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Inhibition of Innate Co-Receptor TREM-1 Signaling Reduces CD4$^+$ T Cell Activation and Prolongs Cardiac Allograft Survival

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Correction made after online publication March 6, 2013: author names have been updated.

The innate receptor "triggering-receptor-expressed-on-myeloid-cells-1" (TREM-1) enhances downstream signaling of "pattern recognition receptor" (PRR) molecules implicated in inflammatory responses. However the mechanistic role of TREM-1 in chronic heart rejection has yet to be elucidated. We examined the effect of TREM-1$^+$ antigen-presenting cells (APC) on alloreactive CD4$^+$ lymphocytes. Bm12 donor hearts were transplanted into wild-type MHC-class-II-mismatched C57BL/6J recipient mice. Progressive allograft rejection of bm12-donor hearts with decreased organ function, severe vasculopathy and allograft fibrosis was evident within 4 weeks. TREM-1$^+$CD11b$^+$MHC-II$^+$F4/80$^+$CCR2$^+$ APC and IFNγ-producing CD4$^+$ cells were detected during chronic rejection. Peptide inhibition of TREM-1 attenuated graft vasculopathy, reduced graft-infiltrating leukocytes and prolonged allograft survival, while being accompanied by sustained low levels of CD4$^+$ and CD8$^+$ cell infiltration. Remarkably, temporary inhibition of TREM-1 during early immune activation was sufficient for long-term allograft survival. Mechanistically, TREM-1 inhibition leads to reduced differentiation and proliferation of IFNγ-producing Th1 cells. In conclusion, TREM-1 influences chronic heart rejection by regulating the infiltration and differentiation of CD4$^+$ lymphocytes.

Key words: Allograft survival, chronic rejection, heart transplantation, innate immunity, TREM-1

Abbreviations: APC, antigen presenting cells; IFNγ, interferon-gamma; IL, interleukin; LP17, synthetic peptide for TREM-1 inhibition; MHC, major histocompatibility complex; Myd88, myeloid differentiation primary response gene 88; PRR, pattern recognition receptor; TNF, tumor necrosis factor; TREM-1, triggering-receptor-expressed-on-myeloid-cells-1.

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Introduction

Despite advances in surgical technique, donor organ preservation and immunosuppressive agents, cardiac allograft vasculopathy caused by chronic rejection remains a major limitation to long-term survival in heart transplant recipients (1,2). Although the cellular mechanisms and cascade of events that initiate chronic rejection and vasculopathy remain poorly defined, the innate immune response appears to play an important role (3,4). The key cellular players for translating innate information into adaptive immunity are antigen-presenting cells (APC), such as dendritic cells and macrophages (5,6). APC express "pattern recognition receptors" (PRRs) which are an important family of innate receptors. Until now, the influence of innate immunity on allograft rejection, particularly that mediated by PRRs, remains poorly understood. A crucial function in PRR-dependent activation of the immune system is mediated by the co-receptor "triggering-receptor-expressed-on-myeloid-cells-1" (TREM-1). TREM-1 belongs to the immunoglobulin superfamily, is facultatively expressed on monocytes/macrophages and neutrophils, and upregulated by various proinflammatory stimuli such as pathogen-associated molecular patterns (PAMPs; Ref.7). Hence, it has been suggested that TREM-1 is involved in monocytic activation and inflammatory responses. Although no specific ligand has yet been identified, indirect evidence suggests that TREM-1 can recognize PAMPs secreted in response to tissue degradation or microbial infections by the host, amplifying immunological events and potentiate cytokine production (8–10). In fact, silencing of the TREM-1 gene significantly alters expression of several genes, including Myd88, CD14, IκBα, IL-1β, MCP-1 and IL-10 in response to treatment with LPS in macrophages (8,11). In a variety of inflammatory disorders, the selective inhibition of TREM-1 can be both therapeutically beneficial and serves as an important diagnostic marker (12). The functional activities of TREM-1 in chronic inflammatory diseases have yet to be elucidated; in particular their

influence on T cell differentiation and cytokine expression. Therefore, we have investigated the role of TREM-1 in the development of chronic allograft rejection.

Methods

Human tissues

All heart and kidney tissue specimens were obtained from surgical biopsies carried out 2 years posttransplantation, and recorded in the archives of the Department of Pathology in Regensburg. Inflammatory or infectious complications were absent in selected patients. Studies with human samples were approved by the local ethics committee (No. 04/56).

Animals

Ten- to 12-week-old female C57BL/6J (B6), Balb/c and female B6.C-H-2bm12/KhEg (bm12) mice, were purchased from The Jackson Laboratory (Bar Habor, MA, USA). B6.Myd88-deficient mice (Myd88$^{-/-}$) were provided by S. Akira (Osaka University, Osaka, Japan). All mice were housed at the animal facility in University Hospital Regensburg (Germany) under specific pathogen-free conditions. The mouse strains bm12 and B6 (wild-type or Myd88$^{-/-}$) differ at one locus of MHC-class-II antigen (I-A), but are otherwise identical. The mouse strain Balb/c has a class-I and class-II disparity to B6 mice.

Heart transplantation

Heterotopic heart transplantation was performed according to the method of Hasegawa et al. (13), with some modifications. To increase the rejection intensity, we performed a reduced donor heart perfusion via the abdominal vena cava with cold 0.9% saline (3 mL) containing 500 IE heparin (Ratiopharm, Ulm, Germany; Ref.14). Graft function was assessed by palpation of the abdomen and rejection was defined as cessation of cardiac contractility. All donor hearts had palpable contractions at the time of recovery (20, 30, 50 or 120 days). For the model Balb/c-B6, recipients were treated on day –1, 0 and 7 with 1 mg i.p. of αCD4 antibody (clone GK1.5; BioXCell, West Lebanon, NH, USA).

Inhibition of TREM-1 and treatment

TREM-1 inhibitory peptide: Starting on day 3 after transplantation mice were treated with either an antagonistic TREM-1 peptide, LP17 (LQVTDSGLYRCVIYHPP) or a sequence-scrambled control-peptide, (TDSRCVIGLYHPPLQVY), as previously described by Gibot et al. (15). The peptides were chemically synthesised (Pepscan Systems, Lelystad, Netherlands). Mice were treated once daily with 100-μg peptide, injected i.p. in 100 μL PBS.

TREM-1 antibody: Starting on day 3 after transplantation, recipients were treated with an αTREM-1 antibody (R&D Systems, Minneapolis, MN, USA). One hundred micrograms of αTREM-1 antibody in 100 μL PBS was administered per transplanted recipient by daily i.p. injection.

In vitro *analyses*

Naïve CD11b$^+$ cells from the spleen were isolated with MACS CD11b MicroBeads (Miltenyi Biotech, Gladbach, Germany) and cultured with LP17 to analyze the function of this selective peptide. Secretion of pro-inflammatory cytokines IL-6, IL-12(p70) and TNF following stimulation with LPS (1 μg/mL) or PGN (10 μg/mL) was measured (Figure S1A). In addition, induced cytokine expression following TREM-1 inhibition was also shown by siRNA transfection of J774 macrophages using a TREM-1 (#2697369, GeneID: 58217) and negative control siRNA (#2445448), both chemically synthesized by Qiagen (Hilden, Germany). J774 cells were stimulated with LPS or PGN and maintained in DMEM supplemented with 10% fetal calf serum (FCS), and 1% penicillin/streptomycin (both from Biochrom, Berlin, Germany) for 72 h. For transfection HiPerFect from Qiagen was used. We demonstrated a reduced expression of the cytokines IL-6, IL-12(p70) and TNF by J774 macrophages following incubation with TREM-1 siRNA (Figure S1B).

Histology and immunohistochemistry

Formalin-fixed and paraffin-embedded samples were used and sectioned (2–3 μm). Human: For immunohistochemical TREM-1 staining, sections were incubated with antibody-dilution-buffer (DCS Innovative Diagnostic Systems, Hamburg, Germany) for 1 h at room temperature. After washing with PBS (containing 0.3% Triton-X-100; Sigma, Munich, Germany), sections were stained with goat anti-human TREM-1 antibody over night at 4°C. Mouse: Prepared sections were stained with hematoxylin and eosin (H.E.) or Masson's trichrome. Immunohistological staining for TREM-1 was performed on paraffin sections. Frozen sections were used for CD11b, CD4, CD8 and Ki67 staining. Sections were blocked with 1% BSA (biomol, Hamburg, Germany), 10% goat serum (Sigma) or an antibody-dilution buffer.

