INTRODUCTION

Solid allograft transplantation can be lifesaving at the point of organ failure. However, long-term allograft and patient survival depends on sustained drug-induced immunosuppression. Acute cellular rejection (ACR) occurs in around 25% of heart transplant recipients in the first-year posttransplant despite optimal levels of currently available immunosuppressive therapy, accounting for 10% of mortality in the first-year posttransplant. Organ transplantation is often lifesaving, but the long-term deleterious effects of combinatorial immunosuppression regimens and allograft failure cause significant morbidity and mortality. Long-term graft survival in the absence of continuing immunosuppression, defined as operational tolerance, has never been described in the context of multiple major histocompatibility complex (MHC) mismatches. Here, we show that miR-142 deficiency leads to indefinite allograft survival in a fully MHC mismatched murine cardiac transplant model in the absence of exogenous immunosuppression. We demonstrate that the cause of indefinite allograft survival in the absence of miR-142 maps specifically to the T cell compartment. Of therapeutic relevance, temporal deletion of miR-142 in adult mice prior to transplantation of a fully MHC mismatched skin allograft resulted in prolonged allograft survival. Mechanistically, miR-142 directly targets Tgfbr1 for repression in regulatory T cells (T_{REG}). This leads to increased T_{REG} sensitivity to transforming growth factor – beta and promotes transplant tolerance via an augmented peripheral T_{REG} response in the absence of miR-142. These data identify manipulation of miR-142 as a promising approach for the induction of tolerance in human transplantation.

KEYWORDS

animal models: murine, basic (laboratory) research/science, immunobiology, molecular biology: micro RNA, organ transplantation in general, T cell biology, tolerance: experimental, tolerance: mechanisms
first-year posttransplant and increasing the risk of 5-year mortality.\(^1\)
Effector CD4\(^+\) T cells (T\(_{\text{EFF}}\)) are critical mediators of allograft responses resulting in allograft rejection. Induction of tolerance is a major goal in transplantation, potentially allowing withdrawal of immunosuppression and indefinite allograft survival.\(^2\)
 Regulatory T cells (T\(_{\text{REG}}\)) are a subset of CD4\(^+\) T cells that exert dominant suppression of T\(_{\text{EFF}}\) responses.\(^3\)
Peripherally induced T\(_{\text{REG}}\) cells are critically dependent on transforming growth factor – beta (TGF-β) for the induction of their key transcription factor, FoxP3.\(^5\)
The vital role of T\(_{\text{REG}}\) in transplant tolerance is apparent where deletion of T\(_{\text{REG}}\) in mice already tolerant of kidney allografts leads to allograft rejection.\(^6\)
In human studies, the expression of FoxP3 in transplant infiltrating T cells is associated with donor-specific hyporesponsiveness and improved graft histological findings.\(^6\)

MicroRNAs (miRNAs) are short noncoding RNAs that regulate gene expression posttranscriptionally by binding to target sequences on mRNAs, leading to their degradation and/or translational inhibition.\(^7\)
The role of miRNAs in the regulation of both innate and adaptive immune responses is increasingly recognized, with aberrant expression of miRNAs contributing to autoimmune diseases and malignancies.\(^7,8\)
Recently, we demonstrated a critical role for MicroRNA-142 (miR-142) in regulating peripheral immune tolerance via its action in CD4\(^+\) T cells.\(^9\)
MiR-142 is highly expressed in cells of hematopoietic lineages, including CD4\(^+\) T cells\(^10\) and is conserved across vertebrates, including between humans and mice, supporting the validity of murine models in the investigation of miR-142 function in human disease and therapeutic development.\(^11\)
This microRNA exists as two functional isoforms (miR-142-3p and miR-142-5p), capable of binding to different mRNA targets but processed from a single pre-miRNA hairpin. In the context of acute cellular rejection, levels of miR-142-3p and miR-142-5p are consistently increased in cardiac allograft tissue.\(^12\)
Serum miR-142-3p levels are also significantly higher in the context of biopsy proven ACR in heart transplant recipients.\(^13\)
MiR-142 over-expression is highly predictive of acute T cell-mediated rejection in renal allograft biopsies and is increased in peripheral blood mononuclear cells (PBMC) of patients with chronic antibody-mediated rejection.\(^14,15\)
However, miR-142 has also been shown to be up-regulated in B lymphocyte subsets of PBMCs in operationally tolerant renal transplant patients.\(^16,17\)
Whether miR-142 actually plays a functional role in transplant rejection and what that role may be, has not previously been explored.

