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Modulation of Tissue-Specific Immune Response to Cardiac Myosin Can Prolong Survival of Allogeneic Heart Transplants

Eugenia V. Fedoseyeva,²* Koji Kishimoto,²† Hillary K. Rolls,* Ben M.-W. Illigens,* Victor M. Dong,† Anna Valujskikh,³ Peter S. Heeger,‡ Mohamed H. Sayegh,† and Gilles Benichou³*

The role of immune response to tissue-specific Ags in transplant rejection is poorly defined. We have previously reported that transplantation of cardiac allografts triggers a CD4+ Th1 cell response to cardiac myosin (CM), a major contractile protein of the heart, and that pretransplant activation of proinflammatory CM-specific T cells accelerates rejection. In this study, we show that administration of CM together with IFA (CM/IFA) can prevent acute rejection of an allogeneic heart transplant. Prolongation of cardiac graft survival is associated with activation of CM- and allo-specific T cells secreting type 2 cytokines (IL-4, IL-5) and reduction of the frequency of proinflammatory IFN-γ-secreting (type 1) alloreactive T cells. Blocking of IL-4 cytokine with Abs abrogates the prolongation. CM/IFA treatment prevents acute rejection of MHC class I-mismatched, but not fully mismatched grafts. However, if donor heart is devoid of MHC class II expression, CM-IFA administration delays rejection of fully allogeneic cardiac transplants. This finding suggests that the effect of CM modulation depends on the type (direct vs indirect) and strength of recipient’s CD4+ T cell alloreponse. Our results underscore the important role of host immunity to tissue-specific Ags in the rejection of an allograft. This study demonstrates that modulation of the immune response to a tissue-specific Ag can significantly prolong cardiac allograft survival, an observation that may have important implications for the development of novel selective immune therapies in transplantation. The Journal of Immunology, 2002, 169: 1168–1174.

The immune response to allogeneic transplants is initiated by T cells that recognize MHC molecules present on donor cells. T cell allorecognition is mediated via two distinct pathways: 1) the direct pathway in which T cells interact with intact donor MHC proteins displayed on grafted cells, and 2) the indirect pathway in which T cells recognize processed allo-MHC-derived peptides bound to self MHC molecules on host APCs (1–4). Either of these types of T cell responses to donor MHC is sufficient to cause acute rejection of skin allotransplants (5). Other polymorphic proteins called minor transplantation Ags contribute to immune-mediated destruction of an allograft (6). In addition, nonpolymorphic tissue-specific proteins may play an important role in antigen immunity. Indeed, several studies showed the presence of T and B cell responses to these Ags in transplanted individuals (7–11). However, little is known regarding the exact contribution of these responses to the allograft rejection process.

We have previously identified a heart-specific protein, cardiac myosin (CM), which triggers de novo CD4+ Th1 and B cell autoimmune responses after transplantation of cardiac allografts in mice (9). CM also represents the target autoantigen in experimental autoimmune myocarditis, a mouse model of autoimmune heart disease (12). This suggests that anti-CM immunity could cause tissue damage to transplanted heart in a fashion similar to that observed in autoimmune myocarditis. Supporting this view, induction of an autoimmune response to CM in recipients before transplantation accelerated the rejection of an allogeneic heart transplant (9). Furthermore, host sensitization to CM was sufficient to cause the rejection of syngeneic grafts in the absence of alloresponse (9). De novo posttransplant autoreactivity has also been observed in kidney, skin, and liver transplant models (13–15). Interestingly, Yasufuku et al. (10) have reported that oral administration of lung Ag, collagen type V, can reduce the pathogenesis associated with lung transplant rejection. Together with our data, this suggests that this type of response represents a general phenomenon in transplantation and that it is relevant to the rejection process. However, it remains to be established whether modulating tissue-specific immunity can prolong the survival of an allogeneic transplant.

