MicroRNA-31 regulates T-cell metabolism via HIF1α and promotes chronic GVHD pathogenesis in mice

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Abstract:
Chronic graft-versus-host disease (cGVHD) remains a major obstacle impeding successful allogeneic hematopoietic cell transplantation (allo-HCT). MicroRNAs (miRs) play key roles in immune regulation during acute GVHD development. Preclinical studies to identify miRs that impact cGVHD pathogenesis are required to develop these as potential life-saving interventions. Using oligonucleotide array, we identified miR-31 that was significantly elevated in allogeneic T cells following HCT in mice. Using genetic and pharmacological approaches, we demonstrated a key role for miR-31 in mediating donor T-cell pathogenicity in cGVHD. Recipients of miR-31-deficient T cells displayed improved cutaneous and pulmonary cGVHD. Deficiency of miR-31 reduced T-cell expansion and Th17 differentiation, but increased generation and function of Tregs. MiR-31 facilitated Neuropilin-1 down-regulation, Foxp3 loss and IFNγ production in alloantigen-induced Tregs. Mechanistically, miR-31 was required for Hypoxia-inducible Factor 1α (HIF1α) upregulation in allogeneic T cells. Hence, miR-31-deficient CD4 T cells displayed impaired activation, survival, Th17 differentiation and glycolytic metabolism under hypoxia. Upregulation of Factor Inhibiting HIF1 (FIH1), a direct target of miR-31, in miR-31-deficient T cells was essential for attenuating T-cell pathogenicity. However, miR-31-deficiency CD8 T cells maintained intact glucose metabolism, cytolytic activity and graft-versus-leukemia response. Importantly, systemic administration of a specific inhibitor of miR-31 effectively reduced donor T-cell expansion, improved Treg generation, and attenuated cGVHD. Taken together, miR-31 is a key driver for T-cell pathogenicity in cGVHD but not for the anti-leukemia activity. Taken together, miR-31 is essential to drive cGVHD pathogenesis and represents a novel potential therapeutic target for controlling cGVHD.

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1. Loss of miR-31 in allogeneic T cells alleviates the progression of scleroderma and lung dysfunction in cGVHD but not GVL response

2. MiR-31 inhibition attenuates CD4+ T-cell activity in hypoxia environments
Abstract

Chronic graft-versus-host disease (cGVHD) remains a major obstacle impeding successful allogeneic hematopoietic cell transplantation (allo-HCT). MicroRNAs (miRs) play key roles in immune regulation during acute GVHD development. Preclinical studies to identify miRs that impact cGVHD pathogenesis are required to develop these as potential life-saving interventions. Using oligonucleotide array, we identified miR-31 that was significantly elevated in allogeneic T cells following HCT in mice. Using genetic and pharmacological approaches, we demonstrated a key role for miR-31 in mediating donor T-cell pathogenicity in cGVHD. Recipients of miR-31-deficient T cells displayed improved cutaneous and pulmonary cGVHD. Deficiency of miR-31 reduced T-cell expansion and Th17 differentiation, but increased generation and function of Tregs. MiR-31 facilitated Neuropilin-1 down-regulation, Foxp3 loss and IFNγ production in alloantigen-induced Tregs. Mechanistically, miR-31 was required for Hypoxia-inducible Factor 1α (HIF1α) upregulation in allogeneic T cells. Hence, miR-31-deficient CD4 T cells displayed impaired activation, survival, Th17 differentiation and glycolytic metabolism under hypoxia. Upregulation of Factor Inhibiting HIF1 (FIH1), a direct target of miR-31, in miR-31-deficient T cells was essential for attenuating T-cell pathogenicity. However, miR-31-deficiency CD8 T cells maintained intact glucose metabolism, cytolytic activity and graft-versus-leukemia response. Importantly, systemic administration of a specific inhibitor of miR-31 effectively reduced donor T-cell expansion, improved Treg generation, and attenuated cGVHD. Taken together, miR-31 is a key driver for T-cell pathogenicity in cGVHD but not for the anti-leukemia activity. Taken together, miR-31 is essential to drive cGVHD pathogenesis and represents a novel potential therapeutic target for controlling cGVHD.
Introduction

Chronic graft-versus-host disease (GVHD) remains a major cause of mortality and morbidity following allogeneic hematopoietic cell transplantation (allo-HCT). cGVHD is characterized by systemic inflammation, multi-organ fibrosis and increased risks of infection. Uncontrolled donor T-cell activation and expansion in target organs contributes to the initiation of cGVHD. By differentiating into effector T helper (Th) subsets, including Th1, Th17, extrafollicular T cells and follicular helper T-cell (Tfh) et al., CD4 T cells are key mediators of cGVHD. Loss of immune tolerance caused by impaired differentiation and homeostasis of regulatory T cells (Tregs) perpetuates cGVHD. Eventually, aberrant production of cytokines and autoantibodies leads to fibroblast activation and tissue fibrosis.

