Absence of miR-182 Augments Cardiac Allograft Survival

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Abstract

Background—MicroRNAs (miRNAs) are small noncoding RNA molecules that regulate the posttranscriptional expression of target genes and are important regulators in immune responses. Previous studies demonstrated that the miRNA, miR-182 was significantly increased during allograft rejection. Further, the transcription factor Forkhead box (FOX) protein 1, (FOXO1) was shown to be a target of miR-182. The aim of this study is to further examine the role of miR-182 in alloimmune responses.

Methods—Transplantation of BALB/c cardiac allografts was performed in C57BL/6, miR-182$^{-/-}$, B6.129S-H2$^{dlAb1-Ea}$ (MHC II$^-$ and CD4$^+$ T cell-deficient) and B6.129S2-Tap1^{tm1Arp} (MHC I$^-$ and CD8$^+$ T cell-deficient) mice, with or without CTLA-4Ig administration. T cell phenotype, FOXO1 protein levels and graft infiltrating lymphocytes were determined in C57BL/6 or miR-182$^{-/-}$ mice by flow cytometric analysis, western blot and immunohistochemistry, respectively.

Results—We now show that T cells, mainly CD4$^+$ are the main cellular source of miR-182 during allograft rejection. In the absence of miR-182, CTLA-4Ig treatment significantly increased allograft survival (31.5 days C57BL/6 vs. 60 days miR-182$^{-/-}$, P<0.01). Further, CTLA4-Ig treatment inhibits miR-182 expression, increases FOXO1 levels, and reduces the percentage of

CD4$^+$CD44^{hi} T cells after transplantation. Fewer T cells infiltrate the cardiac allografts and memory T cells are significantly decreased in allograft recipients deficient in miR-182 with CTLA4-Ig Treatment P<0.01).

Conclusions—Our findings suggest miR-182 contributes to the T cell responses to alloantigen especially under costimulation blockade. Therapeutics that target specific miRNAs may prove beneficial in transplantation.

Introduction

MicroRNAs (miRNAs) are small noncoding RNA molecules that regulate the posttranscriptional expression of target genes[1-4]. There is ample evidence that miRNAs are involved in the regulation of the immune response including after transplantation[5-7]. In previous studies, we demonstrated that miR-182 was significantly increased in mononuclear cells that infiltrate rejecting allografts[8]. Furthermore, as miR-182 increases after transplant, there is a concomitant posttranscriptional decrease in the mRNA target, FOXO1[8]. FOXO1 acts as a master cellular regulator of a variety of cellular processes and plays a critical role in the homeostasis of cells of the immune system including neutrophils, T and B cells[9-12]. We, and others, have demonstrated that miR-182 is induced by IL-2 and represses FOXO1 to promote clonal expansion of activated helper T lymphocytes. Expression of miR-182 is dependent on combined T cell receptor (TCR) and IL-2 signaling through STAT5[13]. Further, recent studies have demonstrated that miR-182 in increased in both antibody-mediated rejection and delayed graft function of human renal allografts[14]. Since miR-182 has been demonstrated to affect T cell responses and to be increased during graft rejection in both heart and kidney in experimental models and human transplants, we sought to further probe the role of this miRNA in alloimmune responses.

Costimulation blockade of T cell – antigen presenting cell (APC) responses has been identified as an effective treatment strategy for a variety of conditions[15-18]. Biologics that target either CTLA-4 (monoclonal antibody Human IgG1, Ipilimumab) or CD80/CD86 (recombinant fusion protein CTLA-4 human IgG1, Abatacept or Belatacept) are being successfully used for metastatic melanoma, rheumatoid arthritis (RA) and renal transplantation, respectively[19-23]. We now demonstrate that combined costimulation blockade and absence of miR-182 is superior to costimulation blockade alone, in decreasing alloimmune T cell responses, leading to a significant prolongation of allograft survival. Thus, our findings demonstrate a role for miR-182 in T cell activation during allograft rejection.

