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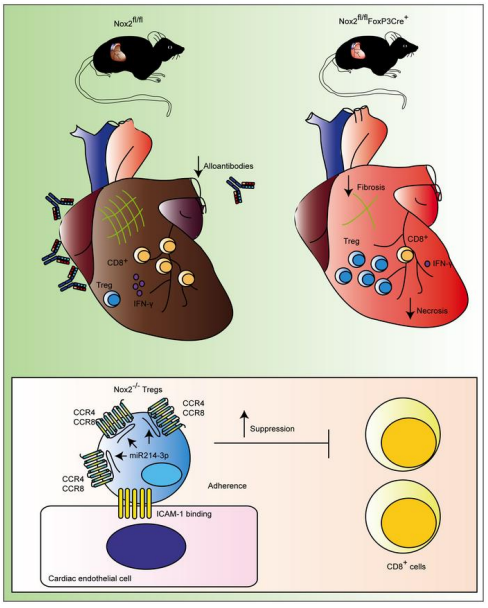
Graphical abstract



Nox2-deficient Tregs improve heart transplant outcomes via their increased graft recruitment and enhanced potency

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Abstract

Nox2 is a ROS-generating enzyme, deficiency of which increases suppression by Tregs in vitro and in an in vivo model of cardiac remodelling. Since Tregs have emerged as a candidate therapy in autoimmunity and transplantation, we hypothesised that Nox2 deficiency in Tregs in recipient mice may improve outcomes in a heart transplant model. A novel B6129 mouse model with Treg-targeted Nox2 deletion ($Nox2^{fl/fl}FoxP3Cre^{+}$) was generated and transplanted with hearts from CB6F1 donors. As compared to littermate controls, $Nox2^{fl/fl}FoxP3Cre^{+}$ mice had lower plasma levels of alloantibodies and troponin-I, reduced levels of IFN-γ in heart allograft homogenates and diminished cardiomyocyte necrosis and allograft fibrosis. Single cell analyses of allografts revealed higher absolute numbers of Tregs and lower $CD8^{+}$ T cell infiltration in Nox2-deficient recipients compared to Nox2-replete mice. Mechanistically, in addition to a greater suppression of $CD8^{+}CD25^{-}$ T effector cell proliferation and IFN-γ production, Nox2-deficient Tregs expressed higher levels of CCR4 and CCR8, driving cell migration to allografts; this was associated with increased expression of miR214-3p. These data indicate that Nox2 deletion in Tregs enhances their suppressive ability and migration to heart allografts. Therefore, Nox2 inhibition in Tregs may be a useful approach to improve their therapeutic efficacy.

Keywords: Nox2, heart transplant, chemotaxis, regulatory T cells, miR214-3p.

Introduction

Cardiac transplantation remains the only available “curative” therapy for end-stage heart failure. However, the average survival after surgery is less than 10 years due to immune-mediated allograft rejection and side effects of immunosuppressive drugs (1). This provides the impetus to manipulate the immune system to achieve heart allograft tolerance (2).

Tregs are a subset of T cells expressing the transcription factor FoxP3 and the surface molecules CD4 and CD25 (3). In addition to maintaining self-tolerance (4), Tregs can recognize allogeneic MHC molecules and inhibit allograft rejection through different mechanisms (5). Tregs are currently under intensive investigation as an adoptive cell-based therapy to prevent transplant rejection and treat autoimmune diseases (6). Polyclonal Treg-based cell therapy approaches yielded promising early results for the prevention of graft-*versus*-host disease (GvHD) (7), and for the cure of type I diabetes (8, 9). We have also completed two Phase I/II clinical trials, the ONE Study (NCT02129881) (10) and ThRIL (NCT02166177) (11), assessing the safety and feasibility of adoptive transfer of ex vivo expanded polyclonal Tregs in renal and liver transplant patients (12-14).

Manipulation of Tregs in vitro can enhance their beneficial therapeutic effect (15, 16). Recently, our group showed that murine Nox2-deficient Tregs have higher suppressive activity in vitro on $CD4^+$ T effector cell (Teff) proliferation than WT Tregs, which was attributed to increased nuclear levels of FoxP3 and NF-κB, which led to increased expression of CD25, CTLA-4, CD39 and CD73, key molecules linked to Treg suppressive function (17). An increased potency of Nox2-deficient Tregs was also manifest in vivo by reducing inflammation in a model of angiotensin II-(ANGII)-induced cardiovascular remodelling.

Having shown that Nox2 impairs Treg suppressive function, we hypothesized that targeting its deletion in $FoxP3^+$ T cells of recipient mice could improve heart allograft survival.

Results

Generation of mice with Nox2 deletion in Tregs

To analyse the contribution of Nox2 in Tregs to allograft protection, mice with FoxP3-targeted Nox2 deletion ($Nox2^{fl/fl}FoxP3Cre^{+}$) were generated by crossing male B6129S-Tg(FoxP3eGFP/iCre)1aJbS/J (18) with female $Nox2^{fl/fl}$ mice(19) (Supplementary Figure 1A). $Nox2^{fl/fl}FoxP3Cre^{+}$ mice were confirmed to have Nox2 DNA recombination (Supplementary Figure 1B) and the $EGFP^{+}$ cells in $Nox2^{fl/fl}FoxP3Cre^{+}$ were 95% $CD25^{+}FoxP3^{+}$ (Figure 1A). $Nox2^{fl/fl}FoxP3Cre^{+}$ mice also had lower Nox2 mRNA levels (Figure 1B) in lymph nodes and reduced Nox2 protein levels in $CD4^{+}FoxP3^{+}$ but not $CD4^{+}FoxP3^{-}$ cells (Figure 1C-D). Purified Tregs from $Nox2^{fl/fl}FoxP3Cre^{+}$ mice did not increase ROS production after stimulation with anti-CD3ε and anti-CD28 Abs unlike cells from control $Nox2^{fl/fl}$ mice. In fact, after stimulation, Tregs from $Nox2^{fl/fl}FoxP3Cre^{+}$ mice produced comparable ROS levels to Tregs from littermate controls treated with a Nox2 flavoprotein inhibitor, diphenyleneiodonium (DPI; Figure 1E-F).

