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## Role of T cell Transforming Growth Factor $\beta$ Signaling and IL-17 in Allograft Acceptance and Fibrosis Associated with Chronic Rejection<sup>1</sup>

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### Abstract

Chronic allograft rejection (CR) is the main barrier to long-term transplant survival. CR is a progressive disease defined by interstitial fibrosis, vascular neointimal development, and graft dysfunction. The underlying mechanisms responsible for CR remain poorly defined. Transforming growth factor  $\beta$  (TGF $\beta$ ) has been implicated in promoting fibrotic diseases including CR, but is beneficial in the transplant setting due to its immunosuppressive activities. To assess the requirement for T cell TGF $\beta$  signaling in allograft acceptance and the progression of CR, we used mice with abrogated T cell TGF $\beta$  signaling as allograft recipients. We compared responses from recipients that were transiently depleted of CD4<sup>+</sup> cells (that develop CR and express intra-graft TGF $\beta$ ) to responses from mice that received anti-CD40L mAb therapy (that do not develop CR and do not express intra-graft TGF $\beta$ ). Allograft acceptance and suppression of graft-reactive T and B cells were independent of T cell TGF $\beta$  signaling in mice treated with anti-CD40L mAb. In recipients transiently depleted of CD4<sup>+</sup> T cells, T cell TGF $\beta$  signaling was required for the development of fibrosis associated with CR, long-term graft acceptance, and suppression of graft-reactive T and B cell responses. Further, IL-17 was identified as a critical element in TGF $\beta$  driven allograft fibrosis. Thus, IL-17 may provide a therapeutic target for preventing graft fibrosis, a measure of CR, while sparing the immunosuppressive activities of TGF $\beta$ .

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## Keywords

T cells; Th1/Th2; transplantation; suppression

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## Introduction

With the advent of immunosuppressive therapies, a decline in graft loss due to acute rejection has established chronic allograft rejection (CR)<sup>3</sup> as the leading cause of late graft failure (1). CR is an irreversible disease characterized by deteriorating graft function, interstitial fibrosis, and occlusive neointima (2-5). Despite continued investigation, the underlying mechanisms responsible for these disease manifestations remain poorly defined and no therapies exist to prevent or treat CR except re-transplantation (6).

The immune system evolved to combat pathogens while maintaining tolerance to self, and TGF $\beta$  plays a pivotal role in regulating immune responses (7). The importance of TGF $\beta$  in immune regulation is underscored by the severe autoimmunity observed in knockout mice that lack TGF $\beta$  or are unable to signal through the TGF $\beta$  receptor (8-11). TGF $\beta$  controls T cell mediated self-reactivity by regulating lymphocyte proliferation and survival, inhibiting Th1/Th2-cell differentiation, and dampening effector function (reviewed in (12)). TGF $\beta$  signaling in B cells inhibits proliferation and survival, prevents activation, and inhibits IgG class switching (12). TGF $\beta$  is also critical in both the development and function of T regulatory cells (Treg) (12-17). TGF $\beta$  signaling in Treg is essential for peripheral maintenance of this cell subset (7,18) and for the induction of FoxP3 expression and Treg function in CD4+CD25- T cells (19).

In addition to TGF $\beta$ 's anti-inflammatory activities, TGF $\beta$  also mediates pro-inflammatory as well as pro-fibrotic activities. A reciprocal developmental pathway exists for the generation of pathogenic effector Th17 and Treg (20-22). TGF $\beta$  in association with IL-6 or IL-21 favors the commitment of CD4+ T cells to the Th17 lineage (20,21,23-26). IL-17 stimulates stromal cells, such as fibroblasts, endothelial cells, and epithelial cells to produce IL-6, IL-8, granulocyte CSF (G-CSF), and PGE2 and up-regulates critical chemoattractants, such as CXCL1 and CXCL2 (27-29). IL-17 serves to amplify the inflammatory responses and has also recently been implicated as a pro-fibrotic cytokine (30-37).

A critical role for TGF $\beta$  in transplant acceptance has been described (38,39), and early studies revealed the importance of TGF $\beta$  in donor-specific transfusions and allograft acceptance (40). Subsequent studies investigating skin allograft acceptance have also identified TGF $\beta$  as a protective factor against allograft rejection (41,42). Further, transduction of cardiac allografts with active TGF $\beta$  prolongs graft survival (43) and is associated with the induction of graft-reactive Treg (44).

While TGF $\beta$  mediates many beneficial anti-inflammatory activities in the immune system (7), we have previously reported an association between TGF $\beta$  and fibrosis associated with CR using the mouse vascularized cardiac allograft model (45). Intra-graft transcript levels of TGF $\beta$  were readily detectable in the CR grafts from recipients transiently depleted of CD4+ T cells, but not in the grafts of anti-CD40L treated recipients, which remain free of CR (45). Allograft transduction with active TGF $\beta$  resulted in CR in anti-CD40L treated recipients that do not normally exhibit CR, but was not observed in TGF $\beta$  transduced syngeneic grafts. This

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<sup>3</sup>Abbreviations used in this paper: anti-CD40L, anti-CD40 ligand; CD4-DNR, transgenic mice with a T cell specific dominant negative TGF $\beta$  receptor; CR, chronic allograft rejection; GIC, graft infiltrating cells; IL-17<sup>-/-</sup>, IL-17 deficient; TGF $\beta$ , transforming growth factor-beta; TGF $\beta$ RII, transforming growth factor-beta receptor II; Treg, T regulatory cell; WT, wild type.

supported a critical role for TGF $\beta$  and alloantigen in the progression of CR. In this study, we sought to determine the role of TGF $\beta$  signaling on alloreactive effector cells and IL-17 induction by using T cell-specific dominant negative TGF $\beta$  receptor type II (CD4-DNR) (10) and IL-17 deficient (IL-17 $^{-/-}$ ) mice (46) as transplant recipients. Further, we identify both TGF $\beta$  dependent and independent pathways to allograft acceptance.

## Materials and Methods

### Mice

C57BL/6 CD4-DNR (10), C57BL/6 wild type (WT) and BALB/c mice were purchased from the Jackson Laboratory. CD4-DNR mice express a dominant-negative form of the human TGF $\beta$  receptor II (TGF $\beta$ RII) under the direction of the mouse CD4 promoter, which lacks a CD8 silencer. TGF $\beta$  signaling is abrogated in both CD4 $^{+}$  and CD8 $^{+}$  T cells in these transgenic mice. CD4-DNR were propagated by breeding C57BL/6 WT females with CD4-DNR males. The genotyping of transgene expressing CD4-DNR mice was carried out using the following PCR primers: primer WT forward 5'CTAGGCCACAGAATTGAAAGATCT-3'; primer WT reverse 5'-TAGGTGGAAATTCTAGCATCATCC-3'; primer CD4-DNR transgene (TG) forward 5'-GCTGCACAT CGTCCTGTG-3'; primer TG reverse 5'-ACT TGACTGCACCGT TGTTG-3'. Primers WT forward and WT reverse were used to detect the internal control, IL-2 (324 bp). Primers TG forward and TG reverse were used to detect the transgenic allele (100 bp). CD4-DNR mice exhibit an autoimmune phenotype and immunopathology with age, resulting in the development of multi-focal inflammation best characterized by inflammatory bowel disease between 3-5 months of age (10). To avoid potential complications of age-related autoimmunity, mice were transplanted at 6 weeks of age and at the termination of each experiment the colons were examined macroscopically and histologically for autoimmune manifestations. None of the mice used in this study exhibited an autoimmune phenotype. IL-17 $^{-/-}$  mice (46) were generated by Dr. Yoichiro Iwakura and provided by Dr. Weiping Zou in collaboration. All mice were housed under specific pathogen-free conditions in the Unit for Laboratory Animal Medicine at the University of Michigan. These experiments were approved by the University Committee on Use and Care of Animals at the University of Michigan.