MicroRNAs (miRs) are short, non-coding RNAs that repress gene expression at the post-transcriptional level via binding to target mRNAs, subsequently promoting degradation or impeding translation. MiRs play critical roles in regulating T-cell response and dendritic cell function in GVHD development. We previously reported that miR-17-92 enhances T- and B-cell pathogenicity in GVHD, yet was dispensable for T-cell mediated GVL effect. Recently, miR-31 emerged as an important regulator of T-cell response. Downregulation of miR-31 in human CD4 T cells contributes to immunosuppression during sepsis. MiR-31 expression was downregulated by Bcl6 that caused stabilized Tfh program in tonsils. In mice, miR-31 enhances CD4 T-cell pathogenicity in experimental autoimmune encephalomyelitis (EAE) while reducing CD8 T-cell potency in controlling lymphocytic choriomeningitis virus (LCMV) infection. However, whether or how miR-31 impacts allogeneic T-cell responses is essentially unknown. Using genetic and pharmacological approaches, we uncovered a key role for miR-31 in promoting T-cell expansion, Treg/Th17 imbalance, and augmenting donor T-cell pathogenicity in cGVHD yet dispensable for CD8 T-cell function and GVL response. At a molecular level, by inhibiting factor inhibiting HIF1 (FIH1), miR-31 enhances hypoxia-inducible factor 1α (HIF1α) expression and promotes glycolytic metabolism and T-cell function under hypoxic conditions.
Materials and Methods

Mice. CD45.1\(^+\) B6, BALB/c and B6D2F1 were purchased from the National Cancer Institute (NCI, Frederick, MD). B10.BR and B10.D2 mice were purchased from Jackson Laboratory (Bar Harbor, ME). MiR-31\(^{floxed/floxed}\) mice on B6 background were provided by Dr. Honglin Wang\(^{17}\) and were bred with CD4Cre mice to generate miR-31 KO (miR-31\(^{floxed/floxed}\) CD4Cre\(^+\)) and WT (miR-31\(^{floxed/floxed}\) CD4Cre\(^-\)) mice. All mice were housed in a pathogen-free facility at the American Association for Laboratory Animal Care–accredited Animal Resource Center at Medical University of South Carolina (MUSC). All animal studies were carried out under protocols approved by the Institutional Animal Care and Use Committee at MUSC.

Experimental procedures and materials

Bone marrow transplantation (BMT), GVHD Scoring, lung function measurement, miR detection, locked nucleic acid (LNA) antagonir treatment, T-cell differentiation \textit{in vitro}, flow cytometry, western blot, seahorse assay and histopathology and trichrome staining are described in previous published work\(^{7,19,20}\) and in the Supplemental Methods.

Statistics. GraphPad Prism was used to perform statistical analysis. The log-rank test was used to determine statistical significance in recipient survival. Normality of data was assessed and the statistical significance was determined with two-tailed unpaired Student’s t-test.
Results

MiR-31 expression in donor T cells is critical for the induction of cutaneous and pulmonary cGVHD in mice

To search for candidate miRs that regulate allogeneic T-cell responses, we used microarray to profile miR expression of donor T cells in allogeneic or syngeneic recipients after BMT in mice. We found many miRs that were differentially expressed in T cells during allogeneic (alloantigen-driven) vs. syngeneic (homeostasis-driven) response including miR-17-92, 155, 146, 181 and 142, which were tightly associated with GVHD pathogenesis as previously demonstrated by us and others. We also identified novel miR candidates that potentially impact allogeneic T-cell response, among which miR-31 was significantly upregulated in allogeneic T cells (Fig. 1A). Increased expression of miR-31 in allogeneic T cells compared with either syngeneic or naïve T cells was confirmed by qPCR (Fig. 1B).

To investigate how miR-31 contributes to T-cell pathogenicity in cGVHD, we used B6 mice that had miR-31 conditionally knocked out in T cells (miR-31creCD4Cre+). No significant differences were observed in the baseline composition of splenocytes of miR-31 deficient mice and WT controls, with respect to the frequencies of CD4, CD8, B220, CD25, Foxp3, CD44 and CD62L positive cells (Fig. S1). To study this, we used allogeneic BMT (allo-BMT) models that have cutaneous fibrosis as a feature because it is also a common clinical manifestation of cGVHD (B6 → BALB/c and B6 → B6D2F1). Consistent with a low intensity of acute inflammation, most recipients with cGVHD survived long term, yet the recipients of miR-31 KO grafts had improved survival (Fig. S2). In the acute-to-chronic GVHD transition model (B6 → BALB/c)22, the recipients of BM and splenocytes from miR-31 KO donors showed significantly attenuated clinical scores and body weight loss compared to those with WT grafts (Fig. 1C). In addition, cutaneous manifestations, including pathological scores and collagen deposition, were alleviated in KO graft recipients (Fig. 1D-E). Using another clinically relevant, haploidentical model of cutaneous cGVHD (B6 → B6D2F1)23, we consistently found improved clinical scores, body weight maintenance and attenuated cutaneous...
manifestations in the recipients of miR-31-deficient T cells compared to those of WT controls (Fig. 1F-H). In addition, salivary glands, prototypical targets of cGVHD, showed milder pathological damage in the BALB/c recipients of miR-31 grafts (Fig. 1I). GVHD delays the reconstitution of donor lymphocytes and increases the risk of life-threatening infections in patients. Compromised donor T-cell reconstitution is partially attributed to impaired T-cell development in recipient thymus, a direct target organ of alloreactive T cells. Consistent with attenuated cGVHD severity, reconstitution of donor CD4+, CD8+ T- and B220+ B-lymphocytes was significantly improved in the recipients of miR-31 KO grafts (Fig. S3A-D). These mice also displayed improved thymic regeneration and positive selection reflected by significantly increased cell numbers of total- and CD4+CD8- thymocytes (Fig. S3E-G).

To extend our findings, we used an additional preclinical cGVHD model (B6 → B10.BR) characterized by lung cGVHD to further evaluate how miR-31 regulates T-cell pathogenicity in pulmonary cGVHD. Recipients of miR-31 KO T cells showed significantly better body weight maintenance and lung function preservation, indicated by reduced alveolar constriction and elastance and increased compliance (Fig. 1J-K). Therefore, in three preclinical cGVHD models, our data consistently demonstrate that lack of miR-31 reduces T cell pathogenicity in the skin and lung damage.