After staining with the appropriate secondary antibody sections were incubated with SensiTek-HRP (ScyTec Laboratories, Logan, Utah, USA) and positive signals were visualized using a DAB-kit (3,3′-diaminobenzidine-tetrahydrochlorhydrate; Merck, Darmstadt, Germany) or AEC+ High-Sensitivity-Substrate-Chromogen-kit (Dako, Hamburg, Germany). For antibody specifications see Table S1. Images were captured using an Axio-Observer-Z1 microscope (Carl Zeiss, Oberkochen, Germany). For quantifying graft-infiltrating leukocytes, five high-power fields (HPF) were counted per slide. Histological evaluation of cardiac allograft rejection was performed according to the revised 2004 ISHLT-grading-system (16). Ki67 labeling index was defined using the ratio between Ki67$^+$CD4$^+$ and all CD4$^+$ cells on each slide.

Extraction of lymphocytes from cardiac grafts

Cardiac tissue was minced with a sterile razor blade and placed in 10 mL of RPMI-1640 medium (Gibco, Darmstadt, Germany) containing 10% FCS, 600 U/mL collagenase-II (Roche Diagnostics, Mannheim, Germany) and desoxyribonuclease-I (DNAse; Sigma). This mixture was slowly shaken at room temperature for 2 h and supernatant was flushed through a 100-μm-nylon cell strainer (Schubert & Weiss, Munich, Germany). Remaining tissue was again digested in RPMI-collagenase-DNAse solution at 37°C, strained and red blood cells were lysed with ACK lysis buffer (BioWhittacker, Lonza, MD, USA) The final solution containing cells (diluted in HBSS; Gibco) was passed through a 40-μm-nylon cell strainer.

Coculture experiments

CD4$^+$ cells were obtained and purified from allogeneic donor hearts (bm12-B6) following *in vivo* treatment with LP17 or control-peptide. Enrichment of CD4$^+$ lymphocytes was performed using the CD4$^+$ T cell Isolation Kit II (Miltenyi Biotech). Cells were cultured in RPMI-1640 medium (containing 0.1% β-mercaptoethanol (Gibco), 10% FCS and 1% penicillin/streptomycin). Splenocytes from a bm12 mouse were isolated and depleted of CD4$^+$ cells using MACS CD4$^+$ (L3T4) MicroBeads (Miltenyi Biotech) to obtain CD4$^-$ stimulator cells. 200 000 naïve CD4-depleted splenocytes were irradiated (30 Gy), mixed with 100 000 CD4$^+$ cells (isolated and purified from allografts) and kept at 37°C for 72 h without further stimulation. Cells were recovered and analyzed by flow cytometry.

RNA Isolation and real-time PCR

Cardiac allografts were recovered on day 20, 50 or 120 posttransplantation, and homogenized in 1 mL TRI-reagent (Sigma) for isolation of RNA. One microgram of total RNA was reverse transcribed using the AffinityScript^{TM}-QPCR-cDNA-Synthesis-Kit (Agilent Technologies, Böblingen, Germany). Real-time PCR was performed using the Roche LightCycler480 System. Primer sequences are listed in Table S2.

Flow cytometry

Leukocytes from recovered grafts were isolated and stained using fluorochrome-conjugated mouse-specific antibodies against CD45, CD3, CD4, CD8, CD11b, MHC-class-II (Isotype: rat-$IgG_{2B\kappa}$), F4/80 (Isotype: rat-$IgG_{2A\kappa}$), Foxp3, IFNγ and IL-17A (all from eBiosience, Frankfurt, Germany), GR-1 (Miltenyi Biotech), CCR2 (Isotpye: rat-IgG_{2B}) and TREM-1 (R&D, Wiesbaden-Nordenstadt, Germany). Intracellular cytokines were detected using a fixation and permeabilization protocol from eBiosience. Analyses were performed using a FACS Calibur or FACS-Canto-II flow cytometer (BD, Heidelberg, Germany). Data were obtained using BD CellQuest-Pro acquisition software (BD) and analyzed via FlowJo software (Tree Star Inc., Ashland, OR, USA).

Statistics

Graft survival comparisons were made using the log-rank (Mantel–Cox) test. All other data, unless otherwise specified, are shown as the mean $\pm$ standard error of the mean (SEM), and were compared using a two-tailed Student's test.

Results

Myd88 Deficiency prolongs allograft survival during chronic heart rejection

To investigate the effect of innate immune signaling during chronic heart rejection, we transplanted B6.C-H-2bm12/KhEg (bm12, allogeneic) or C57BL/6J (B6, syngeneic) donor hearts into B6 recipients. In this allogeneic situation bm12 grafts were rejected with a median survival time (MST) of 28 days, compared to a syngeneic control group where all grafts survived for >50 days (Figure S2A). Intimal proliferation, graft vasculopathy and allograft fibrosis were detected in the allogeneic combination (Figures S2B–D). Extensive leukocyte infiltration ($CD45^+$ cells), including $CD11b^+F4/80^+$ macrophages was observed in allografts analyzed on day 20 (Figure S3A). Although the proportion of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells in transplanted allografts was similar to those from syngrafts, the total number of graft-infiltrating $CD45^+$ cells was less in syngrafts compared to allografts (Figure S3B). In addition, a similar percentage of graft-infiltrating $CD4^+$ T cells from syngrafts and allografts were $Foxp3^+$ (Figure S3C). The absolute number of graft-infiltrating $CD4^+$ and $CD8^+$ cells was found to be significantly enhanced in allografts on day 20, as well as the proliferation (Ki67) of $CD4^+$ T cells (Figure S4).

Based on this model, we initially explored the importance of Myd88-mediated innate immune signaling during chronic allograft rejection. Innate immune responses are known to initiate and regulate inflammatory responses, as well immune homeostasis and underlie regulation by cell intrinsic factors. In this regard, there is evidence that TREM-1 activation regulates Myd88-signaling and amplifies proinflammatory responses (8,11,17). We found that in Myd88-deficient mice receiving bm12-donor hearts, allograft survival was prolonged in a significant proportion of recipients (Figures S5A and B). In addition, a reduced number of graft-infiltrating TREM-1^+ cells from $Myd88^{-/-}$ mice were observed (Figure S5C). This initial observation led to the conclusion that innate immune responses influence allograft survival. A detailed analysis of TREM-1 as an amplifying molecule in innate immune responses during organ rejection was then performed.

TREM-1 is upregulated during chronic rejection of transplanted organs in humans

Due to the likelihood that Myd88 depletion in terms of clinical application may not be practicably feasible, regulatory molecules influencing innate immune signaling via Myd88 were considered as potential targets. One potentially targetable molecule known to amplify Myd88-mediated intracellular signaling is the cell membrane bound co-receptor TREM-1. Indeed, TREM-1 has shown its importance in the development of human inflammatory disorders (9,18–20). Our analyses of rejecting human organ transplants undergoing rejection revealed TREM-1^+ cells in tissue biopsies of donor-hearts from patients with allograft vasculopathy and chronic allograft rejection (Figure 1A). In contrast, there was no detectable TREM-1^+ cell infiltration in heart transplant biopsies lacking an allograft rejection signature (Figure 1B). A similar pattern of TREM-1^+ cell infiltration was identified in biopsies from patients undergoing chronic kidney rejection (Figure S6).

Increased TREM-1⁺ APC following allograft rejection in mice

The finding in human transplant biopsies was corroborated in murine grafts undergoing chronic rejection, where a marked and progressive infiltration of TREM-1^+ cells in rejecting organ transplants was observed (Figures 1C and S7A). Determination of TREM-1^+ cells in spleen and peripheral blood revealed no appreciable differences between syngeneic or allogeneic transplanted recipients following rejection (Figure S7B). Further characterization of the cellular infiltrate in the grafts revealed that chronic rejection was closely associated with an increased appearance of $CD11b^+GR\text{-}1^{intermediate}TREM\text{-}1^+$ cells with co-expression of MHC-class-II, F4/80 and CCR2 (Figures 1D and S8). $CD11b^+GR\text{-}1^{high}$ cells (granulocytes) show limited TREM-1 expression. Collectively, these data indicate that TREM-1^+ APC are frequently present in the graft during chronic rejection.

