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Allochimeric MHC I-Conditioned T Cells Attenuate Chronic Rejection in Rat Cardiac Model System

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Abstract

Background: We have shown previously that the mutated class I MHC molecules abrogate acute and chronic rejection and attenuate transplant vascular sclerosis (TVS) through the early changes (1-7 days post-transplantation) in T cell and dendritic cell molecular response. Here we studied a cohort of long-term (100 days) graft survival recipients for changes in T cell molecular response, and the role of regulatory T cells in the abrogation of chronic rejection in adoptive transfer experiments.

Methods: Heterotopic cardiac transplants were performed between donor Wistar Furth (WF) and ACI recipient rats. Controls received no treatment or 6 days therapeutic dose of cyclosporine (CsA 10mg/kg). The experimental group of primary ACI recipient received, peri-operatively, the allochimeric [[α]$_{1h}^{l/u}$]-RT1.A^{a} MHC I molecule (1mg/kg) in conjunction with the sub-therapeutic dose of CsA for 3 days. Splenic T cells were isolated from ACI recipient at 100 days post-transplantation and either assessed for changes in the expression of chosen protein markers or, in adoptive transfer experiments; they were injected into lightly irradiated secondary ACI recipients grafted with WF hearts. Secondary cardiac grafts were harvested at 100 days of post-transplantation for assessment of chronic rejection, neointimal index (NI) and apoptosis.

Results: Secondary cardiac grafts from recipients exposed to allochimeric MHC I-conditioned splenic total T cells or CD4^{+} T cells showed significantly reduced NI and apoptosis, and were selectively infiltrated with CD4^{+}Foxp3^{+} (T regulatory, Treg) cells.

Conclusion: Adoptive transfer of allochimeric MHC I-conditioned T cells promotes development of Treg cells and attenuates chronic rejection in rat cardiac model system.

Keywords: Allograft, Chronic rejection, Rat, Treg cells, T Cells, Adoptive transfer

Introduction

Peter Medawar's studies on the induction of acquired tolerance to foreign tissue using fetal exposure to donor-derived antigens led to numerous studies on protein therapy in abrogating graft rejection and promoting tolerance induction [1]. Despite the success of the current immunosuppressive armamentarium for transplantation, chronic rejection continues to plague graft survival and outcome. T cells recognize allogeneic MHC either through the "direct" or "indirect" pathways of antigen presentation. Numerous studies indicate that the indirect antigen recognition pathway and CD4^{+} T cells play a major role in both acute and chronic rejection and the development of tolerance [2,3]. Studies on MHC molecules showed that the altering of critical amino acid residues in the immunodominant regions of MHC molecule promotes inhibition of T cell proliferation, T cell anergy, and alters host response toward naïve antigens [4-6].

Our previous studies in rat cardiac model system demonstrated that the peri-operative treatment of recipients with an allochimeric MHC I abrogated acute and chronic rejection of the allograft, decreased perivascular inflammation, concentric vascular intimal hyperplasia, necrotizing arteritis and progressive luminal narrowing [7]. We also showed that allochimeric MHC I treatment caused the inhibition of T cell migration into the graft, restriction of their Vβ-TCR repertoire [3], and induced changes in T cell actin cytoskeleton and down-regulation of numerous molecules involved in actin organization (RhoA, HIPP55), cell polarity (PAR6) and intracellular antigen trafficking (KDEL, GM130; 8-11).

Numerous studies indicate the existence of a subset of regulatory CD4^{+}CD25^{+} T cells, which express the Forkhead wing-helix protein, Foxp3 that plays a role in maintaining immune tolerance [12-15]. Foxp3 is expressed predominantly in T regulatory cells (Tregs) and is necessary for their development and function [16]. Tregs are capable of inhibiting autoimmunity and maintaining peripheral tolerance [16]. Following portal vein injection of antigen, liver dendritic cells induce the generation of regulatory T cells from naïve CD4^{+} T cells [17]. Adoptive transfer of immune tolerance to allografts is governed, at least in part, by T regulatory cells (Tregs) through the peripheral regulation of naïve CD8^{+} T cells and other CD4^{+} T lymphocytes [13].

In our present study, we used adoptive transfer of T cells conditioned with allochimeric MHC molecule to elucidate the mechanism of chronic

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graft injury and the role of Tregs in the maintenance of tolerance and in the attenuation of chronic rejection.

Materials and Methods

Animals

Adult male inbred Wistar Furth (WF; RT1.Au) and ACI (RT1.Aa) rats were purchased from Harlan Sprague Dawley (Indianapolis, IN) and housed in standard rat cages. Heterotopic cardiac transplants were placed intra-abdominally as described previously [18]. Transplantation was performed as follows: 1) transplantation control group that received no treatment, 2) transplantation in the presence of high dose cyclosporine (CsA) delivered by gavage feed (10mg/kg, day 0-6) and 3) transplantation in the presence of sub-therapeutic dose of CsA (10mg/kg, day 0-2) in conjunction with allochimeric protein [$\alpha_{1h}^{l/u}$]-RT1.Aa (1mg/kg) which was injected as a single dose into the portal vein of recipient at the time of transplantation. Graft survival was assessed by abdominal palpation for a heartbeat daily for 2 weeks and then once weekly until no heartbeat was palpable. To assess early and long- term gene expression changes, three animals from each group were euthanized at 1, 3, and 100 days survival. A second cohort of WF donors to ACI transplanted recipients was used for the adoptive transfer of lymphocytes at the time of graft implantation. These animals were irradiated at a low dose (130 Rad) one day prior to transplant and adoptive transfer. The adoptive transfer experiments consisted of the following: 1) injection of 10^7 naïve T cells, 2) injection of 10^7 primed T cells from previously transplanted untreated animals 3) injection of 10^7 T cells isolated from previously transplanted animals treated with high dose CsA and 4) injection of 10^7 T cells isolated from previously transplanted animals treated with a sub-therapeutic dose of CsA plus allochimeric protein. Additionally, T cell subpopulations ($CD4^+$ and $CD8^+$ T cells) were isolated from previously transplanted CsA and CsA+allochimeric protein-treated animals and adoptively transferred into secondary recipients. These animals were assessed for graft survival, as described above, and euthanized at >100 days or when heartbeat had ceased. Graft survival time (GST) for all transplants was calculated from the day of transplant to the last day a palpable heartbeat could be felt. Animals were housed postoperatively according to standard protocols and all experiments were conducted under the Methodist Hospital Research Institute's animal care guidelines and the NIH standards set forth in the "Guide for the Care and Use of Laboratory Animals" (DHHS publication No. (NIH) 85-23 Revised 1985).