In the present study, we show that pretransplant administration of CM in IFA significantly prolongs the survival of cardiac allografts in the absence of MHC class II mismatch between donor and recipient. This treatment activates CM-specific T cells producing type 2 cytokines. Prolongation of graft survival in CM/IFA-treated animals correlates with reduction of proinflammatory Th1-mediated responses to donor MHC. The mechanisms by which modulation of CM-specific response can influence alloreactivity and graft survival are discussed.

*Abbreviations used in this paper: CM, cardiac myosin; HEL, hen egg white lysozyme; KO, knockout.
Materials and Methods

Mouse heart transplantation

A/J (K^a A^b E^b D^b), A.TL-H2^d (K^a A^b E^b D^b), BALB/c (K^d A^b E^o D^o), C57BL/6 (K^b A^o E^o D^o) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). B6.129-Abb^tm1 N5 MHC class II-deficient mice (K^b A^o E^o D^b) were obtained from Taconic (Germantown, NY). The care of animals was in accordance with institutional guidelines. Vascularized heterotopic cardiac transplantation was performed as described by Corry et al. (16). Transplanted hearts were monitored daily by palpation through the abdominal wall. Heart beat intensity was graded on a scale of 0 (no palpable impulse) to 4 (strong impulse). Rejection was defined by the loss of palpable cardiac contractions and verified by autopsy and pathological examination.

Immunizations and anti-IL-4 mAb treatments

Mouse CM was purified from the hearts of mice of recipient strains (BALB/c, A.TL), as described by Shiverick et al. (17). The purity of preparations (>95%) was determined by SDS-PAGE. Mice were injected i.p. with 300 μg of either mouse CM or hen egg white lysozyme (HEL; Sigma-Aldrich, St. Louis, MO) emulsified in 0.5 ml IFA (Life Technologies,

![FIGURE 1. Administration of CM along with IFA activates CM-specific T cells producing type 2 cytokines. Recipient A.TL mice were injected i.p. with CM emulsified in IFA (A), HEL (control Ag) in IFA (B), or CM in CFA (C). Ten days later, spleen cells were harvested and tested for their response to CM (filled bars) and to HEL (hatched bars) or to medium (open bars). The production of cytokines was assessed by ELISPOT. Data are expressed as mean numbers of IL-2, IL-4, IL-5, and IFN-γ spots ± SE (4–7 mice tested individually in each group).](http://www.jimmunol.org/)
Gaithersburg, MD) or with PBS/IFA. Control group received the same Ags together with CFA (Life Technologies).

For anti-IL-4 mAb treatments, mice were given a single i.p. injection of 4 mg rat mAb specific for mouse IL-4 cytokine (clone 1B11B). Abs were purified from tissue culture supernatants using a protein G column. The hybridoma 1B11 was obtained from American Type Culture Collection (Manassas, VA).

Measurement of alloimmune and CM-specific T cell responses

ELISPOT assays were performed as described elsewhere (18). Briefly, ELISPOT plates (Polytronics, Rockland, MA) were coated with either 3 
\( \mu \)g/ml rat anti-mouse IL-2 (JES6-1A12), 4 
\( \mu \)g/ml rat anti-mouse IFN-\( \gamma \) (R4-6A2), 2 
\( \mu \)g/ml rat anti-mouse IL-5 (TRK-4) capturing mAbs. The plates were then blocked for 1.5 h with PBS containing 1% BSA and washed with sterile PBS. For measuring Ag-induced responses, 10\(^6\) spleen cells from immunized, transplanted, or control (naive) mice were incubated in 0.2 ml AIM-V medium (Life Technologies) containing 1% FCS (Atlanta Biologicals, Norcross, GA) in the presence of Ags (20–40 
\( \mu \)g/ml) or medium alone. To measure the allore-

sponse, 10\(^6\) splenocytes from transplanted or naive mice were cultured with 10\(^6\) irradiated (2000 rad) syngeneic or allogeneic splenocytes. The fre-