TREM-1 inhibition attenuates chronic rejection and reduces graft fibrosis

To evaluate the importance of TREM-1 in mediating rejection, a TREM-1-specific inhibitory peptide, LP17, was injected i.p. into B6 recipients with transplanted donor hearts. Inhibition of TREM-1 with LP17 resulted in a significant increase in allograft survival (>50 days) compared to control-peptide-treated recipients (Figure 2A). Bm12 grafts on day 20 from control-peptide-treated animals showed typical histological signs of rejection; compared to the LP17-treated group on day 20 and 50 (Figure 2B). More specifically, assessment of allograft fibrosis in the allogeneic transplanted group showed that TREM-1 inhibition

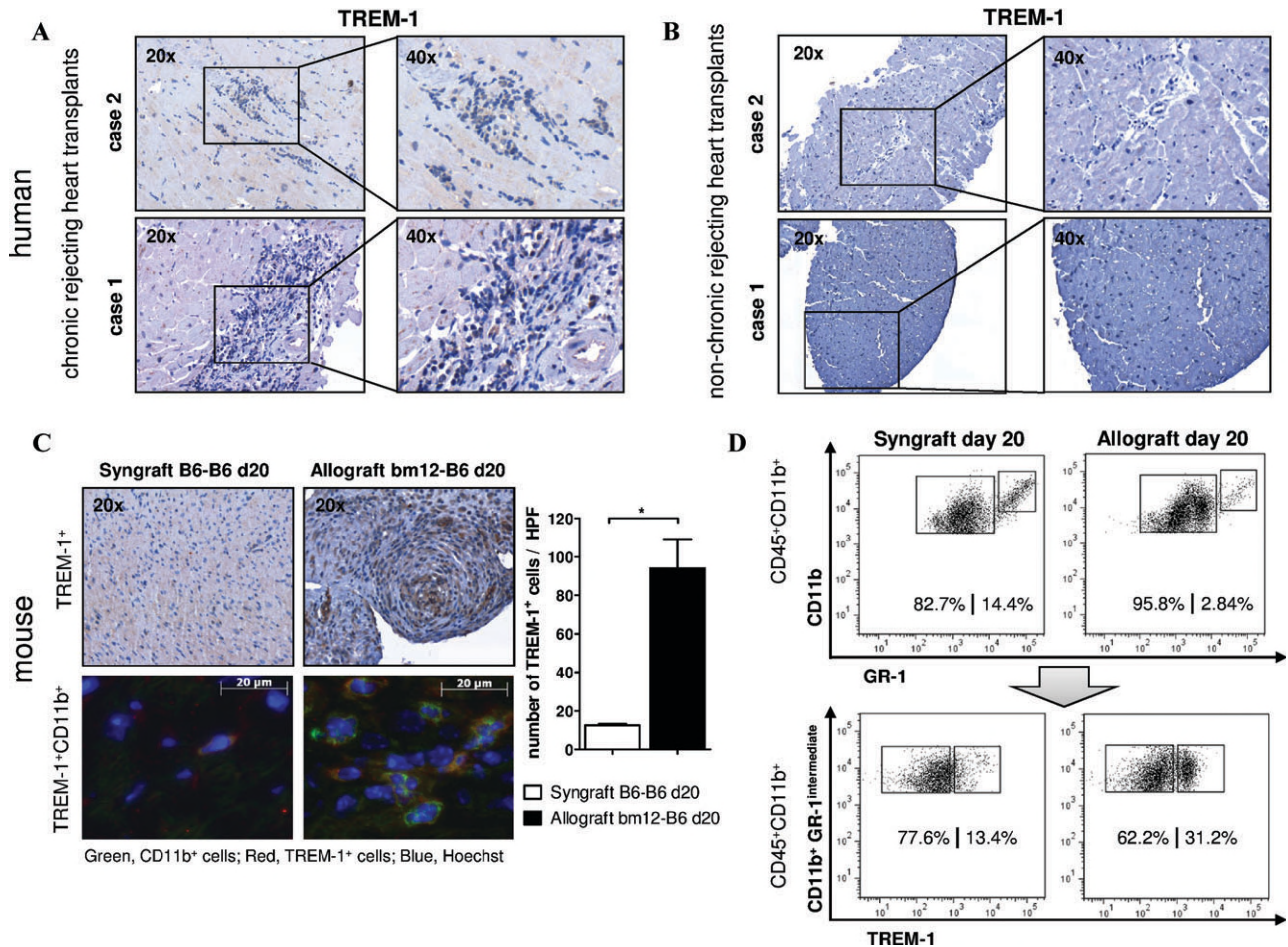

Figure 1: TREM-1$^+$ cells in human and mouse allografts posttransplantation. (A) Heart biopsy from a 46-year-old male. A biopsy was taken 2 years after transplantation and TREM-1$^+$ cell infiltration was found in immunohistochemical (IHC) staining. (B) A biopsy from two male patients after heart transplantation showing no signs of heart rejection and no TREM-1$^+$ cell infiltration (magnification, ×20 and ×40). (C) In the mouse model, donor hearts were recovered on day 20 posttransplantation; number of TREM-1$^+$ cells from syngen (B6-B6) and allogen (bm12-B6) transplanted hearts (n = 5) were counted per high-power field (HPF; n = 5; $^*P \leq 0.05$; ±SEM). Representative IHC of TREM-1$^+$ cells and immunofluorescence double-staining (IF) for TREM-1$^+$CD11b$^+$ cells are shown. (D) Representative flow cytometric data demonstrates CD45$^+$CD11b$^+$GR-1^{intermediate}TREM-1$^+$ cells in the syngraft and allograft on day 20 (n = 5).

results in a significant reduction in collagen deposition (Figure 2B and C). To elucidate the effects of LP17 on long-term cardiac survival, the frequency of TREM-1$^+$ APC was analyzed. It was found that these putative innate immune cells were significantly reduced in the LP17-treated group compared to the control-peptide-treated group on day 20 and 50 (Figures 3 and S9A). Therefore, we suggest that an attenuated inflammatory effect could be achieved by blocking TREM-1 engagement.

TREM-1 inhibition limits T cell responses during chronic rejection

In the bm12 model differentiated T lymphocytes contribute mainly to the development of vascular inflammation and vasculopathy (21–23). Therefore, the potential for TREM-1 inhibition to dampen allograft rejection by innate immune activation consecutively mediated through CD4$^+$ and CD8$^+$ T cells was assessed. As predicted, grafts from allogeneic transplanted recipients in combination with LP17 displayed reduced infiltration by CD4$^+$ and CD8$^+$ cells on day 20 and 50 (Figure 4A). Moreover, the frequency of proliferating Ki67$^+$CD4$^+$ T lymphocytes in allografts was reduced by TREM-1 inhibition (Figure 4B). TREM-1 inhibition led to fewer graft-infiltrating regulatory T cells with an unchanged relative number of Foxp3$^+$ Treg amongst total CD4$^+$ T cells (Figure S9B and data not shown). During continuous LP17 treatment until day 50, the percentage of proliferating CD4 T cells increased slightly, but still remained lower than cells from control-peptide-treated mice on day 20.

