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Class I MHC allochimeric presentation of composite immunogenic and self epitopes induces tolerance to genetically diverse rat strains¹

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Abstract

Functional topography of rat class I major histocompatibility complex (MHC) molecule was studied. The α_1 -helical sequences that are shared by class I RT1.AI and RT1.A^u were substituted in the RT1.A^a molecule to produce the composite [$\alpha_{1h}^{I^u}$]-RT1.A^a MHC class I allochimeric molecule. Dominant immunogenic epitopes that induce accelerated rejection were identified within the hypervariable regions of the α_1 domain of RT1.A^a, RT1.A^I, and RT1.A^u. Peri-transplant portal venous delivery of MHC class I allochimeric proteins, that included composite α_1 helical immunodominant epitopes of RT1.A^u and RT1.A^I, induced donor-specific tolerance to RT1^u (Wistar Furth, WF) and RT1^I Lewis, LEW) disparate cardiac allografts in ACI (RT1^a) hosts. Allochimeric generated tolerance was characterized by absence of T cell deletion or anergy. Donor specific IgM allo-Abs was not detected, while IgG alloresponse was markedly attenuated in sera of tolerant hosts. Further, long-term allografts in allochimeric-conditioned hosts exhibited moderate B cell infiltration when compared to rejecting controls. Analysis of intra-graft cytokines revealed selective upregulation of IL-10 and marked inhibition of IL-2, IFN- γ and IL-4. Our findings indicate the emergence of a peripherally induced tolerant state, afforded by the novel approach of soluble class I allochimeric conditioning that presents donor immunogenic epitopes in the context of recipient class I determinants.

Keywords

MHC class I; allochimeric protein; allo-antibody; cardiac allograft; tolerance; humoral immunity

Introduction

Multideterminant protein antigens exhibit immunodominant epitopes that elicit host immune responses towards native antigen [1–4]. Alteration of critical a.a. residues in such

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immunodominant determinants inhibited T cell proliferative responses, induced T cell anergy and altered host immune responses toward native antigens [3–7].

Similar to the recognition of multideterminant antigens, strong evidence suggest that murine, rodent and human host immune responses to organ allografts are focused on a restricted number of alloantigenic determinants that correspond to short a.a. peptide residues [8–15], after indirect recognition of processed major histocompatibility complex (MHC) alloantigens [16–18]. Murine T cell proliferative responses to donor MHC, after allogeneic cell transplants or skin grafts, were limited to a single short peptide dominant determinant within the class II molecule in each of three donor/recipient combinations that differed in the class II region [8]. In human recipients of cardiac allografts that were mismatched at the HLA-DR antigens, alloreactivity of T cells rejecting heart allografts was directed against the dominant determinant of a single HLA-DR antigen, even if the donor was mismatched from the recipient by two HLA-DR alleles [11]. Such dominant determinant corresponded to a short peptide sequence that was derived from the hypervariable region of the mismatched HLA-DR antigen [11]. T cell receptor (TCR) repertoire of lymphocytes that rejected totally mismatched rodent heart allografts predominantly expressed V β 4 genes [9], indicating a dominant response to a dominant donor MHC determinant. TCR-V β gene usage by T cells responding to dominant allopeptides was also shown to be limited in humans [10, 19]. In rodents, short peptide sequences derived from the α_1 domain polymorphic region of rat class I RT1.A^a induced sensitization against RT1^a allografts [12], provoked alloantibody production and T cell proliferative responses [13–15]. MHC alloantigens are therefore similar to multideterminant protein antigens that bear localized immunodominant a.a. sequences that trigger immune responses toward the whole protein molecule [2, 20–22]. This suggests that indirect allorecognition is amenable to specific immunointervention possibly through manipulation of such dominant immunogenic epitopes, similar to previous experiments that modified immunodominant determinants of multideterminant protein antigens. Thus, recipient treatment with donor MHC antigens that bear modifications of critical alloantigenic determinants may alter the host immune response toward organ allografts [23–25].

We have adopted a molecular strategy to identify the polymorphic immunogenic epitopes of rat class I MHC antigens and induce modifications to such regions in order to deviate host immune responses to donor allografts. This strategy resulted in the construction of allochimeric class I MHC molecules that contained donor-type polymorphic epitopes displayed on recipient-type heavy chains. We have previously demonstrated the ability of class I soluble [$\alpha_{1h}^{1/u}$]-RT1.A^a allochimeric molecule, that contains Lewis (LEW, RT1.A^l) and Wistar Furth (WF, RT1.A^u) α_1 -helical sequences displayed on recipient ACI (RT1.A^a) backbone, to induce tolerance and inhibit chronic rejection of WF heart allografts in ACI hosts [25, 26]. This suggested that tolerance might be induced by functional presentation of donor immunogenic epitopes via self-sequences. This study examined the immunogenic and tolerogenic ability of the α_1 -helical region of RT1.A^a class I MHC in a well established rat cardiac allograft model and further defined the mechanisms associated with long-term allograft acceptance following presentation of dominant donor immunogenic sequences by self-recipient epitopes.

Materials and Methods

Animals

Adult male inbred Wistar Furth (WF; RT1^u), LEW (RT1^l), ACI (RT1^a) and Brown Norway (BN; RT1ⁿ) rats (200–250 gm) were purchased from Harlan Sprague Dawely (Indianapolis, IN) and housed in wire-bottomed cages with controlled light/dark cycles. Rats were given

free access to water and standard rat chow and were cared for according to the guidelines of the American Association of Laboratory Animal Care.

Production of mutant class I MHC molecules

The polymerase chain reaction (PCR)-based method of gene splicing with overlap extension (gene *SOEing*) was utilized to mutate WT-RT1.A^a cDNA as previously performed [25, 27]. Briefly, plasmid pBS3.3/1 containing the WT-RT1.A^a cDNA served as a template for PCR amplification reactions between 5' and 3' flanking "outside" primers that contained EcoRI restriction sites and two internal overlapping, "SOEing" primers, which contained the base substitutions. The first PCR reaction used 1 µl of the 5' outside primer (500ng), 1 µl of the first internal primer (500ng), 1 µl of dNTP (25mM), 1 µl of hot-start DNA polymerase (Amersham, Arlington, IL), and 85 µl of dH₂O. A second PCR reaction used the second internal primer and the 3' flanking outside primer. The PCR reactions were subject to standard denaturation and annealing cycles. PCR products were separated by agarose gel electrophoresis. DNA bands of appropriate size were excised and melted at 60°C. These PCR products were then used as templates in a third *SOEing* PCR reaction using the two outside flanking primers to yield the final 1.6 kb mutant RT1.A^a cDNA. Mutated RT1.A^a cDNA was then subcloned in the eukaryotic expression vector pSG5 containing the SV40 promoter and transfected in BUF Morris hepatoma 7316A cells using lipofectin reagent (Gibco, Grand Island, NY) as previously performed [25]. Lipofectin-plasmid DNA (pSG5-RT1.A^a and pSV2 NEO, 10:1) complexes were added to 2×10⁵ cells in complete RPMI medium (Sigma, St. Louis, MO) in tissue culture dishes. Stable transfectants were selected by addition of 400 mg/ml of Geneticin (G418; Gibco). Surviving colonies were expanded and analyzed for surface expression of chimeric RT1.A^a molecules by flow cytometry analysis. Allochimeric MHC antigen was extracted with 3M-KCl from BUF hepatoma stable transfectants as previously described [26]. Resultant total protein concentration was determined by spectrophotometry (Becton-Dickinson, USA), and stored at -70°C in aliquots. 1mg of soluble allochimeric protein per rat was administered as outlined below.