Under baseline conditions, $Nox2^{fl/fl}$ control and $Nox2^{fl/fl}FoxP3Cre^{+}$ mice had similar numbers of $CD4^{+}$ and $CD8^{+}$ cells in thymus, spleen and mesenteric lymph nodes (Supplementary Figure 2A-C), including naïve ($CD44^{-}CD62L^{+}$), memory ($CD44^{+}CD62L^{+}$), Th17 ($CD4^{+}RORγT^{+}$), $CD4^{+}$Tregs ($CD25^{+}FoxP3^{+}$ cells), $CD8^{+}$Tregs ($CD8^{+}FoxP3^{+}$ cells) and $CD4^{+}CD8^{+}$ T cells (in thymus only). $Nox2^{fl/fl}FoxP3Cre^{+}$ and control $Nox2^{fl/fl}$ mice had similar baseline heart and vascular function parameters (Supplementary Figure 2D-F).

Nox2 deficiency in Tregs improves allograft outcome

Mice with FoxP3-targeted Nox2 deletion ($Nox2^{fl/fl}FoxP3Cre^{+}$; $H\text{-}2^{b}$) and littermate controls ($Nox2^{fl/fl}$; $H\text{-}2^{b}$) were transplanted with hearts from CB6F1 mice ($H\text{-}2^{b/d}$).

Allografts transplanted into $Nox2^{fl/fl}FoxP3Cre^{+}$ mice showed delayed rejection as compared to those into littermate controls (Figure 2A, Supplementary video 1 and 2), along with diminished cardiomyocyte necrosis (Supplementary Figure 3A) and myocardial fibrosis (Supplementary Figure 3B) 7 and 100 days after surgery. To further confirm the relevance of Nox2 deletion in Tregs to the protection from heart allograft rejection, an animal model that more closely resembles the clinical setting was used. Recipient mice were treated with cyclosporin (30mg/kg) for 10 days after heart transplantation. Both $Nox2^{fl/fl}$ and $Nox2^{fl/fl}FoxP3Cre^{+}$ mice had increased allograft survival rates after cyclosporin treatment, but the $Nox2^{fl/fl}FoxP3Cre^{+}$ mice showed a lower rate of rejection (Figure 2A).

Plasma troponin-I levels were lower in $Nox2^{fl/fl}FoxP3Cre^{+}$ mice compared to littermate controls (Figure 2B) 7 days after transplantation. Plasma alloantibody levels were reduced in mice with Treg-targeted Nox2-deficiency 7 and 100 days after transplantation (Figure 2C-D), whilst the inflammatory mediators CCL2, IL-10 and IL-6 in allograft homogenates (Supplementary Figure 3C-E) and CCL1 and CCL22 mRNA levels in heart tissues (Supplementary Figure 3F-G) were equivalent between the two groups of mice 7 days after surgery.

To evaluate the contribution of Tregs to improved heart allograft outcome, the presence of $FoxP3^{+}GFP^{+}$ cells was first analysed in hearts transplanted into B6129S-Tg(FoxP3eGFP/iCre)1aJbS/J mice. The number of recipient-$FoxP3^{+}GFP^{+}$ Tregs in allografts started increasing 3 days after transplant, peaked at day 7 and then decreased by day 14 (Figure 2E). The increased number of Tregs at day 7 coincided with the higher $FoxP3^{+}$ Treg numbers and Treg/Teff ratios observed in allografts transplanted in $Nox2^{fl/fl}FoxP3Cre^{+}$ mice compared to controls $Nox2^{fl/fl}$ (Figure 2F-H). The higher Treg number was associated with reduced $CD8^{+}$ cells but not $CD4^{+}$ T cells in the allografts (Figure 2I-J). Moreover, $Nox2^{fl/fl}FoxP3Cre^{+}$ mice had lower IFN-γ levels in heart homogenates than $Nox2^{fl/fl}$ 7 days after transplantation (Figure 2K). The numbers of

416 $CD4^+$, $CD8^+$ and $FoxP3^+$ Tregs in spleen were similar between $Nox2^{fl/fl}FoxP3Cre^+$ and
417 $Nox2^{fl/fl}$ mice (Supplementary Figure 4).

418 Therefore, Nox2 deficiency in Tregs improves heart transplant outcomes and
419 prevents acute rejection through the reduction of $CD8^+$ cell infiltration and IFN-γ
420 production in the allografts associated with a higher proportion of Tregs.

421

422 *Nox2-deficient Tregs exhibit higher suppression of $CD8^+$ T cell proliferation*

423 We have previously shown that Nox2-deficient Tregs inhibit in vitro $CD4^+$ T Teff
424 proliferation more efficiently than WT Tregs (17). To assess whether the reduced number
425 of $CD8^+$ T cells in the transplanted hearts in $Nox2^{fl/fl}FoxP3Cre^+$ recipient mice was due
426 to a superior inhibitory function of Nox2-deficient Tregs, we purified and co-cultured
427 $CD4^+CD25^+$ Tregs with $CD8^+CD25^-$ Teffs. Tregs deficient in Nox2 inhibited $CD8^+$ WT
428 Teff proliferation (Supplementary Figure 5A-B) more efficiently than WT Tregs (IC_{50} 0.13
429 vs IC_{50} 0.41). Additionally, Nox-2-deficient Tregs abolished the production of IFN-γ by
430 $CD8^+$ T effs whereas a dose-dependent decrease was observed using different ratios of
431 WT Tregs: WT Teffs (Supplementary Figure 5C).

432 Therefore, $Nox2^{-/-}$ Tregs exhibit higher suppression of $CD8^+$ Teff proliferation and
433 IFN-γ production than WT Tregs.