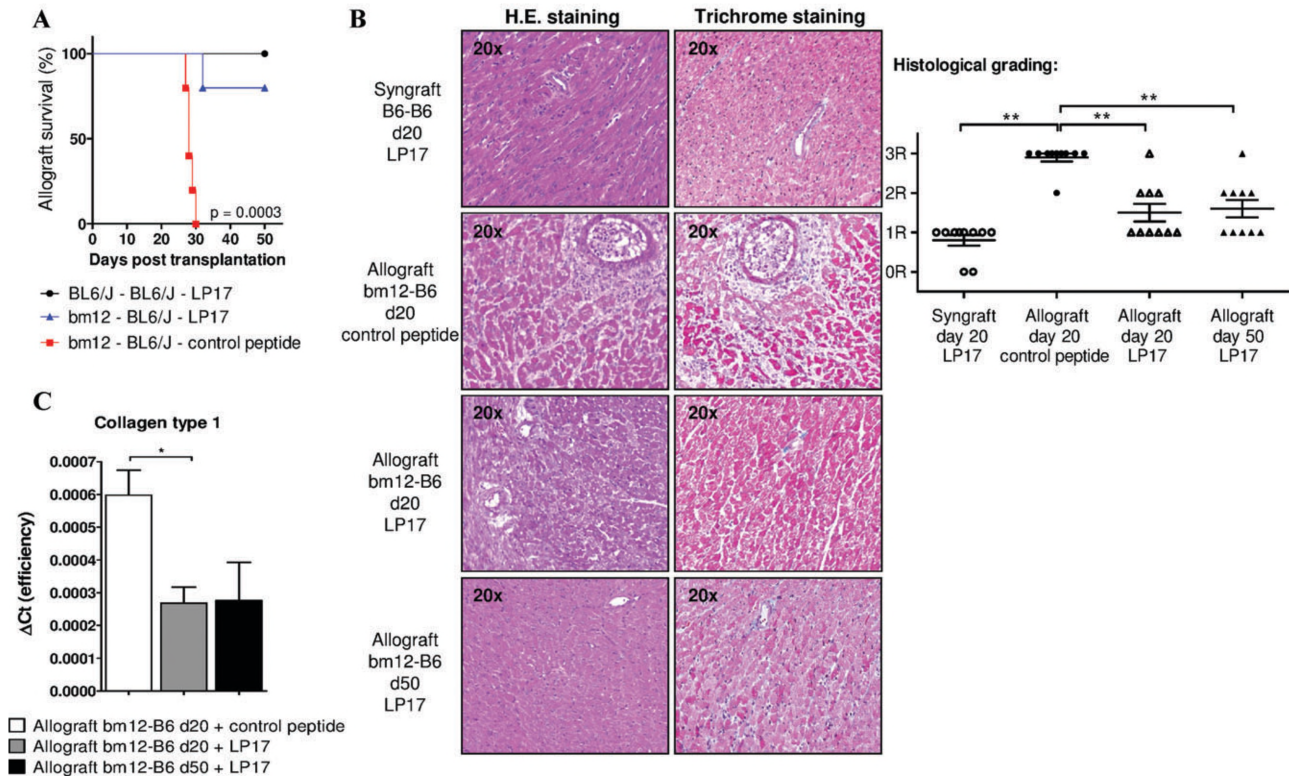

Figure 2: TREM-1 inhibition attenuates chronic heart rejection and reduces graft fibrosis. (A) Allograft survival from LP17 and control-peptide-treated syngeneic and allogenic transplanted hearts (n = 5 per group). (B) Hematoxylin and eosin (H.E.) or Masson's trichrome staining (fibrotic tissue appears blue) of bm12 cardiac allografts from LP17-treated recipients on day 20, 50 and control-peptide-treated mice on day 20 (magnification, ×20). Histological grading of allograft in H.E. stained sections of cardiac allografts treated with LP17 or control-peptide (n = 10; $^{**}P < 0.001$; ±SEM). (C) *Collagen type 1* expression (qPCR) on day 20 and 50 after transplantation in the allograft after LP17 or control-peptide treatment (allogeneic groups n = 4; syngeneic group n = 3; $^{*}P \leq 0.05$; ±SEM). While day 50 demonstrates the sustained effect of TREM-1 inhibition, grafts from control-treated recipients have already rejected at this time. Representative data are relative to β-actin and assessed by qPCR.

TREM-1$^+$ APC induce alloreactive Th1 T cell differentiation

APC are known to induce T-helper cell responses during inflammatory processes (5,24). To further evaluate the question whether TREM-1 inhibition can affect alloreactive T cell differentiation, CD4$^+$ cells were isolated from allografts on day 20 after transplantation following treatment with control-peptide or LP17, and were cocultured with CD4-depleted donor splenocytes (bm12). Expression of IFNγ by graft-infiltrating CD4$^+$ T cells (flow cytometry) was significantly reduced in LP17-treated mice compared to controls. Therefore, blocking TREM-1 by an inhibitory peptide influences the differentiation of alloreactive Th1 cells. Regarding Th17 cell differentiation, we detected a slight increase in T-helper lymphocytes producing IL-17A following TREM-1 inhibition (Figure 4C).

A second approach of blocking TREM-1 activation with an αTREM-1 antibody confirmed our previous findings by showing reduced cardiac vasculopathy associated with reduced graft-infiltrating cells and decreased fibrotic allograft remodeling (Figure 5A). TREM-1$^+$ APC were fewer in grafts of αTREM-1 antibody-treated recipients (Figures 5B and C), while the expression of surface antigens on APC remained unchanged (Figure S9C). The number of CD4$^+$ lymphocytes in transplanted grafts after αTREM-1 antibody treatment was reduced (Figure 6A), similar to the observation with LP17 treatment, and the differentiation of alloreactive Th1 cells was less apparent (Figure 6B). Graft-infiltrating Tregs were fewer in grafts of αTREM-1 antibody-treated recipients compared to the isotype-treated control group, whereas the percentage of Foxp3$^+$ Treg amongst total CD4$^+$ T cells remained unchanged (Figure S9D and data not shown).

We have extended our findings in a model of chronic allograft rejection based on a full antigen mismatch (Balb/c donor hearts into B6 recipients), which includes treatment with αCD4 antibody on day −1, 0 and 7 after transplantation. In this model, allografts from B6 recipients transiently depleted of CD4$^+$ T cells and treated with the control-peptide revealed extensive fibrosis compared to

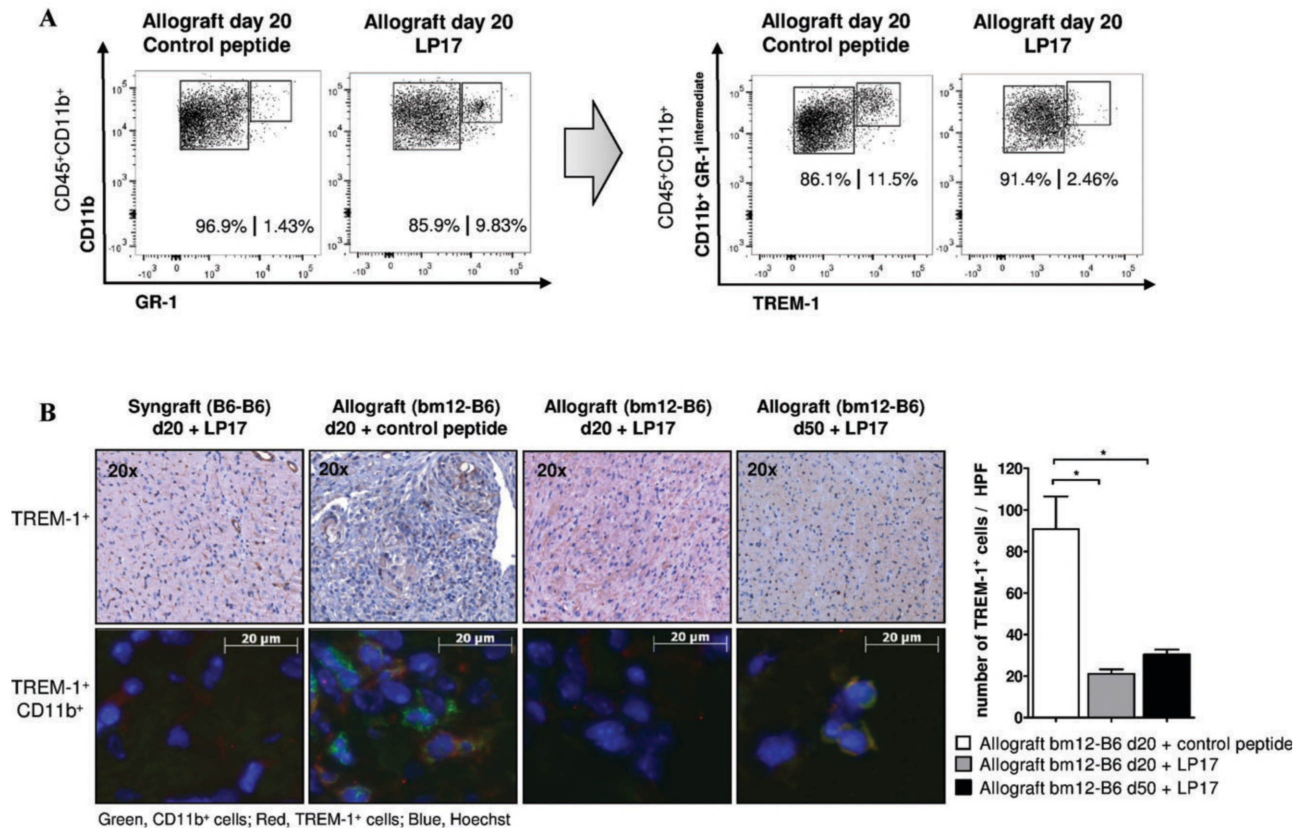

Figure 3: Blocking of TREM-1 leads to reduction of graft-infiltrating antigen-presenting cells. (A) Representative flow cytometric dot plot from CD11b$^+$GR-1$^+$ cells (left-hand side) and CD11b$^+$GR-1^{intermediate}TREM-1$^+$ cells (right-hand side) isolated from transplanted grafts of control-peptide- or LP17-treated recipients on day 20 posttransplantation (n = 5). (B) TREM-1 IHC staining and TREM-1/CD11b double-IF of representative sections on postoperative day 20 and 50 of mice treated with LP17 or control-peptide. TREM-1$^+$ cells per HPF were counted from 5 mice per group (magnification, ×20; $^*P \le 0.05$; ±SEM).

allografts from LP17-treated recipients (Figure 7A and B). While the number of CD11b$^+$GR-1^{intermediate} cells was only slightly reduced with LP17 treatment, graft-infiltrating TREM-1$^+$ cells were significantly fewer (Figure 7C). Regarding CD4$^+$ cells, their numbers were reduced after TREM-1 inhibition (Figure 7D). Together, our results from different blocking treatment strategies and murine models indicate that TREM-1 inhibition *in vivo* reduces the potential of APC to induce effector T cell differentiation and proliferation.