2 | MATERIALS AND METHODS

2.1 | Animals

Mir142\(^{fl/fl}\) mice were generated by homologous recombination in 1295 mouse embryonic stem cells using a targeted vector containing FRT and loxP sites flanking the Mir142 locus and a neomycin resistance cassette (Genoway). Chimeric offspring were bred with C57BL/6J-Flp deleter mice to generate conditional lines, which were fully back-crossed onto a C57BL/6 background. Cd4\(^{Cre}\) mice were kindly provided by Professor Matthias Merkenschlager (MRC London Institute of Medical Sciences, Imperial College London). Rosa26-Cre-ERT\(^2\) transgenic mice were kindly provided by Dr Thomas Ludwig (Columbia University) and generated using the Cre-ERT\(^2\) construct generated by Pierre Chambon at the Institute of Genetics and Molecular Biology (University of Strasberg). Mice were housed in specific pathogen-free conditions. All experiments were performed according to King’s College London and national guidelines, under a UK Home Office Project License (PPL: 70/7869). Appropriate control mice were utilized, with age and sex-matched littermate mice used where possible.

2.2 | Heterotopic heart transplantation

BALB/c (H-2\(^b\)) cardiac allografts were transplanted heterotopically into C57BL/6 (H-2\(^b\)) recipients that is, a major MHC mismatch (class I and II).\(^18\)
Daily palpation of the recipient abdomen for the heterotopic heartbeat, monitoring for signs of slowing or reduced impulse was performed. Allograft rejection was defined as complete cessation of the heterotopic heartbeat. A standard measure of indefinite allograft surgical has been defined as allograft survival of over 100 days.\(^19,20\)

2.3 | In vivo tamoxifen treatment protocol

Rosa26-Cre-ERT2 (ER\(^T2\)Cre) mice were crossed with Mir142\(^{fl/fl}\) animals. Female ER\(^T2\)Cre mice were bred with male ER\(^T2\)Cre\(^{-/-}\) mice. Only ER\(^T2\)Cre\(^{-/-}\) animals were utilized in experiments.

100 μg tamoxifen (Sigma # 156738) diluted in sunflower oil, warmed to 37°C were administered intraperitoneally once daily on 3 consecutive days to ER\(^T2\)Cre x Mir142\(^{fl/fl}\) mice at 5-6 weeks of age.

2.4 | Skin transplantation

Skin transplantation was performed as previously described.\(^21\)
Ears of euthanized donor mice were disinfected with 70% ethanol, excised, and split into ventral and dorsal halves. Ventral tissue was kept in phosphate-buffered saline (PBS) on ice prior to implantation and the collagenous ventral flap discarded. Recipient mice were anesthetized by 3% isoflurane inhalation in 100% oxygen at a flow rate of 2 L/min, then maintained using 1.5%-2% isoflurane at 2 L/min intraoperatively. The recipient site was shaved and swabbed with 70% ethanol. A 1.1-1.5 cm incision was made over the back. The skin graft was then placed atop the graft bed and wrapped in a sterile bandage. The bandage was secured with a single 2-0 silk suture and removed 6-7 days posttransplant.

2.5 | Flow cytometry and intracellular cytokine staining

Spleen and peripheral lymph nodes were processed by tissue disruption and filtration. Following red cell lysis, 5 × 10\(^6\) splenocytes were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μg/mL Ionomycin for 4 hours at 37°C, 5% CO\(_2\), with
the addition of 2 μmol/L Monensin for the last 2 hours (Sigma, St. Louis, MO). Anti-mouse CD16/32 antibody was used for Fc-blocking, Live/Dead dyes (Life Technologies, Carlsbad, CA), fluorochrome-conjugated anti-mouse antibodies (eBioscience, San Diego, CA) and anti-Tgfbr1/ anti-Tgfbr2 (R&D Systems, Minneapolis, MN) were used. Intracellular Staining for cytokines was performed after cell fixation and permeabilization using the FoxP3 staining buffer kit (eBioscience). Labelled anti-mouse antibodies to interferon-gamma (IFN-γ) and interleukin (IL)-17 (eBioscience) were used. Fluorescence minus one controls were used for gating cytokine expression. Cells were acquired on a BD LSRFortessa (BD Biosciences, San Jose, CA) and data analyzed using FlowJo software (TreeStar, Ashland, OR).