434

435 *Nox2 deficiency favours Treg migration and homing into heart allografts*

436 A potential mechanism underlying the increased number of recipient-Tregs in
437 allografts is augmented leukocyte trafficking. Chemokine receptors are essential for the
438 initial phases of leukocyte trafficking (20) and were first analysed in WT and Nox2-
439 deficient Tregs purified from spleen and lymph nodes. These cells were predominantly
440 thymus-derived Tregs, as approximately 70% of them were neurophilin-1^+
441 (Supplementary Figure 6). Of the 17 chemokine receptors evaluated, six had enhanced

mRNA levels in Nox2-deficient Tregs (Figure 3A). Among these, CCR4 is of particular interest as it has been described as a homing receptor for the heart (21). The protein levels of CCR2, CCR4, CCR6, CCR7, CCR8 and CXCR4 were further investigated by multi-colour flow cytometry (Figure 3B-C and Supplementary Figure 7A-B). Nox2-deficient Tregs showed higher CCR4 and CCR8 expression than WT Tregs (Figure 3B-C), along with a higher chemotactic index toward CCL22 and CCL1, respectively (Figure 3D). The difference in chemotaxis was abolished by pre-incubation of Tregs with Ly294002, an inhibitor of phosphoinositide 3-kinases - a known downstream effector of chemokine receptor signalling (22). Nox2-deficient Tregs also showed higher F-actin assembly following incubation with CCL1 and CCL22 (Figure 3E-F). Of note, CCR2, CCR4, CCR7, CCR8 and CXCR4 protein levels in $CD4^+CD25^-FoxP3^-$ and $CD8^+CD25^-FoxP3^-$ Teffs were comparable between WT and Nox2-deficient mice (Supplementary Figure 7C-D). To further confirm the superior migratory capacity of Nox2-deficient Tregs in vivo, WT and Nox2-deficient Tregs ($H\text{-}2^b$), stained in green and orange respectively, were adoptively co-transferred into B6 mice transplanted with CB6F1 hearts (Figure 3G). Supporting the previous results, a greater number of Nox2-deficient Tregs were recovered from the allografts as compared to WT Tregs (Figure 3H).

Chemokines mediate integrin activation via inside-out signalling and consequently induce adhesion of lymphocytes to ECs (23). Therefore, in addition to chemotaxis, an increase in numbers of Tregs in the allograft may also be influenced by their adherence to cardiac ECs. After stimulation with CCL22, Tregs deficient in Nox2 displayed higher binding *in vitro* to ICAM-1 than WT Tregs (Figure 3I-J). Moreover, when WT and Nox2-deficient Tregs stained in contrasting colour dyes were co-perfused over cardiac ECs, the Nox2-deficient cells displayed higher adherence to ECs (Figure 3K).

Taken together, our data suggest that Nox2 expression in Tregs negatively regulates their chemotaxis and EC adherence.

miR-214-3p drives increased CCR4 and CCR8 expression in Nox2-deficient Tregs

The intracellular mechanism by which Nox2 controls transcription of CCR4 and CCR8 in T cells was next investigated. Because miRNAs are important regulators of transcription and a previous study showed that miR-214 deficiency decreases CCR4 expression in T cells (24), we assessed this as a possible mechanism. PCR analyses using primers for these miRs revealed that Nox2-deficient Tregs have higher expression of miR-214-3p but comparable levels of miR-214-5p to WT Tregs (Figure 4A, Supplementary Figure 8A). Accordingly, Jurkat T cells incubated with a specific Nox2 inhibitor, gp91ds-tat, had higher mRNA levels of CCR4 and CCR8 than those incubated with sc-tat peptide control. This increase was prevented in cells transfected with a miR-214-3p inhibitor (Figure 4B-C). Transfection of Jurkat T cells with miR-214-3p mimetic also increased CCR4 and CCR8 mRNA levels (Figure 4B-C) and cells treated with the Nox2 inhibitor showed higher miR 214-3p levels, which was reduced by transfection with the miR inhibitor (Supplementary Figure 8B). The incubation of Jurkat T cells with PEG-SOD and PEG-catalase also increased the levels of miR-214-3p as well as CCR4 and CCR8 mRNAs, indicating that the Nox2 effects were ROS-dependent (Figure 4D-F). We next cloned the 3' untranslated region (UTR) of mouse CCR4 and part of the coding region harbouring binding sites for miR-214-3p in CCR4 and CCR8 mRNAs into a dual-luciferase reporter vector. Jurkat T cells transfected with the CCR4 constructs showed a higher luciferase signal in the presence of gp91ds-tat or the miR-214-3p mimetic (Figure 4G-H), suggesting increased CCR4 mRNA stabilization. The assays using the CCR8 construct showed an increased signal in the presence of gp91ds-tat but not miR-214-3p mimetic, pointing to a possible distinct regulatory mechanism (Figure 4I).

Finally, we studied the expression of mRNA for Nox2, FoxP3 and miR214-3p in heart allografts, 7 days after transplantation. The expression of mRNA for FoxP3 in the allografts directly correlated with the Treg counts (Figure 5A), and inversely correlated

with the expression of mRNA for Nox2 (Figure 5B). In agreement with the results presented in figures 2F-G and 4A, Nox2 mRNA expression inversely correlated with Treg cell counts and with miR214-3p expression in the heart allografts (Figure 5C-D).

Taken together, our results indicate that Nox2 deficiency in Tregs improves heart allograft outcomes due to a greater suppression of $CD8^+$ Teff proliferation and IFN-γ production. Additionally, Nox2-deficient Treg migrate more efficiently into the allografts due to their increased expression of CCR4 and CCR8 mRNAs mediated via miR214-3p.

Discussion

The development of improved methods to suppress cardiac transplant rejection is a major goal to enhance the effectiveness of this life-saving therapy. Previous work, including early-phase clinical studies, suggests that the administration of Tregs may be one way to induce immune tolerance and improve allograft outcome (7, 11). We focused on the ROS-generating enzyme Nox2, as we have recently found it to reduce Treg suppression of $CD4^+$ Teff proliferation (17) and thereby increase cardiovascular inflammatory responses. Here, a novel mouse model with Treg-specific deficiency of Nox2 in the recipient showed improved allograft outcomes, which were accompanied by reduced cardiomyocyte necrosis, lower myocardial fibrosis and diminished circulating levels of alloantibodies. The mechanisms underlying these improved outcomes were increased chemotaxis and adherence of Tregs in the transplanted hearts as well as an enhanced suppression of $CD8^+CD25^-$ Teff proliferation by Nox2 deficient $CD4^+CD25^+$ cells. Additionally, Nox2-deficient Tregs downregulated IFN-γ production in cultures with $CD8^+$ Teff cells, which could also have contributed to increase survival of the heart allografts.