### Culture medium

Culture medium consisted of the following: RPMI 1640 supplemented with 2% FCS, 1 mM sodium pyruvate, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, 1.6 mM L-glutamine, 10 mM HEPES buffer (all from Invitrogen), 0.27 mM L-asparagine, 1.4 mM L-arginine HCl, 14  $\mu$ M folic acid, and 50  $\mu$ M 2-ME (all from Sigma-Aldrich).

### Vascularized cardiac transplantation

CD4-DNR, IL-17 $^{-/-}$ , and WT mice were transplanted with intact BALB/c cardiac allografts, as described (47). Briefly, the aorta and pulmonary artery of the donor heart were anastomosed end-to-side to the recipient's abdominal aorta and inferior vena cava, respectively. Upon perfusion with the recipient's blood, the transplanted heart resumes contraction. Graft function is monitored by abdominal palpation.

### In vivo mAb treatment

The hybridoma secreting anti-CD4 (clone GK1.5) was obtained from American Type Culture Collection. The hybridoma secreting anti-CD40L (clone MR1) was provided by Dr. Randy Noelle (Dartmouth, Lebanon, NH). Anti-CD4 and anti-CD40L mAb were purified and resuspended in PBS by Bio X Cell (West Lebanon, NH). Mice received 1 mg i.p. of anti-CD4 mAb on days -1, 0, 7 (45,48,49). In recipients transiently depleted of CD4 $^{+}$  T cells, CD4 $^{+}$  T

cells begin to repopulate the periphery 3-4 weeks post-transplantation (48-50). Mice received 1 mg of anti-CD40L i.p. days 0, 1, and 2 (45). All doses are relative to day of transplant.

## Histology

Allografts were recovered at the times indicated post-transplantation, fixed in formalin, and embedded in paraffin. Sections were stained with hematoxylin-eosin (H & E) to assess myocyte viability (presence of cross striation and myocyte nuclei), and the nature and intensity of graft infiltrating cells.

## Recovery of graft infiltrating cells (GIC)

Groups of three transplanted hearts were removed, pooled, minced, and digested with 1 mg/mL collagenase A (Roche) for 30 min at 37 °C. Tissue debris were allowed to settle at  $1 \times g$  and the suspension containing GIC was harvested by pipette. RBC were lysed by hypotonic shock, GIC were passed through a 30- $\mu$ m pore size nylon mesh, and viable leukocytes were enumerated by trypan blue exclusion.

## ELISPOT assays for cytokine-producing cells

ELISPOT assays were performed as previously described (51). Capture and detection antibodies specific for IFN $\gamma$  (R4-6A2, XMG1.2), IL-4 (11B11, BVD6-24G2) and IL-17 (TC11-18H10.1, TC11-8H4.1) were purchased from Pharmingen (San Diego, CA). PVDF-backed microtiter plates (Millipore, Bedford, MA) were coated with unlabeled mAb and blocked with 1% BSA in PBS. Irradiated (1000 rad) donor splenocytes ( $4 \times 10^5$ ) and  $1 \times 10^6$  recipient splenocytes were added to the plates. After washing, a 1:1000 dilution of anti-biotin alkaline phosphatase (AP) conjugate (Vector Laboratories, Burlingame, CA) was added to IFN $\gamma$  and IL-17 plates, and a 1:2000 dilution of horseradish peroxidase-conjugated streptavidin (SA-HRP; Dako, Carpinteria, CA) was added to IL-4 plates. Plates were washed and spots visualized by addition of nitroblue tetrazolium (NBT; Biorad, Hercules, CA) / 3-bromo-4-chloro-inolyl-phosphate (BCIP; Sigma) to IFN $\gamma$  and IL-17 plates, or 3-amino-9-ethylcarbazole (AEC; Pierce, Rockford, IL) to IL-4 plates. Color development continued until spots were visible and was stopped by adding H $_2$ O. Plates were dried and spots quantified with an Immunospot Series 1 ELISPOT analyzer (Cellular Technology Ltd., Cleveland, OH).

## Flow cytometry

Splenocytes were isolated by mechanical dissociation followed by lysis via hypotonic shock and blocked in PBS containing 0.1% BSA, 0.025% NaN $_3$  and 10% FBS. After washing,  $1 \times 10^6$  cells were stained with fluorochrome-conjugated anti-mouse CD4 (clone GK1.5), CD3 (clone 145-2C11) and CD8 (clone 53-6.7) (all from BD Biosciences, San Jose, CA). Three-color flow cytometry was performed with a FACS Calibur (BD Biosciences) equipped with Cell Quest software.

## RNA isolation and RT-PCR

Cardiac allografts were homogenized in 1 mL TRIzol® (Invitrogen Life Technologies, Carlsbad, CA) and RNA was isolated as per manufacturers protocol. 5  $\mu$ g of total RNA were reverse transcribed using 10 $\times$  PCR buffer (Roche), 10 mM dNTPs, Oligo (dt), M-MLV-RT (all from Invitrogen), and RNasin (Promega). Products were then cleaned with 1:1 phenol/chloroform/isoamyl (25:24:1) and re-precipitated with 7.5 M NH $_4$ OAC in pure EtOH overnight at -80 °C.

Real-time PCR was performed on cDNA using a Rotor-Gene 3000 TM (Corbett Life Science, CA). Primer binding to DNA was detected by SYBR Green ITM dye (Roche, Indianapolis, IN). Relative expression of the gene of interest was expressed as the comparative concentration

of the gene product to the GAPDH product as calculated by accompanying Rotor-Gene software. Significance was determined with an unpaired Student t-test.

Primer sequences:

IL-17 sense: 5' GGACTCTCCACCGCAATGA

IL-17 anti-sense: 5'GACCAGGATCTCTTGCTGGA

FoxP3 sense: 5'CCAAGGTGAGCGAGTGTC

FoxP3 anti-sense: 5'AAGGCAGAGTCAGGAGAAGT

GAPDH sense: 5'CTGGTGCTGAGTATGTCGTG

GAPDH anti-sense: 5'CAGTCTTCTGAGTGCCAGTG

### Donor-reactive Ab determination

As described (48,50,52) P815 cells (H-2<sup>d</sup>) were stained for flow cytometric analysis using diluted (1:50) sera obtained from mice as the primary antibody, followed by FITC-conjugated isotype specific anti-mouse IgG, IgG1, or IgG2a secondary antibodies (The Binding Site, San Diego, CA, USA) at a 1:50 dilution. Data are reported as the mean channel fluorescence determined on a Becton Dickinson FACSCaliber (San Jose, CA, USA).

### Immunohistochemistry

To detect IgG deposition within the graft, frozen sections of grafts were fixed in cold acetone and incubated with 1:150 dilution of goat anti-mouse IgG-HRP (Southern Biotech, Birmingham, AL) followed by AEC staining (50). To detect C3d and C4d deposition (50), sections of paraffin embedded tissue were fixed in methanol. A 1:20 dilution of goat anti-mouse C3d (R&D Systems, Minneapolis, MN) was added followed by secondary detection antibodies added as per R&D System's anti-goat cell and tissue staining kit. Slides were stained with rabbit anti-mouse C4d (kindly provided by Dr. William Baldwin, Cleveland Clinic) at a 1:500 dilution, followed by DAB development using the SuperPicTure™ Polymer Detection Kit (Zymed). Specificity of staining was ensured by staining of native hearts.