quency of T cells producing IL-2 and IL-4 was determined 24 h later. For detection of IFN-\( \gamma \) and IL-5 spots, plates were incubated for 40 h. After removal of cells from the plates and washing, 2 
\( \mu \)g/ml biotinylated rat anti-mouse IL-2 mAb (JES6-5H4), rat anti-mouse IFN-\( \gamma \) mAb (XMG1.2), rat anti-mouse IL-4 mAb (BVD6-24G2), or rat anti-mouse IL-5 mAb (TRK-5) was used, followed by incubation with streptavidin D HRP (Vector, Burlingame, CA) diluted at 1/2000 in PBS/0.025% Tween. All mAbs were obtained from BD PharMingen (San Diego, CA). After washing, the plates were developed using 0.8 ml 3-amino-9-ethylcarbazole (Pierce, Rockford, IL; 10 
\( \mu \)g dissolved in 1 ml dimethyl formamide) mixed with 24 ml 0.1 M sodium acetate, pH 5.0, containing 12 
\( \mu \)l H\( \text{2} \)O. The resulting spots were counted using a computer-assisted enzyme-linked immu-

nospot image analyzer (T Spot Image Analyzer; Cellular Technology, Cleveland, OH).

Morphology

Cardiac grafts from untreated and CM-treated animals were fixed in 10% buffered Formalin (Sigma-Aldrich), embedded in paraffin, coronally sec-

tioned, and stained with H\&E for evaluation of cellular infiltrates by light microscopy.

Statistical analysis

All statistical analyses were performed using STATView software (Abacus Concepts, Berkeley, CA). Values of \( p \) were calculated using an unpaired \( t \) test analysis with a two-tailed distribution and unequal variance. Value of \( p < 0.05 \) was considered statistically significant.

Results

Administration of CM with IFA activates CM-specific T cells secreting type 2 (IL-4 and IL-5) cytokines

Immunity to CM detected in heart-grafted mice is consistently mediated by CD4\(^+\) T cells releasing type 1 (IL-2, IFN-\( \gamma \)), but not type 2 cytokines (9). Sensitization of recipient mice to CM before transplantation induces a potent proinflammatory Th1 anti-CM immunity resulting in accelerated transplant rejection (9). Based upon these findings, we postulated that stimulation of CM-specific T cells producing type 2 cytokines before grafting could antagonize the effects induced by anti-CM Th1 cells, thereby improving heart transplant survival in recipient mice.

Inoculation of autoantigens in IFA can lead to prevention of autoimmune diseases via activation of anti-inflammatory T cells displaying type 2 cytokine patterns (IL-4, IL-5, IL-10) (19, 20). In this study, we first tested whether injection of mice with CM emul-

sified in IFA could induce CM-specific type 2 T cell response. A.TL mice were injected i.p. with CM/IFA. Ten days after immunization, the frequencies of type 1 (IL-2, IFN-\( \gamma \)) and type 2 (IL-4, IL-5) cytokine-producing CM-specific T cells were evaluated using ELISPOT. High frequencies of IL-4- and IL-5-producing T cells were detected (Fig. 1A). In turn, type 1 immunity was strongly reduced. Induction of type 2 immunity to CM was Ag specific in that no activation of type 2 cytokine-secreting CM-specific T cells was found after injection of an irrelevant Ag, HEL.

![FIGURE 2](http://www.jimmunol.org/)

FIGURE 2. Pretreatment injection of recipient mice with CM/IFA prolongs survival of MHC class I-disparate cardiac allografts. Survival of A/J donor hearts was monitored in A.TL recipients that received no treatment, CM in IFA 10 days before transplantation or IFA alone. Prolongation of allograft survival was abrogated by in vivo administration of neutralizing anti-IL-4 mAb. Data are expressed as percentage of surviving allografts (6–13 mice tested in each group).
emulsified in IFA (Fig. 1B). Control mice immunized with CM together with proinflammatory adjuvant CFA displayed vigorous type 1, but not type 2 T cell response to CM (Fig. 1C). No response to CM was observed in naive mice (<5 spots/well). Our data thus show that CM/IFA injection elicited activation/expansion of CM-specific T cells producing type 2 cytokines.