Mutated class I molecule production

Polymerase chain reaction based method of gene splicing with overlap extension (gene SOE ing) was used to construct a mutated class I RT1.A. The resultant [$\alpha_{1h}^{l/u}$]-RT1.Aa molecule bears both donor $RT1.A^u$ and recipient $RT1.A^a$ immunogenic epitopes by altering the hypervariable α1-helical region (a.a. 51-90) of ACI ($RT1.A^a$) to WF ($RT1.A^u$) sequences as described previously [7,19] and the allochimeric protein molecule was produced by GenWay, San Diego CA.

T cell isolation and adoptive transfer

T cells were isolated from spleens harvested at 100 days of post-transplantation. The cell suspension was made by passing splenic tissue through a cell strainer using a 3-cc syringe. Cells were treated with an erythrocyte-lysing reagent (Becton Dickinson, Franklin Lakes, NJ) and washed three times with complete media (10% FCS/1640 RPMI). Total T lymphocytes and subpopulations of $CD4^+$ and $CD8^+$ T cells were purified via positive T cell isolation kit using magnetic pan-T cell, anti-$CD4^+$, and anti-$CD8^+$ microbeads (Miltenyi Biotech, Auburn, CA). Purity of the T cell populations was confirmed using FACS analysis. T cells were stained with PE-conjugated mouse anti-rat CD3 antibody (BD Pharmingen, San Diego, CA), FITC-conjugated mouse anti-rat CD4 and CD8 antibodies (Invitrogen, Camarillo, CA), and FITC-conjugated mouse anti-rat Foxp3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were incubated for 15 minutes, washed three times in 1% PBS, and analyzed using a FACS flow cytometer (Becton Dickinson, Franklin Lakes, CA). Following isolation, 10^7 cells were adoptively transferred via injection into the portal vein of newly transplanted animals as described above.

Histopathology and Immunostaining

Rat hearts were fixed in formalin, embedded in paraffin and 5μM sections were cut at the mid-heart region. Sections were stained with Hematoxylin & Eosin (H&E), Trichrome or Verhoeff-van Gieson (VVG) using standard protocols. H&E staining was used to examine heart global architectural integrity and inflammation.

Immunohistochemistry was performed using primary antibodies against IgG (#SC-2041, Santa Cruz Biotechnology, Santa Cruz, CA) and IgM (#31476, Thermo Scientific, Waltham, MA), horseradish peroxidase-conjugated secondary antibodies and DAB staining. Results were reported as negative (-), low positive (+), moderate positive (++) and high positive (+++) staining. Averages were calculated by assigning the following numerical values: (-)=0, (+)=1, (++)=2 and (+++)=3. Apoptosis test was performed using the Apoptec kit (Millipore, Billerica, MA) according to the manufacturer's protocol. Apoptosis score was expressed as the number of apoptotic cells per heart cross-section. A blind observer performed analysis of 24 rat hearts, and the results were de-coded after the completion of the analysis.

Immunofluorescence

Cardiac allografts from the secondary recipients were harvested from the three experimental groups. Tissue was fixed in formalin and embedded in paraffin blocks. The 5μM sections were stained with anti-CD4 and -Foxp3 goat polyclonal antibodies as well as anti-IL-2rα (CD25) rabbit polyclonal antibody (all from Santa Cruz, Santa Cruz, CA) at a 1:1000 dilution. FITC-conjugated secondary antibodies were used for CD4 and Foxp3, and a Rhodamine-conjugated secondary antibody was used for IL-2rα (Abcam, Cambridge, MA).

Quantitative RT-PCR

Total RNA was isolated from purified splenic T cells using the RiboPure kit (Applied Biosciences, Foster City, CA) Complementary DNA (cDNA) was made from RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosciences, Foster City, CA) per manufacturers protocol. Primers for RhoA, Rock-1 and Spred1 were purchased from SA Biosciences (Frederick MD, USA). cDNA was amplified and compared to the housekeeping gene, β-actin, using the previously reported protocol [10].

Western blotting

Splenic T cells were homogenized on ice in RIPA buffer (0.15 M NaCl, 1% deoxycholate Na salt, 1% Triton X-100, 0.1% SDS, 0.01 M Tris HCl, pH 7.2) in the presence of complete protease inhibitor (Roche, Indianapolis, IN). The protein concentration was determined using ELISA and BioRad protein assay reagents. Protein samples were electrophoresed on 10% SDS-PAGE, blotted with primary antibodies against RhoA, Foxp3, IL-7, IL-7r, Spred1, and Rock1, blotted with HRP-conjugated secondary antibodies, and then developed using Lumi-Light western blotting substrate (Roche, Indianapolis, IN). Band

intensity for each experimental group was quantified using Quantity One 4.6.1 system (BioRad, Hercules, CA).

Statistical analysis

For RTPCR data the amount of target mRNA relative to housekeeping gene mRNA was expressed as fold increase or decrease. Relative changes were measured using real-time PCR in the 7500 Fast or Standard Real-Time PCR System (version 1.3.1). To calculate the relative quantity (RQ) of particular gene, we used the 2-delta delta ct method in the software. The data are presented as the fold change (RQ values) in gene expression level normalized to an endogenous reference gene. Standard error was calculated in Excel using formula STDEV(range)/SQRT(COUNT(range)) and plotted using Sigma plot.