### Morphometric analysis of cardiac allograft fibrosis

Graft fibrosis was quantified by morphometric analysis of Masson's trichrome stained tissues using iPLab software (Scanalytics Inc., Fairfax, VA) (53). Mean fibrotic areas were calculated from 10 to 12 areas per heart section analyzed at 200× magnification. A minimum of 5 individual hearts were analyzed per group.

### Statistical analysis

Data were analyzed with GraphPad Prism 4.0c software using unpaired Student t-tests. *p* values of  $\leq 0.05$  were considered statistically significant.

## Results

### Experimental System

In the mouse cardiac allograft model, prolonged allograft survival can be achieved by transiently depleting recipients of CD4<sup>+</sup> cells or by disrupting CD40-CD40L interactions. However, these two inductive therapies differ with respect to the development of CR. Allografts in mice treated with anti-CD40L mAb remain free of CR, while grafts in mice transiently depleted of CD4<sup>+</sup> cells develop CR, which is associated with intragraft expression of TGF $\beta$  (45). While graft-reactive T cells remain in a hyporesponsive state in both settings,

recall responses of cells from these groups differ in that dominant Th2 responses are mounted by mice that are depleted of CD4<sup>+</sup> cells, while Th1 responses are mounted by recipients given anti-CD40L mAb therapy (54). The current study assessed the requirement for TGFβ signaling in T cells for allograft acceptance, graft-reactive T and B cell hyporesponsiveness, and graft fibrosis as a parameter of CR.

### Effect of T cell TGFβ signaling on transplant acceptance

TGFβ mediates beneficial immunosuppressive activities in the transplant setting (38-40). To assess the requirement for T cell TGFβ signaling in allograft acceptance and the progression of graft fibrosis associated with CR, we used CD4-DNR mice with abrogated T cell TGFβ signaling (10) as allograft recipients. CD4-DNR mice express a dominant-negative form of the human TGFβRII under the direction of the mouse CD4 promoter, which lacks a CD8 silencer. Hence, TGFβ signaling is abrogated in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in these transgenic mice. To rule out alloantigen independent cellular infiltration and tissue damage in CD4-DNR recipients, both WT and CD4-DNR mice were transplanted with syngeneic grafts. Grafts from both groups functioned until the experiment was terminated on day 50 post-transplant (Figure 1A). Histologically, syngeneic grafts from both groups were free of infiltrate, exhibited minimal fibrosis, normal arteries and viable myocytes (data not shown). Similar observations were made in CD4-DNR recipients of syngeneic grafts treated with inductive CD4<sup>+</sup> T cell depletion (data not shown). These results indicate that there were no differences in graft survival or histology between the WT and the CD4-DNR recipients of syngeneic grafts ruling out non-antigen specific inflammatory responses in graft loss in CD4-DNR mice.

Due to the immunosuppressive activities of TGFβ, we predicted that CD4-DNR recipients would mount exacerbated rejection responses when compared to WT mice. Allografts in both WT and CD4-DNR recipients were acutely rejected by day 9 post-transplant (Figure 1A) and histological examination revealed evidence of rejection (data not shown). While the tempo of rejection was not different between these two groups, a more intense infiltration of the grafts was observed in CD4-DNR recipients (total number of GICs per graft: WT =  $0.33 \times 10^6 \pm 0.01$ ; CD4-DNR =  $1.4 \times 10^6 \pm 0.23$  ( $p < 0.01$ )) indicating that T cell TGFβ signaling dampens cell proliferation and/or cellular infiltration in unmodified recipients.

Treatment of both WT and CD4-DNR recipients with inductive anti-CD40L mAb resulted in long-term graft survival (Figure 1B), demonstrating that allograft acceptance following anti-CD40L therapy is independent of TGFβ signaling in T cells. WT allograft recipients treated with inductive CD4<sup>+</sup> T cell depletion also exhibited long-term graft survival (Figure 1C). In contrast, 90% of the allografts from CD4-DNR mice transiently depleted of CD4<sup>+</sup> T cells were rejected by day 40 post-transplantation (Figure 1C). The majority of the allografts were rejected between days 35-40, correlating with CD4<sup>+</sup> T cell repopulation of the periphery (48, 50). A greater percentage of CD4<sup>+</sup> T cells were present in the spleens of CD4-DNR recipients compared to WT suggesting that TGFβ signaling controls CD4<sup>+</sup> T cell proliferation in recipients inductively depleted of CD4<sup>+</sup> T cells (Figure 1D). Rejection could be attenuated in CD4-DNR recipients when CD4<sup>+</sup> T cells were continuously depleted by weekly injections of anti-CD4 mAb (Figure 1C). These results indicate that T cell TGFβ signaling is essential for allograft acceptance in recipients transiently depleted of CD4<sup>+</sup> T cells and that repopulation of CD4<sup>+</sup> cells is required for rejection in CD4-DNR mice.

### TGFβ regulation of donor-reactive Th1, Th2 and Th17 responses

T cell TGFβ signaling inhibits differentiation of Th1 and Th2 cells through the suppression of T-bet/STAT4 and Gata-3/NFAT (7,12). To determine if T cell TGFβ signaling regulates donor-reactive cellular immune responses, we employed ELISPOT to quantify the number of *in vivo* primed donor-reactive Th1 (IFNγ), Th2 (IL-4) and Th17 (IL-17) responses (Figure 2).

Elevated donor-reactive Th1 responses were observed in unmodified WT and CD4-DNR recipients indicating acute rejection is associated with a dominant Th1 response (Figure 2A). While abrogation of TGF $\beta$  signaling in recipients did not result in elevated Th1 responses compared to WT recipients, CD4-DNR recipients did exhibit enhanced IL-4 priming in response to donor antigen. However, this was not statistically significant. Donor-reactive Th17 responses were negligible in both WT and CD4-DNR recipients.

Donor-reactive Th responses were not observed in either WT or CD4-DNR recipients treated with anti-CD40L (Figure 2B). These findings indicate that T cell TGF $\beta$  signaling is not required for the suppression of donor-reactive responses (55) and is dispensable for allograft acceptance following anti-CD40L mAb treatment.

As previously reported (48,49,54), donor-reactive Th1 and Th2 responses are suppressed in recipients transiently depleted of CD4<sup>+</sup> T cells. Consistent with these findings, inductive anti-CD4 mAb therapy resulted in hyporesponsiveness in WT recipients compared to unmodified recipients (Figure 2A and 2C). In contrast, elevated donor-reactive Th1 and Th2 responses were detected in the CD4-DNR recipients transiently depleted of CD4<sup>+</sup> T cells (Figure 2C). These data indicate that TGF $\beta$  plays a pivotal role in suppressing immune responses and in maintaining allograft acceptance in recipients initially depleted of CD4<sup>+</sup> T cells.

### **Cardiac fibrosis is reduced in mice with abrogated T cell TGF $\beta$ signaling**

TGF $\beta$  has been strongly implicated in many fibrotic diseases (30,56), including CR (3,4,57, 58). To investigate the relationship between T cell TGF $\beta$  signaling and the development of graft fibrosis as a measure of CR, quantitative morphometric trichrome analysis was employed to evaluate allograft fibrosis in WT and CD4-DNR recipients treated with inductive anti-CD40L or anti-CD4 mAb. Allografts from WT and CD4-DNR recipients treated with inductive anti-CD40L mAb revealed minimal fibrosis (Figure 3B). Allografts from WT recipients transiently depleted of CD4<sup>+</sup> T cells revealed significant fibrosis compared to allografts from CD4-DNR recipients (Figure 3C). These findings indicate that in recipients transiently depleted of CD4<sup>+</sup> cells, allograft fibrosis is dependent on T cell TGF $\beta$  signaling.