Pretransplantation injection of recipients with CM emulsified in IFA prevents acute rejection of MHC class I-disparate heart allografts

We next examined whether induction of type 2 immunity to CM could impact the rejection of cardiac allografts in mice. Ten days after CM/IFA-treatment, A.TL mice were transplanted with MHC class I-disparate A/I hearts. Remarkably, these mice retained donor hearts for >100 days (100 ± 25, n = 8), while untreated recipients and mice injected with IFA alone rejected their transplants ~10 days after grafting (mean survival time in the untreated group, 9.7 ± 0.8 days, n = 9; mean survival time in IFA-treated group, 11.0 ± 4.9 days, n = 4; Fig. 2). We conclude that in this model, CM/IFA treatment prevented acute allograft rejection.

Acutely rejected hearts from control untreated recipients exhibited generalized interstitial inflammatory cell infiltration and myocyte damage (Fig. 3B). In contrast, donor hearts from CM/IFA-treated mice displayed areas of well-preserved epicardium with minimal or no inflammatory cell infiltrates (Fig. 3C). In these cardiac allografts, cellular infiltration was mostly confined to the endomyocardium. Therefore, the patterns of cell infiltration in hearts were different in CM/IFA-treated animals as compared with untreated mice.

Modulation of CM response in BALB/c mice can prolong the survival of B6 MHC class II-negative, but not normal B6 heart transplants

Fig. 2 shows a striking prolongation of graft survival after CM/IFA treatment in a model in which donor and recipient differ by a single MHC class I molecule. Next, we investigated the influence of CM/IFA treatment on the rejection in a donor/recipient combination with multiple mismatches. To test this, BALB/c mice were injected with CM/IFA and 10 days later transplanted with C57BL/6 (B6) hearts, which differ for both class I and class II MHC as well as minor transplantation Ags. As shown in Fig. 4A, CM/IFA treatment had no effect on graft survival in this donor/recipient combination (rejection at 8.0 ± 0.6 days vs 8.4 ± 0.5 days in untreated recipients). We hypothesized that CM/IFA therapy is not efficient in BALB/c-B6 model due to the presence of allogeneic MHC class II molecules on the graft. MHC class II Ags are known to induce a potent polyclonal direct CD4+ T cell alloresponse that may be difficult to alter. We used B6 mice devoid of MHC class II expression (B6.II knockout (KO)) as donors of allogeneic hearts. In the absence of CM/IFA treatment, hearts from MHC class II-deficient mice were acutely rejected 11.7 ± 2.0 days after transplantation (n = 6). However, following CM/IFA injection in BALB/c recipients, B6 MHC class II-negative hearts showed prolongation of the allograft survival (mean survival time in this group, 20.4 ± 1.8 days, n = 10; Fig. 4B). Therefore, in the absence of donor MHC class II, CM/IFA treatment leads to significant prolongation of cardiac allograft survival in a donor/recipient combination with multiple mismatches.

Prevention of acute rejection in CM/IFA-treated mice depends on Th1/Th2 balance of allo- and autoimmunity

The effects of CM modulation on graft survival suggested that activation of CM-specific type 2 T cells could affect alloresponse in recipient mice. To address this possibility, we measured the overall (direct and indirect) alloresponse mediated by both CD4+ and CD8+ alloreactive T cells. The frequencies of type 1 (IFN-γ) and type 2 (IL-5) cytokine-producing alloreactive T cells were assessed in CM-IFA-treated and in control mice transplanted with an allogeneic heart. As shown in Table I, in the A/J-A.TL and BALB/c-B6.II KO combinations, pretransplantation treatment with CM/IFA resulted in the expansion of a population of anti-donor T cells secreting IL-5 and the concomitant reduction of the number of IFN-γ-producing alloreactive T cells. This phenomenon was reflected by the reversal of the ratio between type 1 and type 2 alloreactive T cells in CM-IFA-treated mice as compared with nontreated recipients (Table II). In contrast, in the BALB/c-B6 combination, although the number of type 2 cytokine-releasing alloreactive T cells was increased, there was no decrease in the frequency of activated allospecific IFN-γ-producing T cells, nor was there a reversal of the type 1-type 2 ratio (Table II). From these data, it appears that after CM/IFA modulation, the overall T cell alloresponse is biased towards a type 2 cytokine profile only in the absence of MHC class II disparity between donor and recipient.