Nox2 was previously shown to be involved in leukocyte migration in distinct disease contexts and related to different cell types, including ECs (25), platelets (26) and

neutrophils (27). In the present study, we observed that Nox2 deficiency upregulates CCR4 expression in $CD4^+CD25^+$ Tregs, which favours their infiltration into heart allografts. The importance of Treg chemotaxis toward CCR4 ligands in the context of heart allograft survival is corroborated by previous studies (28-30). Long term allograft survival induced by treatment with tanshinol plus rapamycin was reversed by neutralizing the CCR4 ligand CCL22 (29). Furthermore, Lee et al. (28) showed that upregulation of CCR4 and Treg infiltration of the transplant following combined anti-CD154 monoclonal antibody and donor-specific transfusion induced tolerance, which was not observed in CCR4-deficient recipients nor in mice receiving anti-CD25 antibody treatment.

It is well known that CCR4 inside-out signaling activates the integrin CD11a, which adheres more to ICAM-1 expressed by antigen-presenting cells (APCs) and ECs (31, 32). In fact, we observed that Nox2-deficient Tregs had enhanced binding to ICAM-1 in vitro after CCL22 stimulation as compared to WT Tregs. As a consequence, Tregs deficient of Nox2 had a higher adherence to cardiac ECs, facilitating migration into allografts; the possible increased interaction with APCs, reducing their capacity to provide costimulatory signals, could have contributed to a higher suppression of $CD8^+$Teff proliferation. Corroborating the importance of integrin activation to heart allograft survival, Warren et al.(32), showed that anti-α4 integrin antibody reduced the number of Tregs in transplanted hearts leading to impaired allograft survival. The increased CCR8 expression displayed by Nox2-deficient Tregs could also have contributed to enhanced chemotaxis to allografts and to the higher suppressive function. In support of this, Barsheshet et al.(33) showed that the suppressive function of $CD25^+CD127^{lo}$ Tregs in vitro is upregulated by expression of CCR8 and the presence of its ligand CCL1 (33). Nox2-deficient Tregs showed enhanced expression of mRNA for six different chemokine receptors despite only CCR4 and CCR8 having corresponding increases in protein levels as compared to WT Tregs. These discrepancies could be due to internalization and degradation of chemokine receptors (22).

We further explored the intracellular mechanism through which Nox2 regulates CCR4 and CCR8 expression in T cells and found that miR-214-3p is enhanced in Nox2-deficient Tregs. Our data support an miR-dependent upregulation of target mRNA transcription or stabilization previously reported in the literature (34-36). Consistently, hearts from miR-214-deficient mice displayed lower CCR4 expression compared to WT controls (24), indicating upregulation rather than reduction of mRNA levels. Additionally, Nox2 mRNA levels in heart allografts inversely correlated to miR-214-3p levels, Treg counts and FoxP3 mRNA expression. Our results agree with a previous study showing an association between decreased expression of miR214-3p and increased levels of alloantibodies and development of bronchiolitis obliterans syndrome following lung transplantation (37). Additionally, murine heart allografts had lower levels of miR214-3p compared to isografts (38).

The higher Treg infiltration in recipient mice with Treg-targeted Nox2 deletion was associated with lower necrosis and fibrosis of heart allografts and with lower plasma levels of troponin-I as early as 7 days after surgery. Indeed, increased troponin-I levels correlated positively in patients with acute heart transplant rejection (39), which was also observed in the murine heterotopic heart transplant model used in this study.

The lower interstitial fibrosis observed in allografts transplanted into recipient mice with Nox2 deletion agrees with our recent published data showing that the adoptive transfer of Nox2-deficient Tregs induced lower cardiac fibrosis in a model of ANGII-induced cardiovascular remodelling (17). It also agrees with another study showing Treg depletion using anti-CD25 antibody aggravated cardiac fibrosis in a model of virus-induced myocarditis whereas adoptive transfer of Tregs prevented it (40).

In addition to impaired T cell mediated alloresponses, there was a significant decrease in levels of alloantibodies in mice with Nox2-deficiency in Tregs as compared to controls with preserved Nox2 activity. Since post-transplantation reactive anti-HLA antibodies in humans are associated with the development, frequency and severity of

cardiac allograft vasculopathy (CAV), it would be of interest to assess whether Nox2 deficiency in Tregs also impacts on this serious complication after heart transplantation (41).

We did not see differences in $CD4^+$ T cell infiltration between allografts transplanted into $Nox2^{fl/fl}$ and $Nox2^{fl/fl}FoxP3Cre^+$mice. This response differs from the pattern of T cell infiltration in a model of ANGII-induced cardiac remodelling, in which Nox2-deficient mice had a decrease in both $CD4^+$ and $CD8^+$ T effector cells (17). These differences could be attributed to the distinct importance of $CD4^+$ and $CD8^+$ cells in different animal models. In fact, cytotoxic $CD8^+$ T-cell responses against mismatched MHC Class I alloantigen are the principal arm of the cellular response against the transplanted organ (42) whereas cardiac remodelling is modulated mainly by $CD4^+$ T cells producing IL-17 (43).

Nox2-deficient Tregs express higher levels of CCR4 and CCR8, but other chemokine receptors were not affected (CCR2, CCR7, CCR6 and CXCR4- Supplementary Figure 7A-B). Therefore, the presence of CCL1 and CCL22 in the heart transplant microenvironment increases the potential therapeutic effects of Nox2-deficient Tregs in this context. Because Nox2-deletion does not reduce the expression of other chemokine receptors, other disease-contexts where CCL1 and CCL22 have a secondary role probably would not be negatively affected by Nox2-deletion in Tregs.

In conclusion, we observed that Nox2-deficiency increased the suppressive capacity and chemotaxis of Tregs in vitro and in vivo. Therefore, Nox2 could be used as a target to potentiate Tregs with clinical application.

Material and methods

Mice and in vivo studies. Nox2$^{fl/fl}$FoxP3Cre^{+} mice were generated by crossing B6129S-Tg (FoxP3-EGFP/iCre)1aJbs males with Nox2 homozygous floxed females(19). Heterotopic heart transplants were performed as previously described (44).

ELISA. Troponin-I and IFN-γ levels were determined by ELISA according to manufacturer's recommendations.

Treg purification. CD4^{+}CD25^{+} cells were purified from spleen and lymph nodes using a commercial kit (Dynabeads^{TM} FlowComp^{TM} Mouse CD4^{+}CD25^{+} Treg, Cat. 11463D).