### **Donor-reactive IgG production in WT and CD4-DNR recipients**

Since donor-reactive antibodies have been implicated in both acute and chronic rejection (59,60), we analyzed WT and CD4-DNR recipients for donor-reactive IgG antibodies (Figure 4). At the time of rejection, both WT and CD4-DNR unmodified recipients exhibited comparable donor-reactive IgG production (Figure 4A). These results indicate that TGF $\beta$  signaling does not overtly influence IgG alloantibody production in unmodified recipients.

It is well established that CD40-CD40L interactions are critical for antibody isotype switch (61). Consistent with these findings, both WT and CD4-DNR recipients treated with anti-CD40L mAb did not generate donor-reactive IgG production compared to unmodified recipients (Figure 4B). Hence, CD40-CD40L interactions are required for IgG isotype switching in the presence or absence of TGF $\beta$  signaling in T cells.

CD4-DNR recipients inductively depleted of CD4<sup>+</sup> T cells produced significantly elevated levels of donor-reactive IgG compared to WT controls (Figure 4C). To further characterize the donor-reactive antibody production in these recipients, both Th1- and Th2- dependent IgG2a and IgG1 isotypes were measured (62). Consistent with an increase in total IgG level, the CD4-DNR recipients exhibited significantly elevated levels of both IgG1 and IgG2a alloantibody production compared to WT recipients (Figure 4D). Collectively, these findings demonstrate that in the absence of TGF $\beta$  signaling, T cells acquire effector functions, secrete both Th1 and

Th2 cytokines, and provide help for alloreactive B cells in recipients transiently depleted of CD4<sup>+</sup> T cells.

### **Evidence of antibody-mediated rejection in CD4-DNR recipients treated with anti-CD4 mAb**

The diagnostic criteria for antibody-mediated rejection include histologic evidence of acute tissue injury, serologic evidence of circulating donor-reactive antibodies, and the deposition of complement C3d and C4d within allografts (reviewed in (59,63,64)). Hence, we investigated the deposition of IgG and C3d and C4d within the grafts from WT and CD4-DNR recipients transiently depleted of CD4<sup>+</sup> T cells (Figure 5). Intense IgG staining was localized mainly to the capillaries and arteries of allografts from CD4-DNR recipients, while allografts from WT recipients were free of IgG deposition (Figure 5A). Further, both C3d and C4d were deposited within the vasculature structures of allografts from CD4-DNR recipients, but were not present in control allografts (Figure 5B). These data indicate that in recipients transiently depleted of CD4<sup>+</sup> T cells, antibody mediated rejection of allografts occurs when T cells are unresponsive to TGF $\beta$ .

### **Anti-CD4 treated CD4-DNR recipients exhibit reduced intragraft FoxP3 and IL-17 levels compared to WT controls**

TGF $\beta$  induces expression of FoxP3 (65), leading to the differentiation of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells from CD4<sup>+</sup>CD25<sup>-</sup> T cells (19,66,67). However, a dichotomy exists in which TGF $\beta$  in the context of IL-6 or IL-21 promotes pathogenic Th17 cell development (20,21,23,24,26,68). To investigate the role of TGF $\beta$  signaling in Treg and Th17 induction and the contribution of these subsets in CR, we assessed intragraft FoxP3 and IL-17 transcript levels from WT and CD4-DNR recipients inductively depleted of CD4<sup>+</sup> T cells (Figure 6). Allografts from the CD4-DNR recipients exhibited significantly reduced intragraft FoxP3 gene expression compared to WT (Figure 6). This may indicate a failure to induce Treg from naïve CD4<sup>+</sup> T cells in the absence of T cell TGF $\beta$  signaling, poor maintenance of peripheral Treg, and/or reduced Treg trafficking into the graft (reviewed in (69)). In addition, intragraft IL-17 was detected in allografts from WT but not CD4-DNR recipients (Figure 6). IL-17 was not detected by ELISPOT in the spleens of the WT mice (Figure 2), which may suggest a compartmentalized immune response against donor antigen. Indeed, similar polarization of Th17 responses solely within target tissue has been observed in murine models of allergic lung disease and hypersensitivity pneumonitis and lung fibrosis (37,70). IL-17 is best recognized as a cytokine that mobilizes neutrophils and coordinates local tissue inflammation through the induction of various pro-inflammatory cytokines (27,28). Numerous recent reports implicate IL-17 as a pro-fibrotic cytokine in the context of T cell dependent fibrosis (31-37,71), capable of inducing fibroblast proliferation and collagen deposition in cardiac tissue (31,32). Our observation that allografts from CD4-DNR recipients treated with anti-CD4 exhibited minimal fibrosis (Figure 3) led us to hypothesize that IL-17 plays a pro-fibrotic role in CR.

### **Allograft fibrosis is reduced in anti-CD4 mAb treated IL-17<sup>-/-</sup> recipients**

To investigate the role of IL-17 production in fibrosis associated with CR, IL-17<sup>-/-</sup> mice (46) were used as transplant recipients. Both WT and IL-17<sup>-/-</sup> allograft recipients treated with anti-CD4 exhibited long-term graft survival, while unmodified recipients rejected their grafts by day 9 post-transplant (Figure 7A). In IL-17<sup>-/-</sup> recipients transiently depleted of CD4<sup>+</sup> T cells, fibrosis was markedly reduced relative to WT counterparts (Figure 7B and 7C). This reduction of allograft fibrosis in IL-17<sup>-/-</sup> recipients implicates IL-17 as a cytokine responsible for TGF $\beta$ -mediated fibrosis and CR.

To investigate the effect of IL-17 deficiency on intragraft gene expression, we assessed both IL-17 and FoxP3 transcript levels from WT and IL-17<sup>-/-</sup> recipients that were transiently depleted of CD4<sup>+</sup> T cells. Allografts from IL-17<sup>-/-</sup> recipients exhibited comparable FoxP3



gene expression to WT counterparts, while IL-17 transcripts were not detected in the IL-17<sup>-/-</sup> recipients (Figure 7D). These results suggest that reduced fibrosis in IL-17<sup>-/-</sup> recipients transiently depleted of CD4<sup>+</sup> T cells is not a result of enhanced Treg number as assessed by intragraft FoxP3 transcript levels.

## Discussion

We have previously reported an association between TGF $\beta$  and graft fibrosis associated with CR using the mouse vascularized cardiac model (45). Intragraft transcript levels of TGF $\beta$  are readily detectable in the CR grafts from recipients transiently depleted of CD4<sup>+</sup> T cells, but not in the grafts of anti-CD40L treated recipients, which remain free of CR. In the current study, we used inductive anti-CD4 or anti-CD40L mAb therapy and CD4-DNR recipients to evaluate the role of T cell TGF $\beta$  signaling in graft acceptance and the progression of fibrosis associated with CR. Collectively, our data suggest that in WT allograft recipients transiently depleted of CD4<sup>+</sup> cells, allograft acceptance, T and B cell hyporesponsiveness, and fibrosis of the graft are dependent on TGF $\beta$  signaling in T cells. We further demonstrate that IL-17 is involved in the development of graft fibrosis in recipients transiently depleted of CD4<sup>+</sup> T cells.