It was important to ascertain whether production of type 2 cytokines was involved in blockade of acute rejection following CM modulation. To test this, we blocked in vivo IL-4 cytokine that serves as a growth and differentiation factor for Th2 cells (21).
IL-4 was neutralized at the time of activation of type 2 CM-specific T cells using a protocol previously described by others (22). Recipient A.TL mice were injected i.p. with 4 mg anti-IL-4 mAb 1 day before CM/IFA injection. Ten days after CM/IFA administration, mice were grafted with A.TL heart. As shown in Fig. 2, anti-IL-4 mAb treatment prevented the prolongation of graft survival by CM/IFA (mean survival time in this group, 15.5 ± 0.8 days). These data show that IL-4 cytokine is a key element of the prevention of acute rejection following CM modulation.

Taken together, our data lead us to conclude that activation of a CM-specific anti-inflammatory type 2 immune response polarizes alloreactivity toward a type 2 response in the absence of MHC class II mismatch, and thus results in prolongation of heart transplant survival.

Table I.  Frequencies of type 1 and type 2 allo- and CM-reactive T cells in CM-IFA-treated mice grafted with an allogeneic heart

<table>
<thead>
<tr>
<th>Donor-Recipient Combination</th>
<th>Alloresponse&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CM-Specific Response&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type 1</td>
<td>Type 2</td>
</tr>
<tr>
<td>A/J→A.TL</td>
<td>1492 ± 42</td>
<td>32 ± 12</td>
</tr>
<tr>
<td>B6.II KO→BALB/c</td>
<td>&gt;2000</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>B6→BALB/c</td>
<td>&gt;2000</td>
<td>32 ± 14</td>
</tr>
</tbody>
</table>

<sup>a</sup> Allospecific T cell responses were measured by ELISPOT following incubation of recipient splenocytes with irradiated donor-derived splenocytes.

<sup>b</sup> CM-specific T cell responses were measured by ELISPOT following incubation of recipient splenocytes with purified mouse CM. Data are presented as mean numbers of cytokine spots/well ± SE. In each group, three to eight mice were analyzed individually.
Discussion

In this study, we show that pretransplant administration of CM/IFA can induce type 2 immune responses to both CM and alloantigens and prolong heart transplant survival. After treatment with CM in IFA, the type 2 T cell response to the injected Ag (CM) becomes predominant, as well as to donor MHC Ags. Therefore, the type 2 response to CM spreads to another set of Ags, a phenomenon referred to as intermolecular spreading. Ag spreading of tissue-specific Th2 responses has been observed in autoimmune disease models (19, 20). Our finding suggests that it may also represent a fundamental mechanism of regulation of alloreactivity after transplantation.

There are several possible mechanisms that could explain how spreading of CM-specific type 2 response to alloreactivity could occur in our model. Th1 and Th2 cytokines support the expansion of the corresponding T cell subset while suppressing activation of the other (21). It is possible that type 2 cytokines released by activated CM-reactive T cells directly promote the differentiation of naive alloreactive T cells into Th2 cells while preventing the activation/expansion of the alloreactive Th1 subset. Alternatively, the type 2 cytokines could mediate their effects on alloresponse by influencing APC functions, as described for IL-4 and IL-10 in other models (23).

The effect of CM/IFA on graft survival was detected only in the absence of MHC class II mismatch. This is consistent with the idea that prolongation of graft survival under these conditions requires presentation of both the tissue-specific Ag (CM) and alloantigens by self-MHC class II. It has been known for some time that tolerance generated to one Ag can suppress the response to an unrelated Ag presented on the same APCs (linked suppression) (24). Both indirect allore cognition and type 2 cytokines have been implicated in this phenomenon (25–27). Therefore, linked suppression represents one potential mechanism to explain the requirement for MHC class II matching in our model.