| 2.6 | Real-time PCR |

RNA was extracted using the RNeasy Micro Kit (Qiagen, Hilden, Germany). cDNA was prepared using TaqMan advanced miRNA cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA). TaqMan fast advanced master mix and TaqMan advanced microRNA Assays were used (Thermo Fisher Scientific) and run on a 7900HT Fast Real-Time PCR system (Thermo Fisher Scientific). U6 small nuclear RNA, miR-191-5p and miR-361 were used as endogenous controls.

| 2.7 | Alloantibody detection |

Serum samples were prepared from 60 μL tail vein bleeds taken at the time of transplant and 14-day intervals. Samples were incubated with anti-CD3-PE (eBioscience) labelled donor (BALB/C) splenocytes (after Fc blockade) with 3-fold serial dilutions of heat-inactivated recipient serum. Cells were then labelled with fluorescein isothiocyanate-labeled goat anti-mouse IgG (Poly4053) (BioLegend, San Diego, CA) and acquired on a FACS Canto machine (BD Biosciences). Sera from wild-type recipients of fully allogeneic allograft and naïve animals were used as positive and negative controls respectively.

| 2.8 | Histology |

Samples were fixed in 10% neutral buffered formalin for 48 hours before paraffin-embedding and sectioning. Histological scoring was performed blinded according to the International Society for Heart and Lung Transplantation standardizing grading criteria for the diagnosis of heart rejection and the Banff 2007 working classification of skin allograft pathology. Microscopy was performed with an Olympus BX51 microscope.

| 2.8.1 | Hematoxylin & eosin staining |

5 mm thick sections were dewaxed, rehydrated with water, then stained with Mayer's hematoxylin for 5 minutes, washed and incubated with 1% Eosin stain for 5 minutes, rinsed briefly with tap water, dehydrated rapidly through graded methanol, cleared in Xylene and mounted in DPX.

| 2.8.2 | Masson trichrome staining |

5 mm thick sections were stained with Celestine Blue for 5 minutes, washed, incubated in hemotoxylin for 5 minutes and washed in tap water for 5 minutes. Slides were then incubated with Acid Fucsin for 5 minutes, rinsed quickly in distilled water, differentiated in Phosphomolybdic acid (1%) for 3-5 minutes, then rinsed briefly in water prior to staining in Methyl Blue for 2-3 minutes. Slides were washed briefly with distilled water and dehydrated in ascending series of methanol, cleared in xylene and mounted in DPX (VWR).

| 2.9 | Immunohistochemistry |

| 2.9.1 | Immunoperoxidase staining |

5 μm thick paraffin wax sections were dewaxed and rehydrated with water. Endogenous peroxidases were blocked with 3% hydrogen peroxide for 5 minutes. Antigen retrieval was carried out in 0.1 mol/L citrate buffer (pH 6) with microwaving for 10 minutes. Samples were washed in water and incubated with 3% Bovine serum albumin (BSA; Sigma) for 30 minutes, then incubated overnight in a moist chamber with antibody against C4d (rabbit polyclonal, Hycult biotech, HP8033) or Foxp3 (rabbit polyclonal, Novus Biologicals, Littleton, CO, NB100-39002). Negative controls consisted of 3% BSA in PBS. After washing, samples were incubated with biotinylated goat anti-rabbit IgG for 60 minutes followed by Avadin-Biotin-peroxidase Complex (ABC) (Vector Laboratories, Burlingame, CA) for 30 minutes. Specimens were incubated with DAB (Sigma) for 5 minutes, washed with water and stained with Hematoxylin for 1 minute and mounted using Aquatex (VWR). Slides were viewed on a Zeiss Axioskop microscope and digital micrographs taken using a Nikon D MX1200 camera. Five random areas of the skin were imaged at ×40 magnification and manually counted using image J software (nonblinded).