Flow cytometry. Levels of alloantibodies in plasma and single cell analyses of heart allograft digests were determined in a LSRFORTESSA flow cytometer (BD Biosciences, Oxford, UK) and analysed using FlowJo software 9.7.5 (Ashland, USA). Superoxide production was estimated using 10 μM dihydroethidium(17).

Quantitative PCR. RNA was extracted using Trizol^{TM} Reagent. SYBR green real-time PCR was performed using the ΔΔCt method and GAPDH for normalization. cDNA synthesis and q-PCR for miR-214-3p and miR-214-5p were done using an miCury LNAtm MiRNA PCR starter kit, mmu-miR-214-3p and mmu-miR-214-5p.

Cell transfection. Jurkat T cells were transfected with microRNA mimic miR-214-3p or hsa-miR-214-3p miRCURY LNA miRNA Inhibitor or miRVana^{tm} miR mimic negative control by electroporation.

626

627 *Statistics*. Analyses were performed using GraphPad Prism software v9.0. Comparisons
628 were undertaken using Kruskal-Wallis followed by Dunn's post-test or a Mann Whitney
629 t-test two tailed or 2-way ANOVA followed by Bonferroni post-test, as appropriate. A
630 Mantel-Cox test was used to compare survival rates. $P<0.05$ was considered significant.
631
632 *Study approval.* All animal procedures were undertaken in accordance with the Guidance
633 on the Operation of the Animals (Scientific Procedures) Act, 1986 (UK Home Office) and
634 institutional ethics approval from King's College London, London, United Kingdom.
635
636 See Supplementary Methods for additional information.
637
638 **Author Contributions**
639 A.M.S. and G.L. supervised the study. A.M.S., G.L. and S.C.T. conceived the study and
640 contributed to experimental design. S.C.T., A.Z., A.I., A.B., L.S., R.K and G.S. performed
641 experiments and interpreted data. F.M.B and R.L. provided critical intellectual input.
642 S.C.T., G.L. and A.M.S. wrote the manuscript.
643
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Figures

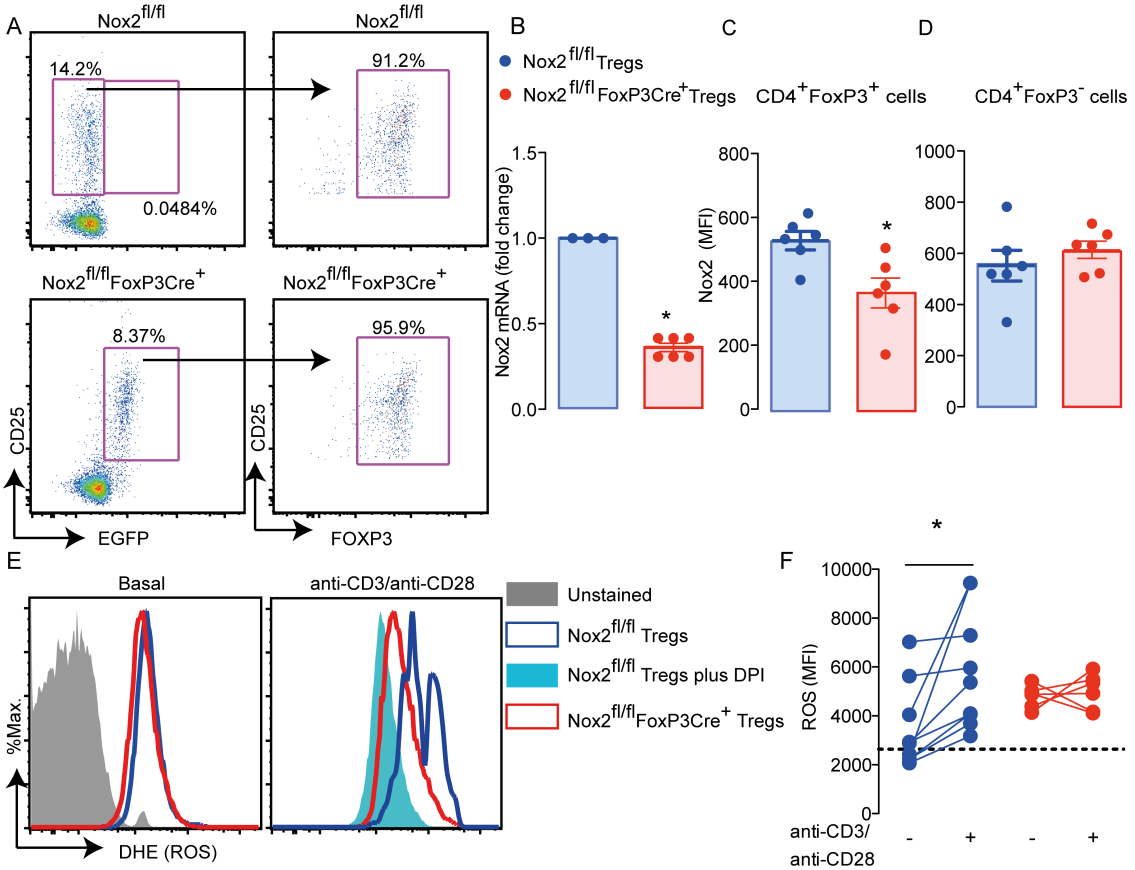


Figure 1. Generation of mice with Treg-targeted Nox2 deletion. (A) EGFP$^+$ cells from lymph nodes of Nox2$^{fl/fl}$FoxP3Cre$^+$ mice stained with CD25 and FoxP3 Abs. Plots are representative of 3 Nox2$^{fl/fl}$FoxP3Cre$^+$ and 3 Nox2$^{fl/fl}$ mice. (B) Nox2 mRNA levels in lymph nodes (n=3-6 per group). (C-D) Nox2 protein levels in CD4$^+$FoxP3$^+$ and CD4$^+$FoxP3$^-$ cells, respectively (n=6 per group). (E-F) ROS estimated by dihydroethidium (DHE) fluorescence in purified Tregs stimulated with anti-CD3 (4 µg/ml) and anti-CD28 (8 µg/ml) Ab (n=4-7 per group). Panels in E show representative histograms and mean data is displayed in F. Dashed line in E represents the MFI of Nox2$^{fl/fl}$ cells pre-incubated with the flavoprotein inhibitor diphenyleneiodonium (DPI, 10 µM) 30 minutes before stimuli. Data are shown as mean ±SEM; *P<0.05 for indicated comparisons; Mann Whitney t-test (two tailed) in B and C; Kruskal Wallis followed by Dunn's post-test in D.