Collagen deposition was quantitated from Trichrome stained slides using Image-Pro plus and is presented as % of stained area/total area. VVG staining was used to analyze the neointima and is presented as the neointimal index (NI= (intimal area)/ (luminal area + intimal area) x100). Five animals from each experimental group were analyzed. Five sections from each animal and 8 vessels from each section were analyzed. To avoid erroneously high indices, only vessels of a minimum size of 85 μM or larger diameter were analyzed. A cardiac pathologist blindly reviewed the slides in parallel and confirmed the findings using standard pathological based criteria for neointimal involvement. Standard error was calculated in Excel using formula STDEV(range)/SQRT(COUNT(range)) and plotted using Sigma plot.

Results

Early versus late post-transplantation molecular changes in T cells

We had previously proposed that some of the anti-rejection effects attributed to allochimeric MHC I therapy rely on acute changes (day 1 and 3 post-transplantation) in T cell cytoskeletal organization and in down-regulation of effector molecules involved in actin distribution, T cell motility and Rho GTPases (Rho A and Rock-1) and MAPK signaling pathways [8-10].

In this study, we examined whether these changes in the cellular cytoskeletal signaling pathways persisted in T cells from long-term tolerant primary recipients. We assessed the level of gene and protein expression of the RhoA GTPase at 1, 3 and 100 days post-transplantation in splenic T cells. We found, in agreement with previously published observations, that in allochimeric MHC-treated animals the level of RhoA RNA and protein expression were significantly lower at day 1 and 3 post-transplantation compared to untreated and CsA treated controls (Figure 1A and Supplementary Figure 1A). However, at 100 days of post-transplantation there was no difference in the levels of RhoA RNA or protein expression between CsA - treated and allochimeric MHC-treated groups, and in both groups, the relative levels of expression of RHoA were much higher than at 1-3 days of post-transplantation (Fig.1B and Suppl. Fig.1B). Comparison of the level of expression of Rock-1 and MAPK pathway (Spred1) showed the same trend: they were significantly lowered at day 1 and 3 (Figure 2A, C), and elevated at 100 days of post-transplantation (Figure 2B, D).

The finding that the levels of signaling molecules were decreased early post-transplantation (day 1 and 3) but returned to or surpassed control baseline levels over time suggests that the early molecular and cellular changes acquired during allochimeric MHC treatment contribute to early attenuation of graft injury and to graft survival.

We were also interested in finding out what is the difference between the expression of RNA and protein for Treg cell markers Foxp3 and IL-7r (CD127) in splenic T cells from allochimeric MHC- and CsA-treated animals at 100 days of post-transplantation. We found that the Foxp3 RNA and protein expression was markedly increased, and the IL-7r (CD127) expression was decreased in splenic T cells from allochimeric MHC- treated animals (Figure 3A and Supplementary Figure 2A). In contrast, the Foxp3 RNA and protein levels were markedly decreased, and IL-7r (CD127) expression was increased in splenic T cells from CsA- treated animals at 100 days post-transplantation (Figure 3 and Supplementary Figure 2B). Interestingly, the IL-7r protein expression was also found to be suppressed in splenic and thymic T cells at days 1 and 3 post-transplantation isolated from allochimeric MHC-treated animals (Supplementary Figure 3).

Allochimeric MHC treatment attenuates chronic rejection

To study the role of allochimeric MHC treatment in inhibition of graft rejection we performed adoptive transfer experiments. The

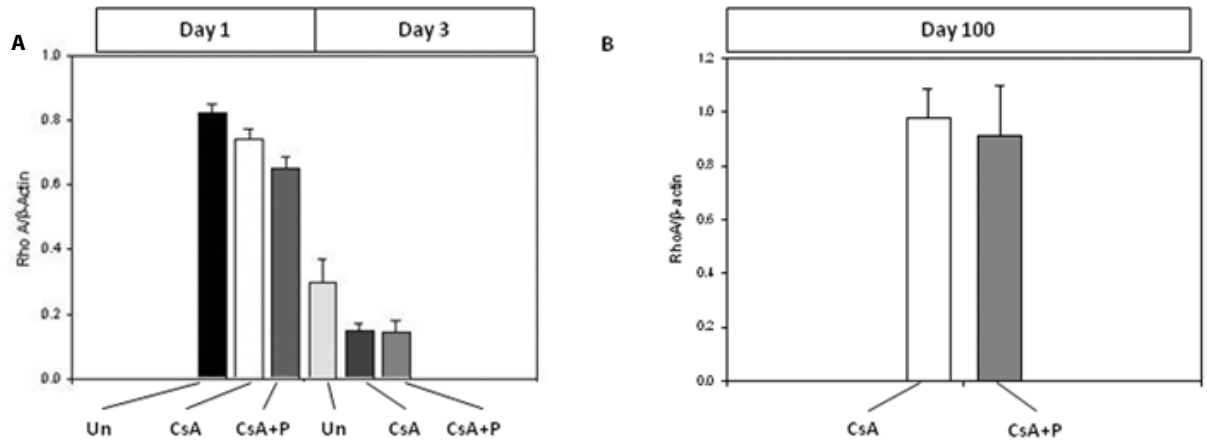

Figure 1: Down regulation of Rho A in splenic T cells. (A) RT-PCR analysis shows significant reduction in transcriptional expression of RhoA at days 1 and 3 post transplantation with allochimeric MHC treatment (CsA+P) when compared to untreated (Un) and cyclosporine (CsA) treated controls (3 animals averaged per group). (B) The acute changes in splenic T cells return to or surpass baseline untreated controls at 100 day graft survival. (n=3). The data are presented as the fold change (RQ values) in gene expression level normalized to an endogenous reference gene. Standard error was calculated in Excel using formula STDEV(range) / SQRT(COUNT(range)) and plotted using Sigma plot.