Cardiac Transplantation

For tolerance induction, allochimeric molecules were administered through the portal vein (pv) or intravenously (iv, 1mg/rat) into ACI recipients of WF hearts at the time of transplantation in addition to a 3 day course of oral cyclosporine delivered by gavages feed (CsA, 10mg/kg/day; day 0–2). CsA elixir was obtained from Sandoz and diluted in purified olive oil to a final concentration of 10mg/ml. Controls included transplantation of allogeneic hearts in the presence or absence of subtherapeutic CsA. Heterotopic cardiac transplants were placed intra-abdominally [28]. Cardiac function was assessed by abdominal palpation. The hearts were palpated daily for 14 days and then weekly thereafter. The day on which beating was last palpable was defined as the day of rejection. Data are presented as mean survival time ± SD and were compared using Student's *t* test. *P* values <0.05 were considered statistically significant.

Mixed lymphocyte reaction (MLR)

Lymph node T cells harvested from ACI rats were used as responders toward stimulator cell populations harvested from the spleens of syngeneic ACI, donor WF, donor LEW, and third party BN rats. Lymph nodes were aseptically harvested from naïve, rejecting and tolerant ACI rats. Responder T cells (1 × 10⁵) were incubated on 96 well plate U-bottom for 5 days with 1×10⁵ 20 gray-irradiated stimulator cells at 37°C/5% CO₂, cultured in DMEM medium (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated FCS (Hyclone, Logan, UT), L-Glutamine 2mM, non-essential amino acids, sodium pyruvate (1 mM), penicillin-streptomycin (100 U/ml), 2-mercapthoethanol (50 µM), Hepes buffer (10mM), and 1% heat inactivated rat serum [29]. Wells including stimulator cells from

syngeneic ACI rats served as control for nonspecific cell proliferation. Lymph node T cells from naïve and rejecting ACI rats served as positive controls. Proliferation was assessed by ^3H -thymidine incorporation (specific activity 5.0 Ci/mM; Amersham, Arlington, IL) added 1.0 μCi per well on fourth day of incubation with following 18 hours of incubation at 37°C/5% CO_2 . Results of triplicate cultures were expressed as the mean cpm $\pm$ SD.

Limiting dilution analysis

Twelve replicates of each serial dilution of responder cells from 1:25,600 to 1:400 were cultured for 7 days with 5×10^4 irradiated (2000 rads) spleen cell stimulators per well in complete DMEM medium supplemented with 10% heat inactivated FCS and 20 U/ml of purified IL-2 (Collaborative Research Inc., Bedford, MA) as previously performed (25). Cytotoxic activity of each well was assessed by a 6 h incubation with 1×10^4 ^{51}Cr -labeled (($\text{Na}_2^{51}\text{Cr}$) O_4 , specific activity 200–500 mCi/mg Cr; Amersham, Arlington, IL) ConA (2.5 $\mu\text{g}/\text{ml}$) activated stimulator T cell blasts. ^{51}Cr release from the supernatants was measured in a gamma counter. Positive results were defined as values exceeding the mean spontaneous chromium release by more than 3 SD. Minimum chi-square estimates with 95% confidence limit of the frequency of cytotoxic T cells were obtained by the Poisson distribution relationship between responder-cell number and the natural logarithm of the fraction of negative cells.

Flow cytometry analysis

Sera from naïve, rejecting controls and tolerant ACI recipients with engrafted WF and LEW hearts were analyzed for the presence of binding anti-RT1A^u and anti-RT1A^l alloantibodies, at 120 days posttransplantation, as previously described [26]. A collected serum was heat inactivated (56°C/30 minutes) and stored at –80°C until the time of analysis. In brief, target LN cells (3×10^5) cells were suspended in Hank's balanced salt solution (HBSS) containing 1% fetal calf serum and 0.1% sodium azide. Serially diluted experimental sera (1:4, 1:16 and 1:64) were added to the cells in 100 μl and incubated for 30 min. at 4°C. After washing, fluoresceinated (FITC-conjugated) rabbit anti-rat IgG (1:20 dilution; Zymed labs, San Francisco, CA), and mouse anti-rat IgM (1 μg per 1×10^6 cells; PharMingen BD Biosciences, San Diego, CA) were added in 100 μl and incubated for additional 30 min. at 4°C. Thereafter cells were washed and fixed with 2.0% paraformaldehyde (Scientific Products, McGaw Park, IL), before analysis on a Becton-Dickinson Laser Flow Cytometry System (Mountain View, CA). Background non-specific fluorescence was assessed by staining with the second FITC-conjugated antibody only. Data are presented as percent of positive staining.

RNA extraction and competitive template RT-PCR

Total RNA was extracted from homogenized frozen tissue samples and concentration determined by spectrophotometer. Total of 5 μg of RNA was reverse-transcribed using oligo (dT) primers and superscript reverse transcriptase according to the manufacturer's instructions (Invitrogen, USA). Oligonucleotide primer pairs for the 5' and 3' rat cytokine regions were selected based on published sequence [30]. To compare relative level of each cytokine in different samples, competitors for IL-2, IFN- γ , IL-4, IL-10 and β -actin were constructed, and the competitive template RT-PCR amplification was performed, as described [30, 31]. According to the varying contents of specific DNA and varying amplification efficiencies, the samples were subjected to different cycle numbers at the annealing temperature that was optimized for each primer pair: 40, 63°C (IL-2), 45, 63°C (IL-4), 40, 55°C (IL-10), 35, 60°C (IFN γ), and 32, 63°C (β -actin), respectively. PCR products were analyzed in ethidium bromide-stained 2% agarose gel and photographed with Polaroid film under ultraviolet light. The PCR results were scanned, and the densities of WT cDNA and gene-specific competitor bands were analyzed using Kodak Digital Science 1D

Analysis Software (Version 2.0). All samples were normalized against respective β -actin WT cDNA/CT cDNA ratio.

Immunopathology

Cardiac allografts were examined by immunocytochemistry for mononuclear cell infiltration as previously described [32]. Briefly, tissues were embedded in tissue Tec OCT compound (Miles, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at -70°C . Cryostat sections (5mm) were fixed in acetone and then endogenous peroxidase activity was blocked by incubating the slides with 0.3% H_2O_2 . Appropriate primary antibody against rat B cells (OX33), IL-4 (OX82) and IL-10 (A5-4) were purchased from PharMingen (San Diego, CA). Bound primary antibody was detected using biotinylated rabbit anti-mouse IgG or goat-anti rabbit IgG and streptavidin peroxidase-conjugated complexes obtained from Vector Laboratories (Carpinteria, CA). The control sections were performed by replacing the primary mAb with either dilution buffer, normal mouse serum, or normal rabbit serum. The peroxidase reaction was developed with 3,3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO). The sections were evaluated blindly by counting the labeled cells in triplicates within 10 high-power fields (HPF)/section. Some antigens were analyzed in a semi-quantitative fashion where the relative abundance of each one was judged as (–) negative, (+) little, (++) moderately abundant, and (+++) very abundant.