| 2.9.2 | Immunofluorescence colocalization staining |

5 mm thick paraffin wax sections of skin tissue were dewaxed and rehydrated with water. Antigen retrieval was carried out by immersing the slides in Tris-EDTA buffer (pH 9) and microwaving for 10 minutes. Samples were left in the same buffer for 20 minutes followed by tap water wash. Slides were incubated with 3% Bovine serum albumin (BSA -Sigma) for 30 minutes and incubated overnight in a moist chamber with antibodies against CD4 (rabbit monoclonal, 1:300 dilution, Abcam) and FoxP3 (rat monoclonal, 1:50 dilution, Invitrogen). Negative controls consisted of matched rat and rabbit
antibody isotypes. After washing, specimens were incubated with goat anti-rabbit AlexaFluor 488 and goat anti-rat AlexaFluor 594 respectively (Life Technologies) for 1 hour. After washing three times with PBS, sections were incubated with DAPI (sigma) and mounted using Permafluor (Thermo Fisher). Stained sections were imaged with a Zeiss LSM 880 confocal microscope.

2.10 Western blot

FACS purified naïve CD4+ T cells were activated with plate-bound anti-CD3 (2 μg/mL) and anti-CD28 (3 μg/mL) antibodies (Bio X Cell), and cultured for 30, 60 and 120 minutes in serum-free medium at 37°C in the presence of recombinant human IL-2 (20 ng/mL) (R&D Systems) and TGF-β1 (3 ng/mL) (Promokine). Cells were prepared as previously described and probed with rabbit anti-mouse Smad2/3, Smad4, Smad 7, phospho-Smad2/3 and beta-actin (Cell Signaling). HRP-conjugated goat anti-rabbit IgG was used for secondary detection (GE Healthcare, Chicago, IL). Blots were developed using enhanced chemiluminescence (Thermo Scientific/Pearce). Intensity was quantified using GeneTools software (Syngene) and expressed as fold increase above basal level.

2.11 Statistical analysis

Statistical analysis was carried out using GraphPad Prism 7 (GraphPad Software). P < .05 was considered statistically significant and represented in figures as (*), with P < .01 represented in figures as (**), P < .001 represented as (***), and P < .0001 represented as (****).

3 RESULTS

3.1 miR-142 plays a critical role in the tolerogenic response to an allograft, specifically via the T cell compartment

MIR-142 levels are raised in the context of acute rejection of renal and cardiac transplants in humans. Therefore, we assessed miR-142 levels in allograft tissue in a murine heart transplant model of acute humoral and cellular rejection known to result in robust acute humoral and cellular rejection, with high circulating IgG alloantibody levels, and graft failure within 10 days.

At day 4 posttransplant, miR-142-3p and miR-142-5p were increased in the allografts of wild type (WT) recipients, with a further increase at day 8 posttransplant (Figure 1A). To understand how rejection might take place in an environment deficient for this miRNA, we generated and validated constitutive miR-142 deficient mice via Cre-mediated recombination (MiR-142−/−) (Figure S1A-D). When these animals were subjected to heterotopic heart transplantation, recipients demonstrated indefinite allograft survival (>100 days) in the absence of exogenous immunosuppression, with WT recipients rejecting at the expected time of under 10 days (Figure 1B).

Importantly, we observed the same phenomenon when mice with a T cell-conditional deletion of miR-142 (Cd4cre+Mir142−/−) (Figure S1A-D) were subjected to the same model, demonstrating that the effects of miR-142 mapped specifically to the T cell compartment. Monitoring of donor-specific antibody response revealed absent donor-specific IgG alloantibody in both Cd4cre+Mir142−/− and the Mir142−/− recipients. In contrast, WT recipients exhibited significantly increased alloantibody responses at the time of rejection (Figure 1C; Figure S2B). Furthermore, histological evidence of perivascular infiltrate, myocyte damage and hemorrhage seen in WT allografts were entirely absent in both Cd4cre+Mir142−/− and Mir142−/− recipients (Figure 1D; Figure S2A). Immunohistochemical staining for C4d also revealed no deposition in myocytes and the endothelium of Cd4cre+Mir142−/− recipient allografts, supporting an absence of a humoral response to the allograft in the absence of T cell intrinsic miR-142 expression (Figure 1E). Additionally, to determine if this phenomenon was due to donor or recipient effects, allografts from Mir142−/− donors were transplanted into BALB/c recipients. Importantly, Mir142−/− donors were rejected at the same rate as WT donors (Figure S3).