Temporary TREM-1 inhibition is sufficient to prolong allograft survival

It is well established that innate immune responses in initial phases of host protection are necessary to orchestrate further effects mediated by the adaptive immune system. Therefore, we were interested in determining whether temporary inhibition of TREM-1 is sufficient to reduce rejection when applied only during the initial phase following cardiac transplantation. Recipients received LP17 posttransplantation up to day 50, and then were further treated with control-peptide out to 150 days; a second group of mice continued to receive treatment with LP17 until day 150 (Figure 8A). As shown in Figure 8B, continuous TREM-1 inhibition resulted in 100% allograft survival, while withdrawal of treatment from day 50 on resulted in 80% allograft survival. Both groups revealed no detectable differences regarding histological signs of heart rejection or fibrosis (Figure 8C and D), but significantly fewer TREM-1$^+$ cells were detected on day 120 after transplantation (Figure 9A). The numbers of CD4$^+$ and CD8$^+$ T cells were not substantially affected by stoppage of TREM-1 blockade (Figure 9A). Finally, graft-infiltrating CD4$^+$ cells following both initial and continuous TREM-1 inhibition showed decreased proliferation rates compared to rejecting allografts on day 20 without treatment (Figure 9B).

Discussion

In this study it is shown that expression of TREM-1 is upregulated during allograft rejection in humans and mice. In addition, TREM-1 appears to be a key molecule in promoting inflammatory cell recruitment into allografts.

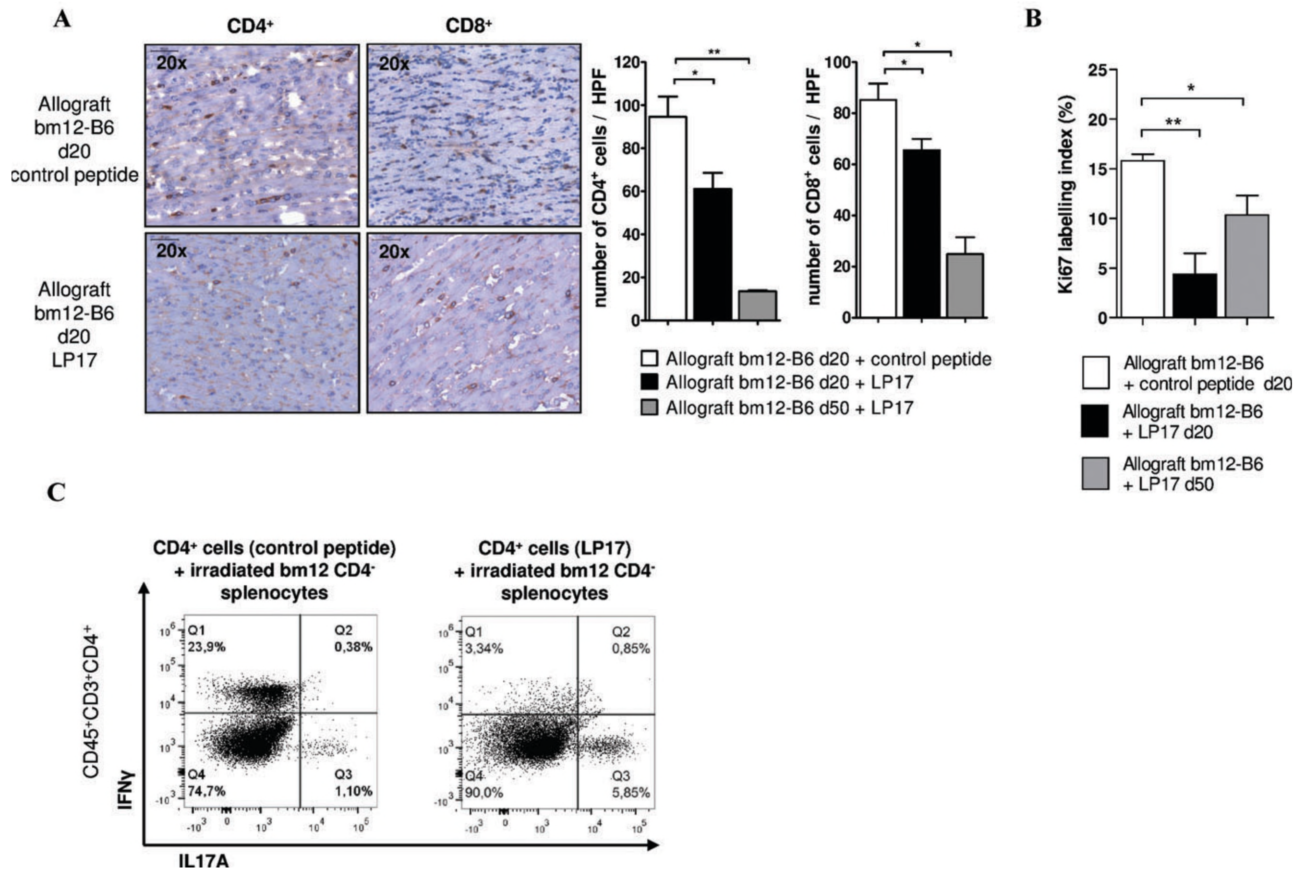

Figure 4: Inhibition of TREM-1 results in diminished T cell infiltration and reduction of IFNγ expression. (A) Staining of graft-infiltrating $CD4^+$ and $CD8^+$ cells on day 20 and 50 posttransplantation (magnification, ×20). $CD4^+$ and $CD8^+$ cells per HPF were counted from 5 mice per group ($^*P \leq 0.05$; $^{**}P < 0.001$; ±SEM). (B) Ki67 labeling index of graft-infiltrating $CD4^+$ T cells n = 5; $^*P \leq 0.05$; $^{**}P < 0.001$; ±SEM) on day 20 and 50. Recipients were treated with LP17 or control-peptide. (C) Representative cytokine production of $CD4^+$ cells from donor hearts of LP17- or control-peptide-treated recipients. $CD4^+$ graft-infiltrating cells obtained on day 20 after transplantation were pooled (three mice per treatment group) and stimulated with irradiated CD4-depleted donor bm12 splenocytes for 72 h; cytokines were measured by flow cytometry. One of two independent experiments is shown.

Mechanistically, inhibition of TREM-1 leads to APC-mediated reduction of T cell proliferation and cytokine expression.

Although the exact pathogenesis of chronic rejection remains unclear, there is strong evidence that innate immune responses leading to chronic rejection are initiated by both extracellular (e.g. TLR) and intracellular factors (e.g. Myd88; Refs.25,26). Consistent with this idea, it has been proposed that Myd88 has a crucial function in the pathogenesis of allograft rejection (27–29). In our experimental study we could extend this theory by demonstrating that Myd88 deficiency attenuates chronic forms of cardiac allograft rejection in mice. A feature of the Myd88 deficiency in this model is a decreased presence of cell infiltration and vasculopathy. While these results suggest Myd88 would be a good target to potentially reduce chronic transplant rejection, its indispensability in a variety of inflammatory and homeostatic processes expose substantial risks if the molecule were directly inhibited. However, modulating the innate immune system by influencing cell intrinsic regulatory mechanisms (26,30–33), such as TREM-1 (7,9,18,19,34) is feasible. TREM-1 expression is upregulated in response to microbial products and inflammation caused by bacteria and fungi. Although crosslinking TREM-1 alone induces cellular activation and proinflammatory cytokine secretion, it can act as a TLR pathway amplifying molecule (17,34,35). Silencing TREM-1 in LPS-treated macrophages alters expression of several genes including, i.e. Myd88 (8,11). It is logical, therefore, that considering a role for Myd88 in heart rejection and graft fibrosis (28,36), a reduced number of graft-infiltrating TREM-1^+ cells were found in $Myd88^{-/-}$ transplant recipients. Following this view, a key initial observation in the present study was that chronic allograft rejection in humans is accompanied by elevated numbers of graft-infiltrating TREM-1^+ cells, identifying a possible novel target for therapeutic intervention against chronic rejection.