Results

Characterization of allochimeric $[\alpha_{1h}^{l/u}]$ -RT1.A^a class I MHC molecules

Figure 1A demonstrates the differences between a.a. sequences of RT1.A^a, RT1.A^l and RT1.A^u. The hypervariable α_1 -helical region (a.a. 51-90) of wild type (WT)-RT1.A^a was altered to LEW (RT1.A^l) sequences by changing nucleotides encoding 10 amino acids: (Glu⁵⁸⇒ Asp, Gln⁶²⇒ Arg, Gln⁶³⇒ Glu, Arg⁶⁵⇒ Gln, Ile⁶⁶⇒ Lys, Glu⁶⁹⇒ Gly, Trp⁷⁰⇒ Asn, Ile⁷³⇒ Asn, Asp⁷⁷⇒ Ser, Thr⁸⁰⇒ Asn). As shown in Figure 1A, the four underlined a.a. at positions 62, 63, 65 and 70 are identical between Wistar Furth (WF, RT1.A^u) and Lewis (LEW, RT1.A^l) strains, but are different from the ACI (RT1.A^a) sequences at the corresponding positions. Thus, the designed $[\alpha_{1h}^{l/u}]$ -RT1.A^a molecule bears both RT1.A^l and RT1.A^u α_1 -helical epitopes.

Presence of immunogenic determinants in the polymorphic α_1 -helical region of class I MHC

In BUF (RT1^b) hosts, pre-transplant s.c. injection (day -7) of WT-RT1.A^a bearing transfectants (20×10^6), which exhibit α_{1h}^a epitopes, caused accelerated rejection of ACI (RT1^a) cardiac allografts (mean survival time, MST $\pm$ SD 3.75 ± 0.9 days), compared to non-immunized BUF hosts that rejected ACI cardiac allografts in 6 ± 0.0 days ($P < 0.001$). In contrast, transfectants bearing $[\alpha_{1h}^{l/u}]$ -RT1.A^a, which lack α_{1h}^a determinants, failed to immunize recipients against ACI (RT1^a, 5.75 ± 0.7 versus 6.0 ± 0.0 days in controls; *PNS*) hearts, indicating the presence of RT1.A^a immunogenic epitopes in the α_{1h}^a region (Table 1). Additionally, allochimeric $[\alpha_{1h}^{l/u}]$ -RT1.A^a that lack RT1.Aⁿ α_1 -helical epitopes failed to induce accelerated rejection of Brown Norway (BN, RT1ⁿ; 6.0 ± 0.0 versus 6.0 ± 0.0 days; *PNS*) hearts in BUF hosts. Further, as we have previously demonstrated, allochimeric $[\alpha_{1h}^{l/u}]$ -RT1.A^a bearing transfectants, which exhibits α_{1h}^l and α_{1h}^u determinants (Figure 1), immunized hosts toward LEW (RT1^l, 4.5 ± 0.5 versus 6.75 ± 0.5 days; $P < 0.01$) and WF (RT1^u, 4.25 ± 0.5 versus 6.0 ± 0.0 days; $P < 0.01$; Table 1) [25].

To further examine the immunogenic ability of the α_1 -helical region, the frequency of cytotoxic T cells (fTc) was analyzed in BUF hosts 7 days following s.c. administration of $[\alpha_{1h}^{l/u}]$ -RT1.A^a bearing transfectants. As shown in Table 2, immunization by $[\alpha_{1h}^{l/u}]$ -

RT1.A^a, which lacks the α_1 -helical epitopes, failed to increase the frequency toward ACI (RT1a, $1:11,713 \pm 139$) when compared to non-immunized hosts ($1:11,804 \pm 261$, *PNS*; Table 2). In contrast, immunization of BUF hosts with WT-RT1.A^a, which bears the α_1 -helical epitopes but otherwise identical to [$\alpha_{1h}^{l/u}$]-RT1.A^a, significantly increased the anti-ACI (RT1a) fTc to $1:7839 \pm 588$ ($P < 0.001$). Additionally, [$\alpha_{1h}^{l/u}$]-RT1.A^a significantly increased the fTc toward WF (RT1^u, $1:9750 \pm 21$) and LEW (RT1^l, $1:9035 \pm 39$) in RT1^b hosts when compared to untreated controls ($1:13,717 \pm 273$, $P < 0.01$ and $1:13,508 \pm 1910$, $P < 0.02$, respectively; Table 2). Further, the specificity of the CTL response was demonstrated by the inability of [$\alpha_{1h}^{l/u}$]-RT1.A^a, which lacks α_1 -helical RT1.Aⁿ epitopes, to increase the fTc toward BN (RT1ⁿ) targets (Table 2). Thus, immunogenic determinants that cause accelerated allograft rejection and increased anti-donor cytotoxic T cell frequency are present in the α_1 -helical region of class I MHC RT1.A^a, RT1.A^l and RT1.A^u.

Induction of tolerance by [$\alpha_{1h}^{l/u}$]-RT1.A^a to WF (RT1A^u) and LEW (RT1A^l) allografts in ACI (RT1A^a) recipients

Our previous results demonstrated that peri-transplant portal venous (p.v.) administration of [$\alpha_{1h}^{l/u}$]-RT1.A^a allochimeric molecules, which include RT1.A^u immunogenic epitopes flanked by self recipient ACI sequences, induced tolerance to WF (RT1^u) heart allografts in ACI (RT1^a) recipients, when administered with subtherapeutic CsA [25]. A critical question therefore arises as to the ability of [$\alpha_{1h}^{l/u}$]-RT1.A^a allochimeric molecule to induce tolerance to RT1^l (LEW) allografts in RT1^a (ACI) recipients since its α_1 -helical sequence, which is essentially derived from the RT1.A^l antigen, contains the immunogenic RT1.A^l epitopes flanked by RT1.A^a self-sequences. As shown in Table 3, administration of [$\alpha_{1h}^{l/u}$]-RT1.A^a allochimeric or subtherapeutic CsA alone modestly prolonged WF and LEW heart allograft survival in ACI recipients. In marked contrast, peri-transplant p.v. administration of [$\alpha_{1h}^{l/u}$]-RT1.A^a antigen, in the presence of subtherapeutic CsA, induced indefinite survival of WF hearts in 85% and more than 90% of LEW cardiac allografts in ACI recipients (MST > 120 days, $P < 0.001$; Table 3), respectively. Recipients bearing LEW hearts for more than 100 days indefinitely accepted WF or LEW but rejected third-party BN skin allografts (9.3 ± 0.6 and 8.3 ± 0.6 days, $n = 3/\text{group}$) simultaneously applied to the same recipient on either side of the abdominal wall, thereby documenting donor-specific transplantation tolerance. The specificity of the allochimeric approach to induce peripheral tolerance was demonstrated by the inability of [RT1.Aⁿ immunogenic epitope, to prolong the BN (RT1ⁿ) heart $\alpha_{1h}^{l/u}$]-RT1.A^a, which lacks allografts (MST 21.0 ± 0 days, Table 3) in ACI recipients.

