², Hung Nguyen², Steven Schutt², Yuejun Liu²,⁴, Junfei Jin²,⁵, Chen Liu⁶, Qi-Jing Li⁷, Changqing Xia*¹,⁶ and Xue-Zhong Yu*²,⁸

¹Department of Hematology, Xuanwu Hospital, Capital Medical University, Beijing, China; ²Microbiology & Immunology, Medical University of South Carolina, Charleston, SC; ³Department of Pathology and Cell Biology, University of South Florida, Tampa, FL; ⁴Department of Hematology, First Affiliated Hospital of Soochow University, Suzhou, Jiangsu, China; ⁵Laboratory of Hepatobiliary and Pancreatic Surgery, Affiliated Hospital of Guilin Medical University, Guilin, China; ⁶Pathology, Immunology and Laboratory Medicine, University of Florida, Gainesville, FL; ⁷Department of Immunology, Duke University Medical Center, Durham, NC; ⁸Department of Medicine, Medical University of South Carolina, Charleston, SC.

Address correspondence to: Dr. Xue-Zhong Yu, M.D., Department of Microbiology & Immunology, Medical University of South Carolina, HCC350, MSC 955, 86 Jonathan Lucas Street, Charleston, SC 29425, USA. Phone: (843) 792-4756; Fax: (843) 792-9588; Email: yux@musc.edu.

* These authors share correspondence.

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Running Title: miR-17-92 is essential for T cells to induce GVHD

Key points:

1. miR-17-92 is required for T cells to mediate GVHD but not the GVL effect

2. Targeting miR-17-92 with antagomirs efficiently alleviates GVHD
Abstract

MicroRNAs (miRs) play important roles in orchestrating many aspects of the immune response. The miR-17-92 cluster, which encodes 6 miRs including 17, 18a, 19a, 20a, 19b-1 and 92-1, is among the best characterized of these miRs. The miR-17-92 cluster has been shown to regulate a variety of immune responses including infection, tumor, and autoimmunity, but the role of this cluster in T-cell response to alloantigens has not been previously explored. By using MHC-matched, mismatched, and haploidentical murine models of allogeneic bone marrow transplantation (allo-BMT), we demonstrate that the expression of miR-17-92 on donor T cells is essential for the induction of GVHD, but dispensable for the GVL effect. The miR-17-92 plays a major role in promoting CD4 T cell activation, proliferation, survival, and Th1 differentiation, while inhibiting Th2 and iTreg differentiation. On the other hand, miR-17-92 may promote migration of CD8 T cells to GVHD target organs, but has minimal impact on CD8 T-cell proliferation, survival, or cytolytic function, which could contribute to the preserved GVL effect mediated by T cells deficient for miR-17-92. Furthermore, we evaluated a translational approach and found that systemic administration of antagomir to block miR-17 or miR-19b in this cluster significantly inhibited alloreactive T-cell expansion, IFN\(\gamma\) production, and prolonged the survival in recipients afflicted with GVHD while preserving the GVL effect. Taken together, the current work provides a strong rationale and demonstrates the feasibility to target miR-17-92 for the control of GVHD while preserving GVL activity after allo-BMT.
Introduction

Despite the significant improvements in the field of allogeneic hematopoietic cell transplantation (allo-HCT), graft-versus-host disease (GVHD) remains the major cause of transplant related morbidity and mortality.\(^1\) Multiple cell types, cytokines, chemokines, and signaling pathways involved in the innate and adaptive immune response are implicated in the development of GVHD.\(^2\) Further understanding of the molecular mechanisms that regulate the pathophysiology of GVHD is highly desirable.

MicroRNAs (miRs) are endogenous single-stranded and non-coding RNAs of 19-22 nucleotides.\(^3,4\) The seed sequence in miRs can bind to the partially complimentary sequence in their target mRNAs, resulting in degradation of these target mRNAs and translational repression.\(^3,4\) The miRs regulate almost every known cellular process and play crucial roles in numerous biological and pathological responses. Pertaining to miRs relation to GVHD, an elegant pre-clinical study demonstrated that a specific miR-mRNA network regulates allogeneic T-cell responses.\(^5\) A recent clinical study illustrated miR-423, miR-199a-3p, miR-93, and miR-377 were up-regulated in the plasma of patients with acute GVHD, which were then validated as biomarkers to predict GVHD occurrence.\(^6\) Other studies have indicated that miR-100,\(^7\) miR-34a,\(^8\) and miR-155\(^9\) play a potentially significant role in GVHD. Specific targeting of miR-155 using locked nucleic acid (LNA)–modified oligonucleotides (also known as antagonirmrs) effectively mitigates GVHD,\(^9\) leading to a current clinical trial to further establish the significance of miR-155 in GVHD (ClinicalTrials.gov identifier: NCT01521039). Evidently, miRs not only regulate the development of GVHD, but may also potentially be used as biomarkers and therapeutic targets.\(^10\)