Loss of miR-31 attenuates the expansion of pathogenic donor T cells following allo-BMT

T lymphocytes in the recipients are derived from two sources: expansion of mature alloreactive T cells in donor grafts; and generation from donor hematopoietic stem cells. Thymic negative selection during de novo T-cell development largely eliminates alloreactive T cells, and therefore, mature T cells in donor grafts are considered the key mediators of GVHD. To examine how miR-31 impacts the expansion of mature alloreactive T cells, we performed BMT using congenic donor mice to distinguish mature alloreactive (CD45.2+) vs. BM-derived (CD45.1+) donor T cells. We first examined frequencies of donor-type (H2Kb+) T cells and confirmed a successful engraftment of
donor cells in recipient splenic and single-positive thymic T cells (Fig. S4). Across three models, recipients of KO T cells displayed significantly attenuated expansion of CD45.2\(^+\) T cells in spleens (Fig. 2A, S5A, S6A). Consistent with improved thymic regeneration, thymic infiltration of CD45.2\(^+\) donor T cells was significantly reduced in the recipients of KO grafts (Fig. 2B). Furthermore, these recipients had fewer CD45.2\(^+\) T cells in skin-draining lymph nodes (LN) and lungs (Fig. S5B, S6B). Mechanistically, miR-31 KO T, especially CD4\(^+\) cells displayed reduced proliferation and cell survival after being transferred into haploidentical recipients (Fig. S5C-D). Consistently, in model of pulmonary cGVHD, miR-31 KO T cells showed increased cell death in recipient spleens and lungs (Fig. S6C-D), which may be attributed to increased expression of the death receptor Fas (Fig. S6E).

Reduced Ki67 expression in KO CD4 T cells in recipient spleen and increased Lag3 expression on KO T cells in spleen and lungs were observed (Fig. S6F-H), indicating less activation and more exhaustion in these cells following allo-BMT.

Recent studies showed a critical role for B cells and autoantibodies in cGVHD pathogenesis in mice as well as in humans. Tfh cells instruct germinal center (GC) B cells to differentiate into antibody-secreting plasma cells, a process inhibited by follicular regulatory T cells (Tfr). In contrast, recent studies reveal extrafollicular CD4\(^+\) T-B interactions as a key mechanism for cGVHD induction, in that germinal center formation is dispensable. To further study whether miR-31 expression impacts Tfh differentiation in cGVHD, we examined Tfh by flow cytometry and found significantly reduced frequencies while increased absolute numbers of splenic follicular T cells (CXCR5\(^+\)PD-1\(^{hi}\)) among total donor T cells in the recipients of miR-31 KO grafts (Fig. 2C, Fig. S7). Among these cells, greater proportion of Tfr (Foxp3\(^+\)) and smaller proportion of Tfh (Foxp3\(^{-}\)) cells was observed in KO cells compared to WT counterparts (Fig. 2D). Furthermore, the frequencies of GC B cells and plasma cells, but not their absolute numbers, were reduced in the recipients of KO grafts (Fig. 2C, E). The structure of B-cell follicle in spleens was similar between WT and KO graft recipients (Fig. S8). In addition, less proinflammatory cytokines, including IFN\(\gamma\), IL-17A and TNF\(\alpha\) were produced by total donor T cells in the recipients of KO grafts (Fig. 2F, S9A-B). Although the frequencies of
naïve/memory populations were comparable between pre-transplant WT and KO cells (Fig. S1), fewer effector memory, but more naïve and central memory populations were observed among KO donor T cells after allo-BMT (Fig. S9C-D). In addition, majority of CD45.2+ donor T cells were CD44+ memory cells after allo-BMT (Fig. S10C). This data indicates that miR-31 is required for the proliferation and survival of alloreactive T cells, and deficiency of miR-31 attenuates the expansion of pathogenic T cells following allo-BMT.

MiR-31 contributes to Th17/Treg imbalance, extrafollicular CD4 T-cell differentiation, and iTreg instability post-BMT

Since mature alloreactive T cells are the major source of proinflammatory cytokines, we evaluated how miR-31 impacts the differentiation of mature donor T cells (CD45.2+CD45.1+). A significant reduction of IL-17A expression in CD45.2+ KO CD4 T cells than their WT counterparts was observed in recipient skin-draining LNs (Fig. 3A). Furthermore, increased Treg differentiation from CD45.2+CD4+ KO T cells were found in recipient spleen and skin-draining LNs (Fig. 3A-B). No difference regarding Tfh differentiation was observed within CD45.2+CD4+ WT vs KO T cells (data not shown). However, there were significantly lower frequencies of extrafollicular cells (CD4+CD44hiCD62LloPSGL-1hi)31,32 within splenocyte-derived KO T cells compared to WT counterparts (Fig. 3B). The IFNγ expression was comparable in CD45.2+CD4 T cells, while increased in CD45.2+ KO CD8 T cells than their WT counterparts in recipient spleens (Fig. S10 A-B).