Intact proliferative and cytotoxic T cell responses in long-term tolerant allochimeric conditioned recipients

T cell responses were investigated in allochimeric conditioned ACI recipients of long-term surviving WF or LEW heart allografts. *In vitro* stimulation of lymph node (LN) T cells harvested from long-term tolerant hosts (day 120), exhibited potent proliferative responses to donor WF (Figure 2A) or LEW (Figure 2B) and third-party BN alloantigens. Additionally, LN T cells of tolerant hosts generated potent anti-donor cytotoxic T cell activity (Table 4). The frequency of anti-WF- cytotoxic T cells, in LNs of tolerant hosts ($1:11,373 \pm 1410$) was similar to naive animals ($1:10,579 \pm 1215$, *PNS*), but was significantly lower than ACI animals that rejected WF hearts ($1:5821 \pm 203$, $P < 0.001$). Similarly, the anti-LEW fTc in LN of ACI hosts rendered tolerant to LEW hearts by allochimeric therapy ($1:15,289 \pm 1405$), was significantly lower than rejecting controls ($1:7025 \pm 996$, $P < 0.001$), but not different from naive animals ($1:16,647 \pm 6530$, *PNS*).

Allochimeric tolerance is associated with suppression of IgM and depression of IgG alloantibody response

Sera obtained from allochimeric-treated LEW heart engrafted ACI recipients were tested for the presence of anti-LEW alloantibodies at day 120 post-transplantation (Figure 3). Sera of ACI recipients, which rejected LEW hearts, displayed both IgM and IgG anti-LEW alloantibodies. In contrast, sera from tolerant ACI recipients did not exhibit detectable levels of IgM anti-donor alloantibodies (Panel A), and very low levels of IgG alloantibodies were detected (Panel B). This correlated with marked reduction of B lymphocytes (Panel D) in allografts of tolerant recipients, when compared to naive hearts (Panel C) or rejector controls (Panel E). Thus, tolerance induction by peripheral peri-transplant p.v. administration of allochimeric class I MHC molecules appears to inhibit the generation of anti-donor alloantibodies and reduce allograft B cell migration.

Allochimeric induced tolerance is accompanied by altered Th1- and Th2-type cytokine responses

Based on previous observations of the role of Th1/Th2 differentiation in allograft rejection versus survival [33–35], intra-graft cytokine mRNA expression patterns for Th1- (IL-2 and interferon IFN- γ) and Th2- (IL-4 and IL-10) responses were analyzed in long-term allochimeric conditioned ACI tolerant recipients that accepted WF or LEW hearts for > 100 days. WF cardiac allografts demonstrated a markedly reduced IL-2 and interferon IFN- γ and upregulation of IL-10, when compared to WF hearts that were rejected by ACI hosts. Although IL-4 levels were higher than in rejecting controls, they were comparable to those observed in naive ACI animals (Figure 4 A). Almost identical cytokine patterns were detected in long-term tolerant LEW heart allografts in allochimeric-condition ACI hosts (Figure 4 B). Immunohistochemical analysis of cytokine translation products, in long-term WF hearts allografts, confirmed absence of IL-4 and increased IL-10 expression (Figure 4 C). Thus, IL-10 is the predominant cytokine associated with the allochimeric induced tolerant state.

Discussion

In this study, we examined the determinants of class I MHC molecules that can induce tolerance to cardiac allografts by utilization of peri-transplant peripheral administration of soluble class I allochimeric therapy. Two principal findings have emerged. First, tolerance can be induced as a function of presentation of donor type immunogenic epitopes in the context of recipient class I MHC sequences. Second, allochimeric conditioning by peripheral administration of soluble class I allochimeric therapy provides a unique tolerant state.

Allochimeric class I MHC molecules were employed as a tool to examine the structure-function relationship of class I MHC molecules. This strategy has previously suggested that the polymorphic sequences on the α_1 -helical regions RT1.A^u and RT1.A^l included immunogenic epitopes that induced accelerated rejection of RT1^u and RT1^l cardiac allografts in RT1^b recipients [25]. In concert with previous studies that utilized synthetic peptides derived from the polymorphic RT1.A^a regions [12, 14], current experiments document the critical immunogenic nature of the α_1 -helical region in RT1.A^a alloantigens. Wild type RT1.A^a, but not mutated [α_{1h} ^{l/u}]-RT1.A^a that lacks α_1 ^a helical epitopes, induced accelerated rejection of RT1^a allografts. Further, accelerated allograft rejection correlated with the frequency of alloreactive cytotoxic T cells by the α_1 helical sequences of RT1.A^u, RT1.A^l, and RT1.A^a.

The [α_{1h} ^{l/u}]-RT1.A^a composite allochimeric molecule that displayed class I α_1 -helical donor-type immunogenic epitopes of the two genetically distinct RT1^l and RT1^u strains

displayed on RT1.A^a recipient self-epitopes induced tolerance to both strain combinations. The demonstration of [$\alpha_{1h}^{l/u}$]-RT1.A^a induced tolerance to RT1A^l allografts is critical to the model since its α_1 -helical epitopes are derived from the RT1.A^l sequences. Conversely, [$\alpha_{1h}^{l/u}$]-RT1.A^a which did not display RT1.Aⁿ immunogenic epitopes, failed to prolong the survival of RT1Aⁿ allografts. The critical role of MHC derived immunogenic epitopes in tolerance induction have been well demonstrated. Indeed, *pre-transplant* intrathymic administration of class I or class II immunogenic peptides in an immunomodulated host have resulted in central tolerance induction [24, 39, 40]. Further, *pre-transplant* peripheral administration of syngeneic dendritic cells (DCs) pulsed with immunogenic donor peptide (100 μ g/ml) has induced robust peripheral tolerance [43–45]. In contrast, numerous studies have previously demonstrated that peripheral *peri-transplant* administration of immunogenic class I or class II donor derived alloantigens have consistently failed to induce transplantation tolerance [46–48].

The ability of [$\alpha_{1h}^{l/u}$]-RT1.A^a allochimeric molecule to induce peripheral tolerance is therefore intriguing since the molecule is essentially composed of donor-type immunogenic epitopes and recipient self-sequences. Our studies imply an important role for both donor immunogenic epitopes as well as self-epitopes in peripheral transplantation tolerance by *peri-transplant* administration since portal venous administration of either whole donor or recipient non-mutated class I antigens have not resulted in long-term allograft acceptance [25, 41]. Concurrent presentation of self and dominant donor determinants, may have down-modulated the immune response to donor cardiac allografts. The contribution of self-epitopes to the indirect alloimmune regulation was further emphasized by a recent elegant study, which demonstrated that i.v. administration of a 15-mer peptide encompassing a self-determinant in the α_1 -domain of RT1.Aⁿ diminished the ability of RT1^u recipients to mount an alloantibody response on challenge with intact RT1.A^a alloantigen [36]. Thus, cryptic class I self-determinants may be of relevance in downregulating the indirect helper T cell responses to intact donor alloantigens. Further, the combination of self and immunogenic epitopes may present a ‘neoantigen’ to responding T cells, or self-peptides may compete and limit the presentation of immunogenic epitopes thereby altering allorecognition.