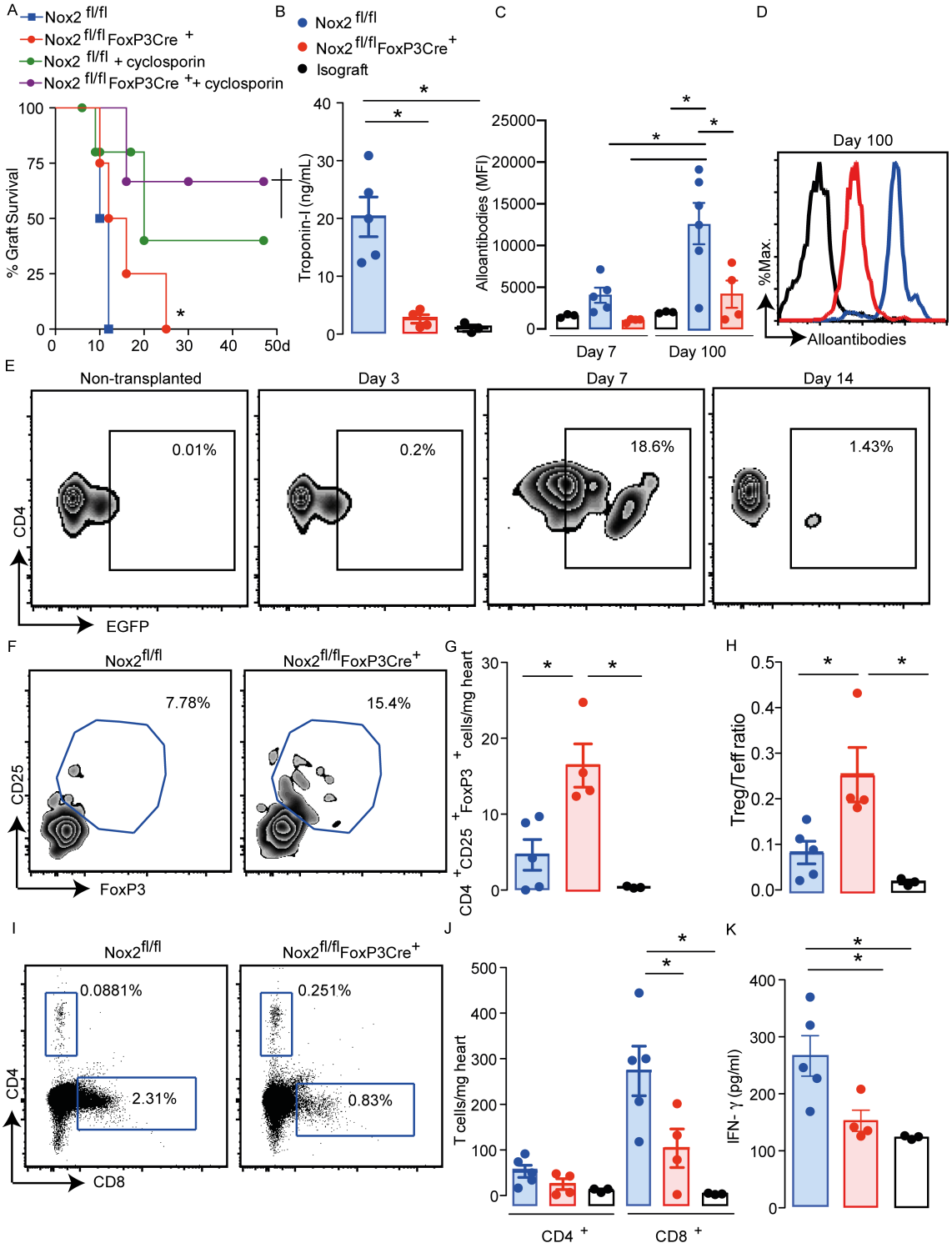


Figure 2. Heart allografts transplanted into Nox2$^{fl/fl}$FoxP3Cre^{+} mice have higher Treg infiltration and better outcome. Nox2$^{fl/fl}$FoxP3Cre^{+} mice and littermate controls (Nox2$^{fl/fl}$) were transplanted with hearts from CB6F1 mice. Mice transplanted with hearts from B6 mice were used as isograft controls. (A) Allograft survival curves. Some mice were treated daily with cyclosporin (30 mg/kg) s.c., for 10 days after transplantation (n=4-

5 per group). (B) Plasma troponin-I levels 7 days after transplant. (C- D) Plasma alloantibodies 7 and 100 days after the transplant. Representative histograms in D show the data obtained 100 days after transplant. (n=3- 5 per group). (E) Representative plots of one experiment realized in triplet of allografts showing the infiltration of $EGFP^+$ recipient Tregs (F-G) Representative plots of $CD25^+FoxP3^+$ cells within the $CD4^+$ cell population (F) and cells/mg of heart allograft 7 days after transplant (G). (H) Treg/Teff ratios. (I-J) $CD4^+$ and $CD8^+$ cells into transplanted hearts. Representative plots are shown in I and cells/mg tissue in J. (K) IFN-γ levels in heart allograft homogenates 7 days after surgery. Data are shown as mean ±SEM; *P<0.05 for indicated comparisons; Kruskal-Wallis followed by Dunn's post-test (n= 3-5 per group). † or *P<0.05 compared to *Nox2*$^{fl/fl}$ mice, Mantel-Cox test (A).