T cell TGF $\beta$  signaling is not required for long term allograft acceptance following anti-CD40L therapy (Figure 1). It is not fully understood how blockade of CD40L results in allograft acceptance, but a number of mechanisms, including donor-reactive T cell anergy and/or deletion, and the induction of Treg have been proposed (69,72). We have reported that anti-CD40L mAb therapy allows for a transient appearance of primed donor-reactive cells (54). Hence, it is possible that these primed T cells express CD40L, which targets these cells for deletion and/or silencing by anti-CD40L mAb therapy. Our study demonstrates that these processes do not require T cell TGF $\beta$  signaling.

In recipients transiently depleted of CD4<sup>+</sup> T cells, our findings support a TGF $\beta$ -dependent mechanism of graft acceptance. Inductive anti-CD4 mAb treatment of recipients results in transient depletion of CD4<sup>+</sup> T cells at the time of transplant (48-50). CD4<sup>+</sup> T cells begin to repopulate the periphery 3-4 weeks post-transplantation (48-50). As CD4<sup>+</sup> T cells return, donor-reactive T cells are functionally distinct from naïve cells in that these repopulating CD4<sup>+</sup> are hyporesponsive toward the graft but mount Th2 recall responses (54). This altered functional T cell capacity is associated with intragraft expression of TGF $\beta$ . Inductive anti-CD4 mAb treatment of CD4-DNR recipients results in allograft rejection between day 35-40 post-transplantation (Figure 1). Th1 and Th2 were maintained in a quiescent state in WT recipients transiently depleted of CD4<sup>+</sup> cells (Figure 2). In contrast, CD4-DNR recipients treated with inductive anti-CD4 mAb mount donor-reactive Th1 and Th2 responses, revealing that the induction of hyporesponsiveness requires that T cells be responsive to TGF $\beta$  (Figure 2). Repopulation of CD4<sup>+</sup> T cells in the periphery is required for rejection in that CD4-DNR transplant recipients that are continuously depleted of CD4<sup>+</sup> T cells did not reject their grafts (Figure 1) and did not mount Th1 and Th2 responses (data not shown). These observations support an essential role for TGF $\beta$  in cellular hyporesponsiveness and allograft acceptance in recipients inductively depleted of CD4<sup>+</sup> T cells.

In the absence of TGF $\beta$  signaling, T cells differentiate into effector cells, secrete cytokines and provide help to B cells in CD4-DNR recipients transiently depleted of CD4<sup>+</sup> cells. CD4-DNR recipients transiently depleted of CD4<sup>+</sup> cells mounted significantly elevated donor-reactive alloantibody levels of both Th2 induced non-complement fixing, IgG1, and Th1 induced complement fixing, IgG2a, compared to wild type controls (Figure 4) (73). Both subclasses have been documented to synergize to cause rejection of cardiac allografts (73). IgG and complement split product C3d and C4d capillary deposition in both human and mouse myocardium is significantly associated with graft loss (74) and these products were detected

in the vessels and surrounding the cardiac myocytes in the CD4-DNR recipient allografts that were rejected (Figure 5). Hence, T cells that are unable to respond to TGF $\beta$  mount cellular responses and provide help to activate alloreactive B cells in recipients inductively depleted of CD4<sup>+</sup> T cells.

Donor-specific hyporesponsiveness is observed in both human and mouse transplant recipients that exhibit prolonged allograft acceptance (15,66,75). In many of these studies, allograft acceptance is strongly associated with Treg infiltration into the graft as detected by high FoxP3 transcript levels (15,66,75,76). Treg are believed to play a critical role within allografts by inhibiting alloreactive T cells responses (15). Studies in skin allograft models reveal Treg enrichment in accepted grafts (77) is dependent on TGF $\beta$  and that this cytokine is important for long-term acceptance (42,66). Consistent with these observations, inductive anti-CD4 mAb treatment of WT recipients exhibit enhanced intra-graft FoxP3 transcript levels compared to CD4-DNR (Figure 6). In CD4-DNR recipients transiently depleted of CD4<sup>+</sup> T cells, reduced FoxP3 expression may indicate impaired maintenance of peripheral Treg, reduced Treg localization within the graft, or a failure in Treg induction (reviewed in (69)). Donor-reactive Th responses observed in CD4-DNR recipients may result from decreased induction of Treg and/or the failure of Treg to control effector cells in the absence of TGF $\beta$  signaling (78). Our data suggest an active regulatory mechanism in which Treg migration into the grafts (Figure 6) and inhibition of alloreactive responses (Figure 2) require T cell TGF $\beta$  signaling (79,80).

While functional T cell TGF $\beta$  signaling prevented allograft rejection in recipients inductively depleted of CD4<sup>+</sup> T cells, it promoted fibrosis associated with CR (Figure 3). In contrast, T cell unresponsiveness to TGF $\beta$  resulted in minimal fibrosis of grafts (Figure 3). It has been reported that sustained production of TGF $\beta$  in tissues is a main contributor to the development of fibrosis (57,81), but we have observed that gene transfer of TGF $\beta$  in syngeneic grafts fail to develop CR (45). Fibrosis was observed only in allografts that adenovirally expressed TGF $\beta$  and were transplanted into anti-CD40L mAb treated recipients (45). This indicates that TGF $\beta$  alone is insufficient to induce fibrosis of the graft, and that alloantigen and elements of the immune system are required for fibrosis induction.

As T cells infiltrate the allograft and respond to TGF $\beta$ , they secrete multiple cytokines, which may influence the local environment to become pro-fibrotic. One TGF $\beta$ -induced cytokine that could potentially mediate fibrosis in this setting is IL-17 (31,32). IL-17 is important in coordinating local tissue inflammation through the induction of various pro-inflammatory cytokines (27,28). IL-17 has also been implicated as a contributor to fibrosis in a number of diseases (30,33-35,37). Allografts from IL-17<sup>-/-</sup> recipients transiently depleted of CD4<sup>+</sup> T cells showed a significant reduction in fibrosis relative to their WT counterparts (Figure 7). These findings are consistent with TGF $\beta$  induced IL-17 promoting interstitial fibrosis in CR allografts.

IL-17 may induce collagen deposition within CR allografts through multiple mechanisms. IL-17 upregulates collagen gene expression in primary mouse cardiac fibroblasts (32). Indirectly, IL-17 induces the production of IL-6 (27,28), which enhances the accumulation of collagen (53,82-84). IL-17 may also play a role in fibrosis by acting as a potent pro-inflammatory cytokine that induces endothelial cells and fibroblasts to secrete additional pro-inflammatory cytokines and chemokines (27,28). These factors may enhance the recruitment of APC and alloreactive T cells into the graft resulting in myocardial damage and extracellular matrix remodeling that favors fibrosis (4).

In summary, TGF $\beta$  is critical for the induction of fibrosis in this model of CR and in a number of fibrotic diseases, including diabetic nephropathy, rheumatoid arthritis, myocarditis, Crohn's disease and radiation-induced fibrosis (reviewed in (30)). As in most immune-mediated

diseases, TGF $\beta$  can have both exacerbating and ameliorating actions making global inhibition of TGF $\beta$  unacceptable and local targeting of TGF $\beta$  or its downstream mediators an attractive therapy. This is evident in recipients transiently depleted of CD4<sup>+</sup> cells in which T cell responsiveness to TGF $\beta$  is critical in maintaining alloreactive T cells in a hyporesponsive state. Our findings that TGF $\beta$  in CR grafts correlates with localized Th17 induction supports findings in other chronic inflammatory diseases (30) and provides a therapeutic target for preventing CR, while sparing the immunosuppressive activities of TGF $\beta$ .