Materials and Methods

Animals and transplantation model

Ten-week-old C57BL/6 and BALB/c mice were purchased from Charles River Laboratories (Hollister, CA). B6.129SH2$^{dlAb1-Ea}$ and B6.129S2-Tap1^{tm1Arp} mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Homozygous miR-182 deficient mice (miR-182$^{-/-}$) on the C57BL/6 background were obtained from Dr. Iwai, National Cerebral and Cardiovascular Center, Japan[24] and confirmed in our hands to not express miR-182

(data not shown). All experimental procedures were performed in accordance with a Stanford Institutional Animal Care and Use Committee approved protocol. Heterotopic heart transplantation was performed in groups (n=4-8; see specific experiments) of syngeneic C57BL/6→C57BL/6 and allogeneic BALB/c→C57BL/6; (wild-type, WT) BALB/c→miR-182[−/−·], BALB/c→B6.129SH2[dlAb1-Ea] and, BALB/c→B6.129S2-Tap1[tm1Arp] mice as reported previously[25]. Some groups of recipients were treated with CTLA4-Ig (Abatacept, a gift from Bristol Myers Squibb) at dose of 0.5 mg i.p. on day 0, followed by a dose of 0.25 mg on days 2, 4, and 6. Function of the grafts was assessed through abdominal palpation and confirmed by histopathological analyses using H&E and Masson Trichrome staining.

Cell isolation and purification

Splenocytes were isolated from transplant recipients on day 5, day 7 or day 28 posttransplant. PBMC and graft infiltrating lymphocytes (GILs) were isolated from blood and heart grafts respectively on day 5 posttransplant as described previously[8]. To isolate GILS, grafts were perfused with PBS before recovery. After removal, the cardiac tissue was minced and placed in RPMI 1640 containing collagenase (2mg/ml), incubated at 37°C for 2 h and strained through a 70μm nylon cell strainer. GILs were purified using Lympholyte (Cedarlane, Ontario, Canada) prior to RNA extraction as described previously[8]. PBMC were isolated from graft recipients using Lympholyte. CD4^{+} T cells were enriched from spleens of mice using EasySep mouse CD4^{+} T cell isolation kit (Stemcell Technologies, BC, Canada). B cells were isolated from spleens of mice using EasySep mouse B cell negative isolation kit (StemCell Technologies, BC, Canada).

CD4^{+} T cell activation

Purified CD4^{+} T cells (2×10^5 cells/well) were plated in 96-well plates precoated with 5 μg/mL anti-CD3 Ab (eBioscience, San Diego, CA), then soluble anti-CD28 Ab (eBioscience, San Diego, CA) was added (final concentration 2μg/mL). Cells were cultured for 48 h and the collected for RNA isolation.

Quantitative RT-PCR

miR-182 expression was quantitated in RNA samples isolated from PBMC, GILs, CD4^{+} T cells, B cells or splenocytes by TaqMan miRNA assays (Applied Biosystems, Carlsbad, CA). SnoRNA202 expression was used for normalization. To determine the expression of mouse IFN-γ, IL-2 and β-actin, cDNA was synthesized from total RNA samples using SuperScript II reverse transcriptase with random hexamers (Invitrogen, Carlsbad, CA). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-rad, Hercules, CA). The primers were as follows (forward and reverse primers): mouse IFN-γ: 5' CAG CAA CAA CAT AAG CGT CAT 3' and 5' ACC TCA AAC TTG GCA ATA CTC 3'; mouse IL-2: 5' GGC ATG TTC TGG ATT TGA CT 3' and 5' TCA TCA TCG AAT GGC CAC TC 3'; mouse β-Actin: 5' CAG CCT TCC TTC TTG GGT AT 3' and 5' GGT CTT TAC GGA TGT CAA CG 3'.

Flow cytometry

Splenocytes were collected and washed with cold FACS buffer (PBS, 1% FBS, 0.1% sodium azide) before staining with immunofluorescent markers. Briefly, 2×10^6 cells were incubated with 0.5 μl LIVE/DEAD® Fixable Aqua Dead Cell Stain (Thermofisher Scientific, Waltham, MA) and incubated for 15-20 min on ice. Cells were then labeled with the following antibodies (as per manufacturer's instructions): FITC rat anti-mouse CD44 mAb, PE rat anti-mouse CD4 mAb (BD PharMingen, San Diego, CA); and BV570 rat anti-mouse CD4 mAb, BV785 rat anti-mouse CD44 mAb and Alexa700 rat anti-mouse CD62L mAb (Biolegend, San Diego, CA). FITC Rat IgG2b, κ mAb and PE Rat IgG2a, κ mAb (BD PharMingen); and BV570 Rat IgG2a κ mAb, BV785 Rat IgG2b κ mAb and Alexa700 Rat IgG2a κ mAb (Biolegend) were used as isotype controls. Cells were washed twice with cell staining buffer (Biolegend), resuspended in 200 μl of cell staining buffer and analyzed on FACScan flow cytometer or LSR II.2 (BD Biosciences, San Jose, CA). Data was analyzed using FlowJo software (Tree Star, Ashland, OR).