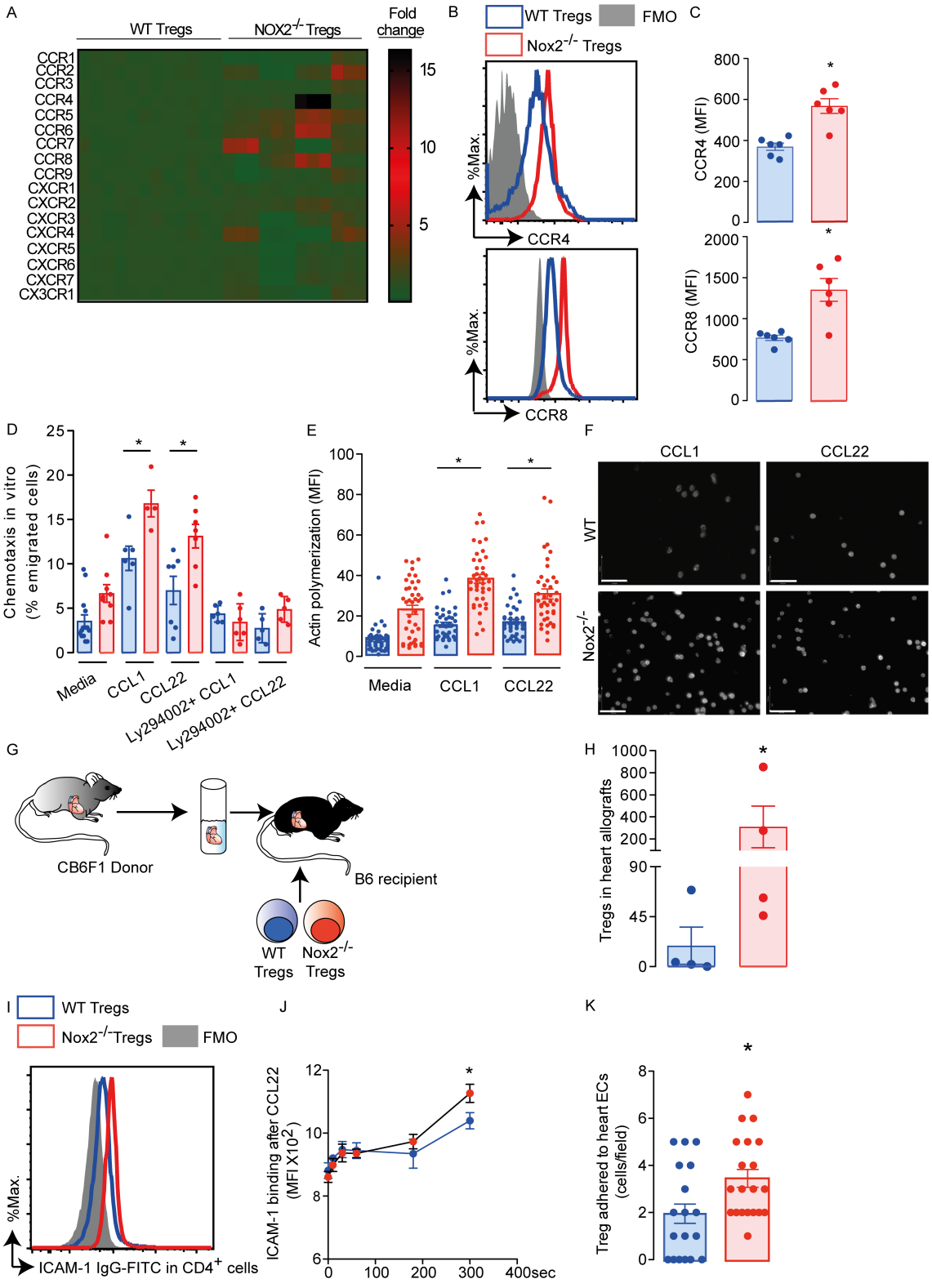
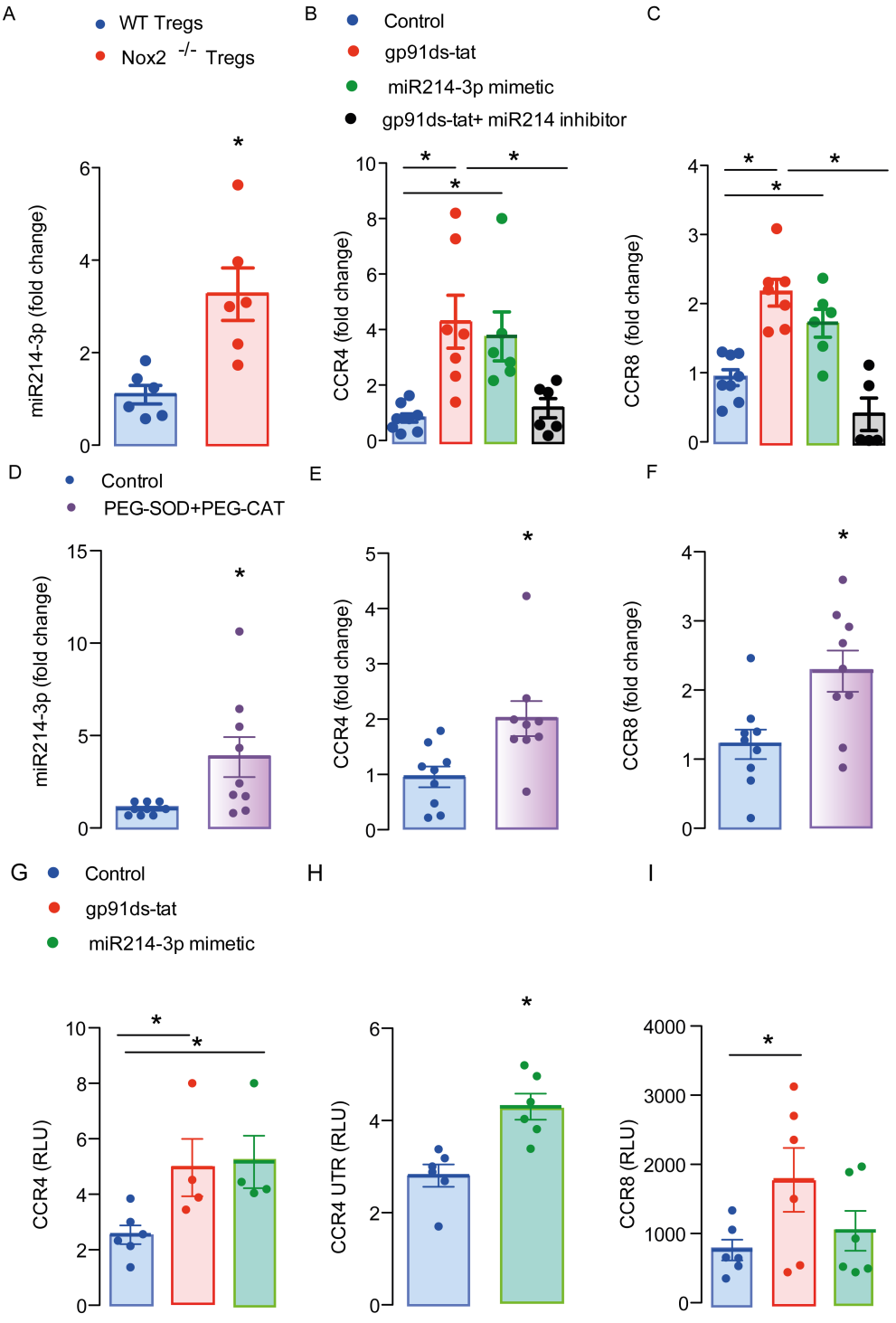