Further characterization of the CD4+ T cell compartment using Cd4cre+Mir142−/− and WT mice demonstrated that in both genotypes, CD4+ T cell populations responded appropriately to an allograft, with an increased peripheral TReg pool (Figure 1F) and a reduction in the CD4+CD25+CD44hiCD62Llo naïve T cell (TNAIVE) population (Figure 1G), concomitant with an increase in T cells displaying an effector/memory phenotype (TEFF/MEM) (Figure 1H). However, at
day 100 posttransplant, Cd4creM142fl/fl mice showed a significant increase in the TREG population (Figure 1F) with a higher proportion of CD4+ T cells retaining a T_{NAIVE} phenotype (Figure 1G) compared with WT mice at the time of rejection (day 8). Critically, direct ex vivo intracytoplasmic cytokine capture (ICC) of splenocytes from WT allograft recipients at the time of allograft rejection revealed robust expression of IFN-γ, in addition to IL-17 production by CD4+ T cells, while there was no increase in CD4+ T cell IFN-γ and IL-17 production in Cd4creM142fl/fl recipients at day 8 or day 100 post-transplant; this was also true of M142−/− animals (Figure 1I).

3.2 | T cell intrinsic miR-142 deficiency prolongs skin allograft survival

Given the striking results observed in our heterotopic heart transplantation model, we opted to examine the outcome of T cell miR-142 deficiency in a fully MHC mismatched (Class I and II) skin transplantation model; difficulties in prolonging allograft survival is a well-documented characteristic of this model.25,26 Despite this, Cd4creM142fl/fl recipients demonstrated prolonged allograft survival (MST 25 days vs 12 days, P < .01) without any exogenous treatment (Figure 2A). Significantly reduced donor-specific antibody responses in Cd4creM142fl/fl recipients compared with control mice at day 8 posttransplant, indicated an attenuated humoral response (Figure 2B). Comparison of histological findings at day 8 posttransplant revealed markedly reduced lymphohistocytic infiltration and hemorrhage in Cd4creM142fl/fl animals24 (Figure 2C). Importantly, immunohistochemical (IHC) analysis revealed CD4+ T cell infiltrate in the allografts of both groups (Figure 2D). However, co-staining for FoxP3 appeared qualitatively far more robust and profuse in allografts of Cd4creM142fl/fl recipients. This was supported by IHC-P analysis showing more extensive and robust FoxP3 expression in skin allografts Cd4creM142fl/fl (Figure 2E), with quantification confirming increased TREG infiltration in Cd4creM142fl/fl recipients compared to WT controls (Figure 2F). In addition, more extensive FoxP3 expression was observed in Cd4creM142fl/fl recipient cardiac allografts taken from the model outlined in Figure 1F (Figure S4). These data suggest that in response to exposure to an allograft, an enhanced TREG response is generated by the absence of miR-142 isoforms.

Deletion of miR-142 either constitutively or conditionally via Cd4-driven deletion in thymocyte T cell precursors, poses the possibility that typical T cell development may be compromised in the absence of this microRNA. To overcome this, we generated a ER^{T2}Cre transgenic27,28 deletion model (ER^{T2}CreM142fl/fl), in which deletion of M142 could be temporally controlled through the administration of tamoxifen in vivo (Figure S5A,B). Temporal deletion of M142 did not grossly alter the peripheral lymphocyte pool, as determined by B:T cell and CD4:CD8 T cell ratios (Figure S6A,B), or negatively impact T cell viability (Figure S6C).

Analogous to our findings in Cd4creM142fl/fl mice, ER^{T2}CreM142fl/fl recipients demonstrated prolonged allograft survival compared to M142fl/fl littermate controls (MST 15 days vs 9.5 days, P < .0001) (Figure 3A,B). Comparison of histological findings at day 8 posttransplant revealed reduced lymphohistocytic infiltration and hemorrhage in ER^{T2}CreM142fl/fl animals (Figure 3C). As before, we found a significant increase in the proportion of CD4+CD25+ T_{REGS} (Figure 3D) and decrease in T_{EFF}/MEM populations in ER^{T2}CreM142fl/fl mice when compared with control mice at day 8 in secondary lymphoid tissue sites (Figure 3D,E). Directly ex vivo ICC revealed significantly reduced CD4+ T cell production of IFN-γ and IL-17 production at day 8 (Figure 3F). Similarly, donor-specific antibody responses were significantly reduced in ER^{T2}CreM142fl/fl mice when compared with control mice (Figure 3G).