Consistently with attenuated GVHD and alleviated lymphopenia in the recipients of miR-31 KO grafts, we found the absolute numbers of total donor CD4 or CD8 T cells, and Foxp3+ Tregs in spleen were significantly increased in these recipients (Fig. S11A-B). The numbers of CD45.2+ Tregs and IFNγ expressing CD8 T cells were significantly elevated in these recipients (Fig. S11C-D). Our data suggest that miR-31 regulates differentiation of mature donor T cells by influencing Th17/Treg imbalance following allo-BMT.
Tregs is essential for controlling excessive effector T-cell (T_{eff}) activity and inducing immune tolerance. Next, to understand how miR-31 regulates Treg function, we analyzed the phenotype of these cells and found similar expression levels of CTLA4, CD28, CD39 and ICOS on WT vs. miR-31 KO Tregs in recipients post-BMT (data not shown). Importantly, KO Tregs expressed a higher level of Neuropilin 1 (Nrp1) that has been reported to interact with semaphorin-4a and enhance stability and function of Tregs. Adoptive transfer of ex vivo generated induced Tregs (iTregs) is a promising therapeutic strategy for controlling inflammatory diseases, including GVHD. However, the instability of iTregs induced by inflammatory environments impedes successful iTreg immunotherapy. To elucidate how miR-31 regulates iTreg stability, we generated alloantigen-reactive CD4+ iTregs in vitro. We consistently found increased iTreg differentiation from miR-31 KO vs. WT CD4 T cells. Again, Nrp1 expression was found increased on KO vs. WT iTregs before and after 3-day culturing in IL-2, -12, or -6 cytokine that favors Treg, Th1, or Th17 differentiation, respectively. Importantly, miR-31 KO iTregs had improved Foxp3 retention and stability after exposure to these cytokines. When co-cultured with T_{eff} cells (CD45.1+) and allogeneic antigen presenting cells, KO iTregs produced significantly lower levels of IFNγ than WT iTregs, indicating they were more stable during allogeneic response. MiR-31 KO iTregs showed improved survival and superior suppressive function for controlling CD8 T_{eff} cell proliferation. Moreover, KO iTregs significantly reduced IFNγ production and increased CD25 expression compared to WT iTregs following transfer into allogeneic recipients, indicating a more stable and activated phenotype of KO iTregs. Therefore, miR-31 negatively regulates Nrp1 expression on Tregs and miR-31 deficiency increases the generation, stability and function of iTregs.
Loss of miR-31 in donor T cells alleviates cGVHD while sparing GVL activity through maintaining CD8 T-cell function

Given that persistent GVL response is essential for successful allo-HCT, we further studied how miR-31 impacts the T-cell mediated anti-leukemia response. We performed a haploidentical BMT and transferred luciferase-transduced P815 leukemia cells into these recipients on the day of BMT. We observed that the recipients of WT T cells presented clinical features of cGVHD including alopecia and skin abrasions (Fig. 4A-B, grey arrow pointed) starting around day 20 after BMT. Recipients of miR-31 KO T cells showed attenuated cGVHD scores and skin manifestations and had improved overall survival (Fig. 4A-C). The recipients of BM only grafts had uncontrolled tumor growth, as reflected by strong bioluminescence signaling, and 100% lethality within 15 days post-BMT (Fig. 4D). Importantly, intact anti-leukemia ability persisted long term after allo-BMT in the recipients of miR-31 KO grafts, despite attenuated cGVHD in these mice (Fig. 4D). Compared with WT controls, KO CD8 T cells expressed similar levels of cytolytic activity-related markers, including IFNγ, TNFα, Granzyme B, Perforin and CD107a after allo-BMT (Fig. 4E, S13). Therefore, our data strongly indicate that miR-31 expression in allogeneic donor T cells is crucial for T-cell induction of cGVHD, but dispensable for the T-cell-mediated anti-leukemia effect after allo-BMT.

MiR-31 is critical for the activation, survival and differentiation of CD4 T cells under hypoxic environment via targeting FIH1

We found that miR-31 KO CD4 T cells had compromised survival, elevated Treg generation and attenuated Th17 differentiation during cGVHD development. It is known that HIF1α signaling enhances cell survival, promotes RORγt expression and Foxp3 degradation \(^{37,38}\). Interestingly, FIH1, an asparaginyl hydroxylase that suppresses HIF1α activity, is negatively regulated by miR-31 in cancer cells \(^{39,40}\). We hypothesize that miR-31 inhibits FIH1 expression thus enhancing HIF1α signaling in T cells, which further augments their response. A highly conserved binding site of miR-31’s seed sequence was presented in the 3'UTR of FIH1 as predicted by TargetScan (Fig. 5A). Given that FIH1 hydroxylates and controls HIF1α activity in an oxygen-dependent manner \(^{41}\), we
examined FIH1 expression in WT vs. miR-31 KO CD4 T cells activated under normoxic or hypoxic condition. WT T cells reduced FIH1 expression in 3% vs. 21% O2 conditions as expected, however KO T cells maintained FIH1 expression in 3% O2 (Fig. 5B). Consistently, WT T cells upregulated HIF1α in 3% O2, while KO T cells failed to do so (Fig. 5C). Consequently, miR-31 KO T cells exhibited impaired activation compared to WT cells in 3% but not 21% O2 environment, reflected by reduced expression of CD69, ICOS, CD25, CD44 and Ki67 (Fig. 5D, S14).

To address whether miR-31 affects T-cell response through regulating HIF1α, CD4 T cells were activated in 3% O2 with the presence of dimethyloxallyl glycine (DMOG), which enhances stability and activity of HIF1α by inhibiting enzymes that catalyse the hydroxylation reaction of HIF1α, including FIH1 and prolyl hydroxylase domain proteins (PHDs). MiR-31 KO cells had significantly increased cell death than WT cells upon activation (Fig. 5E). Adding DMOG in culture partially restored survival of the KO cells, but had no impact on WT cells. To evaluate whether HIF1α is involved in miR-31-mediated regulation of Th17/Treg differentiation, CD4 T cells were activated under 3% O2 in the presence of TGFβ and IL-6, where they differentiated into RORγt+, Foxp3+ or Foxp3+RORγt+ subsets (Fig. 5F). Fewer RORγt+, but more Foxp3+ and Foxp3+RORγt+ cells were observed in KO vs. WT T cells. Adding DMOG increased RORγt+, but decreased Foxp3+ and Foxp3+RORγt+ subsets leading to comparable RORγt and Foxp3 expression in WT vs. KO cells. Further, DMOG restored the activation of KO CD4 T cells under hypoxia (Fig. S15). Taken together, these results demonstrate that miR-31 is critical for HIF1α upregulation through inhibiting FIH1 in low O2 condition, which contributes to increased T-cell activation, survival and Th17/Treg imbalance.