One can argue that tolerance may be induced by administration of the immunogenic epitopes only [24, 37], as opposed to delivery of whole multideterminant class I allochimeric protein. However, other studies have demonstrated the requirement of multiple epitopes for deviation of the immune response as opposed to short peptide sequences. *In vivo* pretreatment with whole soluble protein OVA or with OVA peptide 323-339 inhibited the Th1 development of naive TCR transgenic T cells specific for the chicken OVA peptide 323-339, in response to priming with OVA. However, only protein that contains multiple epitopes, but not single peptide, administration caused the deviation of the T cell population from the Th1 to the Th2 pathway [38]. Additionally, immediate epitope flanking residues, as well as, downstream sequences have been shown to play a crucial role in epitope recognition by responding T cells [42]. Although analysis of T cell responses to nominal protein or transplantation antigens has shown that constraints in antigen processing and presentation usually limit the T cell responses to one or two dominant determinants [1, 8], there is also evidence highlighting the hierarchy of the polyclonal nature of the indirect T cell responses, suggesting the participation of multiple T cell determinants [12, 13, 20–22, 36]. We therefore hypothesize that [$\alpha_{1h}^{l/u}$]-RT1.A^a allochimeric molecules containing both dominant donor-type immunogenic epitopes and cryptic subdominant self-epitopes together orchestrate peri-operative transplantation tolerance through manipulation of the indirect pathway. Nevertheless, our results do not rule the possibility of altering the immune response through direct presentation.

An important aspect of our study is the investigation of the mechanisms of allochimeric-induced long-term allograft acceptance. Long-term engraftment following intrathymic inoculation of class II immunogenic sequences was shown to be mediated by clonal deletion of alloreactive T cells [39]. In contrast, our results in allochimeric conditioned recipients, that are tolerant to either WF or LEW heart allografts, argue against anergy or deletion as underlying mechanisms for long-term graft acceptance. Tolerant hosts mounted potent anti-donor proliferative responses and demonstrated anti-donor cytotoxic T cell frequencies that were equivalent to naive animals. Additionally, our results of intragraft cytokine elaboration patterns contrast those observed in tolerant recipients following anti-CD4 mAb therapy which is IL-4 predominant [34], or following intrathymic inoculation of class I allopeptides which induces dichotomized reduction of Th1 (IL-2 and IFN- γ) and predominant Th2 (IL-4 and IL-10) patterns [40]. Allochimeric conditioned recipients demonstrated predominant elaboration of IL-10, but not IL-4, and downregulation of Th1 (IL-2 and IFN- γ) cytokines. Nevertheless, other studies using different allochimeric sequences elicited a more dichotomized Th1/Th2 responses [49].

Absence of IL-4 in the allochimeric conditioned hosts may underscore limited T-B cell help and thus, in part, explain the observed inhibition of IgM allo-Ab, diminished allo-IgG production, and limited intragraft B cell infiltration. However, the low levels of alloantibodies in sera of tolerant recipients may reflect absorption of alloantibodies in the allografts rather than reduced production. Our current observation of IL-10 deposition coupled with our previous findings of inhibition of chronic rejection in allochimeric conditioned tolerant hosts [26] may argue an important role for IL-10 in abrogation of chronic rejection. Further, the previous demonstration of IL-10 producing CD4⁺ T cells with regulatory properties upon stimulation with immature DCs [50, 51] may argue for a regulatory role for the CD4⁺, IL-10⁺ T cells that were demonstrated in the allografts of long-term allochimeric conditioned tolerant hosts.

During the development of the alloimmune response, T cell specificity spreads to include new additional epitopes that were not targeted at the induction phase of the immune response [52]. B cells may play a pivotal role in such epitope spreading because of their potential in capturing and presenting additional donor or self epitopes required for T cell activation [53]. Since cryptic self epitopes were directly demonstrated to provide help for alloantibody production, it has been postulated that in the context of chronic rejection, presentation of subdominant epitopes by B lymphocytes results in the recruitment of additional T cell clones, and such spreading of alloreactivity contributed to chronic rejection [36]. However, subdominant self-determinants were also shown to inhibit alloantibody production [36]. We have previously demonstrated that allochimeric therapy mitigated the development of chronic rejection [26]. Further, allochimeric therapy is associated with abrogation of IgM and attenuation of IgG alloantibody responses, and marked reduction of intragraft B cellular infiltrates. It is therefore attractive to speculate that allochimeric determinants may have mitigated B cell epitope spreading to responding T cells to afford such quiescent intragraft milieu and inhibition of chronic rejection in long-term tolerant recipients. Alternatively, allochimeric conditioning may have abrogated helper T cell responses to B cells. In agreement with this hypothesis is the abrogation of intragraft IL-4 production, a prototypic Th2 B cell inducing cytokine, may indicate diminished T cell help for alloantibody production.

In conclusion, our present findings demonstrate the successful manipulation of the alloimmune response for induction of a unique tolerant state induced by both donor immunogenic and recipient self-sequences. Such novel immunotherapeutic approach proffers strong rationale for understanding the complexity of the indirect immune response following alloantigen administration.