The differential effect of CM/IFA treatment in A/J-A.TL vs BALB/c-B6 combinations may be also due to the variation in the frequencies of alloreactive CD4+ T cells in these two strains. Indeed, the frequency of activated alloreactive CD4+ T cells producing IL-2 was low (15 ± 3 spots per million of splenocytes) in A.TL recipients grafted with A/J hearts and high (276 ± 17 spots) in BALB/c recipients of B6 grafts (5 mice tested in each group). The rejection in mice grafted with MHC class I-disparate hearts is initiated by indirect CD4+ T cell alloresponse, an immune process that is oligoclonal in nature (28). Conversely, the rejection of fully allogeneic B6 cardiac allografts occurs via a polyclonal direct CD4+ T cell alloresponse that is difficult to block or alter. This may explain why graft rejection initiated only through CD4+ T cell indirect allore cognition is blocked following CM modulation. Further supporting this view, B6.II KO donor hearts, which do not evoke a direct CD4+ T cell alloresponse (5), enjoyed prolonged survival in BALB/c recipients after CM/IFA treatment (Fig. 4B). It is noteworthy that the graft prolongation observed in this combination was less striking than in A/J-A.TL model. The strength of alloresponse to HIC and the presence of minor Ag mismatches in BALB/c-B6 model may account for this observation. Indeed, indirect alloresponse in BALB/c mice transplanted with B6.II KO hearts is directed to a large number of peptides derived from MHC class I and multiple minor Ags. In contrast, the indirect alloresponse in A.TL mice that received A/J hearts is restricted to a single or few dominant peptide determinants on Kk MHC class I molecule. We conclude that, even in the presence of minor Ag disparities and multiple MHC class I mismatches between BALB/c recipients and B6 donor mice, modulation of CM response had a striking effect on heart graft survival.

The influence of Th2-mediated immunity on transplant rejection remains a controversial issue. Although some investigators have demonstrated that alloreactive Th2 cells can reject an allograft (29), others have reported that Th2 alloresponse may promote transplantation tolerance (22, 30). The observation that induction of Th2-mediated tolerance requires not only type 2 cytokines, but also blocking of Th1 responses may explain this discrepancy (31, 32). In our model, long-term allograft survival tightly correlates with induction of type 2 immunity to both tissue-specific and allo-Ags and with a drastic reduction in the frequency of proinflammatory type 1 T cells. Moreover, in vivo blocking of IL-4, the hallmark cytokine of a type 2 response, abrogated the salutary effect of CM-IFA treatment. These results support the view that activation/expansion of graft-specific T cells producing type 2 cytokines can be beneficial for transplant survival.

How does a type 2 microenvironment created by CM/IFA immunization exert its protective effect on a cardiac allograft? First, type 2 cytokines may have affected activation/differentiation of CD8+ allospecific cytolytic T cells, thereby preventing graft destruction, as has been reported for IL-10 (33). Second, CM/IFA treatment may have activated regulatory cells. Indeed, recent studies show that regulatory CD4+CD25+ T cells can share gene expression transcripts with Th2 cells. Hence, it is suggested that transplantation tolerance may represent a unique form of Th2-like differentiation (27). Although we cannot rule out the involvement of immune suppression, our preliminary studies show no expansion of CD4+CD25+ T cells and no production of the regulatory cytokine TGF-β in CM/IFA-treated mice with prolonged graft survival (data not shown).

In summary, we have shown that induction of an anti-inflammatory type 2 response to a single tissue-specific graft Ag, CM, resulted in the spreading of type 2 immunity to alloresponse and prolongation of cardiac allograft survival in the absence of MHC class II disparity between the donor and recipient. This represents, to our knowledge, the first demonstration that modulation of the T cell response to a tissue-specific Ag can ensure long-term cardiac allograft survival in the absence of any immunosuppression. Our finding underscores that immune reactivity to tissue-specific Ags expressed by the transplant may be an important component of the
process of allograft rejection. This implies that modulation of tissue-specific response together with MHC class II matching of donor and recipient or suppression of direct CD4⁺ T cell alloresponse may represent an effective strategy for the prevention of rejection of cardiac allotransplants.

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