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Dual effects of the alloresponse by Th1 and Th2 cells on acute and chronic rejection of allotransplants

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Abstract

The contribution of direct and indirect alloresponses by $CD4^+$ Th1 and Th2 cells in acute and chronic rejection of allogeneic transplants remains unclear. In the present study, we addressed this question using a transplant model in a single MHC class I-disparate donor–recipient mouse combination. BALB/c-dm2 (dm2) mutant mice do not express MHC class I L^d molecules and reject acutely L^{d+} skin grafts from BALB/c mice. In contrast, BALB/c hearts placed in dm2 mice are permanently accepted in the absence of chronic allograft vasculopathy. In this model, $CD4^+$ T cells are activated following recognition of a donor MHC class I determinant, L^d 61–80, presented by MHC Class II A^d molecules on donor and recipient APC. Pre-transplantation of recipients with L^d 61–80 peptide emulsified in complete Freund's adjuvant induced a Th1 response, which accelerated the rejection of skin allografts, but it had no effect on cardiac transplants. In contrast, induction of a Th2 response to the same peptide abrogated the $CD8^+$ cytotoxic T cells response and markedly delayed the rejection of skin allografts while it induced *de novo* chronic rejection of heart transplants. This shows that Th2 cells activated *via* indirect allorecognition can exert dual effects on acute and chronic rejection of allogeneic transplants.

Keywords

Chronic graft rejection; Heart transplantation; Skin graft; T-cell allorecognition; Th1/Th2 cells

Introduction

Transplantation of allogeneic tissues triggers a vigorous inflammatory immune response that is initiated *via* the recognition of donor Ag by the recipient $CD4^+$ T lymphocytes. Such T-cell allorecognition is mediated *via* two distinct pathways: the direct pathway in which T cells are stimulated by intact allogeneic MHC II molecules displayed on donor APC [1,2] and the indirect pathway in which T cells interact with processed alloantigen peptides presented by self-MHC II molecules at the surface of recipient APC [3–5].

In direct alloreactivity, donor bone marrow-derived passenger leukocytes present a multitude of peptides in an MHC class II context thereby eliciting a polyclonal $CD4^+$ T-cell

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response in the host's lymphoid organs. Likewise, it is traditionally accepted that this reaction represents the driving force behind early acute graft rejection. Alternatively, the indirect T-cell alloresponse is oligoclonal in that it is mediated by a selected set of alloreactive T-cell clones recognizing a few dominant peptides presented by self-MHC [6,7]. Despite its low frequency, the indirect alloresponse is sufficient to ensure acute rejection of skin allotransplants [8,9]. However, it is unclear whether, in the absence of a direct $CD4^+$ T-cell alloresponse, indirect alloreactivity can mediate on its own acute rejection of less immunogenic vascularized solid organ transplants, including kidneys and hearts. This presumably depends upon the strength of the indirect response and its ability to promote the differentiation of functional alloreactive $CD8^+$ cytotoxic T cells (CTL).

Early acute allograft rejection of solid organ transplants can be prevented with immunosuppressive drugs such as calcineurin inhibitors. However, a large portion of heart, lung and kidney transplants ultimately succumb to chronic rejection, a slow process involving perivascular inflammation, fibrosis and arteriosclerosis associated with intimal thickening and subsequent luminal occlusion of graft vessels [10–12]. It is believed that the $CD4^+$ T-cell direct alloresponse gradually fades away after transplantation as donor passenger leukocytes vanish. In contrast, the indirect alloresponse is perpetuated *via* spreading to formerly cryptic determinants on alloantigens and graft tissue-specific Ag [13–18]. Also, indirect alloreactivity is thought to play a critical role in the production of alloantibodies [19–21] that are known mediators of the chronic rejection process [22–26]. Therefore, there is strong circumstantial evidence suggesting that indirect rather than direct type of alloreactivity initiates chronic rejection of allografts. In support of this view, some correlation between the presence of indirect alloreactivity and chronic rejection has been reported [27–31]. Additionally, a study by Madsen's group shows that immunization with a donor MHC peptide can accelerate the onset of chronic allograft vasculopathy in heart-transplanted miniature swines [32]. However, there is still no formal demonstration that the indirect alloresponse by $CD4^+$ T cells is necessary and/or sufficient to induce *de novo* chronic rejection of organ allotransplants.