Deficiency of miR-31 reduces HIF1α activity and glucose metabolism in allogeneic T cells following BMT

Hypoxic environments have been observed in a range of organs during inflammation damage, including gastrointestinal tract, epidermis of skin, thymus and even in regions of lymphoid organs. HIF1α signaling was reported to augment T-cell pathogenicity in murine GVHD. Hypoxia was
reported to be involved in pathogenesis of autoimmune disease by creating a proinflammatory environment. Having identified the critical role of miR-31 in regulating HIF1α expression and hypoxic response of T cells ex vivo, we further examined how miR-31 impacts HIF1α expression in donor T cells during cGVHD development. We found that HIF1α expression was cell-type and organ dependent: expression was higher in CD4 than in CD8 T cells and higher in skin-draining LNs than in spleens (Fig. 6A-B). Importantly, KO donor T cells expressed significantly lower levels of HIF1α than WT T cells in recipient spleen and skin-draining LNs.

Our previous work demonstrates that glycolysis is the predominant metabolic pathway in T cells activated by alloantigens; glycolysis is essential for donor T cells to mediate GVHD. It was reported that HIF1α promotes T-cell glycolysis through regulating genes encoding glucose transporter and glycolytic enzymes. We found that miR-31 KO CD4 T cells had reduced Glut1 expression and glucose uptake following allo-BMT (Fig. 6C-D). However, similar expression levels of Glut1 and 2NBDG were observed in KO CD8 T cells compared to WT counterparts. We further examined how miR-31 impacts glycolysis in CD4 T cells activated in 3% O2, close to the physiological oxygen levels in lymphoid and target organs. Consistently, KO T cells had significantly reduced glucose uptake, Glut-1 expression, extracellular acidification rate (ECAR) and glycolysis (Fig. 6E-F). Thus, miR-31 is important for Glut1 expression, glucose uptake and glycolysis in CD4 T cells, which may provide essential metabolites and bioenergy required for increased T-cell expansion and Th17 differentiation during cGVHD development.

**MiR-31 blockade by LNA antagonim attenuates cGVHD after allo-BMT**

Using genetic models, our data strongly support a key role for miR-31 in T-cell expansion and differentiation towards Th17 while away from Tregs, and thus potentiating T-cell pathogenicity in cGVHD induction. To extend our finding, we tested this approach in a classic model of cGVHD after MHC-matched BMT (B10.D2 → BALB/c), in which cutaneous cGVHD is developed and mediated by donor IL-17-producing cells. Consistently, systemic administration of anti-miR-31 from day of BMT...
attenuated cGVHD severity (Fig. 7A). Strikingly, delayed treatment started on day 21-post BMT also effectively alleviated disease severity (Fig. 7B). Similar to the observation in the genetic approach, pharmacological blocking of miR-31 also attenuated pathological damage and collagen deposition in recipient skin during cGVHD development (Fig. 7C-D). Recipients with delayed anti-miR-31 treatment showed reduced frequencies of donor T-cell and increased frequencies of B-cell in spleens (Fig. 7E). Increased numbers of B cells while comparable numbers of T cells were found in the recipients with delayed anti-miR-31 treatment compared to those with vehicle control (Fig. S16A-B). In addition, delayed anti-miR-31 treatment improved Treg generation in recipients (Fig. 7F). Blockade of miR-31 started on day 0, but not on day 21 of BMT, attenuated Th17 differentiation in recipient skin-draining LNs (Fig. 7F, data not shown). Pharmacological inhibition of miR-31 could also increase FIH1 expression in T cells under hypoxia conditions (Fig. S16C), consistent with the data observed in miR-31 KO T cells in the same experimental setting (Fig. 5B). Furthermore, donor CD4 but not CD8 T cells expressed significantly lower HIF1α in recipients with anti-miR-31 treatment (Fig. 7G). Our data demonstrates that systemic blockade of miR-31 can alleviate cGVHD. Similar to genetic deficiency, blocking miR-31 controls T-cell expansion, Treg differentiation and HIF1α expression.
Discussion

Through miR profiling in murine T cells during alloreactive vs. homeostatic responses, we identified miR-31 as a potential immunoregulator. We revealed a key role for miR-31 in augmenting donor T-cell pathogenicity in cGVHD induction but not in anti-leukemia response. MiR-31-deficient CD4 T cells had defects in proliferation and survival; differentiating more towards Tregs and less towards Th17 cells. MiR-31 promoted hypoxic adaptation of T cells by inhibiting FIH1, and therefore enhancing HIF1α signaling and glucose metabolism in T cells. To the best of our acknowledge, the current study is the first time to uncover the biology of miR-31 in regulating hypoxic adaption of T cell response through FIH1/HIF1α pathway.