The miR-17-92 cluster, one of the best characterized miRs, encodes 6 miRs including miR-17, 18a, 19a, 20a, 19b-1, and 92-1 (Fig. S1). Originally, this miR cluster was found to be involved in
tumorigenesis. However, recent research has uncovered unexpected roles of its members in a wide variety of settings including normal development, cardiovascular disease, and aging. Furthermore, the miR-17-92 cluster plays critical roles in early B-cell development, homeostasis, and T-cell differentiation, survival, and function and its regulatory functions have been explored in anti-infection, anti-tumor, and autoimmune responses. However, the role this cluster plays in modulating T-cell alloresponses in GVHD and graft-versus-leukemia (GVL) activity has not been elucidated. In the current study, we have investigated how the miR-17-92 cluster regulates T cell response to alloantigens in murine models of allogeneic bone marrow transplantation (allo-BMT), and revealed that absence or blockade of miR-17-92 significantly reduces GVHD while preserving the GVL effect.
Material and Methods

Mice. C57BL/6 (B6; H-2b, CD45.2), B6.Ly5.2 (CD45.1), BALB/c (H-2d), and (B6×DBA2)F1 (BDF1, H-2b/d) were purchased from National Cancer Institute (NCI, Frederick, MD). The founders of C.B10-H2b/LilMcdJ (BALB.B; H-2b) and Thy1.1 B6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). BALB.B mice were bred at Medical University of South Carolina (MUSC, Charleston, SC). T-cell conditional miR-17-92 knock-out (KO) strain (miR-17-92fl/fl CD4-Cre+) was previously generated and crossed to the B6 background in Q.-J. Li’s laboratory at Duke University14, and the colony was maintained at MUSC. All animals were housed in the American Association for Laboratory Animal Care–accredited Animal Resource Center at MUSC. Experiments were carried out under protocols approved by the Institutional Animal Care and Use Committee of MUSC.

BMT models. T-cell purification from spleen and lymph nodes was done by negative depletion using magnetic beads as previously described.18 Major histocompatibility complex (MHC)-mismatched (B6→BALB/c), MHC-matched but minor histocompatibility antigen (MiHA)-mismatched (B6→BALB.B), and haploidentical (B6→BDF1) BMT models were used as previously established.19 Recipients were monitored for survival and body weight loss twice/week. The GVL models with A20 B-cell lymphoma or p815 mastocytoma were established as previously described.20 Representative samples of GVHD target organs were excised from recipients 14 days post-BMT and subjected to pathology scoring as previously described.18,20

Treatment with LNA antagonirs. The anti-miR-17 and anti-miR-19 antagonirs are 10-mer seed-targeting LNA oligonucleotides, which were designed as shown in supplemental figure 1 and synthesized in Exiqon (Woburn, MA). Recipient mice were treated with anti–miR-17, anti-miR-19, or
scrambled at a loading dose 25 mg/kg at day 0 followed by 5 mg/kg intravenously twice weekly up to day 21 after BMT.

**Flow cytometry and serum cytokine detection.** Mononuclear cells were isolated from recipient spleen, liver, gut, and lung as previously described and stained for surface molecules and intracellular cytokines using standard flow cytometry protocols. Stained cells were analyzed using Diva software, LSR II (BD Biosciences, San Jose, CA), and FlowJo (TreeStar, Ashland, OR). Cytokine levels in recipient serum were quantified using a cytometric bead assay according to the manufacturer's instructions (BD Biosciences, San Jose, CA).

**Statistics.** For comparison of recipient survival among groups in GVHD experiments, the log-rank test was used to determine statistical significance. To compare body weight changes, pathology scores, cytokine levels, and receptor levels, a Student t test was performed.
Results

miR-17-92 promotes allogeneic T-cell responses in vivo

The miR-17-92 cluster promotes T-cell proliferation, enhances Th1 differentiation, protects T cells from activation-induced cell death (AICD), and suppresses the generation of induced regulatory T cells (iTregs) under polyclonal stimulation in vitro. Therefore, we hypothesized that this miR cluster plays an essential role in T-cell alloreponses. To test this, we utilized B6 mice with miR-17-92 conditional KO on the T cell lineage (miR-17-92fl/fl CD4-Cre+). The T cell subsets including CD4, CD8, Tregs, naïve, and memory T cells were comparable between WT and KO mice (data not shown). We then compared the responses of WT and KO T cells after adoptively transferring them into lethally irradiated allogeneic recipients. We observed that the KO T cells had a substantially reduced ability to proliferate and produce IFNγ as compared to WT counterparts, reflected by percentage and number of donor T cells (Fig. 1A-B), CFSE dilution (Fig. 1C-D), and percentage and number of IFNγ+ cells in donor T cells (Fig. 1E-F). Interestingly, the KO CD4 T cells had an increased rate of cell death among fast-dividing cells (CFSElow) but a decreased rate of cell death among slow-dividing cells (CFSEhigh) compared to their WT counterparts (Fig. 1G-H). Decreased rate of cell death in KO CD4 T cells was also observed after being transferred into syngeneic recipients where T cells were undergoing homeostatic proliferation (data not shown). On the other hand, miR-17-92 had no effect on cell death of CD8 T cells, regardless of cell division (Fig. 1G-H). These results suggest that miR-17-92 enhances T-cell proliferation and activation in response to alloantigens. Furthermore, miR-17-92 differentially regulates CD4 vs. CD8 T cells and homeostatic vs. antigen-driven responses, which mainly protects antigen-driven CD4 T cells from undergoing cell death.