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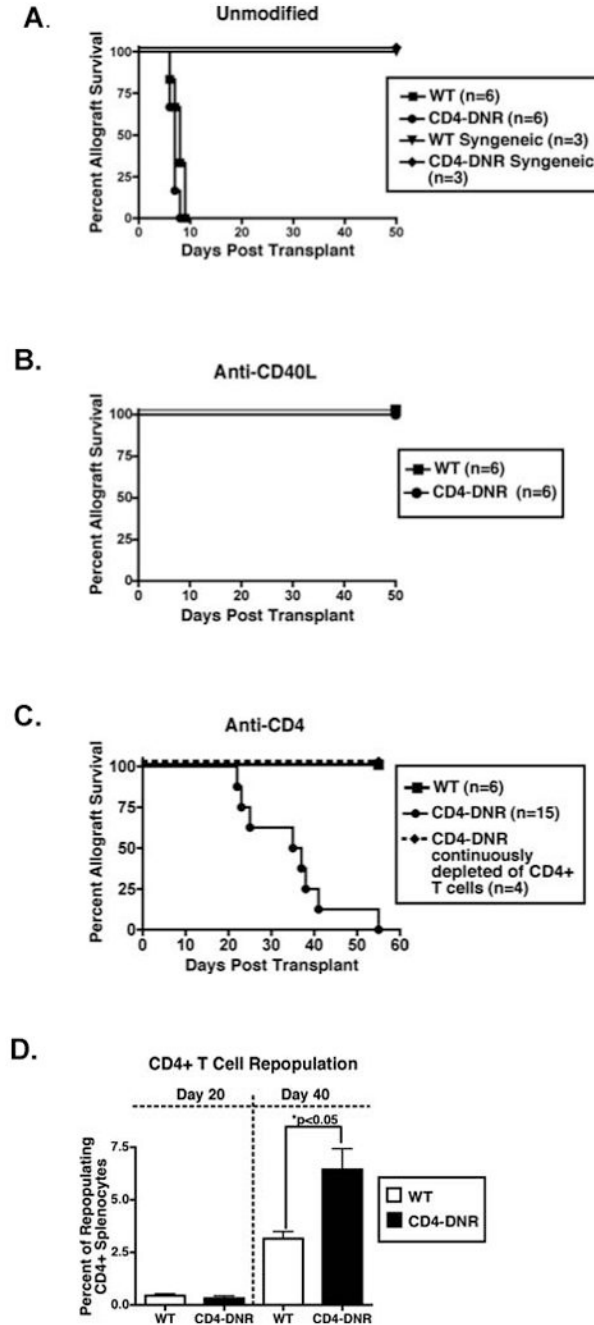
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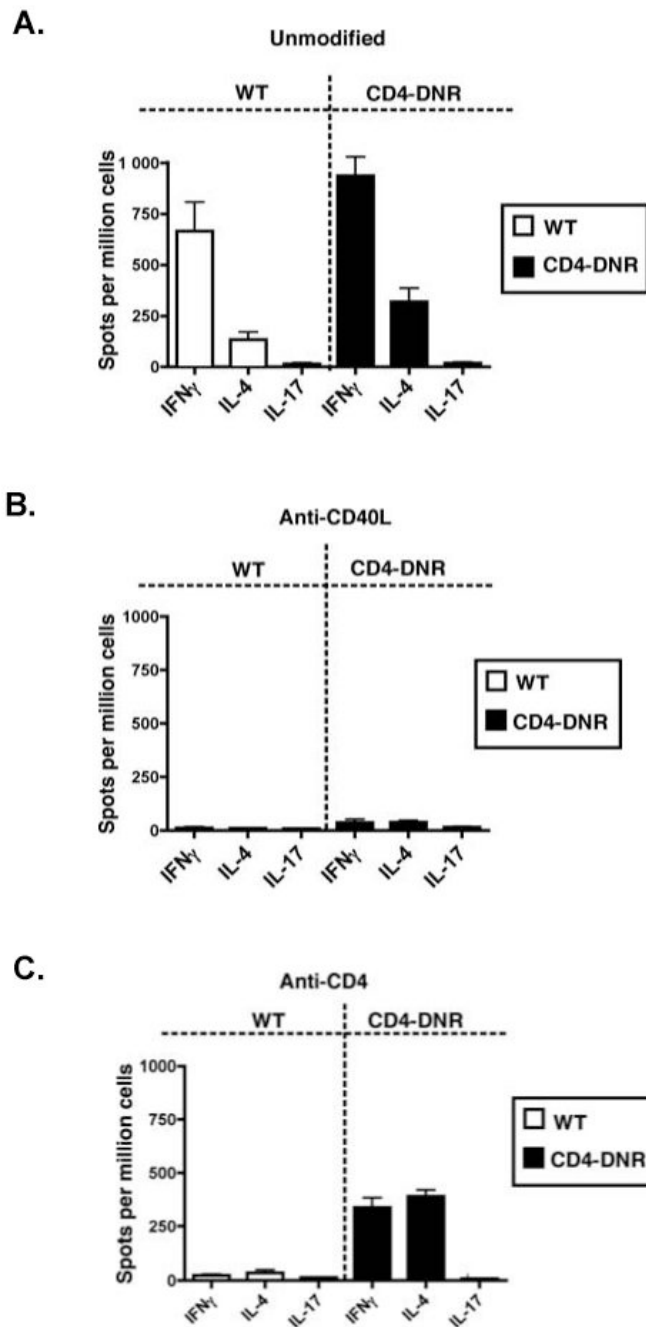
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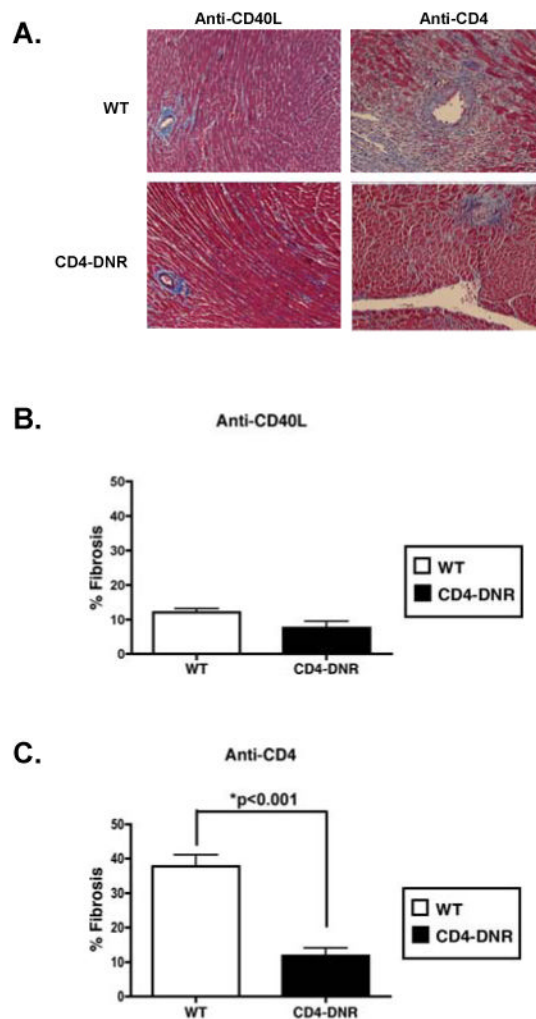
**Figure 1. TGFβ dependent and independent mechanisms of allograft acceptance**

WT (squares and triangles) and CD4-DNR (circles and diamonds) mice were transplanted with allogeneic or syngeneic cardiac grafts and were either left untreated (A), given inductive anti-CD40L mAb (B), or transiently depleted of CD4+ cells (C). Graft function was monitored by palpation and recipients were recovered either at the time of rejection or 50 days post-transplant. (D) Flow cytometric analysis of CD4+ splenocytes that were harvested from WT or CD4-DNR allograft recipients on day 20 and day 40 post-transplant. Allograft recipients were treated inductively with anti-CD4 mAb.