Here, we investigated the contribution of alloreactivity by Th1 and Th2 cells to the acute and chronic allograft rejection of skin and heart transplants in a single MHC class I-disparate donor/recipient (BALB/c to BALB/c-dm2 (dm2)) mouse combination. In this model, skin allografts are acutely rejected within 10 days post-transplantation while heart transplants are accepted permanently without signs of chronic rejection. We observed that induction of a pro-inflammatory Th1 response to a dominant MHC class I allopeptide (L^d 61–80) accelerated acute rejection of skin but it did not provoke the rejection of heart allotransplants. In contrast, while immune deviation of the alloreactivity toward a Th2 response markedly delayed acute rejection of skin allografts by Th1 cells, it triggered chronic rejection of cardiac transplants. This shows that Th2 cells can exert dual effects on acute and chronic forms of allograft rejection. The implications of these findings for the design of selective immune therapies in allotransplantation are discussed.

Results

Immunogenicity of L^d 61–80 allopeptide in transplanted and naive BALB/c-dm2 mice

dm2 mice are BALB/c mice lacking expression of the L^d MHC class I molecule due to a mutation in the 3′ region of the corresponding gene. Consequently, dm2 mice recognize the L^d molecule as foreign and reject acutely WT BALB/c skin grafts. Here, we used this model to study the immune physiology of transplant rejection in a single MHC class I-mismatched donor/recipient combination.

We have previously shown that the third polymorphic region of MHC class I glycoproteins (region 61–80) contains a dominant determinant that is presented in indirect fashion, *i.e.* by recipient dm2 APC to recipient $CD4^+$ T cells after allotransplantation in mice [33,34]. In addition, the L^d 61–80 peptide is naturally processed by BALB/c APC and continuously presented by A^d MHC class II molecules. Therefore, $CD4^+$ T cells from dm2-transplanted mice could also recognize the L^d peptide on donor APC *via* a process that can be referred to as "semi-direct allorecognition."

First, we measured the immunogenicity of the L^d 61–80 peptide in naive and transplanted dm2 mice. Naive dm2 mice were immunized subcutaneously with the peptide L^d 61–80 emulsified in CFA. Ten days later, $CD4^+$ T cells from regional lymph nodes were isolated and incubated *in vitro* for 24 h with the immunizing peptide L^d 61–80 or a control hen eggwhite lysozyme (HEL) binding peptide, HEL 105–120. The frequencies of activated T cells producing IL-2 and IFN-γ (type 1 cytokines) and IL-4 and IL-5 (type 2 cytokines) were determined by ELISPOT. As shown in Fig. 1A, immunization with L^d allopeptide induced a potent inflammatory type 1 cytokine $CD4^+$ T-cell response in dm2 mice (> 100 spots/million T cells). A few activated type 2 IL-4-producing T cells were also detected (15 ± 3 spots *per* million T cells). No response was found in non-immunized mice or in the L^d 61–80 peptide-immunized mice restimulated *in vitro* with the irrelevant HEL 46–61 peptide (<3 spots/million T cells).

Next, $CD8^+$ T cells from L^d 61–80-immunized mice were isolated and tested for their ability to kill target cells presenting the allopeptide. These $CD8^+$ T cells could also kill allogeneic BALB/c target cells in the absence of any exogenously added peptide (Fig. 1B). No CTL response was detected with syngeneic L^d-deficient dm2 targets or with irrelevant B6 ($H\text{-}2^b$) allogeneic cells. Non-immunized dm2 mice (Fig. 1C) as well as dm2 mice immunized with control OVA 323–339 peptide (Fig. 1D) did not mount a CTL response to BALB/c allogeneic cells. It is noteworthy that, in this model, dm2 targets pulsed with the L^d peptide were also killed by $CD8^+$ T cells, a result we previously reported [35]. This suggests that the L^d peptide can be presented by K^d or D^d on dm2 APC.