miR-17-92 is essential for T cells to induce GVHD
Because miR-17-92 is critical for T-cell proliferation, IFNγ production, and survival in response to alloantigens (Fig. 1), we further hypothesized that KO T cells would fail to induce GVHD. Using a MHC-mismatched B6→BALB/c model and titrating the dose of donor T cells, we found that recipients of WT T cells developed severe and lethal GVHD in a dose-dependent manner, while recipients of KO T cells all survived long-term (Fig. 2A, p < .001) with significantly less weight loss (Fig. 2B) and milder pathological injuries in the liver and gut (Fig. 2C-D). Thirty days after BMT, recipients of KO T cells had significantly improved donor-derived B-cell reconstitution in the peripheral blood as compared with recipients of WT T cells (Fig. S2A). While the majority of donor-derived T cells originated from mature T cells (H2Kb+Ly5.1−) in the recipients with WT T cells, the majority of donor T cells were BM-derived T cells (H2Kb+Ly5.1+) in the recipients with KO T cells (Fig. S2B).

Consistently, 80 days post-BMT, the recipients of KO T cells had significantly more donor BM-derived T and B cells but much fewer transferred T cells in their spleens than those of survived WT T cell recipients (Fig. S3), indicating that recipients of KO T cells survived long-term without severe GVHD.

In clinical HCT, most patients receive grafts from MHC-matched but MiHA-mismatched donors. In an effort to extend our studies to better mimic a clinical scenario, we used the B6→BALB.b (both H2b) model with at least 29 different MiHA loci.23 While the recipients of WT T cells suffered modest GVHD with less than 60% survival and severe weight loss, the recipients of KO T cells had mild GVHD with 100% long-term survival (p < .05) and minimal weight loss (Fig. 2E-F). In parallel, the reconstitution of donor BM-derived T and B cells in spleen was significantly better in the recipients of KO T cells as compared to those of WT T cells (Fig. S4A-B). In addition, a significantly improved thymus cellularity and function, reflected by the number of total and CD4+CD8+ thymocytes, were found in recipients of KO T cells. Given the thymus is a sensitive GVHD target organ, which is
important for immune reconstitution, our data indicates that KO T cells did not cause severe damage in recipient thymus or obvious immune deficiency that is typically associated with GVHD. Taken together, these data suggest miR-17-92 is required for donor T cells to induce GVHD.

**miR-17-92 regulates T-cell expansion and migration**

Development of GVHD requires donor T-cell expansion in lymphoid organs and migration into target organs through up-regulating chemokine receptors and integrins.\(^2\)\(^4\) We examined the presence of donor T cells in recipient spleens (secondary lymphoid organ) and livers (GVHD target organ) 14 days following allo-BMT. We found that KO CD4 T cells were fewer than WT CD4 T cells in the recipient spleen (**Fig. 3A**), and the difference was much more dramatic in the liver (**Fig. 3B**). While WT and KO CD8 T cells were comparable in the spleen, KO cells were significantly fewer than WT cells in the liver (**Fig. 3A-B**). We observed that both CD4 and CD8 KO T cells had significantly reduced expression of CXCR3 and \(\alpha 4\beta 7\) as compared to WT counterparts (**Fig. 3C-F**). In addition, KO T cells expressed significantly lower levels of CXCR3 in the liver (data not shown) and of \(\alpha 4\beta 7\) in the gut and mesenteric lymph node (MLN) (Fig. S5A). These data suggest that miR-17-92 is required for CD4, but not CD8, T-cell expansion after allo-BMT. In addition, miR-17-92 may promote T-cell migration to GVHD target organs.

**miR-17-92 controls T-cell differentiation**

Naïve CD4 T cells can differentiate into various T cell subsets including Th1, Th2, Th17, and Treg cells. With regards to GVHD, Th1 and Th17 cells are considered to be pathogenic, whereas Tregs are suppressive.\(^2\)\(^5\) We therefore evaluated how miR-17-92 affects T-cell differentiation after allo-BMT. We found that WT T cells differentiated predominately into IFN\(\gamma\)-producing Th1 cells, with quite few IL-4/5-producing Th2 or Foxp3\(^+\) iTregs presented in recipient spleens (**Fig. 4A-B**). On the contrary,
KO T cells differentiated into significantly less Th1 cells and toward Th2 and iTregs cells. The effect of miR-17-92 on T-cell differentiation was also evident in GVHD target organs such as the liver (Fig. S6). In the absence of miR-17-92, KO CD4 T cells also produced significantly lower levels of IFNγ and IL-17 in recipient gut and MLN, which is consistent with the alleviated gut damage in the recipients of KO T cells (Fig. S5B-C). The intracellular cytokine expression was further supported by a similar cytokine production pattern in the serum. The levels of IFNγ were significantly reduced while the levels of IL-10 were significantly increased in recipients transferred with miR-17-92 KO T cells, compared to those with WT T cells (Fig. 4C). We reason that decreased Th1 but increased Th2 and iTregs contributed to the reduced ability of KO T cells to induce GVHD. To test whether iTregs deficient for miR-17-92 are functional, we generated iTregs from WT or KO CD4+CD25− T cells under iTreg-polarizing culture conditions in vitro, and found that the rate of iTreg generation in KO T cells was comparable in TGFβ condition, yet lower in TGFβ plus retinoic acid (RA) condition as compared with WT T cells. Importantly, the suppressive ability of KO iTregs was similar to that of WT iTregs (Fig. S7). Thus, we conclude that iTregs generated from KO T cells likely contributed to the reduction of GVHD.