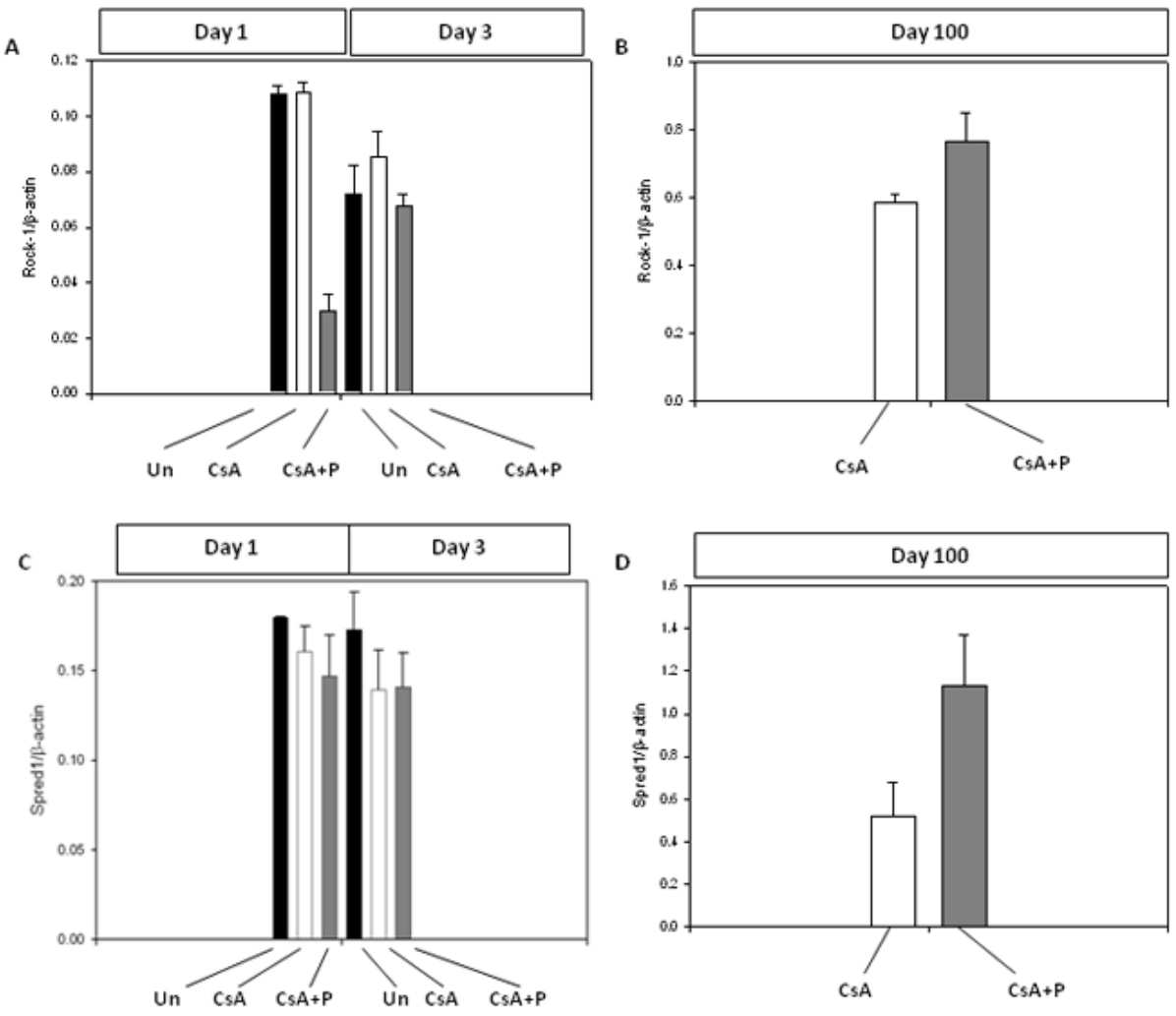

Figure 2: Western blot analysis of splenic T cells. (A) Decreased ROCK-1 protein expression at day 1 and 3 post transplantation in allochimeric MHC (CsA+P) treated animals compared to controls. (B) Increased ROCK-1 protein expression at 100 days post transplantation in CsA+P treated animals. (C) Decreased Spred1 protein expression at day 1 and 3 in CsA+P treated animals compared to controls. (D) Increased Spred1 protein expression at 100 days post transplantation in CsA+P treated animals compared to controls. (n=3) Standard error was calculated in Excel using formula STDEV(range) / SQRT(COUNT(range)) and plotted using Sigma plot.

Donor	$[[\alpha]^{l/u}_{1h}]$-RT1.A^{a}	Low dose CsA (3 days)	High dose CsA (7 days)	Primed T cells	Naïve T cells	Total T cells	CD4^{+} T cells	CD8+ T cells	# of Transferred T cells (secondary recipient)	Mean Survival Time
WF	-	-	-	+	-	-	-	-	1x10^{7}	6 days
WF	-	-	-	-	+	-	-	-	1x10^{7}	14.4 days
WF	-	-	+	-	-	+	-	-	1x10^{7}	>100 days
WF	+	+	-	-	-	+	-	-	1x10^{7}	>100 days
WF	-	-	+	-	-	-	+	-	1x10^{7}	>100 days
WF	+	+	-	-	-	-	+	-	1x10^{7}	>100 days
WF	-	-	+	-	-	-	-	+	1x10^{7}	87 days
WF	+	+	-	-	-	-	-	+	1x107	28 days

Primary recipients of WF grafts were conditioned with allochimeric class I MHC, [[α]1hl/u]-RT1.Aa (1mg/kg), in addition to 3 days of sub-therapeutic CsA (10mg/kg) or 7 days of high dose CsA alone (10mg/kg). Secondary recipients transplanted with WF grafts then received 1x107 T cells (total, CD4+, or CD8+), peri-operatively, isolated from a naïve animal or from primary recipients that were either untreated (primed T cells) or conditioned with CsA or CsA+allochimeric class I MHC (CsA+P). Table shows expected decreased graft survival in primed and naïve T cells treated groups. There was no difference in graft survival between animals receiving CsA+P - compared to those that received CsA alone - conditioned cells, nor was there a difference in those that received CD4+ cells. Survival was significantly less in secondary recipients that had received CD8+ T cells conditioned with CsA+P.