In another set of experiments, (L^{d-}) dm2 mice were transplanted with skins from (L^{d+}) BALB/c mice. At the time of rejection (10–12 days post-transplantation), T cells were isolated from the spleen and lymph nodes of recipients. $CD4^+$ T-cell response to L^d 61–80 peptide was assessed by ELISPOT. As shown in Fig. 2A, BALB/c skin transplantation induced a vigorous inflammatory type 1 alloresponse to L^d 61–80 peptide. In this setting, $CD4^+$ T cells were activated by recipient dm2 APC and the L^d 61–80 allopeptide, *i.e.* through the indirect allorecognition pathway. The response to L^d 61–80 represented approximately 60% of the overall indirect alloresponse as recorded with donor BALB/c sonicates (Fig. 2A). Some direct $CD4^+$ T-cell response was detected, a result presumably reflecting the previously reported presentation of endogenously processed L^d 61–80 by MHC class II A^d molecules on BALB/c APC [33]. A potent $CD4^+$ T-cell direct response was found in dm2 mice transplanted with fully mismatched B6 skins (Fig. 2B). Finally, a low but significant $CD8^+$ T-cell-mediated CTL response to BALB/c targets could be recorded in dm2 mice engrafted with BALB/c skins (Fig. 2C). This CTL response was responsible for acute skin graft rejection given that *in vivo* treatment of recipients with anti-CD8 mAb prevented rejection (data not shown). These results show that in dm2 mice, the $CD4^+$ T-cell alloresponse directed to the dominant L^d 61–80 allopeptide is sufficient to induce a CTL direct response by $CD8^+$ T cells and the subsequent acute rejection of BALB/c skins (depicted in Fig. 3).

Influence of immunization of dm2 mice with L^d 61–80 peptide on the rejection of BALB/c skin grafts

To induce a type 1 cytokine inflammatory indirect alloresponse, dm2 mice were injected i.p. with L^d 61–80 peptide emulsified in CFA (i.p.-CFA). Ten days later, these mice were engrafted with BALB/c skins and tested for their alloresponse and graft rejection. As shown in Fig. 4A, L^d peptide immunization in CFA was associated with a significant increase in anti-donor CTL response as compared with unprimed mice (Fig. 3A) and an accelerated rejection of BALB/c skin allografts (Fig. 4B).

In another set of experiments, dm2 mice were administered i.p. with L^d 61–80 peptide emulsified in incomplete Freund's adjuvant (i.p.-IFA), a procedure known to polarize T-cell response toward type 2 cytokine (Th2: IL-4, IL-5, IL-10) immunity [36–38]. Ten days after immunization, these mice received a BALB/c skin graft and were tested for their alloresponse (ELISPOT), CTL response against BALB/c targets and rejection. Such i.p.-IFA peptide administration resulted in a marked increase in the Th2 response and a reduction of the Th1 response (Table 1). In addition, these mice failed to mount a CTL response to BALB/c allotarget cells (Fig. 5A). Most importantly, rejection of skin allografts was delayed to 60–80 days in all treated mice (Fig. 5B). Control mice injected with PBS or an irrelevant peptide in IFA rejected skin grafts in 10–14 days. Finally, some dm2 mice were treated with anti-IL-4 Ab (mAb: 11B11, 2 mg given i.p.) 1 day before i.p.-IFA L^d 61–80 peptide administration. These mice rejected BALB/c skin grafts like control mice (Fig. 5B). Taken together, these results show that pre-transplantation sensitization of indirect Th1 alloresponse results in accelerated acute rejection of skin transplants correlating with an increase in CTL alloreactivity. In contrast, activation of L^d 61–80 allospecific Th2 cells prior to placement of skin allografts significantly prolonged transplant survival, a phenomenon associated with a reduced inflammatory Th1 alloresponse and the abrogation of the anti-donor cytotoxic T-cell activity.