FOXO1 Quantitation

Protein was extracted from splenocytes using Cell Extraction Buffer (Invitrogen, Carlsbad, CA) containing 1.0 mM Phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor cocktail (Sigma, St. Louis, MO). The following antibodies were used: rabbit anti-mouse FOXO1 mAb (clone C29H4, Cell Signaling Technology, Beverly, MA), anti-β-Actin mAb (clone AC-15, Sigma), donkey anti-rabbit, and donkey anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Blots were visualized with SuperSignal West Pico Substrate (Thermofisher Scientific, Waltham, MA) and quantification was performed using ImageJ software (NIH, Bethesda, MD).

Immunohistochemistry

Infiltrating CD4$^+$ or CD8$^+$ cells in heart grafts were detected by immunofluorescence as described previously [8]. Rat anti-mouse CD4 mAb (clone GK1.5) or rat anti-mouse CD8 mAb (clone 53-6.7) (eBioscience, San Diego, CA) were used as primary Abs. Alexa Fluor 488 goat anti-rat IgG (H+L) mAb was used as secondary Ab (Invitrogen, Carlsbad, CA). 4', 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) was used to label nuclei. Representative images (63X magnification) were photographed using a digital camera attached to a Zeiss LSM 510 META inverted laser scanning confocal microscope (Zeiss, Oberkochen, Germany). Infiltrating cells were counted in different fields of vision (7-12 fields) using ImageJ software (NIH, Bethesda, MD).

Statistical Analysis

All of the data are presented as mean±SEM (mean ± standard error of the mean). GraphPad Prism software was used for generating Kaplan-Meier survival curves and data analysis using Student's t test, and p values of <0.05 were considered statistically significant.

Results

T cells are the cellular source of miR-182 during alloimmune responses

Previously we have demonstrated that cells that infiltrate rejecting cardiac allograft express miR-182[8]. Further, miR-182 was increased in both the PBMC and splenocytes isolated from recipients of cardiac allografts (BALB/C→C57BL/6) as compared to syngeneic (C57BL/6→C57BL/6) grafts on day 7 posttransplant indicating that miR-182 is increased both within the allograft and the periphery during graft rejection (**Figure 1, SDC and SDC, Materials and Methods**). These findings support a role for miR-182 in regulating immune cell functions during allograft rejection. To define the role of miR-182 in graft rejection, groups (n=5) of WT C57BL/6 or miR-182$^{-/-}$ mice received cardiac grafts from BALB/c mice and were monitored for graft survival. miR-182$^{-/-}$ recipients had a modest yet statistically significant, 2-day prolongation of graft survival (6.4 days, vs. 8.2 days, P<0.01; **Figure 1A**). To establish the cellular source of miR-182, BALB/c hearts were transplanted into B6.129S-H2$^{dlAb1-Ea}$ (MHC II$^-$ and CD4$^+$ T cell-deficient), B6.129S2-Tap1^{tm1Arp} (MHC I$^-$ and CD8$^+$ T cell-deficient) or C57BL/6 recipients (n=4 per group) and miR-182 levels in PBMC and GILs quantitated on day 5 posttransplant. As expected, and consistent with our previous studies[8], expression of miR-182 was increased significantly in both the PBMC and GILs of C57BL/6 recipients of allografts as compared to recipients of syngeneic grafts (**Figure 1B**). In PBMC from B6.129S2-Tap1^{tm1Arp} recipients, the miR-182 levels were similar to that seen in the control allogeneic transplant group of wild-type C57BL/6 recipients indicating that miR-182 was still elevated in the absence of CD8$^+$ T cells. In contrast, in B6.129S-H2$^{dlAb1-Ea}$ recipients that lack CD4$^+$ T cells, miR-182 expression was similar to that detected in the syngeneic transplant group. When GILs were analyzed there was a similar significant decrease in mIR-182 in the absence of CD4$^+$ T cells but the levels of miR-182 were increased over syngeneic suggesting a cell type other than a CD4$^+$ T was also expressing miR-182. We have now added an additional, complementary, approach where we isolated B and T cells from the spleens of syngeneic and rejecting recipients of cardiac allografts and quantitated miR-182 by q-RT-PCR (**SDC, Materials and Methods**). We clearly demonstrate that all the enhanced miR-182 is from T cells and not from B cells (**Figure 2, SDC**). Taken together, these results support that miR-182 expression is dependent upon the presence of T cells and suggest that CD4$^+$ T cells are the major cellular source of miR-182 during alloactivation.