Table 1: Allograft survival following adoptive transfer after class I MHC or high dose CsA conditioning.

10^7 splenic total T cells or $CD4^+$ T cell subset isolated from naïve, transplanted-untreated, CsA - treated or allochimeric MHC - treated primary ACI recipients, were injected into slightly irradiated secondary ACI recipients of WF allografts (Table 1). As expected, the secondary recipients that received naïve total T cells promptly rejected the allograft within 14 days (Table 1). Graft survival time was further reduced (to 6 days) in secondary recipients that received total T cells from WF allograft primed primary host (Table 1). The transfer of total T cells or $CD4^+$ T cells from primary allograft recipients treated with CsA alone or with allochimeric MHC resulted in long-term (>100

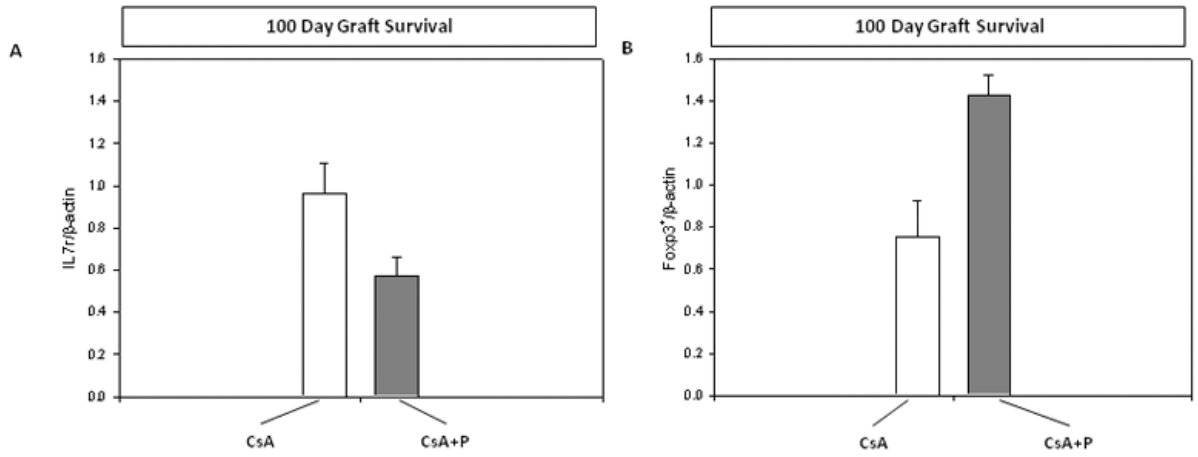

Figure 3: IL-7r and Foxp3 protein expression in splenic T cells isolated at 100 days post transplantation following high dose CsA treatment or allochimeric class I MHC therapy (CsA+P). (A) RT-PCR showed significantly reduced expression of IL-7r in recipients treated with CsA+P compared to recipients treated with CsA alone. (B) RT-PCR showed elevated expression of Foxp3 in CsA+P treated recipients compared to recipients treated with CsA alone. (n=3). The data are presented as the fold change (RQ values) in gene expression level normalized to an endogenous reference gene. Standard error was calculated in Excel using formula STDEV(range) / SQRT(COUNT(range)) and plotted using Sigma plot.