References

1. Katz M, Maizels R, Wicker L, Miller A, Sercarz EE. Immunological focusing by the mouse MHC: mouse strains confronted with distantly related lysozymes confine their attention to a very few epitopes. *Eur J Immunol.* 1982; 12:535–540. [PubMed: 6180905]
2. Clayton JP, Gammon GM, Ando DG, Kono DH, Hood L, Sercarz EE. Peptide-specific prevention of experimental allergic encephalomyelitis. Neonatal tolerance induced to the dominant T cell determinant of myelin basic protein. *J Exp Med.* 1989; 169:1681–1691. [PubMed: 2469764]
3. Liu Z, Williams KP, Chang YH, Smith JA. Immunodominance: A single amino acid substitution within an antigenic site alters intramolecular selection of T cell determinants. *J Immunol.* 1993; 151:1852–1858. [PubMed: 7688387]
4. Sloan-Lancaster J, Evavold BD, Allen PM. Induction of T-cell anergy by altered T-cell receptor ligand on live antigen presenting cells. *Nature.* 1993; 363:156–159. [PubMed: 8483498]
5. Sloan-Lancaster J, Allen PM. Altered peptide ligand-induced partial T cell activation: Molecular mechanisms and role in T cell biology. *Annu Rev Immunol.* 1996; 14:1–27. [PubMed: 8717505]
6. Oki A, Sercarz E. T cell tolerance studied at the level of antigenic determinants. Latent reactivity to lysozyme peptides that lack suppressogenic epitopes can be revealed in lysozyme-tolerant mice. *J Exp Med.* 1985; 161:897–911. [PubMed: 2580937]
7. Singh DP, Kikuchi T, Singh VK, Shinohara T. A single amino acid substitution in core residues of S-antigen prevents experimental autoimmune uveitis. *J Immunol.* 1994; 152:4699–4705. [PubMed: 8157981]
8. Benichou G, Fedoseyeva E, Lehmann PV, Olson CA, Geysen HM, McMillan M, Sercarz EE. Limited T cell response to donor MHC peptides during allograft rejection. *J Immunol.* 1994; 153:938–945. [PubMed: 7517977]
9. Shirwan H, Chi D, Makowka L, Cramer DV. Lymphocytes infiltrating rat cardiac allografts express a limited repertoire of T cell receptor V β genes. *J Immunol.* 1993; 151:5228–5238. [PubMed: 8228221]
10. Liu Z, Sun YK, Xi YP, Hong B, Harris PE, Reed EF, Suci-Foca N. Limited usage of T cell receptor V β genes by allopeptide-specific T cells. *J Immunol.* 1993; 150:3180–3186. [PubMed: 8468463]
11. Liu Z, Colovai AI, Tugulea S, Reed EF, Fisher PE, Mancini D, Rose EA, Cortezini R, Michler RE, Suci-Foca N. Indirect recognition of donor HLA-DR peptides in organ allograft rejection. *J Clin Invest.* 1996; 98:1150–1157. [PubMed: 8787678]
12. Fangmann J, Dalchau R, Fabre JW. Rejection of skin allografts by indirect allorecognition of donor class I major histocompatibility complex peptides. *J Exp Med.* 1992; (175):1521–1529. [PubMed: 1588278]
13. Benham A, Fabre JW. Fine specificity of peptide determinants for indirect T cell recognition of class I MHC alloantigens. *Transplantation.* 1994; 58:1236–1240. [PubMed: 7527604]
14. Fangmann J, Dalchau R, Sawyer GJ, Priestley CA, Fabre JW. T cell recognition of donor major histocompatibility complex class I peptides during allograft rejection. *Eur J Immunol.* 1992; 22:1525–1530. [PubMed: 1601039]
15. Shirwan H, Leamer M, Wang HK, Makowka L, Cramer DV. Peptides derived from α -helices of allogeneic class I major histocompatibility complex antigens are potent inducers of CD4⁺ and CD8⁺ T cell and B cell responses after cardiac allograft rejection. *Transplantation.* 1995; 59:401–410. [PubMed: 7871571]
16. Shoskes DA, Wood KJ. Indirect presentation of MHC antigens in transplantation. *Immunol Today.* 1994; 15:32–38. [PubMed: 8136009]
17. Vella JP, Ferreira M, Murphy B, Alexander S, Sayegh MH, Carpenter CB. Indirect allorecognition of MHC allopeptides in human renal allograft recipients with chronic graft dysfunction. *Transplantation.* 1997; 64:795–800. [PubMed: 9326400]
18. Sayegh MH, Carpenter CB. Role of indirect allorecognition in allograft rejection. *Int Rev Immunol.* 1996; 13:221–229. [PubMed: 8782743]
19. Liu Z, Braunstein NS, Suci-Foca N. T cell recognition of allopeptides in context of syngeneic MHC. *J Immunol.* 1992; 148:35–40. [PubMed: 1530797]

20. Gammon G, Klotz J, Ando D, Sercarz EE. The T cell Repertoire to multideterminant antigen. Clonal heterogeneity of the T cell response, variation between syngeneic individuals, and in vitro selection of T cell specificities. *J Immunol.* 1990; 144:1571–1577. [PubMed: 1689749]
21. Gammon G, Shastri N, Cogswell J, Wilbur S, Sadegh-Nasseri S, Krzych U, Miller A, Sercarz E. The choice of T-cell epitopes utilized on a protein antigen depends on multiple factors distant from, as well as at the determinant site. *Immunol Rev.* 1987; 98:53–73. [PubMed: 2443442]
22. Solinger AM, Ultee ME, Margoliash E, Schwartz RH. T lymphocyte response to cytochrome c. I. Demonstration of a T-cell heteroclitic proliferative response and identification of a topographic antigenic determinant on pigeon cytochrome c whose immune recognition requires two complementing major histocompatibility complex-linked immune response genes. *J Exp Med.* 1979; 150:830–848. [PubMed: 92520]
23. Ghobrial RR, Stepkowski SM, Kahan BD. Molecular engineering of the major histocompatibility complex for immunomodulation. *Clin Transplantation.* 1995; 9:237–245.
24. Murphy B, Krensky AM. HLA-derived peptides as novel immunomodulatory therapeutics. *J Am Soc Nephrol.* 1999; 10:1346–1355. [PubMed: 10361875]
25. Ghobrial RR, Hamashima T, Wang M, Wang M, Stepkowski SM, Kahan BD. Induction of transplantation tolerance by chimeric donor/recipient class I RT1.A^a molecules. *Transplantation.* 1996; 62:1002–1010. [PubMed: 8878396]
26. Singer JS, Mhoyan A, Fishbein M, Shen XD, Gao F, Reemtsen B, Zhao D, Amersi F, Coito A, Busuttill RW, Kupiec-Weglinski JW, Ghobrial RM. Treatment with allochimeric class I MHC molecules inhibits chronic rejection and attenuates alloantibody responses in rat cardiac allograft recipients. *Transplantation.* 2001; 72:1408–1416. [PubMed: 11685113]
27. Horton RM, Cai ZL, Ho SN, Pease LR. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *Biotechniques.* 1990; 8:528–535. [PubMed: 2357375]
28. Ono K, Lindsey ES. Improved technique of heart transplantation in rats. *J Thorac Cardiovascular Surg.* 1969; 57:225–229.
29. Ferrareso M, Tian L, Ghobrial R, Stepkowski SM, Kahan BD. Rapamycin inhibits production of cytotoxic but not noncytotoxic antibodies and preferentially activates T helper 2 cells that mediate long-term survival of heart allografts in rats. *J Immunol.* 1994; 153:3307–3318. [PubMed: 8089500]
30. Siegling A, Lehmann M, Platzer C, Emmrich F, Volk HD. A novel multispecific competitor fragment for quantitative PCR analysis of cytokine gene expression in rats. *J Immunol Methods.* 1994; 177:23–28. [PubMed: 7822831]
31. Onodera K, Volk HD, Ritter T, Kupiec-Weglinski JW. Thymus requirement and antigen dependency in the “infectious” tolerance pathway in transplant recipients. *J Immunol.* 1998; 160:5765–5772. [PubMed: 9637486]
32. Coito AJ, Binder J, Brown LF, de Sousa M, Van De Water L, Kupiec-Weglinski JW. Anti-TNF- α antibody treatment downregulates the expression of fibronectin and decreases cellular infiltration of cardiac allografts in rats. *J Immunol.* 1995; 154:2949–2958. [PubMed: 7876561]
33. Chen N, Gao Q, Field EH. Prevention of Th1 response is critical for tolerance. *Transplantation.* 1996; 61:1076–1083. [PubMed: 8623189]
34. Onodera K, Hancock WW, Graser E, Lehmann M, Sayegh MH, Strom TB, Volk HD, Kupiec-Weglinski JK. Type 2 helper T cell-type cytokines and the development of “infectious” tolerance in rat cardiac allograft recipients. *J Immunol.* 1997; 158:1572–1581. [PubMed: 9029092]
35. Sayegh MH, Akalin E, Hancock WW, Russell ME, Carpenter CB, Linsley PS, Turka LA. CD28-B7 blockade after alloantigenic challenge in vivo inhibits Th1 cytokines but spares Th2. *J Exp Med.* 1995; 181:1869–1874. [PubMed: 7536798]
36. Lovegrove E, Pettigrew GJ, Bolton EM, Bradely JA. Epitope mapping of the indirect T cell response to allogeneic class I MHC: sequences shared by donor and recipient MHC may prime T cells that provide help for alloantibody production. *J Immunol.* 2001; 167:4338–4344. [PubMed: 11591757]
37. Nisco S, Vriens P, Hoyt G, Lyu SC, Farfan F, Pouletty P, Krensky AM, Clayberger C. Induction of allograft tolerance in rats by an HLA class-I-derived peptide and Cyclosporine A. *J Immunol.* 1994; 152:3786–3792. [PubMed: 8144948]