miR-17-92 regulates T-cell response intrinsically

Our data indicates that deficiency of miR-17-92 in T cells strongly inhibits CD4 T-cell expansion, Th1 differentiation, and T-cell migration to target organs. In order to test the endogenous defects of these KO T cells, we utilized a competitive assay to directly compare the responses of WT vs. KO T cells in the same recipient after allo-BMT. While WT and KO T cells were mixed 1:1 prior to adoptive transfer, there were more than 90% of WT cells (Ly5.1+) among total donor CD4 T cells in recipient spleen 2 weeks post-BMT, indicating that the WT CD4 T cells ousted their KO counterparts under the competitive environment (Fig. 5A). In contrast, the deficiency of miR-17-92 had much less effect on
the expansion of CD8 T cells (Fig. 5A). Consistently, the percentage of BrdU-labeled cells was significantly reduced in KO CD4 T cells, but comparable in KO CD8 T cells compared to WT counterparts in recipient spleens (Fig. 5B). We also calculated the ratio of KO/WT T cells in recipients in order to evaluate the impact of miR-17-92 on donor T-cell expansion in the spleen and migration into GVHD target organs including the liver, gut, and lung. The ratio of KO/WT CD4 T cells was dramatically reduced in allogenic recipients, but the ratio in each of those target organs was comparable to that in the spleen (Fig. 5C), suggesting that miR-17-92 affects CD4 T-cell expansion. On the other hand, the ratio of KO/WT CD8 T cells was significantly reduced in gut and lung as compared to the spleen (Fig. 5C). Together with reduced expression of CXCR3 and of α4β7 on the KO T cells (Fig. 3 and S5), these data suggest that miR-17-92 may also promote CD8 T-cell migration.

Under the same inflammatory environment, KO CD4 T cells still had significantly reduced IFNγ+ and increased IL-4/5+ cells (Fig. 5D). These data indicate that deficiency of miR-17-92 in T cells mainly impairs the expansion of CD4 T cells and modifies their differentiation. On the other hand, KO CD8 T cells might impair migration, but did not have substantially reduced expansion ability or cytolytic potential (TNFα, IFNγ, granzyme B, and CD107, data not shown). These data suggest that miR-17-92 affects T-cell responses intrinsically and regulates CD4 vs. CD8 in a different manner.

**miR-17-92 is dispensable to the GVL activity and CD8 T-cell cytolytic function**

We next asked the critical question as to whether T cells deficient for miR-17-92 maintain their GVL activity. To test this, we infused host-type B cell lymphoma (A20) together with BM or BM plus T cells into lethally irradiate allogeneic recipients. As expected, all the recipients without T cell infusion died from lymphoma relapse within 30 days after BMT (Fig. S8A). When receiving 0.5×10⁶/mouse T cells, all the recipients of WT T cells died from GVHD (except one from tumor relapse) based on their survival (Fig. S8A), body weight loss (Fig. S8B), and tumor signal (Fig. S8C). In contrast, the
recipients of KO T cells at the same dose survived without GVHD and were largely free from tumor relapse. Furthermore, when receiving 1.0×10^6/mouse of T cells, all the recipients of WT T cells died from GVHD without tumor relapse, whereas all the recipients of KO T cells have long-term survive without severe GVHD or tumor relapse (Fig. S8D-E). Taken together, these data suggest that the KO T cells had preserved GVL activity in the MHC-mismatched BMT model.

To exclude the possibility that the preserved GVL activity is model specific, we utilized haploidentical B6→BDF1 BMT model with more aggressive p815 mastocytoma. All the recipients without T cell infusion died from leukemia relapse within 20 days after BMT (Fig. 6A), whereas the recipients of WT T cells died from GVHD reflected by 100% lethality (Fig. 6A), severe weight loss (Fig. 6B), and no tumor signal (Fig. 6C). In contrast, the vast majority of the recipients transplanted with KO T cells survived without GVHD or tumor relapse. To confirm the GVL activity, we tested higher doses of p815 that caused accelerated tumor relapse in the recipients without T-cell infusion (Fig. 6D-E). Although the mortality due to tumor relapse was comparable regardless of T-cell type (Fig. 6E), the recipients of KO T cells had improved survival (Fig. 6D) compared to those of WT T cells. These results indicate that miR-17-92 is essential for donor T cells to induce GVHD but not to mediate the GVL effect. To understand the potential mechanisms by which T cells deficient for miR-17-92 had impaired ability to induce GVHD while preserving GVL activity, we examined the expression of cytolytic molecules on donor CD8 T cells. As compared with WT T cells, the KO CD8 T cells expressed lower levels of granzyme B or CD107a, similar levels of IFNγ, and higher levels of TNFα proportionally (Fig. 6F). Furthermore, the absolute numbers of KO CD8 T cells expressing those effector molecules in recipient spleens were comparable to WT cells (Fig. 6G). We reason that residual granzyme B, IFNγ, and/or TNFα in KO CD8 T cells are responsible for their preserved GVL activity.
Targeting miR-17 or -19b using specific LNA antagomirs inhibits T-cell alloresponse and alleviates GVHD