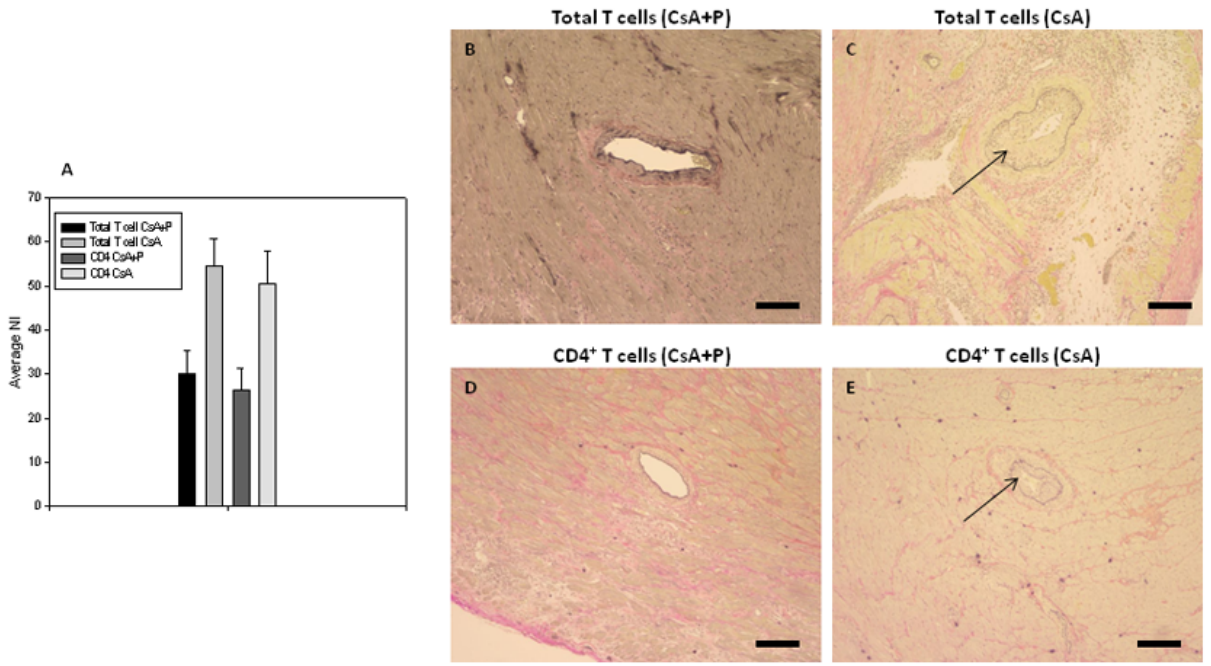

Figure 4: Neointimal Indices (NI) of cardiac allografts from adoptive transfer experiments. (A) Adotpive transfer of allochimeric class I MHC (CsA+P) conditioned total T cell or CD4+ T cells resulted in decreased neointimal hyperplasia. Five animals from each experimental group and five sections from each animal were analyzed. NI was calculated by averaging the degree of neointimal hyperplasia in 8 separate coronary vessels having a minimum diameter of 85μm. (B&C) VVG staining of secondary allografts exposed to CsA+P - or CsA - conditioned total T cells. (B) Cardiac allograft tissue from recipients that received 10^7 total T lymphocytes conditioned with allochimeric protein (CsA+P) showed minimal neointimal hyperplasia when observed at 100 days compared to (C) grafts exposed to total T cells conditioned with CsA alone (30% vs 54.5%, $p<0.05$). (D & E) Secondary allografts that had been exposed to CsA+P or CsA conditioned CD4+ T cells. (D) Allografts from animals treated with CD4+ T cells isolated from CsA+P conditioned host show significant reduction of NI in comparison to (E) grafts from animals exposed to CsA conditioned CD4+ T cells (28.2% vs. 50.5%, $p<0.05$). (C&E) Black arrows point to almost complete occlusion of vessel in grafts from animal exposed to CsA conditioned total T cells or CD4+ T cells. Standard error was calculated in Excel using formula STDEV(range) / SQRT(COUNT(range)) and plotted using Sigma plot. Bar is equal to 50μm.

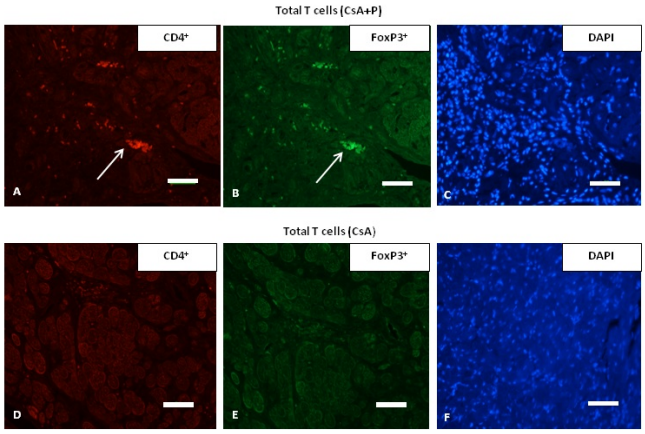

Figure 5: Immunoflourescent staining for regulatory cells (Tregs) in animals conditioned with total T cells. (A, B, C) Cardiac allograft tissue stained with anti-CD4 (red) and anti-Foxp3 (green) antibody and DAPI, respectively, from animals that had received allochimeric protein (CsA+P) - conditioned total T cells. Arrows denote cluster of CD4+Foxp3+ cell infiltration into the graft. (D, E, F) Cardiac allograft tissue from animals treated with CsA - conditioned total T cells, stained with anti-CD4 (red), anti-Foxp3 (green) antibody and DAPI, respectively. There is no infiltration with CD4+ and Foxp3+ cells. Bar is equal to 50μm.

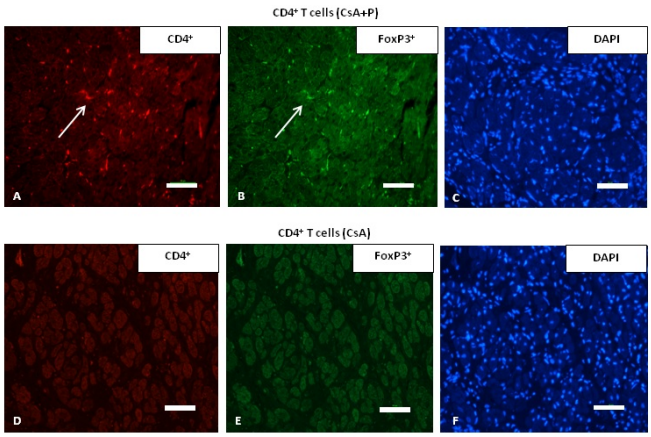

Figure 6: Immunoflourescent staining for regulatory cells (Tregs) in animals conditioned with CD4+ T cells. (A, B, C) Cardiac allograft tissue stained with anti-CD4+ (red), anti-Foxp3+ (green) antibody and DAPI, respectively, from animals that had received CsA+P - conditioned CD4+ T cells. Arrows denote cluster of CD4+ and Foxp3+ cell infiltration into the graft. (D, E, F) Cardiac allograft tissue from animals treated with CsA alone -conditioned CD4+ T cells, stained with anti-CD4 (red), anti-Foxp3 (green) antibody and DAPI, respectively. There is no infiltration with CD4+ and Foxp3+ positive cells. Bar is equal to 50μm.

days) graft survival in the secondary recipients (Table 1). Although these data indicated that allograft survival was prolonged in secondary recipients that received either CsA-conditioned or allochimeric MHC-conditioned T cells, we found (see below) that the attenuation of chronic rejection occurred exclusively in the allochimeric MHC-conditioned group.