Figure 3. Nox2-deficient Tregs (Nox2$^{-/-}$) express higher levels of CCR4 and CCR8 than WT Tregs driving migration into heart allografts. Tregs were purified from spleen and lymph nodes of Nox2$^{-/-}$ or WT mice and assessed for: (A) mRNA levels of

914 chemokine receptors (n=12); (B-C) CCR4 and CCR8 protein levels by flow cytometry
915 (n=6); (D) chemotaxis *in vitro* toward CCL1 and CCL22; (E-F) actin polymerization
916 stimulated by CCL1 and CCL22. Some cells were incubated with Ly294002 (5 μM). Full
917 minus one (FMO) Ab was used as a negative control. Graphs and images represent one
918 from 3 independent experiments. (Scale bar: 32μm) (G-H)) WT and Nox2$^{-/-}$ Tregs were
919 stained with different colour cell tracers and tested for infiltration (after adoptive transfer)
920 into CB6F1 hearts transplanted in B6 recipients (n=4) (G-H); or adherence on cardiac
921 ECs (K). (I-J) In vitro activation and binding of ICAM-1 in Tregs. Histograms and mean
922 data represent one from 2 independent experiments. Data are shown as mean ±SEM;
923 *P<0.05 for indicated comparisons; Mann-Whitney t-test (two tailed) in C, H and K;
924 Kruskal-Wallis followed by Dunn's post- test in D and E; 2-way ANOVA followed by
925 Bonferroni's post -test in J.



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927 **Figure 4. miR-214-3p is up regulated in Nox2$^{-/-}$ Tregs and controls CCR4 and CCR8**
928 **mRNA expression.** (A) Expression of miR-214-3p in Nox2$^{-/-}$ or WT Tregs (n=6). (B-C)
929 CCR4 and CCR8 mRNA expression in Jurkat T cells transfected with miR-214-3p
930 mimetic or inhibitor. Some cells were treated with the Nox2 inhibitor gp91ds-tat (30 μM)
931 for 24 hours. Control cells were transfected with miR-negative control and incubated with

sc-tat for 24 hours. Graphs represent 2 independent experiments. (D-F) Levels of miR-214-3p, mRNA coding for CCR4 and CCR8 in Jurkat T cells incubated 24 hours with PEG-SOD (20 IU/ml) and PEG-catalase (300 IU/ml). Graphs represent 3 independent experiments (G-I) Reporter assay using CCR4, CCR4 UTR and CCR8 constructs. Graphs represent 2 independent experiments. Data are shown as mean ±SEM; *P<0.05 for indicated comparisons; Mann-Whitney t-test (two tailed) in A, D-F and H; Kruskal-Wallis followed by Dunn's post-test in B-C, G and I.

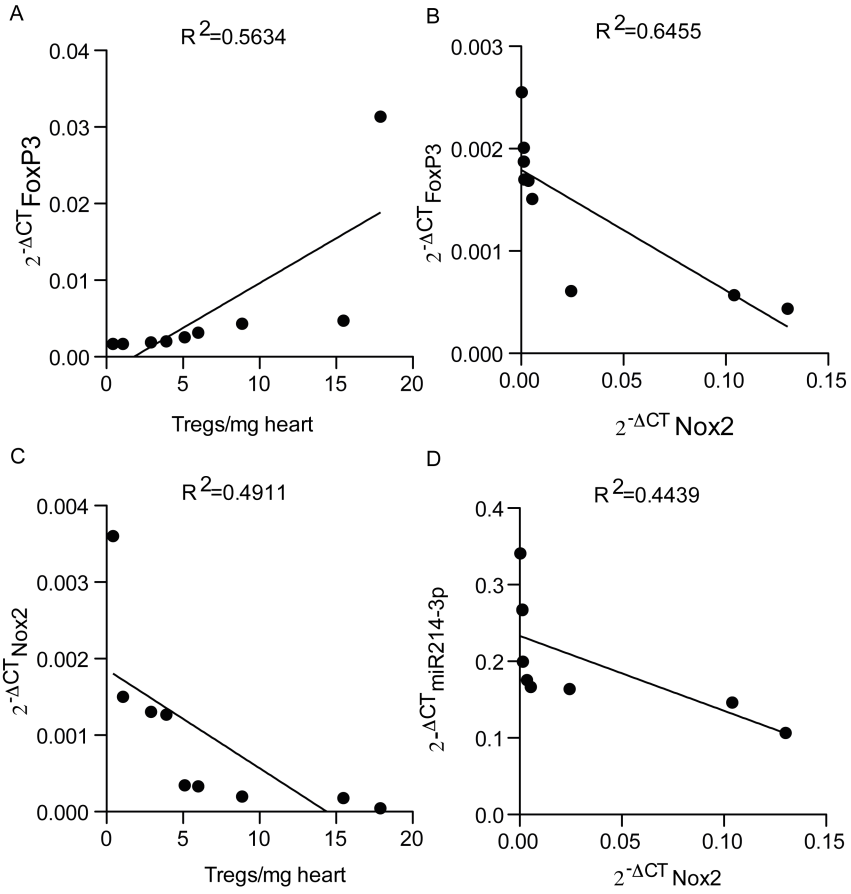

Figure 5. Nox2 expression inversely correlates with expression of FoxP3 and miR 214-3p. (A-D) Linear correlations between FoxP3 mRNA, Nox2 mRNA and miR 214-3p expressions and Treg counts in heart allografts (n=9), 7 days after transplantation. Values of cycle threshold ($2^{-\Delta CT}$) for miR214-3p were normalized by miR-let-103; Nox2

and FoxP3 were normalized by GAPDH. The R^2 values are displayed on the top of each correlation.