In multiple murine models of BMT, targeting miR-31 by genetic mutation or pharmacologic inhibition can significantly attenuate cGVHD symptoms, including sclerodermatous and pulmonary cGVHD manifestations. MiR-31 deficiency attenuates the expansion of mature alloreactive T cells, therefore reducing the amount of pathogenic T cells, including IFNγ-, IL-17A-producing, and effector memory T cells in the recipients. MiR-31 influences the differentiation of mature/alloreactive T cells by increasing IL-17A production, which has been shown to not only drive dermal fibroblast proliferation and but also enhance chemotaxis of macrophages that facilitate fibrosis by producing TGFβ and PDGFα. Therefore, our data reveal a novel biology of miR-31 in promoting Th17 differentiation and fibrosis development in cGVHD. On the other hand, differentiation of Th17 and Tregs are considered reciprocal as Foxp3 antagonizes RORyt expression. MiR-31 limits Treg generation during cGVHD development, consistent with our previous report in EAE. Importantly, miR-31 negatively regulates the stability and function of Tregs during allogeneic response. In addition, miR-31 also impacts the differentiation of a pathogenic extrafollicular CD4 T-cell population (PSGL-1−CD4+ T cells) that contributes to cGVHD pathogenesis by interacting with B cells in a GC-independent manner. It was reported that extrafollicular CD4 T cells express high levels of ICOS and IL-21, and Stat3- and BCL6 which are required for their expansion. Furthermore, tissue resident extrafollicular CD4 T cells augment autologous memory B cell differentiation into plasma cells and
antibody production during cGVHD development in humanized mice. Thus, extrafollicular T-B interaction is critical for pathogenic B-cell expansion and differentiation, especially where GC formation is inhibited when severe lymphopenia was presented during GVHD development.

At molecular levels, miR-31 deficiency or inhibition hampers HIF1α expression in donor T cells. HIF1α enhances Th17 differentiation through promoting the transcriptional program enforcing glycolytic activity. HIF-1α can directly bind to Foxp3 resulting in proteosomal degradation of Foxp3. HIF1α signaling in T cells can be induced by O2 limitation or T-cell receptor stimulation. We found that miR-31 was required for T-cell upregulation of HIF1α during activating in hypoxia, and thus required for optimal activation, survival and RORγt expression in low O2 conditions. We reason that through regulating FIH1/HIF1α axis, miR-31 promotes alloreactive T-cell expansion and Th17/Treg imbalance in hypoxic environment, such as in GVHD target organs. Since HIF1α is reported to directly inhibit Nrp1, it is possible that attenuated HIF1α expression due to miR-31 deficiency may contribute to Nrp1 upregulation and Treg stabilization in miR-31 KO Tregs, although the mechanism remains to be further developed.

Metabolism is considered a basic housekeeping process that plays intricate roles in regulating T-cell response. Allogeneic T cells displayed higher ECAR, glycolytic intermediates, and Glut1 and Gluts3 transcription in aGVHD. Increased glycolysis as well as elevated glycolysis-driven OXPHOS were also shown in T cells in lupus. As cGVHD shares autoimmune features with lupus, we interpret that reduced Glut1 expression, glucose uptake and glycolysis may be attributed in attenuated pathogenicity of miR-31 KO CD4 T cells in cGVHD induction. Since HIF1α enhances the transcription of glucose transporters and glycolytic enzymes, we reason that, during cGVHD induction, miR-31 promotes glycolysis through upregulating HIF1α, which contributes to fulfill the bioenergetic demand for T-cell expansion and to promote Th17 while limiting Treg transcriptional programing.
Interestingly, miR-31 deficiency appears to enhance the potency of CD8 T-cell-mediated antiviral responses while reducing the pathogenicity of T cells, especially CD4\(^+\), in cGVHD and EAE. We found HIF1\(\alpha\) was downregulated in miR-31-deficient CD8 T cells. However, HIF1\(\alpha\) seems non-essential for metabolic reprogramming of CD8 T cells, consistent with lower expression of HIF1\(\alpha\) in CD8 vs. CD4 T cells after allo-BMT. miR-31 expression was much higher in CD8 vs CD4 T cells after activation, suggesting miR-31 possibly targets different downstream mRNAs. Importantly, the largely maintained CD8 T-cell glucose metabolism and cytolytic molecules likely accounts for the preservation of the GVL effect.

Clinical studies evaluating miRs as non-invasive biomarkers (NCT01521039) and/or therapeutic targets highlight the importance of miR biology in immune regulation and the potential of miR-based interference in allo-HCT. Our study reveals a novel biology of miR-31 in modulating T-cell adaptation to low O\(_2\) and their pathogenicity in cGVHD induction, and therefore provides a rationale to target miR-31 for controlling cGVHD in patients after allogeneic HCT in foreseeable future.
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Author contributions

Y. Wu designed and performed research, collected, analyzed and interpreted data, and drafted and revised the manuscript; C. Mealer collected, analyzed data and edited the manuscript; C. Wilson performed lung function tests and revised the manuscript; S. Schutt, D. Bastian, M. Sofi, M. Zhang, H. Choi, K. Yang, L. Tian and H. Nguyen assisted in collecting data; Z. Luo analyzed microarray data; K. Helke read and scored pathological slides; L. Schnapp provided the Flexivent system and edited the manuscript; H. Wang provided miR-31^flox^ mice and participated in experimental design; X.-Z. Yu designed the research, interpreted data, and revised the manuscript.