Strong evidence demonstrates that LNA antagomirs can specifically and effectively block target miRs.\textsuperscript{9,26} Furthermore, the LNA antagomirs specific for the members in the miR-17-92 cluster have been tested and shown to be effective in blocking the function of this cluster and inhibiting tumor progression.\textsuperscript{27,28} By reconstituting miR-17-92 whole cluster deficient T cells with individual miRs, the study by Jiang et al reveals that miR-17 and miR-19b among miR-17-92 cluster are the key players in controlling Th1 responses.\textsuperscript{14} We thus designed LNA antagomirs that can specifically target miR-17 seed family (miR-17 and miR-20a) or miR-19 seed family (miR-19a and miR-19b-1) (sequences highlighted in Fig. S1). We then tested the effect of these LNA antagomirs on T-cell expansion and activation in allogeneic recipients. In comparison with scrambled control, anti-miR-17 significantly reduced donor CD4 and CD8 T-cell expansion (Fig. 7A-B) and IFN\textgamma production (Fig. 7C-D). The effect of anti-miR-19 was more profound than that of anti-miR-17 (Fig. 7A-D). Furthermore, systemic treatment with anti-miR-17 or anti-miR-19 significantly reduced recipient mortality compared with the scrambled control group (Fig. 7E, $p < .05$, $p < .01$, respectively). Since blocking miR-19 was more effective to alleviate GVHD, we further evaluated the effect of anti-miR-19 on the GVL activity (Fig. 7F-G and Fig. S9). Without T cell infusion, the recipients had similar tumor relapse and mortality after the treatment with the scrambled and anti-miR-19 (Fig. 7F-G and Fig. S9), suggesting anti-miR-19 did not have a direct affect on tumor growth. With T cell infusion, the recipients treated with anti-miR-19 showed prolonged survival (Fig. 7F) and comparable mortality due to tumor relapse as those with scrambled antagomir (Fig. 7G and Fig. S9). These data suggest that pharmacologically blocking the key member of the miR-17-92 cluster efficiently constrains T-cell alloresponses and thus attenuates GVHD while preserving GVL activity.


Discussion

Primarily using a genetic approach, our data demonstrates that the miR-17-92 cluster intrinsically controls multiple aspects of the T-cell response to alloantigens and regulates CD4 and CD8 T cells in a different manner. The miR-17-92 strongly enhances activation, survival, and proliferation of CD4 T cells. In addition, this cluster is critical for Th1 differentiation, but is suppressive for Th2 and iTreg differentiation. In contrast to CD4 T cells, miR-17-92 has no impact on cell survival, decreased effect on proliferation and cytolytic function, but may promote migration of CD8 T cells. As a result, the expression of miR-17-92 on donor T cells is essential for the induction of GVHD but not for the mediation of the GVL effect after allo-BMT. Furthermore, pharmacological blockade of miR-17 or miR-19b is effective in controlling T-cell alloresponses and attenuating GVHD severity.

In the current study, we have provided compelling evidence that miR-17-92 is required for T cells to induce GVHD. T cells deficient for miR-17-92 were essentially unable to mediate GVHD in all 3 allogeneic BMT models tested: MHC-mismatched (Fig. 2A-B), MHC-matched but MiHA-mismatched (Fig. 2E-F), and haploidentical (Fig. 6) models. Mechanistically, we first observed that CD4 T cells deficient for miR-17-92 dramatically decreased expansion both on day 4 (Fig. 1A-B) and 14 (Fig. 3A) after allo-BMT. Decreased expansion likely resulted from reduced proliferation (Fig. 1C-D) and increased AICD (Fig. 1G-H) in alloantigen-driven response in vivo. Unexpectedly, the cell death rate was significantly decreased on KO CD4 T cells that were undergoing slow or no cell division (CFSE<sup>high</sup>) (Fig. 1G-H). Consistently, the KO CD4 T cells had lower rates of cell death and expressed higher levels of Bcl-2 compared with WT T cells once being transferred into irradiated syngeneic recipients (data not shown). Thus, miR-17-92 protects CD4 T cells from extrinsic apoptosis under alloantigen-driven proliferation whereas it promotes intrinsic apoptosis under homeostatic proliferation, which is likely because miR-17-92 negatively regulates PTEN during T-cell activation with antigens.
and may also negatively regulate Bcl-2 during T-cell maintenance.\textsuperscript{12} As a consequence, it is plausible that inhibition of miR-17-92 would specifically or preferentially suppress an alloantigen-driven response while preserving T-cell homeostasis, which would benefit patients with GVHD without causing broad immune suppression.