miR-182 contributes to optimal T cell activation and IFN-γ expression

To understand the role of miR-182 in T cell function, purified CD4$^+$ T cells were isolated from C57BL/6 mice and activated with anti-CD3 and anti-CD28 antibodies. Forty-eight hours after activation, miR-182 and IFN-γ mRNA were significantly increased in CD4$^+$ T cells (**Figures 2A and 2B, respectively**). However, in the absence of miR-182, IFN-γ was significantly decreased as compared to WT controls, suggesting that miR-182 contributes to optimal, IFN-γ production by CD4$^+$T cells (**Figure 2B).**

CTLA4-Ig inhibits miR-182 expression and alters the alloimmune response

To further understand the relationship between miR-182 expression and T cell activation after transplantation, groups of C57BL/6 recipients received BALB/c heart allografts with or

without CTLA4-Ig treatment. CTLA4-Ig blocks the CD28/CTLA4 ligands CD80 and CD86, thus inhibiting costimulation between T cells and APCs. As expected, on day 7 posttransplant, the number of CD4$^+$ T cells was significantly increased in recipients of heart allografts that did not receive CTLA4-Ig, with CD4$^+$CD44^{hi} T cells but not CD4$^+$CD44^{lo} T cells elevated **(Figure 3A, grey bars)**. CTLA4-Ig treatment prevented an increase in the number of CD4$^+$ and CD4$^+$CD44^{hi} T cells, with numbers similar to control group **(Figure 3A, black bars)**. We did not observe any significant changes in the numbers of CD4$^+$CD44^{lo} T cells in either allogeneic transplant groups, without or with CTLA4-Ig treatment, which were not elevated after transplantation as compared to control group.

Further, on day 7 posttransplant, miR-182 was significantly increased in splenocytes from mice that received allografts **(Figure 3B, grey bars)**. Treatment with CTLA4-Ig significantly decreased miR-182 expression **(Figure 3B, black bars)**. Since CTLA4-Ig treatment alters miR-182 expression and FOXO1 is a target of miR-182[8], we determined if FOXO1 protein levels were modulated after transplant in recipients treated with CTLA4-Ig **(Figure 3C)**. Splenocytes from graft recipients treated with CTLA4-Ig had significantly more FOXO1 that untreated allograft recipients consistent with a concomitant decrease in miR-182 and increase of the FOXO1 protein. Taken together, these studies indicate that CTLA4-Ig results in a decrease in CD4$^+$CD44^{hi} T cells, decreased levels of miR-182 and a significant increase in the transcription factor, FOXO1.

Absence of miR-182 augments efficacy of CTLA4-Ig treatment posttransplant

Since the absence of miR-182 prolonged graft survival, we then asked whether co-stimulation blockade would further prolong graft survival in the absence of miR-182. Groups of WT C57BL/6 (n=7) or miR-182$^{-/-}$ (n=8) recipients of BALB/c hearts were treated with CTLA4-Ig and graft survival monitored. In WT, CTLA4-Ig-treated C57BL/6 recipients, graft survival was significantly increased to a mean of over 30 days as compared to 7 days in untreated WT C57BL/6 recipients (see Figure 1A for comparison). Graft survival was further prolonged (mean of 60.0 days miR-182$^{-/-}$ versus 30.5 days WT, P<0.01) in the absence of miR-182 **(Figure 4A)**. Splenocyte lysates analyzed by western blot, demonstrated that FOXO1 protein levels were significantly increased in mice deficient in miR-182$^{-/-}$, that had significantly prolonged graft survival, as compared to WT C57BL/6 recipients of **(Figure 4B)** consistent with the finding that graft survival is enhanced in the presence of increased levels of FOXO1 protein.

Sections of the cardiac allografts were examined at 28 days posttransplant, a time-point when graft survival was 100% in the miR-182$^{-/-}$ CTLA4-Ig treated group. Both CD4$^+$ T and CD8$^+$ T cells were decreased (CD4$^+$T cells, 42.9±13.1%, P<0.05; CD8$^+$ T cells, 36.9±12%, P=0.08) in miR-182$^{-/-}$ allograft recipients as compared to WT C57BL/6 allograft recipients **(Figure 4C and 4D)**. The observation that both CD4$^+$ and CD8$^+$ T cells were reduced in the heart grafts of miR-182$^{-/-}$ recipients, suggest that miR-182 regulation of FOXO1 (increased FOXO1 as a result of the lack of miR-182) reduces the infiltration of lymphocytes into allografts leading to reduced inflammatory response and prolonged graft survival.