Conflict of Interest Disclosures

The authors have declared that no conflict of interest exists.
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**Figure Legend**

**Fig. 1. MiR-31 is upregulated in allogeneic T cells and enhances their pathogenicity in cGVHD induction in mice.** T cells isolated from Ly5.1⁺ B6 mice were injected together with T-cell depleted bone marrow (TCD-BM, Ly5.2⁺) into lethally irradiated BALB/c (allogeneic, 700cGy) or Ly5.2⁺ B6 (syngeneic, 1,200 cGy) mice (A-B). MiR expression was evaluated in H2Kᵇ⁺Ly5.1⁺ donor T cells by oligonucleotide arrays on day 14. The heat map represents the log2 value of the relative amount of each miR (A). The mRNA expression of miR-31 in naïve T cells and activated donor T cells isolated from allogeneic or syngeneic recipients was evaluated by real-time PCR (B). Splenocytes (0.35×10⁶) and TCD-BM (5 ×10⁶) from WT or miR-31 KO B6 mice were injected into lethally irradiated BALB/c mice. Recipients of WT BM cells only without splenocyte injections serve as no GVHD control (white circle). Recipient clinical scores and body weight loss were recorded weekly and displayed along the time after BMT (C). Cutaneous pathological scores (D) and collagen deposition in skin evaluated with Masson’s Trichrome stain (E) are shown on day 50. Pathological scores of salivary glands in recipients were recorded on day 60 after BMT (I). WT or miR-31 KO T cells (2.75×10⁶) plus WT TCD-BM were transferred into lethally irradiated B6D2F1 mice. Clinical scores (F), cutaneous pathology scores (G) and collagen deposition (H) are shown on day 50. Data shown is from one representative of 3 individual experiments with n=5-6 mice/group for each experiment. B10.BR mice were conditioned with Cytoxan (120mg/kg) and irradiation and then transplanted with WT or miR-31 KO T cells (5×10⁶) plus WT TCD-BM. Body weight loss (J) and pulmonary compliance, elastance and constriction analyzed with a Flexivent system (K) are shown on day 50 after BMT. * p < 0.05, ** p < 0.01.

**Fig. 2. Loss of miR-31 attenuates alloreactive T-cell expansion and effector T-cell differentiation during cGVHD development.** Splenocytes (CD45.2⁺) from WT or miR-31 KO B6 donors were injected together with WT TCD-BM (CD45.1⁺) into lethally irradiated BALB/c recipients. Frequencies of CD45.2⁺ cells in gated live donor H2Kᵇ⁺ CD4 or CD8 T cells and their absolute numbers are shown in recipient spleen and thymus on day 50 (A, B). In separate experiments, splenocytes and TCD-BM cells from WT
or miR-31 KO B6 mice were injected into lethally irradiated BALB/c mice. On day 50-60, representative flow figures of CXCR5⁺PD-1⁺ cells gated on live donor H2Kᵇ⁺CD4⁺ cells (upper), GC B cells (Fas⁺GL7⁺) gated on H2Kᵇ⁺B220⁺ cells and frequency of CXCR5⁺PD-1⁺ cells in donor H2Kᵇ⁺CD4⁺ cells and their numbers are shown (C). % Tfr (Foxp3⁺) and %Tfh (Foxp3⁻) in gated H2Kᵇ⁺ CD4⁺ CXCR5⁺PD-1⁺ cells (D), %GC B cells in live H2Kᵇ⁺ B cells, and % CD138⁺B220⁻ plasma cells in live H2Kᵇ⁺ cells (E) and their absolute numbers are displayed. Representative flow figures and bar graphs showing the frequencies and numbers of IFNγ⁺, IL-17A⁺, TNFα⁺ cells in gated live donor H2Kᵇ⁺ CD4 T cells in recipient spleens are displayed (F). Data shown is from one representative of 3 individual experiments with n=4-5 mice/group for each experiment. * p < 0.05, ** p < 0.01, *** p < 0.001.

### Fig. 3. MiR-31 facilitates Th17/Treg imbalance and increase extrafollicular CD4 T cell differentiation while inhibits Treg stability.

Splenocytes (CD45.2⁺) from WT or miR-31 KO B6 mice were injected together with WT TCD-BM (CD45.1⁺) into lethally irradiated BALB/c mice. Frequencies of IL-17⁺ or Foxp3⁺ cells in gated live donor H2Kᵇ⁺CD45.2⁺ CD4 T cells are shown in recipient skin-draining LNs (A) and spleen (B) on day 50-60 after BMT. Frequencies of CD62L⁺PSGL-1⁻ cells in gated live H2Kᵇ⁺CD45.2⁺CD44⁺CD4 T cells are shown in recipient spleen on day 60 after BMT. Histograms for Nrp1, PD-L1 and Bcl2 expression on gated H2Kᵇ⁺CD45.2⁺Foxp3⁺ cells are shown (C). Data shown is from one representative of 3 individual experiments with n=4-5 mice/group in each experiment.

CD4⁺CD25⁻ T cells isolated from WT or miR-31 KO mice were stimulated with CD11c⁺ dendritic cells from BALB/c together with IL-2 (5 ng/mL), TGFβ (5 μg/mL), and retinoic acid (40 nM). Frequency of Foxp3⁺ cells in gated live H2Kᵇ⁺CD4⁺ cells are shown on day 5 (D). CD25hi enriched iTregs were cultured in IL-2 (5ng/ml), IL-12 (5ng/ml) or IL-6 (10ng/ml) in the presence of BALB/c APCs. Frequency of Nrp1⁺ cells in gated live H2Kᵇ⁺CD4⁺Foxp3⁺ cells (E) and frequency of Foxp3⁺ cells in gated live H2Kᵇ⁺CD4⁺ cells (F) are shown. Enriched iTregs (CD45.2⁺) were co-cultured with B6 T cells (CD45.1⁺) at different ratios as indicated on the x-axis in the presence of BALB/c APCs. Frequency of IFNγ⁺ cells and live/dead dye⁺ cells in gated CD45.2⁺CD4⁺ cells and frequency of CFSElow cells in gated live CD45.1⁺ CD8 T cells are shown on day 4 (G). Enriched iTregs were injected together with BM cells (Rag1⁻⁻) into lethally
irradiated BALB/c mice followed by T\textsubscript{eff} cell (CD45.1\textsuperscript{+}) transfer 3-day later. Expression of IFN\textgamma and CD25 on gated H2K\textsuperscript{b}CD45.2\textsuperscript{+}Foxp3\textsuperscript{+} cells is shown 4 days post Teff transfer (H). Data shown is from one representative of 2 individual experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