In accordance with the published report that miR-17-92 promotes IFN\textgreek{g} production \textit{in vitro} and Th1 differentiation in delayed-type hypersensitivity \textit{in vivo},\textsuperscript{14} we found that miR-17-92 was essential for optimal differentiation to Th1 cells following allo-BMT (\textbf{Fig. 4}). Given that GVHD is primarily driven by Th1 cells, we surmised that compromised Th1 differentiation largely accounts for the impaired ability of miR-17-92 KO T cells to induce GVHD. In parallel to compromised Th1 differentiation, T cells deficient for miR-17-92 substantially enhanced differentiation into less pathogenic Th2 cells and suppressive iTregs (\textbf{Fig. 4}). A recent report from Bluestone’s group showed that natural Tregs deficient for miR-17-92 do not retain suppressive function due to loss of IL-10 production.\textsuperscript{29} However, we found that the suppressive function of iTregs generated from KO and WT CD4 T cells \textit{in vitro} had comparable suppressive function (\textbf{Fig. S7}). Thus, we reason that iTregs generated from KO T cells likely contributed to the alleviated GVHD.

Donor T-cell infiltration into target organs is a prerequisite for GVHD development; chemokine receptors and integrins play important roles in T-cell migration to GVHD target organs.\textsuperscript{30} We found that there were significantly fewer KO T cells in recipient livers than their WT counterparts (\textbf{Fig. 3}). Both the expansion and migration ability of T cells contribute to the number of donor T cells present in a target organ. In a competitive environment, the deficiency of this cluster dramatically reduced the expansion on CD4 but less on CD8 T cells, and it may further reduce migration of CD8 T cells (\textbf{Fig. 5A-C}). Furthermore, miR-17-92 deficient T cells expressed significantly lower levels of CXCR3 and
α4β7, the chemokine receptor and integrin that direct T-cell migration to the target organs, such as the liver and gut. It is not clear whether CXCR3 and α4β7 are directly regulated by miR-17-92, or secondary to T-cell activation and Th1 polarization. However, decreased T-cell migration to the target organs likely contributes to the reduced pathogenicity of miR-17-92-deficient T cells in the induction of GVHD.

Of clinical importance, we demonstrated that miR-17-92 was dispensable for T cell-mediated GVL effect in 2 preclinical BMT models (Fig. S8 and Fig. 6). We attribute the preserved GVL effect to largely intact CTL activation and function in the absence of miR-17-92. There are several lines of evidence to support this: 1) miR-17-92 had no effect on the survival of CD8 T cells after BMT (Fig. 1 G-H); 2) although CD8 T cells deficient for miR-17-92 showed decreased proliferation in the early phase of BMT (Fig. 1C-D), their expansion was not significantly compromised in the later phase of BMT (Fig. 3A). Our observation is consistent with published findings that over-expression of miR-17-92 in lymphocytes induces a major expansion in the CD4 but to a lesser extent in the CD8 T cells; 3) miR-17-92 deficiency may impair CD8 T-cell migration rather than substantially reducing proliferation (Fig. 3 and Fig. 5); 4) miR-17-92 had little or no effect on cytolytic activity, reflected by levels of granzyme B and CD107a, and cytokine production, reflected by intracellular expression of IFNγ (Fig. 6F-G). It is worthy to point out that both CD4 (data not shown) and CD8 T cells had increased TNFα production in the absence of miR-17-92 (Fig. 6F), which is consistent with a previous report that TNFα is a target of miR-19a. Given TNFα is a GVL mediator, elevated TNFα production by donor T cells may also contribute to the preserved GVL effect.

Using a translational approach, we demonstrate that blocking miR-17 or miR-19b by using specific LNA antagonirs significantly reduced T-cell proliferation and activation under the stimulation of
alloantigens and further attenuated the severity of GVHD while preserving GVL activity (Fig. 7 and Fig. S9). Although not to the extent of miR-17-92 deficiency, the effect of antagomir-treatment was quite remarkable, given only a single member of this cluster was inhibited. Our study provides the rationale to establish the significance of miR-17-92 in GVHD and to target this cluster for GVHD prophylaxis in the clinic. As a novel therapeutic strategy, several miR modulators have entered into clinical trials. Moreover, antagomirs can be relatively easily delivered into primary lymphocytes ex vivo prior to BMT, which may lead to a higher efficiency and specificity in knocking-down miRs in T and B cells prior to transfer and thus result in effective prevention of GVHD with low toxicity. miR-17-92, also known as polycistronic oncomir-1, is overexpressed in many types of malignancies, and a strategy to silence this cluster to suppress solid tumor growth has been established in preclinical models. Therefore, we anticipate that within the foreseeable future the translational feasibility of silencing miR-17-92 will be achieved, which will significantly benefit cancer patients, especially those with hematologic malignancies following allo-HCT.
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Authorship Contributions and Conflict of Interest Statement

Y. Wu participated in experimental design, performed research, collected, analyzed and interpreted data, performed statistical analysis, and drafted and revised the manuscript; J. Heinrichs, D. Bastian, J. Fu, H. Nguyen, S. Schutt and Y. Liu performed research, collected and analyzed data, and edited the manuscript; C. Liu performed pathological analysis. J. Jin, Q.-J. Li and C. Xia participated in experiment design, interpreted data and edited manuscript; X.-Z.Y. designed research, collected, analyzed and interpreted data, and revised the manuscript.