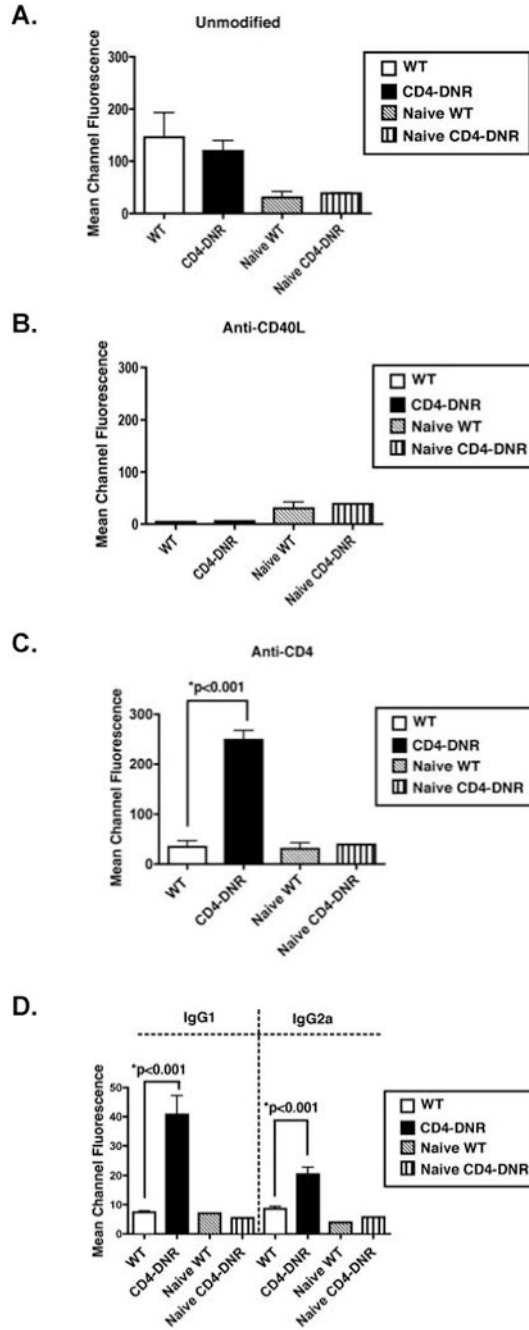




**Figure 2. Donor-reactive Th1, Th2 and Th17 responses in WT and CD4-DNR recipients**  
 WT (open bars) or CD4-DNR (shaded bars) allograft recipients were left untreated (A), treated with inductive anti-CD40L mAb (B), or treated with inductive anti-CD4 mAb (C). Graft function was monitored by palpation and recipients were recovered either at the time of rejection, 50 days post-transplant (for anti-CD40L mAb treated WT and CD4-DNR recipients), or 40 days post-transplant (for anti-CD4 mAb treated WT recipients in order to directly compare to CD4-DNR recipients, which reject between days 35-40). At the termination of the experiment, splenocytes were harvested and processed for ELISPOT assays to quantify primed, donor-reactive IFN- $\gamma$ , IL-4, or IL-17-producing cells. Bars represent the mean number of cytokine producing cells ( $\pm$  S.E.M.) from at least six recipients per group.



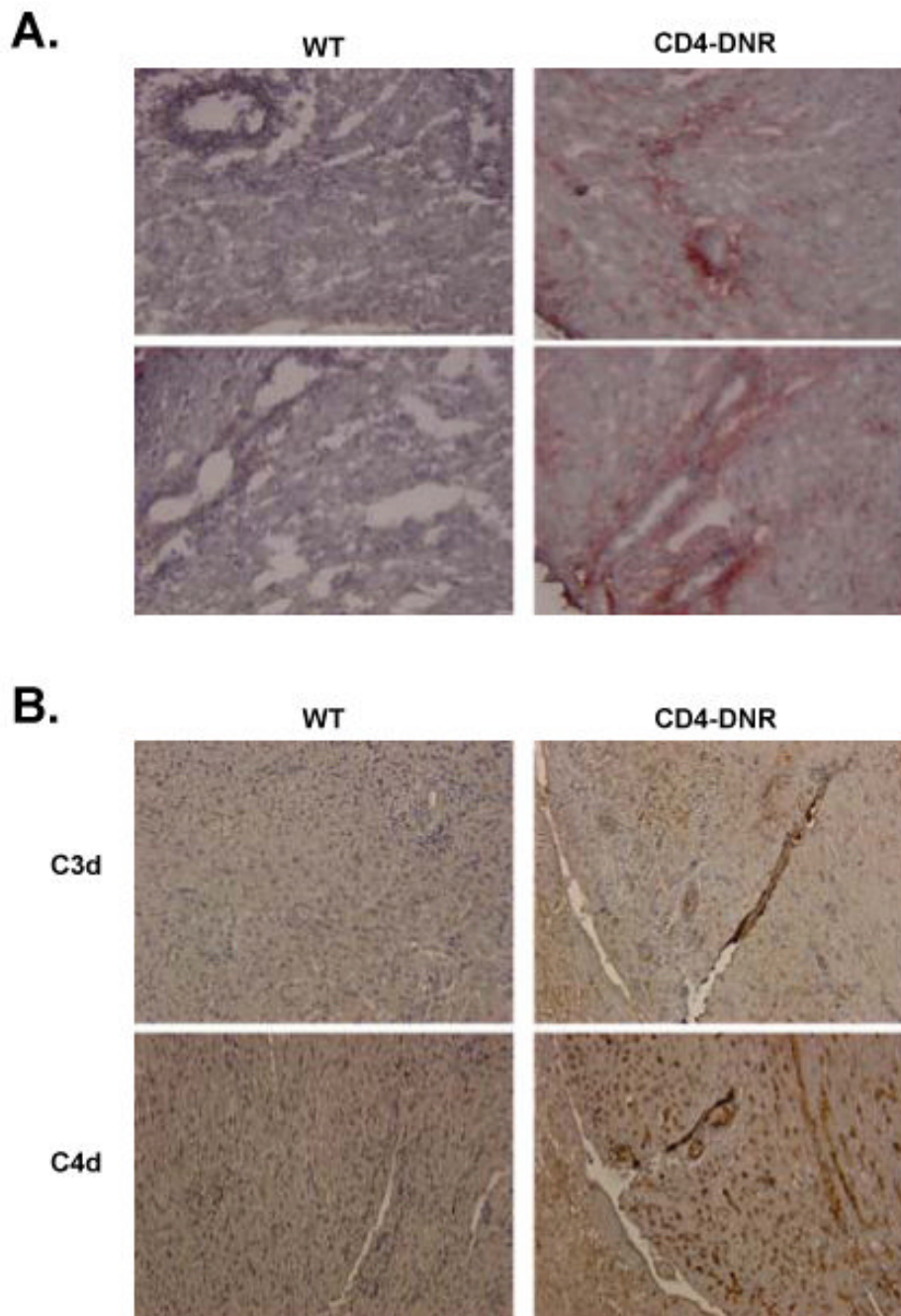
**Figure 3. Allograft fibrosis in WT versus CD4-DNR recipients treated with anti-CD4 mAb**  
 (A) Sections of grafts from recipients treated with inductive anti-CD40L (day 50 post-transplant) or anti-CD4 mAb (between days 35-40 post-transplant due to rejection in CD4-DNR recipients) were stained with Masson's trichrome, which stains fibrotic tissue blue. Frames are of grafts from WT and CD4-DNR recipients and are representative of at least 6 anti-CD40L mAb treated mice and 6-8 mice transiently depleted of CD4+ T cells. 200× magnification. (B) Quantification of mean fibrotic area by morphometric analysis in inductive anti-CD40L and (C) anti-CD4 mAb treated WT and CD4-DNR recipients. Bars represent the average percentage (+/- S.E.M.) of graft area positive for fibrosis in 5 anti-CD40L treated recipients and 6 anti-CD4 treated recipients. WT (open bars) and CD4-DNR (shaded bars).