38. Degermann S, Pria E, Adorini L. Soluble protein but not peptide administration diverts the immune response of a clonal CD4⁺ T cell population to the T helper 2 cell pathway. *J Immunol.* 1996; 157:3260–3269. [PubMed: 8871620]
39. Sayegh MH, Perico N, Gallon L, Imberti O, Hancock WW, Remuzzi G, Carpenter CB. Mechanisms of acquired thymic unresponsiveness to renal allografts. Thymic recognition of immunodominant allo-MHC peptides induces peripheral T cell anergy. *Transplantation.* 1994; 58:125–132. [PubMed: 8042230]
40. Shirwan H, Barwari L, Khan HS. Predominant expression of T helper 2 cytokines and altered expression of T helper 1 cytokines in long-term allograft survival induced by intrathymic immune modulation with donor class I major histocompatibility complex peptides. *Transplantation.* 1998; 66:1802–1809. [PubMed: 9884279]
41. Semiletova N, Shen XD, Fishbein MC, Gao F, Slomowitz SJ, Jiao Q, Mukherjee K, Busuttil RW, Kupiec-Weglinski JW, Ghobrial RM. Posttransplant administration of allochimeric MHC Class I molecules induces true transplantation tolerance. *Transplantation.* 2003; 75:550–553. [PubMed: 12605125]
42. Bergmann CC, Yao Q, Ho CK, Buckwold SL. Flanking residues alter antigenicity and immunogenicity of multi-unit CTL epitopes. *J Immunol.* 1996; 157:3242–3249. [PubMed: 8871618]
43. Oluwale OO, Depaz HA, Gopinathan R, Ali A, Garrovillo M, Jin MX, Hardy MA, Oluwale SF. Induction of donor-specific permanent acceptance of rat islets by adoptive transfer of alloepitope-pulsed host myeloid and thymic dendritic cells. *Diabetes.* 2001; 50:1546–1553. [PubMed: 11423475]
44. Garrovillo M, Ali A, Depaz HA, Gopinathan R, Oluwale OO, Hardy MA, Oluwale SF. Induction of transplant tolerance with immunodominant alloepitope-pulsed host lymphoid and myeloid dendritic cells. *Am J Transplant.* 2001; 1:129–137. [PubMed: 12099360]
45. Gopinathan R, DePaz HA, Oluwale OO, Ali AO, Garrovillo M, Engelstad K, Hardy MA, Oluwale SF. Role of reentry of in vivo alloMHC peptide-activated T cells into the adult thymus in acquired systemic tolerance. *Transplantation.* 2001; 72:1533–1545. [PubMed: 11707742]
46. Didlake R, Kim EK, Kahan BD. Ability of 3M KCl-extracted histocompatibility antigen to potentiate the immunosuppressive effect of cyclosporine to prolong the survival of heterotopic rat cardiac allografts. *Transplantation.* 1988; 46:743–747. [PubMed: 3057696]
47. Goto S, Stepkowski S, Kahan BD. Benefit of multiple over single doses of 3M KCL-extracted histocompatibility antigen in the potentiating cyclosporine-induced prolongation of rat cardiac allograft survival. *Transplantation.* 1992; 53:705–707. [PubMed: 1549876]
48. Ito T, Stepkowski SM, Kahan BD. Soluble antigen and cyclosporine-induced specific unresponsiveness in rats. Frequency of alloantigen-specific T cytotoxic cells in normal, sensitized and unresponsive rats. *Transplantation.* 1990; 49:422–428. [PubMed: 2137656]
49. Stepkowski SM, Kirken RA, Trawick B, Wang M, Tejpal N, Wang ME, Tian L, Clark J, Kahan BD. Allochimeric class I MHC protein-induced tolerance by partial TcR engagement requires activation of both CTL4- and common γ -chain-dependent cytokine signaling. *Transplantation.* 2002; 73:1227–1235. [PubMed: 11981413]
50. Jonuleit H, Schmitt E, Schuler G, Knop J, Enk AH. Induction of interleukin 10-producing, nonproliferating CD4⁺ T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J Exp Med.* 2000; 191:1213–1220. [PubMed: 11067871]
51. Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J Exp Med.* 2001; 193:233–243. [PubMed: 11208863]
52. Ciubotariu R, Liu Z, Colovai AI, Ho E, Itescu S, Ravalli S, Hardy MA, Cortesini R, Rose RA, Suciu-Foca. Persistent alloepitope reactivity and epitope spreading in chronic rejection of organ allografts. *J Clin Invest.* 1998; 101:398–405. [PubMed: 9435312]
53. Mamula MJ. Epitope spreading: the role of self peptides and autoantigen processing by B lymphocytes. *Immunol Rev.* 1998; 164:231–239. [PubMed: 9795779]

A.

51 90

CONSENSUS: W:E:EG::YWE::T::K::E:::R::LR:::YYNQSEG

RT1.A^a: -M-R--PE---QQ-RIA-EW-QIY-VD--TLRG-----

RT1.A^l: -M-R--PD---**RE-QKA-GN-QNY-VS**--NLRG-----

RT1.A^u: -M-R--PE---RE-QGA-GH-QVN-VN--TLRG-----

[$\alpha_{1h}^{l/u}$]-RT1.A^a: -M-R--PD---RE-QKA-GN-QNY-VS--NLRG-----

B. **Figure 1.**

A. Amino acid sequences of the α_1 -helical region (a.a. 51 – 90) of RT1.A^a, RT1.A^l, RT1.A^u and allochimeric [$\alpha_{1h}^{l/u}$]-RT1.A^a class I molecules. This region in RT1.A^l contains 10 polymorphic a.a. which differ from the RT1.A^a sequence (bold). The four underlined a.a. in RT1.A^u represent the polymorphic positions that are shared with RT1.A^l, but differ from RT1.A^a. B. Schematic presentation of [$\alpha_{1h}^{l/u}$]-RT1.A^a allochimeric molecule.

Figure 2.

In vitro proliferative T cell responses of allochimeric conditioned long-term tolerant hosts. Lymph node T cells were tested from naïve and tolerant ACI recipients bearing WF (Panel A) or LEW (Panel B) heart allografts. Lymphocytes were cultured with γ -irradiated syngeneic ACI, allogeneic WF or LEW, and third-party BN stimulator cells. Representative data of three different experiments are shown.

Figure 3.