Allochimeric MHC-conditioned T cells attenuate chronic rejection of the allograft

Comprehensive analysis of the allografts tissue showed dramatic differences in the degree of chronic rejection between allografts from recipients that had received adoptively transferred T cells exposed to allochimeric MHC and those treated with CsA alone. There was a significant reduction of the neointimal hyperplasia (NI) in the allograft from the recipients that received total T cells conditioned with allochimeric MHC in comparison to those conditioned by CsA alone (Figure 4; 30% vs. 54.5%, $p<0.05$). Similar trend persisted in the allografts from the recipients that received isolated $CD4^+$ T cells. The neointimal hyperplasia was markedly reduced in the allografts from the secondary recipients which received $CD4^+$ T cells conditioned with allochimeric MHC in comparison to those conditioned with CsA alone (Figure 4; 28.2% vs. 50.5%, $p<0.05$). Furthermore, using immunofluorescence staining of allografts, we observed a stark increase in the number of cells expressing Treg cell makers $CD4^+$ and $Foxp3^+$ within the grafts of secondary recipients that had received allochimeric MHC-conditioned total T cells and $CD4^+$ cells, in comparison to the grafts from CsA alone-conditioned groups (Figure 5, Figure 6). All these observations indicate the role of allochimeric MHC in the promotion of development of Treg cells during the attenuation of the rejection response.

Allochimeric MHC-conditioned T cells promote vascular accommodation and decrease in allograft apoptosis

Vascular antibody accommodation is believed to play an underlying role in the inhibition of chronic rejection (20). We observed significantly higher levels of IgM deposition on endothelial cells of allografts from the animals treated with allochimeric MHC-conditioned $CD4^+$ T cells when compared to CsA alone-conditioned group (Figure 7A). In addition, we found that allochimeric MHC-conditioned $CD4^+$ T cells promote inhibition of apoptosis in the allograft (Figure 7B). We observed twice as many apoptotic cells per high power field in the grafts from the recipients that received $CD4^+$ T conditioned with CsA alone than from those conditioned with allochimeric MHC (Figure 7C, D).

Discussion

The molecular pathways underlying rejection of incongruous organs are still very poorly understood. We have developed a model system using rat cardiac transplants and allochimeric class I MHC treatment that help to elucidate the molecular mechanisms underlying acute and chronic rejection.

A functional immune response depends on T cell migration toward an antigenic stimulus, adhesive contact with the APC, the ability to scan the surface of the APC and proper segregation of surface proteins involved in functional immunological synapse formation [21-23]. Several studies have reported that allochimeric class I MHC proteins inhibit T cell function by receptor inhibition, restriction of the TCR repertoire, and by induction of T cell apoptosis [24-26]. We have previously demonstrated the inhibition of acute and chronic rejection of allografts in recipients treated with allochimeric class I MHC [$\alpha_{1h}^{l/u}$]-RT1.Aa in combination with low dose of CsA [7,10,19]. We suggested that T cell polarization and motility were impaired through a mechanism involving cytoskeletal structural changes and decreased transcriptional and translational expression of downstream signaling pathways involving actin polymerization and intracellular trafficking (8-10). One such pathway, RhoA and its effectors (ROCK), belong to a family of small GTPases (RhoA, RhoB, Rac1) that are responsible for regulation of T cell shape and proliferation, thymocyte homeostasis and IL-2 production, T cell receptor responses and antigen presentation through structural changes critical for T cell signaling [2,11,27,28-30]. Microarray analysis of splenic T cells demonstrated that the expression of numerous genes involved in actin cytoskeletal polymerization, adhesion and intracellular trafficking, such as Rho