Effects of Th1 and Th2 indirect alloresponses on heart transplant rejection

Cardiac allotransplants are less immunogenic than their skin counterparts. Actually, in certain MHC class I-mismatched mouse donor/recipient combinations, cardiac allografts enjoy long-term survival while skin grafts are acutely rejected. In our model, dm2 mice did not acutely reject BALB/c hearts. Moreover, histological examination of transplanted hearts revealed no signs of chronic rejection in the majority of mice tested even 100 days after heart transplantation (Fig. 6A and B). Two mice out of ten with transplanted hearts exhibited mild interstitial infiltration and fibrosis detectable 120 days post-transplantation (Table 2).

Next, we investigated whether immunization of dm2 mice with the peptide L^d 61–80 would influence their ability to reject BALB/c hearts. First, dm2 mice were injected i.p. with the L^d 61–80 peptide in CFA 10 days prior to heart transplantation. While this procedure induced a Th1-mediated alloresponse (Table 1), it did not cause acute rejection of BALB/c hearts and had no influence on the course of chronic rejection (data not shown). In contrast, dm2 mice which received the L^d 61–80 peptide i.p.-IFA and mounted a Th2 indirect alloresponse (Table 1) displayed severe signs of chronic allograft vasulopathy detectable as early as 50 days after cardiac transplantation (Table 2 and Fig. 6, right panels). No effects were observed with control HEL peptides administered i.p.-IFA (Table 2). Also, no signs of chronic rejection of syngeneic heart transplants were observed in dm2 mice treated with L^d 61–80 peptide given i.p.-IFA (data not shown). Therefore, in this model, activation of Th2 but not Th1 indirect alloreactivity induces chronic rejection of cardiac allotransplants.

Discussion

The vigorous polyclonal direct alloresponse by inflammatory $CD4^+$ T cells recognizing alloMHC class II molecules on donor passenger leukocytes represents the driving force behind acute rejection of fully allogeneic allotransplants [39–41]. However, in the absence of MHC class II mismatch and direct $CD4^+$ T-cell allorecognition, the $CD4^+$ T-cell indirect alloresponse is sufficient to ensure acute rejection of MHC class I-disparate skin allografts [42,43]. Indeed, indirect allorecognition is known to induce DTH, promote CTL activity by $CD8^+$ T cells and trigger the production of anti-donor Ab by B cells [8,19,21,44]. Most importantly, this type of alloreactivity is oligoclonal in that it involves a limited set of T cells specific for a few dominant donor determinants [6,7,45]. This suggests that Ag-based tolerization may be designed to tolerize this pathway of allosensitization. In this study, we tested this possibility using the single MHC class I-disparate combination dm2 (L^d)-BALB/c (L^{d+}) in which the majority of $CD4^+$ T cells recognize a dominant determinant, L^d 61–80. We observed that induction of a Th2 response to the dominant peptide L^d 61–80 significantly delayed the rejection of BALB/c (L^{d+}) skin allografts by dm2 (L^d) mice. This effect was associated with the abrogation of both Th1 inflammatory responses and CTL responses. In our model, third party skin grafts were rejected in 10 days, thereby supporting a model of donor Ag-specific suppression compatible with the activation of Th2 T cells. Matesic *et al*. have previously reported that adoptive transfer of IL-4/IL-5-producing $CD4^+$ T cells, but not $CD8^+$ T cells, can cause alloskin graft rejection associated with an eosinophilic infiltration of the skin [46]. Together with our results, this indicates that Th2 cells activated indirectly can prevent acute rejection of skin grafts but induce delayed rejection of these transplants *via* a non-inflammatory process. In our model, no eosinophils were detected in skin allografts rejected by Th2 cells. We surmise that Th2 cells could ensure the rejection of skin allografts by promoting the production of anti-donor Ab.