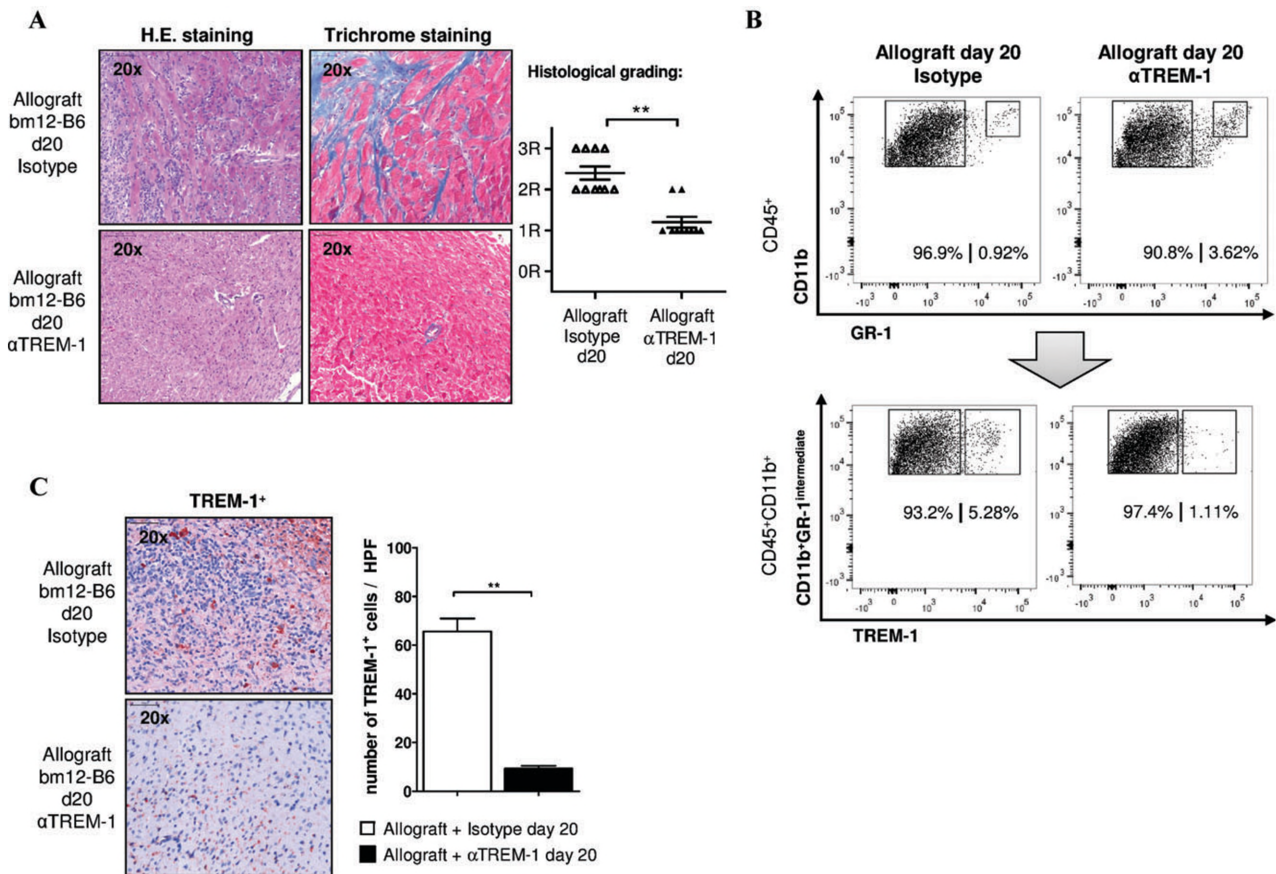

Figure 5: Blocking of TREM-1 with a monoclonal α TREM-1 antibody favors allograft survival. (A) H.E. and Masson's trichrome staining (fibrotic tissue appears blue) of bm12 cardiac allografts from recipients on day 20 receiving either the isotype control or αTREM-1 antibody (magnification, ×20). Histological grading of allografts is shown (n = 10; $^{**}P < 0.001$; ±SEM). (B) Representative flow cytometry plots demonstrate CD11b$^+$GR-1$^+$ cells and CD11b$^+$GR-1^{intermediate}TREM-1$^+$ cells isolated from grafts of αTREM-1 and isotype antibody-treated recipients on day 20. One of two independent experiments is shown. (C) IHC staining of TREM-1$^+$ cells following injection of recipients with αTREM-1 or isotype control antibody (magnification, ×20); grafts were recovered on day 20. TREM-1$^+$ cells per HPF were counted from five mice per group ($^{**}P < 0.001$; ±SEM).

To gain further insight into operative mechanisms and feasibility of therapy, we explored TREM-1 in two murine chronic rejection models. As in the human samples, an increased infiltration by TREM-1$^+$ cells in murine allografts during chronic rejection was identified. Regarding the kinetics of TREM-1$^+$ cell infiltration into transplanted allografts, we demonstrated that the number of graft-infiltrating TREM-1$^+$ cells gradually increased over time. The cellular population expressing TREM-1 was found to have a macrophage phenotype expressing CD11b, GR-1^{intermediate}, CCR2, F4/80 and MHC-class-II. These findings support the general contention that modulation or inhibition of TREM-1 activity might be useful in regulating chronic immune responses mediated by APC during allograft rejection, mindful also of the fact that macrophage depletion has been shown to reduce cardiac allograft vasculopathy and improve long-term transplant outcomes (37). Our approach using the antagonistic TREM-1-derived peptide LP17 (or TREM-1-specific blocking antibody) shows that chronic rejection is substantially reduced following continuous or even temporary TREM-1 blockade, and, interestingly, this effect is associated with a decreased number of graft-infiltrating lymphocytes, as well as macrophages. In summary, TREM-1 is likely most critical during the early-induced immune response due to induction of proinflammatory cytokines by APC, mediating the recruitment, activation and differentiation of lymphocytes. Importantly, TREM-1 intervention during this early period appears to set in motion an immunological process that is sustainable even after TREM-1 blockade is removed.

Regulation of T cell activation and infiltration into the allograft is of utmost importance for maintaining long-term allograft survival (14,24,29,38,39). In this respect, previous reports indicate that chronic rejection is contingent upon the presence of CD4$^+$ T cells (22,40,41). We asked whether TREM-1$^+$ APC were critical for the

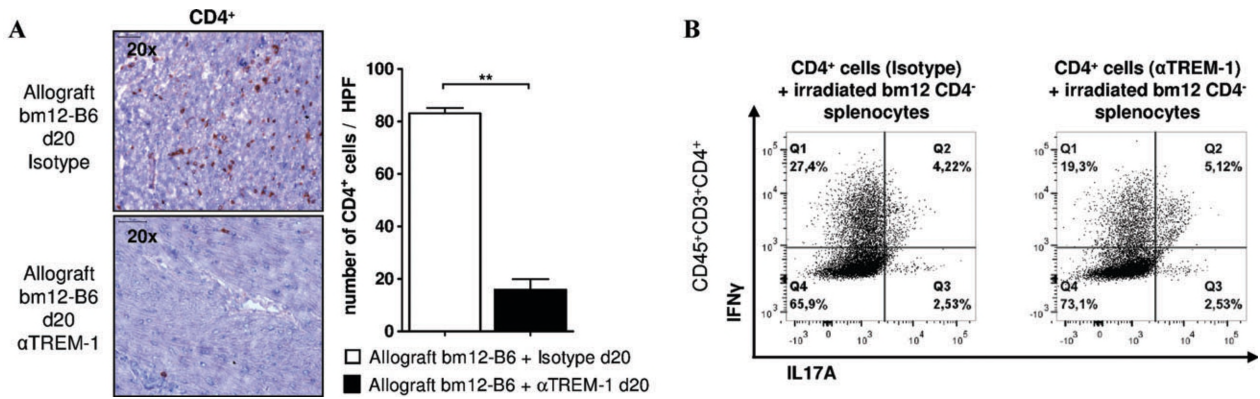

Figure 6: Reduced T cell infiltration in allografts of recipients following α TREM-1 antibody treatment. (A) Representative sections of CD4$^+$ lymphocytes in transplanted hearts on day 20 following αTREM-1 or isotype control antibody treatment (magnification, ×20). Number of cells counted per HPF is shown (n = 10 in each group; $^{**}P < 0.001$; ±SEM). (B) CD4$^+$ cells were purified from allografts on day 20 after transplantation following antibody treatment and were cocultured with CD4$^-$ donor splenocytes (bm12); cytokines were determined by flow cytometry. One of two independent experiments is shown.