The authors declare they have no conflicts of interest.
References


Figure Legends

Figure 1. Effects of miR-17-92 on T-cell proliferation, activation and survival. Purified T cells from WT or miR-17-92 KO mice (miR-17-92^{fl/fl} CD4-Cre^{+}) on B6 background were labeled with CFSE and transferred into lethally irradiated BALB/c mice at 2×10^6/ per mouse. Four days after cell transfer, recipient spleens were collected and subjected to cell counting and FACS staining. Percentage (as showed by representative flow figures) and absolute numbers of donor-derived (H2K^{b+}) CD4 and CD8 T cells on gated live cells (A, B); representative flow figures of CFSE dilution and percentage of CFSE-diluted cells on gated donor cells (C, D); percentage and absolute numbers of IFNγ^{+} cells on gated donor CD4 and CD8 T cells (E, F); Cell death along with cell division on gated donor T cells (G); percentage of dead cells among fast-dividing (CFSE^{low}) or slow-dividing (CFSE^{high}) on gated donor T cells (H). Data were shown of one representative mouse (A, C, E, G) or mean ± SD of 4 mice per group (B, D, F, H). Two replicate experiments were performed with totally 8 mice. Asterisk indicates statistical significance: * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 2. Role of miR-17-92 on donor T cells in the induction of GVHD. BALB/c mice were lethally irradiated and transplanted with 5×10^6/mouse T-cell depleted marrow cells (TCD-BM, Ly5.1^{+}) or plus purified T cells (Ly5.2^{+}) from WT or KO mice on B6 background at doses indicated. Recipient mice were monitored for survival (A) and body weight change (B) until 80 days after transplant. Five to seven mice were included in each group at each cell dose. Statistical analysis was performed to compare the same dose of WT and KO T cells. In separate experiments, pathology injuries (C) and scores (D) were shown 14 days post BMT; N = 14/group. SI: small intestine; LI: large intestine. BALB/b mice were lethally irradiated and transplanted with TCD-BM (Ly5.1^{+}) alone or plus purified T cells at 3 x 10^6/mouse from WT or KO mice on B6 background. Recipient mice were monitored for
survival (E) and body weight change (F) until 90 days after BMT. Data shown were pooled from 2 replicated experiments with 10-12 mice per group. * p < 0.05, ** p < 0.01, *** p < 0.001.

**Figure 3. Role of miR-17-92 on donor T-cell migration and expression of chemokine receptor and integrin.** BMT was carried out as outlined in figure 2A, in which 1×10^6/mouse donor T cells were transplanted. Fourteen days post BMT, recipient spleens and livers were collected and mononuclear cells were isolated and subjected to cell counting and FACS staining. Absolute numbers of donor H2K^b^Ly5.1^−^CD4^+^ or CD8^+^ WT (gray symbols) and KO (black symbols) T cells were shown in spleen (A) or liver (B). % CXCR3^+^ cells were shown on gated H2K^b^Ly5.1^−^CD4^+^ or CD8^+^ T cells in spleen (C, D). % a4β7^+^ cells were shown on gated H2K^b^Ly5.1^−^CD4^+^ or CD8^+^ T cells in spleen (E, F). Data shown were pooled from 2 replicated experiments (N=10/group). * p < 0.05, ** p < 0.01, *** p < 0.001.

**Figure 4. Effects of miR-17-92 on the differentiation of donor T cells after allo-BMT.** BMT was carried out as outlined in figure 3. Two weeks post BMT, recipient spleens were collected and subjected to cell counting and FACS staining. The expression of IFNγ (Th1 cytokine), IL-4/5 (Th2 cytokine), and Foxp3 (Treg markers) were shown on gated donor H2K^b^Ly5.1^−^CD4^+^ cells as showed in the gating strategy (A); Absolute numbers of IFNγ^+^, IL-4/5^+^ or Foxp3^+^ cells among donor H2K^b^Ly5.1^−^CD4^+^ cells were shown (B). Few cells differentiated into IL-17-producing Th17 cells and there was no difference between WT and KO T cells (data not shown). The levels of cytokines in recipient serum were measured using cytokine beads assay and displayed as mean ± SD (C). Data shown were pooled from 2 replicated experiments (N = 10/group). * p < 0.05, ** p < 0.01;
Figure 5. miR-17-92 affects T-cell response intrinsically. Lethally irradiated BALB/c mice were transplanted with 5×10⁶/mouse TCD-BM (Thy1.1⁺) together with 1×10⁶/mouse WT (Ly5.1⁺ Thy1.2⁺) and 1×10⁶/mouse miR-17-92 KO T cells (Ly5.2⁺ Thy1.2⁺) from B6 mice. Fourteen days after BMT, recipient mice were euthanized and spleen, liver, lung and intestine were harvested. Mononuclear cells were isolated from these organs and subjected for flow staining. The percentage of KO (Ly5.2⁺) and WT (Ly5.1⁺) CD4 or CD8 T cells on gated on H2Kb⁺Thy1.2⁺ donor cells were shown in the representative flow figures (A). BrdU was injected into recipients (2mg/mouse) 24 hours before euthanizing of the recipients. Percentage of BrdU⁺ cells in WT and KO cells T cells were displayed on gated Ly5.1⁺ or Ly5.2⁺ cells as shown in panel A (B). Based on the percentage of WT and KO T cells in panel A, we calculated the ratio of KO to WT T cells in spleen as well as in GVHD target organs (C). First bar indicates 1:1 mixture of WT and KO T cells prior to BMT (C). Percentages of IFNγ⁺, IL-4/5⁺, and Foxp3⁺ cells among WT or KO CD4⁺ T cells were shown (D). Data shown were from one of two replicate experiments (N = 10/group). * p<0.05, ** p<0.01, *** p<0.001.