Memory T cells are decreased in recipients deficient in miR-182 after cardiac transplantation and CTLA4-Ig treatment

To further define the mechanism for prolonged graft survival by the combination of CTLA4-Ig treatment and the absence of miR-182, we examined the T cell phenotypes from splenocytes in groups (n=3) of C57BL/6 and miR-182$^{-/-}$, recipients of BALB/c cardiac allografts with and without CTLA4-Ig treatment. As expected, we observed a reduction in the effector (CD4$^+$CD44^{hi}CD62L^{lo}T cells, 23.6±2.11 vs 43.4±2.4; P<0.01) and memory (CD4$^+$CD44^{hi}CD62L^{int}T cells, 2.35±0.23 vs 4.77±0.4; P<0.01) T cell types in CTLA-4Ig-treated C57BL/6 WT allograft recipients (grey bars) as compared to untreated (vehicle) control allograft recipients (black bars). While we observed an increase in the naïve CD4$^+$ T cells (CD4$^+$CD44^{lo}CD62L^{hi}T cells, 67.5±1.76 vs 80.93±2.44; P<0.05), there was a significant decrease in percentages of both effector (CD4$^+$CD44^{hi}CD62L^{lo}T cells, 18.93±2.53 vs 30.93±1.76; P<0.05) and memory (CD4$^+$CD44^{hi}CD62L^{int}T cells, 0.12±0.04 vs 1.42±0.18; P<0.01) T cells in miR-182$^{-/-}$ recipients treated with CTLA4-Ig (diagonal bar) as compared to vehicle control (white bar) **(Figure 5).** Indeed, when compared to transplant recipient's treatment with CTLA-Ig alone (grey bars), the absence of miR-182 did not alter the percentage of effector cells but had a dramatic effect on the memory cells (diagonal bars). Our results show that the absence of miR-182 causes a significant reduction in the memory cells in CTLA-4Ig treated allograft recipients.

DISCUSSION

We demonstrated previously that miR-182 was dramatically increased during rejection and specifically detected in the circulation of allograft recipients[8]. The IL-2–miR-182 axis has been suggested to be important for the clonal expansion of activated naive helper T cells[13]. miR-182 was found to be induced in the late phase of clonal expansion and inhibition of this miRNA limited T helper cell expansion and function. Treatment with an antagomir to miR-182 resulted in diminished severity of disease in a mouse model of ovalbumin-induced arthritis further supporting the role of miR-182 in helper T cell–mediated immune responses. We now show prolongation of cardiac allograft survival in the absence of miR-182 during costimulation blockade.

Since activation of T cells results in increased miR-182 and a concomitant decrease in the transcription factor FOXO1, we asked whether blockade of costimulatory pathways alters miR-182 levels. Indeed, we clearly show that in the presence of CTLA4-Ig, miR-182 in decreased in T cells while increasing FOXO1 protein production. CTLA4, a member of the immunoglobulin superfamily, is expressed on the surface of T cells and transmits an inhibitory signal. Specifically, CTLA4 competes with CD28 for binding to CD80 and CD86 and while CD28 transmits a stimulatory signal, CTLA4 suppresses T cell function[26]. Indeed, 2 pharmacologically modified forms of CTLA4-Ig, Belatacept and Abatacept are FDA approved for treatment of kidney transplantation and rheumatoid arthritis, respectively[27,28].

Treatment with CTLA4-Ig dramatically and significantly prolonged cardiac allograft survival in mice deficient in miR-182; 50% of the allografts survived more than 60.0 days as compared to 30.5 days in mice expressing miR-182, suggesting a role for miR-182 in graft rejection. Analysis of human renal allograft biopsies demonstrated that increased expression

of miR-182 is associated with both antibody-mediated rejection and acute kidney injury[14,29]. Our studies, and others, have demonstrated FOXO1 as a target of miR-182 and indeed FOXO1 was significantly increased in miR-182$^{-/-}$ recipients of cardiac allografts treated with CTLA4-Ig supporting a role for FOXO1 in graft survival[8,13,30]. FOXO1 suppresses proliferation of resting CD4$^+$ T cells by up-regulating the expression of a Cdk inhibitor, p27Kip1[31]. FOXO1 has been reported to control effector to memory conversion and functional maintenance of CD8$^+$ T cells and memory CD4$^+$ T cells[32]. This is consistent with the significant decrease in the memory (CD4$^+$CD44^{hi}CD62L^{int}) CD4$^+$ T cell compartment we observed in the CTLA4-Ig treated miR-182$^{-/-}$ graft recipients.