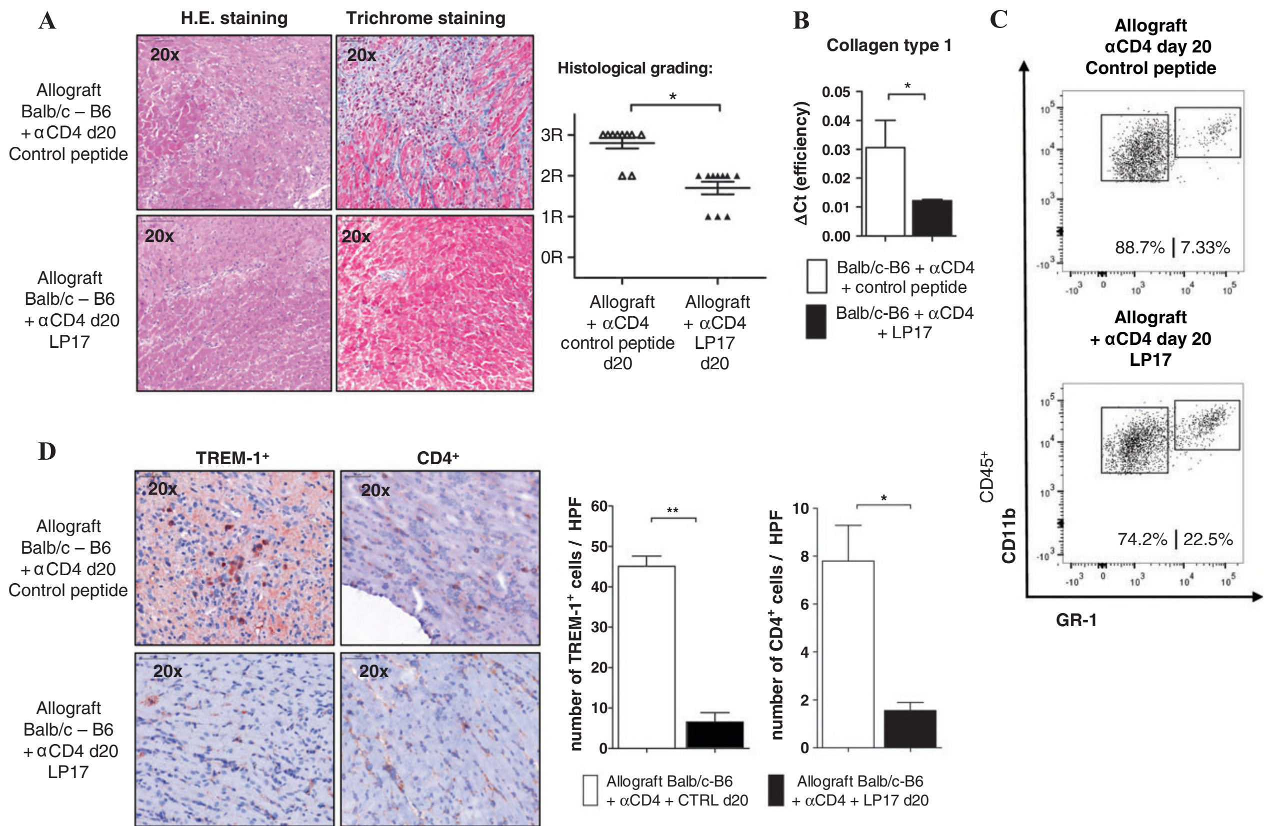

Figure 7: Transient depletion of CD4$^+$ T cells results in fibrotic organ remodeling and is blocked by TREM-1 inhibition. Balb/c donor hearts were transplanted into B6 recipients and treated with αCD4 antibody on day −1, 0 and 7 after transplantation. (A) Representative H.E. sections and trichrome staining (fibrotic fibers appear blue) from recovered allografts on day 20 posttransplantation (magnification, ×20). Histological grading results are shown (n = 10; $^{*}P \leq 0.05$; ±SEM). (B) ΔCT of *collagen type 1* expression on day 20 after transplantation (n = 5; $^{*}P \leq 0.05$; ±SEM). Data shown are relative to β-actin and assessed by qPCR. (C) Representative flow cytometric dot plot from CD11b$^+$GR-1$^+$ cells isolated from transplanted grafts of control peptide or LP17-treated recipients on day 20 posttransplantation. One of two independent experiments is shown. (D) Representative IHC staining of TREM-1$^+$ and CD4$^+$ graft-infiltrating cells (magnification, ×20). Number of cells per HPF is shown quantitatively (n = 5–8 in each group; $^{*}P \leq 0.05$; $^{**}P < 0.001$; ±SEM).

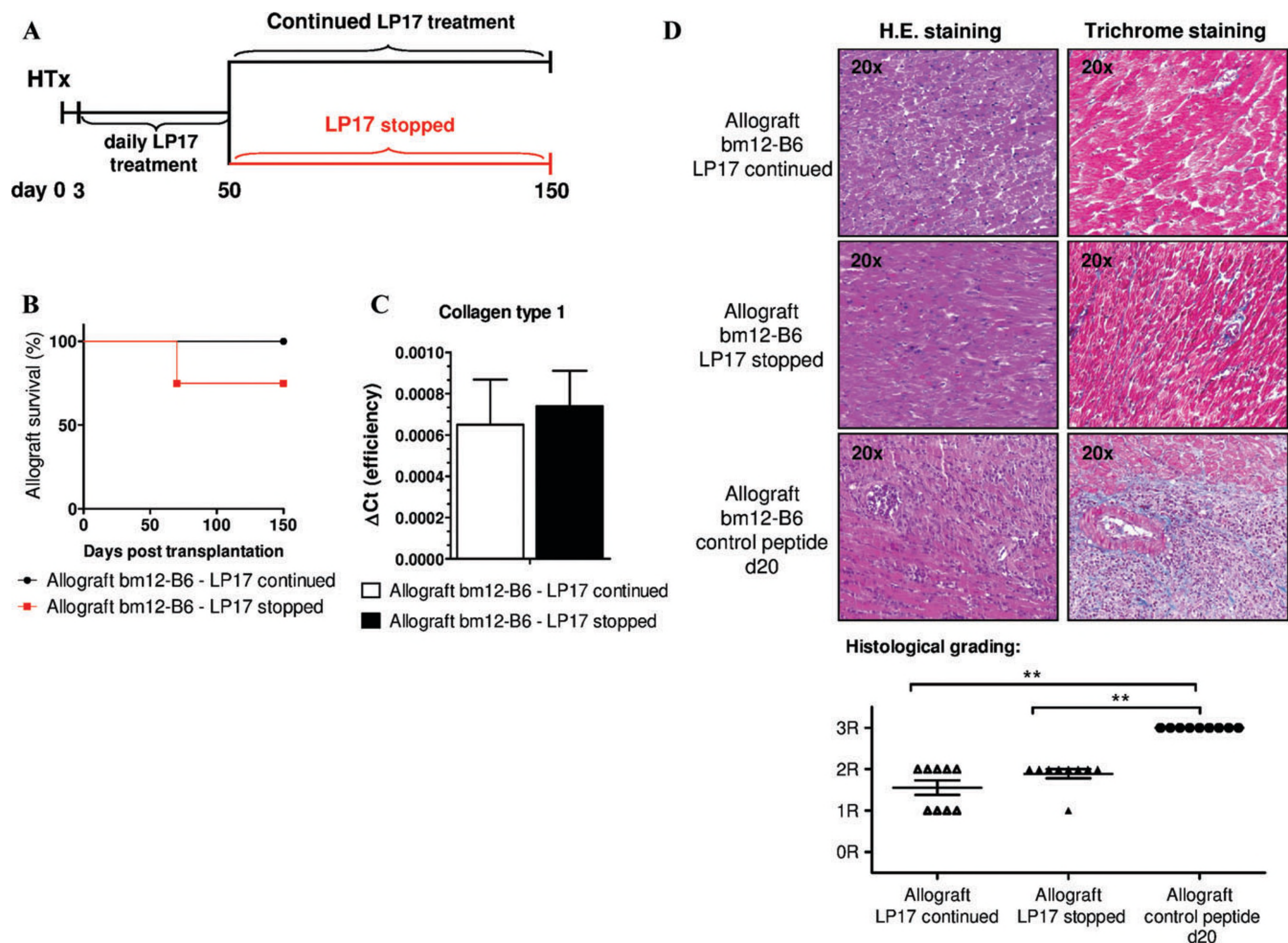

Figure 8: Initial inhibition of TREM-1 during early immune activation resulted in reduction of graft-infiltrating cells. (A) Experimental design. Heart transplantation (HTx) was performed in B6 wild-type recipients; starting on day 3 recipients were treated with LP17 up to day 50. After day 50, the groups were divided and treated either with LP17 or control-peptide out to 150 days (black line, continued TREM-1 inhibition; red line, LP17 stopped; n = 5 in each group). (B) Heart transplant survival (bm12 donor hearts to B6 recipient mice) from mice treated continuously with LP17 or mice that had stopped LP17 treatment (n = 4). (C) *Collagen type 1* expression following initial or early TREM-1 inhibition on day 120 after transplantation. Data represented are relative to β-actin (qPCR; n = 3; ±SEM). (D) H.E. staining of representative cardiac sections in B6 wild-type recipients with continued LP17 treatment or LP17 stopped (magnification, ×20). Histological grading of allograft rejection is shown in H.E. stained sections; Masson's trichrome staining of bm12 cardiac allografts on day 120 is also shown (n = 9; $^{**}P < 0.001$; ±SEM).