Alloantibody production in allochimeric conditioned long-term tolerant recipients. Serum was harvested from allochimeric-treated ACI recipients of LEW hearts at days 120 posttransplantation (▲) and analyzed for alloantibody production by flow cytometry as described in Materials and Methods. Sera from naive ACI rats (◆) or ACI rats that rejected LEW heart allografts (□) served as negative and positive controls, respectively. The results are expressed as % of positive staining. Three rats were included in each group. Immunohistochemical assessment of B cells in long-term WF allografts of allochimeric conditioned tolerant ACI recipients (Panel D). Naive WF hearts (Panels C) and rejected WF hearts (Panels E) were used for comparison. A representative of 3–5 animals/group is demonstrated.

Figure 4.

Competitive template RT-PCR analysis of mRNA coding for Th1 (IL-2 and IFN- γ)- and Th2 (IL-4 and IL-10)-type cytokines in WF (A) or LEW (B) cardiac allografts harvested from tolerant allochimeric conditioned ACI hosts. Each column represents the mean $\pm$ SD of WT cDNA/CT DNA for the cytokine normalized for the ratio of WT cDNA/CT DNA for β -actin (n= 3 samples per group). Panel C illustrates a representative immunohistochemical analysis for IL-10 and IL-4 translation products in WF long-term heart allografts of allochimeric conditioned ACI tolerant hosts.

Table 1Immunogenicity of [$\alpha_{1h}^{I/u}$]-RT1.A^a allochimeric molecule in BUF (RT1^b) hosts

Donor Strain	Immunogen ^a	Survival (days)	Mean $\pm$ SD	P Value ^b
RT1 ^a (ACI)	None	6 $\times$ 3	6.0 $\pm$ 0.0	-
	WT-RT1.A ^a	3 $\times$ 4, 4 $\times$ 2, 5 $\times$ 2	3.75 $\pm$ 0.9	< 0.001
	[$\alpha_{1h}^{I/u}$]-RT1.A ^a	5 $\times$ 3, 6 $\times$ 4, 7 $\times$ 1	5.75 $\pm$ 0.7	NS
RT1 ^a (BN)	None	6 $\times$ 3	6.0 $\pm$ 0.0	-
	[$\alpha_{1h}^{I/u}$]-RT1.A ^a	6 $\times$ 2	6.0 $\pm$ 0.0	NS
RT1 ^l (LEW)	None	6, 7 $\times$ 3	6.75 $\pm$ 0.5	-
	[$\alpha_{1h}^{I/u}$]-RT1.A ^a	4 $\times$ 2, 5 $\times$ 2	4.5 $\pm$ 0.5	< 0.01
RT1 ^u (WF)	None	6 $\times$ 3	6.0 $\pm$ 0.0	-
	[$\alpha_{1h}^{I/u}$]-RT1.A ^a	4 $\times$ 3, 5	4.25 $\pm$ 0.5	<0.01

^aBUF (RT1^b) hosts were untreated or given subcutaneous injection of 20×10^6 of WT-RT1.A^a or [$\alpha_{1h}^{I/u}$]-RT1.A^a transfected BUF hepatoma cells on day -7 prior to transplantation of ACI, WF, LEW or BN cardiac allografts. The last day of palpable heart beats was considered the day of rejection.

^bP value was calculated using Student's t test.

Table 2Frequency of cytotoxic T cells immunized BUF (RT1^b) hosts

Targets ^a	Naive	[$\alpha_{1h}^{l/u}$]-RT1.A ^a immunized	WT-RT1.A ^a immunized	P Value ^b
RT1 ^a (ACI)	1:11,804 $\pm$ 261	1:11,713 $\pm$ 139	1:7839 $\pm$ 588	<0.001
RT1 ^l (LEW)	1:13,508 $\pm$ 1910	1:9035 $\pm$ 39	-	<0.02
RT1 ^u (WF)	1:13,717 $\pm$ 273	1:9750 $\pm$ 21	-	<0.01
RT1 ⁿ (BN)	1:14,104 $\pm$ 375	1:14,767 $\pm$ 119	-	NS

^a BUF (RT1^b) hosts were given subcutaneous injection of 20×10^6 of [$\alpha_{1h}^{l/u}$]-RT1.A^a or WT-RT1.A^a transfected BUF hepatoma cells. Frequency of cytotoxic T cells (fTc) against ACI (RT1^a), LEW (RT1^l), WF (RT1^u), or BN (RT1ⁿ) targets was measured in naive or immunized BUF rats 7 days after injection using limiting dilution analysis as described in materials and methods. At least 3 rats were included in each group.

^b P value was calculated using Student's t test.

Table 3Allochimeric induced long-term survival of WF or LEW allografts in ACI recipients^a

Donor	[$\alpha_{1h}^{l/u}$]-RT1.A ^a	CsA	Survival	Mean $\pm$ SD
WF	–	–	8, 9, 9,10,11	9 $\pm$ 1.0
WF	–	+	10, 19, 20, 20, 21	18 $\pm$ 5.0
WF	+	–	11, 11, 17	13 $\pm$ 3.5
WF	+	+	15, 15, >120 $\times$ 13	>120
LEW	–	–	7, 7, 8, 8, 9	8.0 $\pm$ 0.8
LEW	–	+	11, 11, 13, 19, 23	15.0 $\pm$ 5.0
LEW	+	–	11, 11, 12, 14, 19	13.4 $\pm$ 3.4
LEW	+	+	12, 28, 47, >120 $\times$ 12	>120
BN	+	+	21 $\times$ 3	21 $\pm$ 0.0

^a ACI recipients were not treated, or treated peri-operatively (day 0) with 1mg of [$\alpha_{1h}^{l/u}$]-RT1.A^a allochimeric molecule p.v. administered with or without a subtherapeutic dose of CsA (oral gavages, 10mg/kg at days 0, 1, 2).

Table 4Anti-donor cytotoxic T cell frequency [$\alpha_{1h}^{1/10}$]-RT1.A^a tolerant ACI hosts

ACI Host ^a	Cardiac Allograft	Targets	fTC	P Value ^b
Naive	-	RT1 ^u (WF)	1:10,579 ± 1215	NS
[$\alpha_{1h}^{1/10}$]-RT1.A ^a -treated	RT1 ^u (WF)	RT1 ^u (WF)	1:11,373 ± 1373	NS
WF Rejector	RT1 ^u (WF)	RT1 ^u (WF)	1:5821 ± 203	<0.001
Naive	-	RT1 ^l (LEW)	1:16,647 ± 6530	NS
[$\alpha_{1h}^{1/10}$]-RT1.A ^a -treated	RT1 ^l (LEW)	RT1 ^l (LEW)	1:15,289 ± 1405	NS
LEW Rejector	RT1 ^l (LEW)	RT1 ^l (LEW)	1:7025 ± 996	<0.001

^aFrequency of cytotoxic T cells (fTC) against WF or LEW targets was measured, by limiting dilution analysis, in naive ACI rats, in ACI rats that rejected WF or LEW hearts or in allochimeric-induced tolerant ACI recipients that accepted WF or LEW cardiac allografts for >100 days posttransplantation. At least 3 rats were included in each group.

^bPvalue was calculated using Student's t test.