**Fig. 4. MiR-31 is dispensable for T-cell mediated GVL response.** WT or miR-31 KO T cells (2.75\times10\textsuperscript{6}, CD45.2\textsuperscript{+}) plus WT TCD-BM (CD45.1\textsuperscript{+}) were transferred into lethally irradiated B6D2F1 mice. On the day of BMT, 5,000 luciferase-transduced P815 cells were i.v. injected into these recipients. The clinical scores (A), macroscopic photos of recipients on day 40 (B) and survival (C) are shown. P815 growth was monitored by bioluminescence imaging (BLI) (D). Frequency of IFN\textgamma\textsuperscript{+}, TNF\alpha\textsuperscript{+}, granzyme B\textsuperscript{+}, perforin\textsuperscript{+} and CD107a\textsuperscript{+} cells in gated live H2K\textsuperscript{b}CD45.2\textsuperscript{+}CD8\textsuperscript{+} cells (E) are shown in recipient spleen on day 50 post BMT. Data shown is from one representative of 2 individual experiments with n=4-5 mice/group for each experiment. ** p < 0.01.

**Fig. 5. MiR-31 is critical for the activation, survival and differentiation of CD4 T cells under hypoxic environment via targeting FIH1.** A highly conserved binding site of miR-31 seed sequence in the 3\textapos;UTR of FIH1 was identified using TargetScan website (A). CD4\textsuperscript{+}CD25\textsuperscript{−} WT or KO cells were isolated and stimulated with plate bound anti-CD3 (4μg/ml) and soluble anti-CD28 (2μg/ml) in 3% or 21% O\textsubscript{2} conditions. Three days later, expression of FIH1 protein was determined by western blot (B), and expression of HIF1\textalpha, CD69 and ICOS on gated live CD4 T cells was determined by flow cytometry (C, D). CD4\textsuperscript{+}CD25\textsuperscript{−} WT or KO cells were stimulated with anti-CD3/CD28 in 3% O\textsubscript{2} with 0.1mM DMOG dissolved in PBS or vehicle control. Frequency of Annexin V\textsuperscript{+}7AAD\textsuperscript{+} dead cells were show in gated CD4 T cells on day 3 (E). CD4\textsuperscript{+}CD25\textsuperscript{−} WT of KO cells were stimulated with anti-CD3/CD28 together with anti-IFN-\gamma (1 μg/ml), TGF-\beta (2ng/ml) and IL-6 (1ng/ml) in 3% O\textsubscript{2}. Expression of ROR\gammat and Foxp3 in gated live CD4 T cells was determined by flow cytometry on day 3 (F). Data shown is from one representative of 3 individual experiments. * p < 0.05, ** p < 0.01, *** p < 0.001.
Fig. 6. **Deficiency of miR-31 reduces HIF1α activity and glucose metabolism in allogeneic T cells following BMTs.** Splenocytes (CD45.2+) from WT or miR-31 KO B6 mice were injected together with WT TCD-BM (CD45.1+) into lethally irradiated BALB/c mice. Frequencies of HIF1α+ cells in gated live donor H2Kb+CD45.2+ CD4 or CD8 T cells in recipient spleens and skin-draining LNs are shown on 50-60 days post-BMT (A-B). Data shown is from one representative of 2 individual experiments. Frequency of surface Glut1+ cells (C) and uptake of 2NBDG (D) on gated donor H2Kb+CD45.2+ CD4 or CD8 T cells are shown in recipient spleens. CD4+CD25+ WT or KO cells were stimulated with plate bound anti-CD3 (4μg/ml) and soluble anti-CD28 (2μg/ml) in 3% O2 conditions. 2NBDG-uptake on gated live CD4 T cells is shown on day 3 (E). In the same experimental setting, these activated CD4 T cells were subjected to Seahorse assay and ECAR was measured under basal conditions and following injection of three pharmacologic compounds: glucose (10mM), oligomycin (1μM), and 2-DG (100mM). The diagram of ECAR and glycolysis calculated as increased ECAR after glucose injection are shown (F). * p < 0.05, ** p < 0.01, *** p < 0.001.

Fig. 7. **Blocking miR-31 alleviates cGVHD.** BALB/c mice were lethally irradiated and transferred with 5×10^6 TCD-BM plus 5×10^6 splenocytes from B10.D2 mice. Recipient mice were treated with anti-miR-31 starting on day 0 (A) or day 21 (B) after BMT. The clinical scores with macroscopic photos are shown on day 40 after BMT. Cutaneous pathological scores (C) and collagen deposition in skin evaluated with Masson’s Trichrome stain (D) are shown on day 50. In the experiments where anti-miR-31 treatment started at day 21, frequency of CD4+, CD8+, and B220+ in gated donor Ly9.1+ cells (E), frequency of Foxp3+ in gated donor Ly9.1+ CD4 T cells (F) and frequency of HIF1α+ cells in gated donor Ly9.1+ CD4 or CD8 T cells (G) are shown in recipient spleens on day 50. In the experiments where anti-miR-31 treatment started at day 0, frequency of IL-17+ in gated donor Ly9.1+ CD4 T cells are shown in recipient skin-draining LNs on day 50 (F). * p < 0.05, ** p < 0.01, *** p < 0.001.