Previous studies have also shown that the levels of FOXO1 impact lymphocyte infiltration[33]. Mice with a deletion of FOXO1 in T cells were shown to have increased infiltration of T cells and enhanced Tfh cell differentiation[11]. Consistent with this finding, we show that there is a significant reduction in CD4$^+$ T cells (and a marked trend towards decreased numbers of CD8$^+$ T cells) in CTLA-4Ig treated miR-182$^{-/-}$ mice as compared to miR-182 expressing mice supporting that an increase of FOXO1 is associated with a decrease of lymphocyte infiltration.

Computational analysis identified miR-182 in Th2-Foxp3$^+$ cells as a potential regulatory miRNA hub[34]. Further, it was shown that Tregs isolated from Th2 environments following chronic *Schistosoma mansoni* infection or Th2-driven inflammation downregulated IL-2 associated genes under the control of miR-182[35]. No differences in Treg numbers (CD4$^+$CD25$^+$FoxP3$^+$T cells) were observed in the absence or presence of miR-182 in our (Th1-mediated) model of cardiac allograft rejection (BALB/c to miR-182−/− + CTLA-4Ig, 4.54±0.54 vs; BALB/c to C57BL/6 + CTLA-4Ig, 4.59±0.28; P< 0.9302). Although a recent study suggested that the immune response to experimental *Listeria monocytogenes* infection is intact in miR-182$^{-/-}$ mice [36] our results, using miR-182$^{-/-}$ on the same C57BL/6 background, do suggest some alterations on the early immune response after cardiac allograft transplantation. Further, the recent studies of Li et al demonstrate that miR-182 deficiency (using knock-out mice) does impair early T cell-dependent immune responses[37]. Taken together, these studies suggest that miR-182 does have a role in T cell function, early after stimulation, but suggest after chronic stimulation/exposure the malleability of the miR networks may compensate for the loss of specific microRNAs.

In summary, we have reported for the first time the synergistic effect of absence of a specific miRNA and costimulation blockade on cardiac allograft survival. Further, our results support a mechanism whereby modulation of FOXO1 may be at least partially responsible for the immune alterations that prolong graft survival. Blockade of selective miRNAs may prove to be an efficacious strategy to inhibit the alloimmune response after transplant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Abbreviations

miRNA	microRNA, miR
UTR	untranslated region
AR	acute rejection
FOXO1	Forkhead Box O1
APC	Antigen Presenting Cells
GILs	Graft infiltrating lymphocytes

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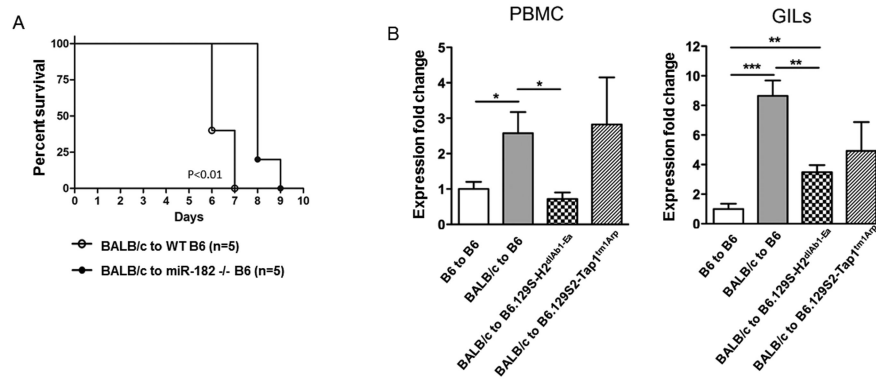
Figure 1. The absence of miR-182, expressed by T cells, prolongs graft survival
(A) Kaplan-Meier survival curves of BALB/c heart allografts in WT C57BL/6 (n=5) or miR-182$^{-/-}$ C57BL/6 (n=5) recipients (p<0.01). (B) BALB/c hearts were transplanted into groups (n=4) of WT C57BL/6, B6.129SH2$^{dlAb1-Ea}$ (MHC II and CD4$^+$ T cell deficient) and B6.129S2-Tap1^{tm1Arp} (MHC I and CD8$^+$ T cell deficient) recipients. C57BL/6 hearts transplanted into C57BL/6 recipients were used as controls. PBMC and GILs were obtained from recipients on day 5 posttransplant and miR-182 expression was detected by quantitative RT-PCR. Expression fold changes of miR-182 were calculated by the 2$^{-\Delta Ct}$ method, normalized to the expression of housekeeping gene snoRNA202 and expression fold changes normalized to the control group. Data shown are the mean ± standard error of all graft recipients, *(p<0.05), ** (p<0.01) or *** (p<0.001).
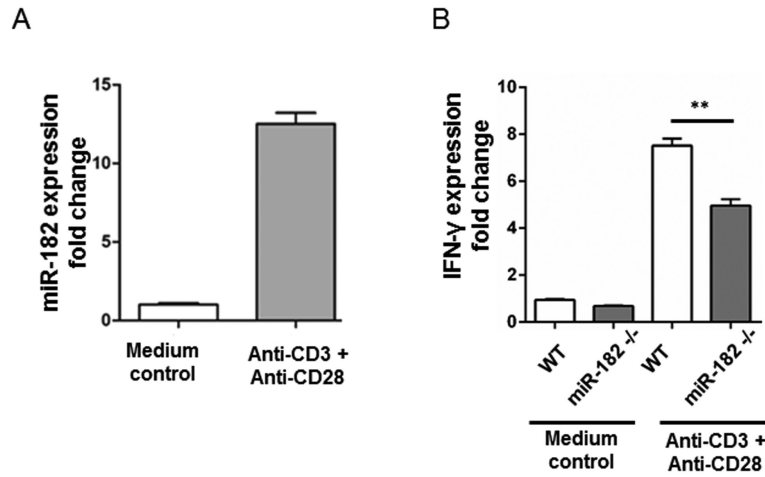
Figure 2. Activation of CD4+ T cells increases miR-182 and IFN-γ expression