3.3 | Expression of the miR-142-3p target Tgfb1 is increased in miR-142 deficient T_{REG} and augments sensitivity to TGF-β signaling

In all the transplantation models we had explored, an enhanced T_{REG} response was apparent in the context of T cell miR-142 deficiency. Therefore, we reasoned that the absence of miR-142 in the context of exposure to an allograft may promote a more tolerogenic environment, via mechanisms which augment T_{REG} development and subsequent dominant tolerance.

Among factors which promote T_{REG} development and function, the TGF-β signaling pathway plays an important role, not least by promoting the expression of Foxp3.29,30 TGF-β signals via a heterodimeric receptor composed of ALK5 (Tgfb1) and the subunit Tgfb2. Interestingly, the 3’UTR of Tgfb1 mRNA contains a highly conserved seed sequence for miR-142-3p and the 3’UTR of Tgfb2 mRNA contains a highly conserved seed sequence for miR-142-5p (Figure S7A,B). miR-142 regulation of the expression of Tgfb1 and Tgfb2 has been validated in several cell types including hematopoietically derived lineages.31–36

To determine whether miR-142 deficient T cells exhibited increased levels of Tgfb1 and Tgfb2, we utilized the T cell-conditional miR-142 deficient model of skin allograft transplantation. At
FIGURE 3 Prolonged skin allograft survival in ER\textsuperscript{T2Cre}\textsuperscript{+MIR142\textsuperscript{fli/fli}} mice. A, Kaplan Meier allograft survival chart of control vs ER\textsuperscript{T2Cre}\textsuperscript{+MIR142\textsuperscript{fli/fli}} littermates (n > 8 per group). Mantel-Cox test **P < .0001). B, Macroscopic appearance of control vs ER\textsuperscript{T2Cre}\textsuperscript{+MIR142\textsuperscript{fli/fli}} skin allografts at days 8 and 12. C, Hematoxylin and eosin (H&E) staining of formalin fixed, paraffin embedded (FFPE) sections from allografts from ER\textsuperscript{T2Cre}\textsuperscript{+MIR142\textsuperscript{fli/fli}} and control recipients at day 8 posttransplant (magnification ×5 and ×20; scale bars 1000 and 100 μm) (left); Banff grading of cell-mediated skin allograft rejection\textsuperscript{23} (n = 5; Student’s t test; **P < .001) (right). D, Percentage of T\textsubscript{REG} (CD4\textsuperscript{+CD25\textsuperscript{+CD44\textsuperscript{low}CD62L\textsuperscript{low}}}) cells (left) and percentage of T\textsubscript{NAIVE} (CD4\textsuperscript{+CD25\textsuperscript{−CD44\textsuperscript{high}CD62L\textsuperscript{high}}}) (right) (n = 4; Mann-Whitney U test. *P < .01). E, Percentage of T\textsubscript{EFF/MEM} (CD4\textsuperscript{+CD25\textsuperscript{−}CD44\textsuperscript{high}CD62L\textsuperscript{low}}) cells (left) and percentage of T\textsubscript{NAIVE} (CD4\textsuperscript{+CD25\textsuperscript{−}CD44\textsuperscript{low}CD62L\textsuperscript{high}}) (right) (n = 4; Mann-Whitney U test. *P < .05). F, Direct ex vivo interferon-gamma (IFN\textgamma) and interleukin (IL)-17 cytokine responses in CD3\textsuperscript{+CD4\textsuperscript{+} and CD3\textsuperscript{+CD8\textsuperscript{+}} T cells at day 8 posttransplant (n = 4 per group; *P < .05, **P < .01; unpaired two-tailed t test). G, Donor-specific antibody responses, control vs ER\textsuperscript{T2Cre}\textsuperscript{+MIR142\textsuperscript{fli/fli}} at day 8 posttransplant (n > 8 per group; **P < .01; unpaired two-tailed t test).