**Figure 4. Effect of TGFβ unresponsiveness on donor-reactive alloantibody levels**

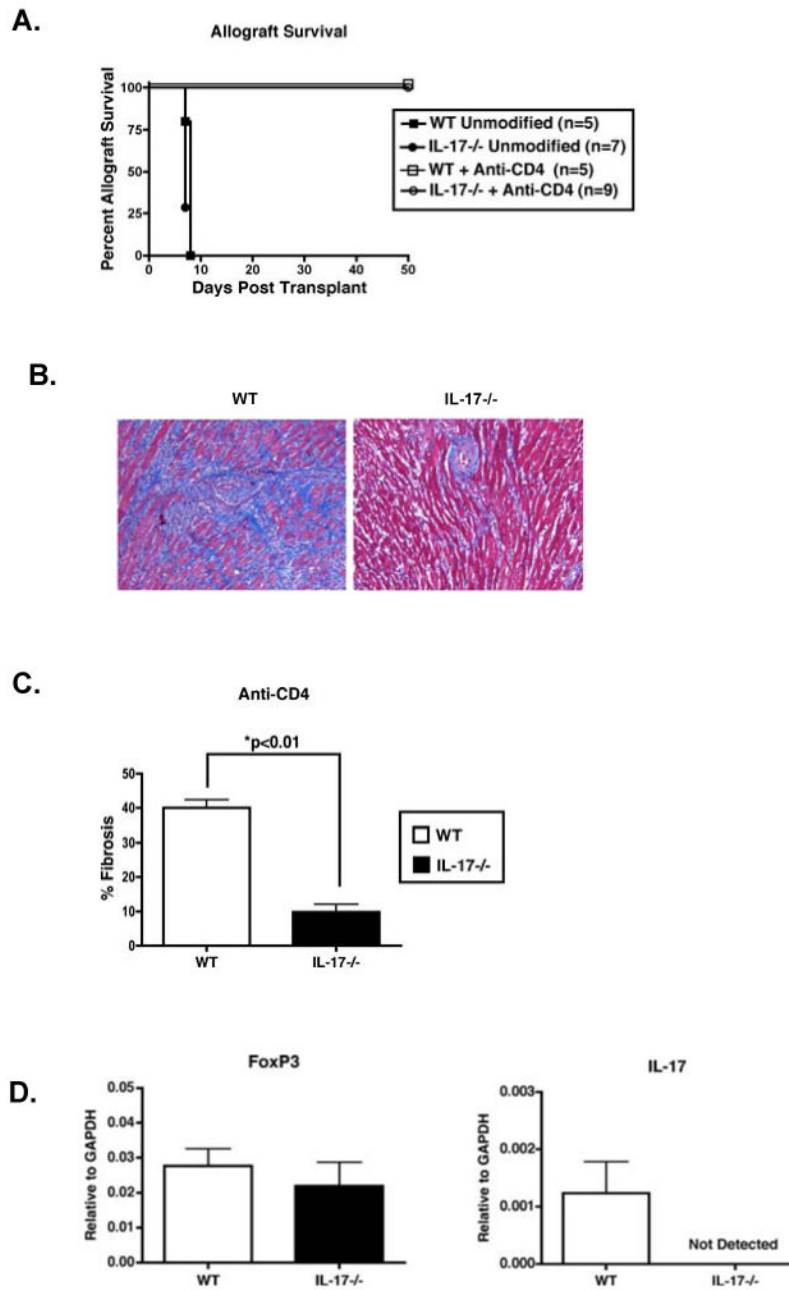
Sera were obtained from WT (open bars) or CD4-DNR (shaded bars) allograft recipients that were left untreated (A), treated with inductive anti-CD40L mAb (B), or treated with inductive anti-CD4 mAb (C). Sera were obtained at the time of rejection for unmodified recipients), 50 days post-transplant (for anti-CD40L mAb treated WT and CD4-DNR recipients), or 40 days post-transplant (for anti-CD4 mAb treated WT recipients). P815 (H-2<sup>d</sup>) cells were incubated with 1:50 dilution of sera and bound donor-reactive Ab were detected by incubation with FITC-tagged anti-IgG, anti-IgG1 or anti-IgG2a Abs. The mean channel fluorescence is indicative of the relative amount of donor-reactive antibodies. Bars represent the average mean channel fluorescence of at least 6 WT and 6 CD4-DNR recipient samples (+/- S.E.M.). IgG1 and IgG2a

donor-reactive antibody levels were analyzed in anti-CD4 mAb treated WT and CD4-DNR recipients (*D*).



**Figure 5. IgG, C3d and C4d deposition in allografts of WT and CD4-DNR recipients**  
 WT (left column) or CD4-DNR (right column) allograft recipients were treated with inductive anti-CD4 mAb therapy. Grafts were recovered at either the time of rejection or 40 days after transplantation. Graft sections were fixed and incubated with goat anti-mouse IgG (A) or the goat anti-mouse C3d or C4d (B) followed by development with 3-amino-9-ethylcarbazole or DAB to visualize mouse Ab and complement deposition. Results are representative of grafts from 6-10 recipients. Magnification, 400 $\times$  (A) and 200 $\times$  (B).





**Figure 7. In recipients that fail to produce IL-17, allografts are protected from fibrosis** (A) WT (squares) and IL-17<sup>-/-</sup> (circles) mice were transplanted with BALB/c cardiac allografts and were either left untreated (closed symbols) or transiently depleted of CD4<sup>+</sup> cells (open symbols). Graft function was monitored by palpation. Unmodified recipients were harvested at time of rejection, while inductive anti-CD4 mAb treated recipients were harvested at day 50 post-transplantation. Numbers in parentheses represent the number of recipients in each group. (B) Sections of grafts from recipients transiently depleted of CD4<sup>+</sup> T cells (day 50 post-transplant) were stained with Masson's trichrome stain. Frames are of grafts from WT and IL-17<sup>-/-</sup> recipients and are representative of at least 6 WT and 9 IL-17<sup>-/-</sup> recipient allografts. 200 $\times$  magnification. (C) Quantification of fibrosis by morphometric analysis. Bars represent the average percentage ( $\pm$  S.E.M.) of graft area positive for fibrosis in at least 5 WT and

IL-17<sup>-/-</sup> recipients treated with anti-CD4 mAb. WT (open bars) and IL-17<sup>-/-</sup> (closed bars).  
(D) On day 50 post-transplant, RNA was harvested from allografts of WT and IL-17<sup>-/-</sup> recipients transiently depleted of CD4<sup>+</sup> T cells. Intra-graft transcript levels of FoxP3 and IL-17 were assessed by real-time RT-PCR. Bars depict the means of RNA from 5 WT and 9 IL-17<sup>-/-</sup> grafts.