CD4+ T cells were isolated from spleens of WT C57BL/6 mice and activated with anti-mouse CD3 and anti-mouse CD28 antibodies (n=6 wells). (A) miR-182 levels were detected by quantitative RT-PCR after 48 h. Expression fold changes of miR-182 were calculated by $2^{-\Delta Ct}$ method, normalized to the expression of housekeeping gene snoRNA202 and compared to the medium (without activation) control group. (B) IFN-γ mRNA levels in CD4+ T cells were detected by quantitative RT-PCR at 48 h after stimulation. Expression fold changes of IFN-γ were calculated by $2^{-\Delta Ct}$ method, normalized to the expression of β-actin and compared to the group without activation. Data shown are the mean ± standard error of the mean of 3 independent experiments, (**, p<0.01).
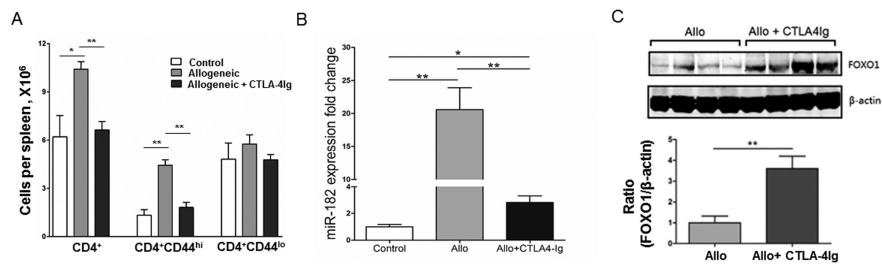
Figure 3. CTLA4-Ig inhibits miR-182 expression, increases FOXO1 levels, and reduces the CD4+CD44hi T cell population

Groups (n=4) of BALB/c→C57BL/6 recipients of heart allografts were left untreated or treated with CTLA4-Ig and splenocytes isolated on day 7 post-transplant, (A) The absolute numbers of CD4+, CD4+CD44hi, and CD4+CD44lo T cells in the spleen were quantitated, by flow cytometry on day 7 post-transplant, (B) miR-182 expression was quantitated in splenocytes as detailed previously and compared to control C57BL/6 mice, (C) Lysates were prepared from splenocytes and FOXO1 protein levels determined by western blot (upper panel) with the densitometry of the ratio of FOXO1 to β-actin shown below (lower panel). Data represent mean ± standard error of the mean (*, P<0.05; **, P<0.01).
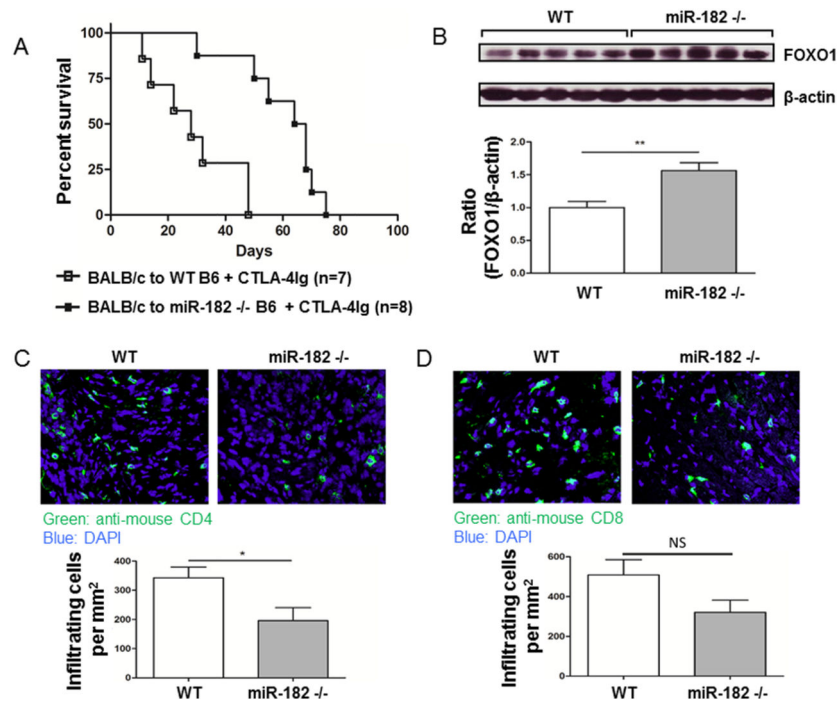
Figure 4. Absence of miR-182 prolongs graft survival by increasing FOXO1, which decreases T cell infiltration into allografts