4 | DISCUSSION

We have demonstrated that the absence of miR-142 induces indefinite cardiac allograft survival across full MHC (Class I and II) mismatches, caused by a T cell specific mechanism, which promotes dominant tolerance and suppression of effector CD4\textsuperscript{+} T cell responses via the enhancement of the peripheral T\textsubscript{REG} response. Diminished T cell numbers in secondary lymphoid organs have been observed in Mir142\textsuperscript{−/−} mice.\textsuperscript{37,58} We also found this to be the case in our constitutive and T cell-conditional knockout lines at baseline (data not shown). However, CD4 and CD8 deficient mice as well as Rag\textsuperscript{−/−} mice, which lack T and B cells, can vigorously reject skin allografts at the same rate as WT mice.\textsuperscript{39-42} B6 μMT\textsuperscript{−/−} mice are B cell-deficient with a paucity of CD4\textsuperscript{+} T cells yet are still capable of rejecting skin and heart allografts.\textsuperscript{41,43} Importantly, as few as 1 × 10\textsuperscript{3} donor-specific T cells are capable of rejecting skin allografts.\textsuperscript{12,13,55} The quantitatively similar responses to the allograft in our mice, which are only partially T cell-deficient and possess T cells in their peripheral tissues, indicates that this is unlikely to contribute significantly to the observed long-term transplant survival.

The strength of T\textsubscript{EFF/MEM} responses in both lymphoid and non-lymphoid tissues is a barrier to transplant tolerance.\textsuperscript{45,46} The presence and number of alloantigen-specific T\textsubscript{EFF/MEM} Cells in humans correlates with worse transplant outcomes.\textsuperscript{47} Despite current immunosuppressive regimens capable of non-specific T cell depletion, certain T\textsubscript{EFF/MEM} subsets have been found to survive treatment with the commonly used depleting induction agents, Campath (anti-CD52 mAb) or anti-thymocyte globulin. Indeed, these cells expand and proliferate in the "empty" host to play a prominent role in acute rejection.\textsuperscript{48} Other attempts to augment allograft survival using co-stimulatory blockade (eg, CD28/B7) have had limited success due to the presence of alloreactive memory T cells. In fact, few strategies have resulted in the induction of transplant tolerance. Chronic allograft rejection is also associated with decreased CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{REG} and reduced Foxp3 transcripts in PBMCs compared with both healthy controls and operationally tolerant recipients, indicating the importance of maintenance of the T\textsubscript{REG} population for allograft survival.\textsuperscript{49} In murine studies, increasing the number of T\textsubscript{REG} can adoptively transfer transplant tolerance and expansion of the T\textsubscript{REG} population has been shown to occur in the periphery to mediate this phenomenon.\textsuperscript{50-52} To address the question of the functional capacity of these T\textsubscript{REG}, three separate studies used autologous, polyclonal or donor-specific T cell stimulation in T\textsubscript{REG} suppression assays. The authors concluded that these T\textsubscript{REG} are fully functional and propose that it is the proportion rather than any altered suppressive ability of T\textsubscript{REG} that determines allograft outcome.\textsuperscript{49,53,54} A number of human and murine studies demonstrated that miR-142 expression is increased in the context of acute allograft rejection\textsuperscript{12,13,55} and, in light of the above studies, the shift in the balance of T\textsubscript{REG} and T\textsubscript{EFF/MEM} populations in response to an allograft in the absence of miR-142 is likely to be of crucial importance.

There have been conflicting reports on the level of TGF-β1 mRNA in operationally tolerant patients. However, the TGF-β signaling pathway has been shown to regulate the function of 27% of the genes that are associated with operationally tolerant patients in blood samples.\textsuperscript{56,57} Previously, TGF-β signaling in T cells has been shown to promote long-term skin allograft acceptance in a model of minor MHC mismatch\textsuperscript{58} and, in conjunction with co-receptor blockade, TGF-β has also been shown to promote induction of Foxp3 and maintenance of transplant tolerance in monospecific TCR-transgenic mice.\textsuperscript{59} Enhanced expression of FOXP3, TGFβ1 and TGFβ1R1 in PBMC of operationally tolerant renal transplant patients compared with renal transplant recipients not exhibiting operational tolerance, indicate the significance of this signaling pathway in transplant tolerance.\textsuperscript{17,57} Several studies have shown that miR-142 plays a critical and highly conserved role in regulating TGF-β receptor signaling in vertebrates.\textsuperscript{31-36} Uregulation of Tgfb1 expression in CD4\textsuperscript{+}CD25\textsuperscript{+}FoxP3\textsuperscript{−} T\textsubscript{REG} from mice with T\textsubscript{REG}-conditional deletion...
of Mir142, in combination with the highly conserved seed sequences for miR-142-3p in the 3’UTR of Tgfrb1 make this a plausible target for miR-142-3p in T REGS.33