Figure 6. Role of miR-17-92 on donor T-cell mediated GVL and cytolytic activities. BDF1 mice were lethally irradiated and transplanted with 5×10⁶/mouse TCD-BM alone or plus purified T cells from WT or KO B6 mice at 4×10⁶/mouse. Recipient mice were also infused with 5×10³ luciferase-transduced p815 cells at the day of BMT. Recipients were monitored for survival (A) and body weight change (B) until 80 days after transplant. Tumor growth was monitored using BLI on the dates indicated (C). Data were pooled from 2 replicate experiments (N = 5-10/group). Using the same BMT model, we increase the dose of p815 cells to 1 or 2.5×10⁴/mouse, respectively. Recipients were monitored for survival (D) and mortality caused by tumor relapse (E) until 80 days after transplant (N=6/group). In a separate experiment, recipient spleens were collected 14 days post-BMT and stained for the expression of granzyme B (GrZB), CD107a, IFNγ, and TNFα on gated CD8⁺ donor T cells.
Representative flow figures were shown on gated donor CD8 T cells (F), and bar graph indicates absolute numbers of total CD8 T cells or CD8 T cells that express each of these molecules (G). *** p < 0.001.

**Figure 7. Effects of anti-miR-17 or anti-miR-19 LNA antagonirs on donor T-cell alloresponse and GVHD.** BALB/c mice were lethally irradiated and transplanted with 2×10^6 purified and CFSE-labeled T cells form WT B6 mice. Recipient mice were injected i.v. with scrambled, anti-miR-17, or anti-miR-19 at 25 mg/kg on day 0 and 5 mg/kg on day 2. Recipients were euthanized on day 4 after cell transfer and splenocytes were counted and stained for surface expression of H2K^b^ (donor marker), CD4, CD8 on gated live cells (A), and intracellular IFN-γ along with CFSE profile gated on H2K^b^+ CD4^+ or CD8^+ cells (C). Absolute numbers of CD4^+ or CD8^+ donor T cells (B), and CFSE-diluted IFN-γ^+ cells (D) were shown. Four mice were used in each group. In a separate experiment, BALB/c mice were lethally irradiated and transplanted with 5×10^6 TCD-BM alone or with T cells from B6 mice at 1×10^6/mouse. Recipient mice were treated with individual antagonirs as indicated twice a week from 25 mg/kg on day 0 and then 5 mg/kg until day 21 after BMT. Recipient survival (E) was shown on 6 mice per group. Using the same BMT model, recipient mice were also infused with 5,000 luciferase-transduced A20 cells at the day of BMT, and treated with individual antagonirs as indicated. Recipient survival (F) and mortality caused by tumor relapse (G) were shown on 4-7 mice per group. * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 3

A

Spleen

\[ \frac{\text{H2K}^b{+}\text{Ly5.1}}{\times 10^5} \]

- WT
- KO

CD4, CD8

B

Liver

\[ \frac{\text{H2K}^b{+}\text{Ly5.1}}{\times 10^4} \]

- WT
- KO

CD4, CD8

C

CXCR3

- WT
- KO

18, 38.7

D

% CXCR3

CD4, CD8

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E

α4β7

- WT
- KO

16.7, 65.7

F

% α4β7

CD4, CD8

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Figure 4

A

B

C

# Cells (x10^4)

IFNγ  IL-4/5  Foxp3

PG/mL

IFNγ  IL-4/5  IL-17  IL-10

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WT  KO

BMM alone
Figure 5

A

Spleen | Liver | Gut | Lung

CD4

3.77 | 3.73 | 2.86 | 4.14

93.8 | 92.6 | 96.1 | 93.3

29.5 | 21.4 | 16.3 | 13.6

CD8

Ly5.2

Ly5.1

B

% BrdU

CD4

*

CD8

C

Before

Spleen | Liver | Gut | Lung

CD4

CD8

% Cells

0.0 | 0.2 | 0.4 | 0.6 | 0.8 | 1.0

Cell Ratio (KO vs. WT)

D

IFNγ | IL-4/5 | Foxp3

WT | KO

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MicroRNA-17-92 controls T-cell responses in graft-versus-host disease and leukemia relapse in mice

Yongxia Wu, Jessica Heinrichs, David Bastian, Jianing Fu, Hung Nguyen, Steven Schutt, Yuejun Liu, Junfei Jin, Chen Liu, Qi-Jing Li, Changqing Xia and Xue-Zhong Yu