differentiation of alloreactive T lymphocytes and found that blocking TREM-1 decreased proliferation and differentiation of IFNγ-producing alloreactive $CD4^+$ T cells, thereby potentially reducing allograft vasculopathy and allograft fibrosis. It is notable that IFNγ-producing Th1 cells are critical effectors of allograft rejection (22,29,42). Besides the effect on T cell activation and consequently allograft survival, TREM-1 inhibition results in reduced cardiac fibrosis. Fibrotic organ remodeling of cardiac allografts represents a critical aspect and a major factor for the progression of heart failure. Fibrosis of heart grafts is characterized by the accumulation of fibers of extracellular matrix (ECM) surrounding cardiomyocytes (43). Although its origins are not fully understood, fibrosis can have detrimental effects on organ function and survival. Ultimately, enhanced ECM accumulation observed in tissue fibrosis is the result of competition between programs that promote ECM degradation and those that promote ECM synthesis (44). Tissue damage during allograft vasculopathy can prompt the production of cytokines, chemokines and growth factors, and these mediators promote infiltration by immune cells, fibroblasts and progenitor cells that further drive fibrotic proliferative responses through cytokine production. In this context $TGF\beta_1$ is implicated in promoting graft fibrosis and graft rejection (45–48). Once $TGF\beta_1$ is induced, it mediates fibrogenesis by activating cardiac fibroblasts to produce excessive amounts of collagen that cause myocardial damage and fibrotic organ remodeling (2,45,46,49,50). $TGF\beta_1$

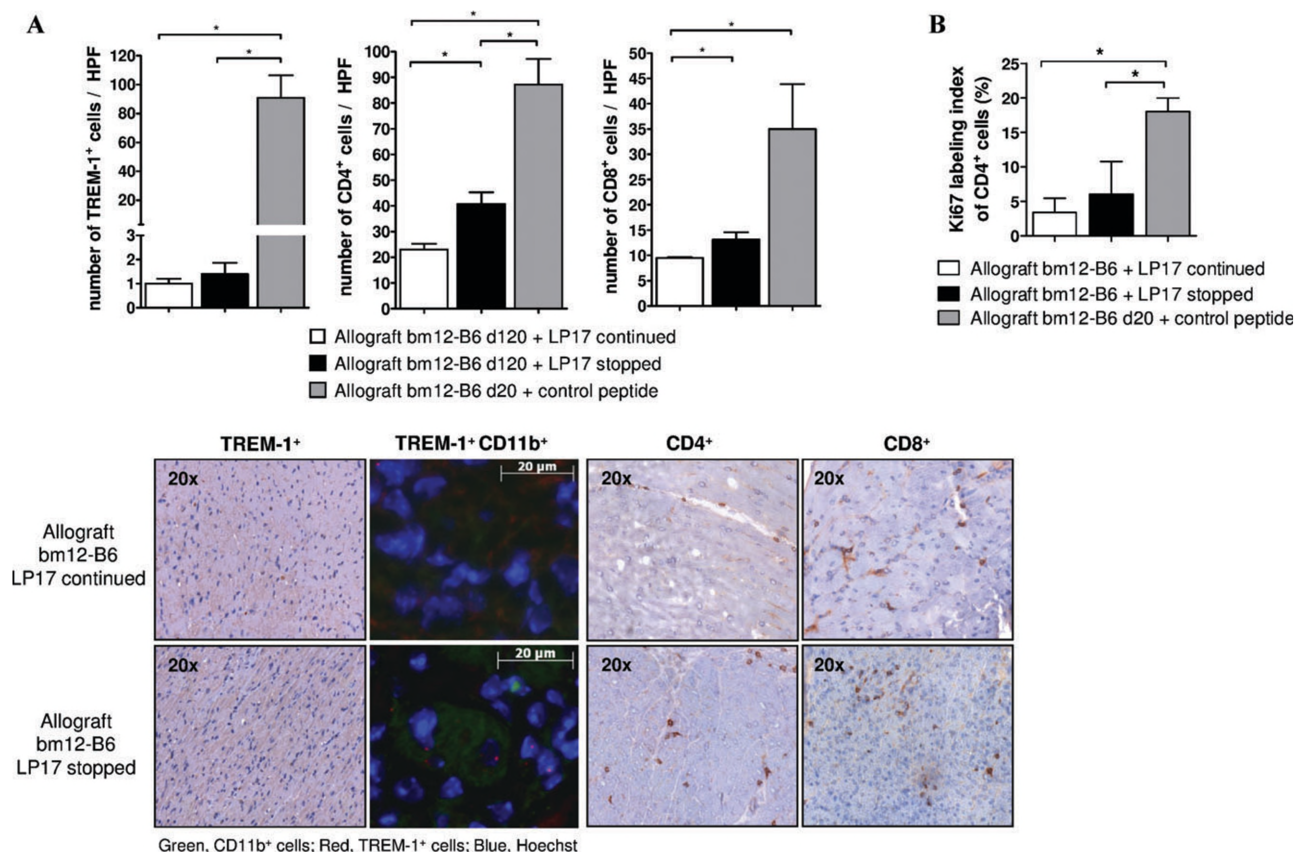

Figure 9: Blocking TREM-1 in the initial innate immune response reduces proinflammatory cell infiltration and T cell responses. (A) TREM-1^{+}, CD4^{+} and CD8^{+} cells per HPF were counted from three mice per group ($^{*}P \leq 0.05$; ±SEM). Representative stainings for TREM-1, TREM-1/CD11b, CD4 and CD8 of cardiac allografts following continued or stopped TREM-1 inhibition (magnification, ×20). (B) Ki67 labeling index of graft-infiltrating CD4^{+} T cells (n = 3; $^{*}P \leq 0.05$; ±SEM) on day 20 and 120 after transplantation. Recipients were treated with LP17 or control-peptide following chronic rejection.

also induces the expression of additional factors, i.e. connective tissue growth factor, to further amplify collagen deposition in the transplanted organ (2). Upregulation of TGFβ_1 during chronic rejection is linked to ongoing Th1, as well as Th17 cell responses in the allograft, which cause tissue inflammation, pro-inflammatory cytokine production and ultimately tissue fibrosis (45,51–53). Importantly, we show that the profibrotic pathway in chronic allograft rejection could be blocked by TREM-1 inhibition, thus cytokine expression, and inhibiting fibrogenesis-triggering in allografts. Early interference of TREM-1 activation in the initial phase of allograft rejection was sufficient to prevent long-term cardiac fibrosis, reinforcing the importance of innate immune system triggering of fibrotic processes leading to transplant failure.

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Disclosure

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

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1: Listing of antibodies used in immunohistochemical stainings

Table S2: Listing of primers and primer-sequences

Figure S1: Functional analysis of TREM-1 inhibition by LP17 or TREM-1 siRNA on cytokine expression and costimulation markers on CD11b+ cells and J774 macrophages.

Figure S2: Heart transplantation in MHC-class-II-mismatched mouse model (bm12 into B6) leads to chronic rejection characterized by severe changes in histology and collagen expression.

Figure S3: Enhanced graft-infiltrating cells from donor hearts transplanted in B6 recipients.

Figure S4: Increased infiltration of T lymphocytes in allografts.

Figure S5: Absence of Myd88 results in prolongation of allograft survival in chronic heart rejection.

Figure S6: TREM-1+ cells in chronic allograft rejection after kidney transplantation in humans.

Figure S7: TREM-1+ cells in allografts, blood and spleen following chronic rejection.

Figure S8: Phenotypic analysis of TREM-1+ cells in syngrafts and allografts.

Figure S9: Determination of TREM-1+ and Foxp3+ cells in allografts after LP17 or αTREM-1 antibody treatment.