In contrast to our previous findings, where we found that the absence of miR-142 specifically in Foxp3+ T REGS results in a lethal, multi-system autoimmune disease and impaired T REG suppressive function,9 here we found that the absence of miR-142 in T cells results in indefinite allograft survival and the augmented generation and/or survival of T REGS. This surprising finding may be explained by putative de novo generation of a large number of alloantigen-specific T REG in the context of a transplant, driven by enhanced TGF-β signaling mediated by the absence of miR-142-3p. Previously we showed aberrant immune responses to nominal self-antigens is mediated by miR-142-5p regulation of Pde3b. Whether this difference reflects the differential expression of the -3p and -5p strand of miR-142 in separate T REG subsets (eg, thymic T REG vs peripheral T REG) in response to self vs nonself-antigens remains to be determined.

In summary, miRNAs have emerged as key regulators of numerous biological processes. Our data identify that miR-142 plays a critical role in solid organ transplantation, modulating expression of its target Tgfrb1 in T REGS, thereby modulating T REG sensitivity to TGF-β and promoting transplant tolerance via augmented T REG development. These findings suggest that targeted T REG therapy aimed at manipulation of miR-142 and its target TGFBR1 could have therapeutic potential in improving allograft survival.

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FIGURE 4 Expression of miR-142-3p target Tgfbr1 is increased in miR-142 deficient CD4+CD25+ T REG following skin allograft transplantation and increases cell sensitivity to transforming growth factor (TGF)-β1 signaling. A, Mean fluorescence intensity (MFI) of anti-Tgfbr1 and anti-Tgfbr2 staining on CD4+CD25− T CONV and CD4+CD25+ T REG from peripheral lymph nodes, 8 d posttransplant with BALB/C skin allografts of wild type (WT; C57B6/J) and Cd4cre−/− Mir142fl/fl mice. B, MFI of anti-Tgfbr1 and anti-Tgfbr2 staining on CD4+CD25− T CONV and CD4+CD25+ T REG from spleen of WT (C57B6/J) and Cd4cre−/− Mir142fl/fl mice, 8 d posttransplant with BALB/C skin allografts. C, Representative western blot images demonstrating Smad7, phospho-Smad2 (Ser465/467)/Smad3(Ser423/425), Smad2/3, and β-Actin protein expression over time (0-120 min) in CD4+ T cells isolated from spleen and peripheral lymph nodes of Mir142+/+, Mir142+/- and Mir142−/− mice treated with exogenous TGF-β1, interleukin (IL)-2 and activated with anti-CD3/anti-CD28 monoclonal antibodies in vitro. D, Cumulative western blot data from 3 independent experiments for Smad2/3 and (E) Smad7 relative intensity to β-Actin expression, combining data from all time points. F, Cumulative western blot data from 3 independent experiments for pSmad2/3 relative to β-Actin over time. All data are represented as mean ± SEM Each data point in (A,B) represents 1 mouse (n = 5). MFI values calculated by subtracting Tgfbr1 and Tgfbr2 fluorescence minus one control MFI values from corresponding sample values. Mann-Whitney U test (A,B)/Student’s t test (C-D,F), *P < .05, **P < .01, n.s. >.05, nonsignificant

DISCLOSURE
The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation.

AUTHOR CONTRIBUTIONS
Study concept and design (GML), acquisition of data (NA, LR, JCDW, PS, FX, IJ), data analysis and interpretation (NA, LR, JCDW, PS, AH), technical support (NA, LR, JCDW, PS, FX, IJ, AH, PK), obtaining funding (NA. JCDW, JKH, RGJ, GML), drafting of manuscript (NA, LR), study supervision (GML), critical revision of the manuscript (JKH, RGJ, GML).

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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