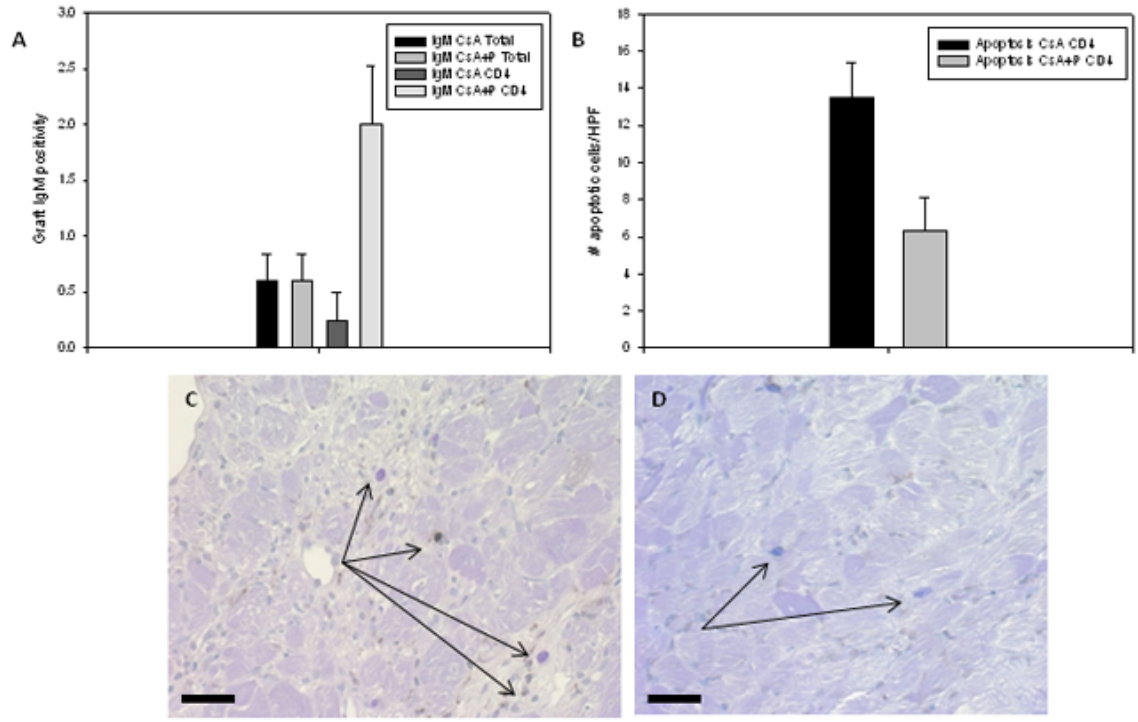

Figure 7: IgM deposition and apoptotic assay of cardiac allografts. (A) Increased level of IgM deposition within allografts exposed to allochimeric protein conditioned CD4+ T cells compared to CsA alone- treated groups; n=5. (B) Tunel assay shows 50% reduction in the number of apoptotic cells in allografts exposed to CsA+P - conditioned CD4+ T cells as compared to CsA alone. (C&D) Cardiac allografts sections show apoptotic cells (black arrows) in the CsA alone - conditioned CD4+ group (C) compared to the CsA+P - conditioned CD4+ group (D), n=5. Apoptosis score was expressed as the number of apoptotic cells per heart cross-section. Standard error was calculated in Excel using formula STDEV(range) / SQRT(COUNT(range)) and plotted using Sigma plot. Bar is equal to 50μm.

GTPases, Rac1, VCAM and genes of the MAPK (Spred1) signaling pathway were decreased following allochimeric MHC treatment [9,31]. In addition, we recently reported that RhoA expression was reduced at day 1-7 post-transplantation following allochimeric MHC treatment, which correlated with longer allograft survival and decreased lymphocyte graft infiltration [10]. Tharaux et al. [32] showed that the application of inhibitors of RhoA pathway improves allograft survival in murine transplant model system and attenuates the proliferation and activation of T cells through the inhibition of actin polymerization and the T cell proliferative response to mitogen. Because our previous study suggested that the reduction of RhoA expression, and the impairment of T cell actin cytoskeleton, T cells motility, graft infiltration and antigen presentation [9,11] induced by the allochimeric MHC treatment are the early (within the first 7 days of post-transplantation) events involved in the immune response, we were interested to see if any of these molecular changes persisted in T cells isolated from long-term surviving allograft recipients treated with allochimeric MHC. Interestingly, we found that the expression of RhoA GTPase and MAPK pathways from T cells acquired 100 days post-transplantation returned or even surpassed the control levels. This suggests that allochimeric MHC treatment influences molecular events responsible for the early graft failure.

We were also interested in finding out what is the participation of Treg cells in allograft accommodation and survival in our model system. We studied the expression of Foxp3 and IL-7r in T cells isolated from allochimeric MHC-treated recipients at 100 days post-transplantation and in adoptive transfer experiments. Foxp3 is a winged helix protein transcription factor that controls the development of a population of anergic $CD4^+CD25^+$ Tregs that suppress the activation and proliferation of other $CD4^+$ and $CD8^+$ T cells [17]. In an effort to define a new biomarker for human Tregs, Weihong et al. [33] showed that the expression of IL-7r (CD127) is inversely related to the expression of Foxp3 and it can be used as a marker for Tregs in humans [13,14,33]. The α chain of the IL-7r, in combination with the α chain of the IL-2 receptor (CD25), can distinguish between regulatory and conventional $CD4^+$ T cells, as conventional cells depend on IL-7 whereas regulatory cells do not [34]. Survival of naïve T cells in the periphery is dependent upon IL-7, and homeostasis of T cells is maintained through maximal sharing of pro-survival signals. IL-7rα expression is reduced on T cells that have received cytokine-mediated survival signals so they do not compete with naïve T cells for remaining IL-7 [35]. IL-7r (CD127) is decreased in cytotoxic $CD8^+$ T cells of HIV positive patients, thus rendering the $CD8^+$ T cells less responsive to immune stimulating cytokines (35). In our present study we found that $CD4^+$ T cells isolated at 100 days post-transplantation from allochimeric MHC-treated recipients showed increased expression of Foxp3 and decreased expression of IL-7r. These data suggest that, in addition to impairing cytoskeletal dynamics, treatment with allochimeric class I MHC stimulates the development of allospecific regulatory cells. This is further supported by our present and previous [36] findings that signs of chronic rejection are markedly reduced following the adoptive transfer of allochimeric MHC -conditioned cells, particularly $CD4^+$ T cells.

Although additional studies are still necessary to elucidate the exact function of allochimeric MHC treatment in attenuation of allograft rejection we can speculate what pathways and mechanisms

are involved in this function. At present, we know that allochimeric MHC treatment in primary host results in a defective host APC antigen processing/presentation machinery [11]. This may lead to presentation of altered allo-peptides, resembling self and donor to the $CD4^+$ T cells resulting in impaired T cell activation signaling as well as the development of Treg cells specific to these allo-peptides [37]. This may explain why we observed a decrease in vascular sclerosis in secondary recipients that received allochimeric-conditioned $CD4^+$ T cells and not $CD8^+$ T cells. Qualitative lesions of chronic rejection include vascular intimal hyperplasia and progressive luminal narrowing that results in compromised blood flow and graft injury [38,39]. In this study, we found significantly reduced neointimal hyperplasia, perivascular inflammation, and fibrosis in grafts that had been exposed to allochimeric MHC-conditioned total T cells and $CD4^+$ cells compared to high dose CsA- conditioned cohorts. Other investigators have also shown alloantibody responses to be protective in late rejection [20]. An accommodation effect has been described in which recipient alloantibodies are produced against alloantigen, yet there is no rejection of the graft [40]. Similar to our findings of increased IgM binding to the myocardial endothelium Semiletova et al. [20] demonstrated increased alloantibody staining for IgG and IgM in cardiac allografts that showed low levels of chronic rejection.

Conclusions

Transplantation of a genetically incongruous organ leads to an elaborate process of antibody- and cell-mediated events, which ultimately lead to graft failure. The factors that contribute to the development of tolerance are multifactorial. Exposure to donor-specific epitopes of class I MHC perioperatively alters the recipient T cell microenvironment such that the effector function of recipient T cells is reduced. In addition to impairing subcellular components, allochimeric MHC conditioning promotes the development of Treg cells that suppress the recipient immune response and maintain immunological tolerance.

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