BALB/c hearts were transplanted into groups of WT C57BL/6 or miR-182−/− recipients and treated with CTLA4-Ig (0.5 mg i.p. on day 0; 0.25 mg on days 2, 4, and 6). (A) Kaplan-Meier survival curves of treated allograft groups (p<0.001). (B) Lysates prepared from splenocytes on day 28 posttransplant from groups (n=5) of WT or miR-182−/− allograft recipients, treated with CTLA4-Ig, were probed for FOXO1 expression by western blot (upper panel). The ratio of FOXO1 to β-actin was determined by densitometry (lower panel). Data shown are the mean ± standard error of the mean (**, P<0.01). (C, D) Heart grafts from groups (n=3) of transplanted mice were sectioned and stained with anti-mouse CD4 (B; clone GK1.5) or anti-mouse CD8 (C; clone 53-6.7) on day 28 posttransplant. Representative images of infiltrating T cells (upper panel) and the average number of infiltrating cell per mm^2 (lower panel) are shown. Data shown are the mean ± standard error of the mean (*, p<0.05; NS, Not Significant).
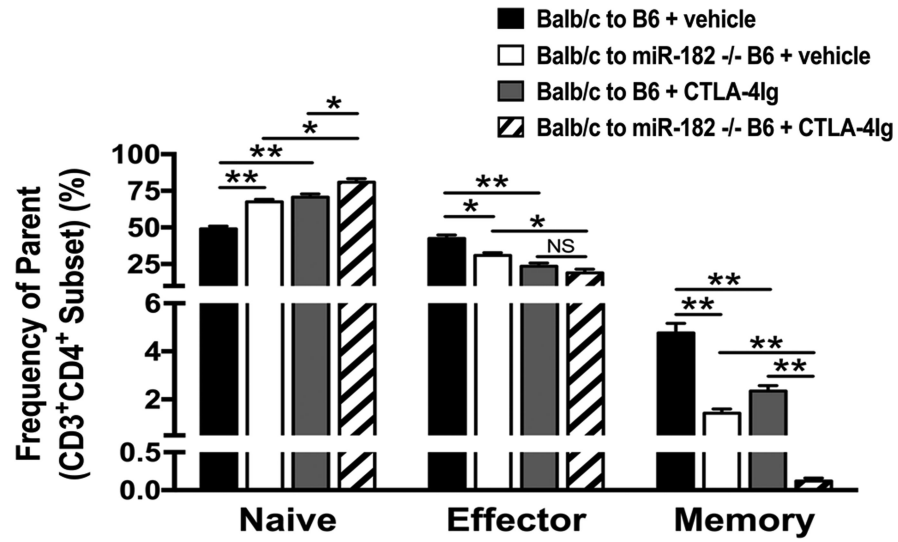
Figure 5. Absence of miR-182 decreases memory T cells in CTLA4-Ig treated allograft recipients
BALB/c→C57BL/6 or BALB/c→miR-182$^{-/-}$ recipients of heart allografts were left
untreated (black, white bars) or treated with CTLA4-Ig (grey, diagonal bars) and splenocytes
isolated on day 7 posttransplant were quantitated by flow cytometry. Percentage of naïve,
effector and memory CD4$^+$ T cells (Frequency of parent population, %). Data shown are the
mean ± standard error of the mean (*, p<0.05; **, p<0.01; NS, Not Significant), n=3.