Unlike their skin counterparts, BALB/c heart transplants are not acutely rejected by dm2 mice. This presumably reflects the weakness of the $CD4^+$ T-cell indirect alloresponse induced by heart transplants. In support of this, we have previously shown that the magnitude of indirect $CD4^+$ T-cell alloreactivity is low after cardiac transplantation as compared with skin and corneal transplants [47]. In the absence of direct alloreactivity, a low indirect alloresponse is likely to result in the lack of differentiation of functional CTL, a phenomenon we previously documented in the case of corneal allotransplants [48]. The poor ability of cardiac transplants to induce an indirect alloresponse is not an intrinsic property of these organs as we recorded vigorous $CD4^+$ indirect alloresponses when the heart was placed under the skin (Gilles Benichou, unpublished data). It is possible that upon immediate vascularization of the transplant, as in classical cardiac transplantation, donor APC migrate to the host's thymus and spleen rather than lymph nodes. This could contribute to the poor immunogenicity of heart and kidney transplants as compared to skin grafts. In addition, the skin contains large numbers of highly immunogenic dendritic cells including Langerhans cells, a feature which presumably accounts for their ability to trigger potent alloimmune reponses. It is noteworthy that not all single MHC class I-disparate vascularized heart transplants escape acute rejection in mice. For instance, A/J mice reject acutely A.TL hearts that differ by a single MHC class I allele (K^k *versus* K^s) [15]. The presence of Th-independent $CD8^+$ cytotoxic T cells in certain mouse strains may account for this heterogeneity [49].

Induction of a Th2 indirect alloresponse to the L^d 61–80 allopeptide induced rapid and severe chronic allograft vasculopathy in dm2 recipients of a BALB/c heart. This finding extends previous studies showing some correlation between chronic rejection and the presence of activated T cells directed to donor HLA peptides in transplant recipients [27–31]. Until now, the most convincing evidence of the potential role of indirect allorecognition

in chronic allograft rejection has been provided by J. Madsen's group, which reported that injection of donor MHC class I peptides could accelerate the course of chronic allograft vasculopathy in heart-transplanted miniature swine [32]. To our knowledge, the present study demonstrates for the first time that induction of an indirect $CD4^+$ T-cell alloresponse can induce *de novo* chronic rejection of an allotransplant.

In our model, the Th2 but not Th1 indirect alloresponse induces *de novo* chronic allograft vasculopathy in heart transplants. This is in agreement with previous studies suggesting the involvement of Th2 cells in cardiac allograft rejection and vasculopathy [31,50–52]. Th2 cells activated *via* indirect allorecognition are likely to mediate their effect on chronic rejection by stimulating the production of Ab displaying non-complement fixing IgG1 isotype [37,53]. Chronic rejection may also result from the activation of autoreactive T cells directed to cardiac tissue Ag including myosin and vimentin Ag, which are known to contribute to cardiac allograft rejection [15,54–56]. It is at first glance surprising that the indirect Th1 alloresponse to the peptide L^d 61–80 activated some cytotoxic T cells while it failed to mediate the acute rejection of cardiac transplants in this model. We surmise that given the low frequency of this response directed to a single peptide, it does not reach the threshold that is necessary to achieve acute graft rejection.

This study shows that the fate of MHC class I-disparate transplants depends upon the type of the tissue grafted and the nature of indirect $CD4^+$ T cell alloresponse. In our model, skin allografts elicit an immediate and potent inflammatory indirect alloresponse that eventuates in acute rejection. Alternatively, induction of an antagonist Th2 indirect response delays graft rejection presumably by suppressing the $CD8^+$ direct cytolytic response. In contrast, transplantation of a vascularized heart induces a poor Th1 indirect alloresponse and fails to achieve acute rejection. In this setting, induction of a Th2 but not a Th1 indirect alloresponse resulted in *de novo* development of chronic rejection. This suggests that although immune deviation of indirect alloreactivity to Th2 type of response could be used to prevent acute allograft rejection, it may induce or exacerbate the chronic rejection of vascularized solid organ transplants.

Materials and Methods

Mouse skin and heart transplantations

Mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The care of animals was in accordance with institutional guidelines. Vascularized heterotopic cardiac transplantation was performed as described by Corry *et al*. [57]. 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. Skin allografts were performed according to the technique previously described by Billingham and Medawar [58].

T-cell and T-cell subsets isolation

T cells as well as $CD4^+$ and $CD8^+$ T-cell subsets were isolated from the spleen and lymph nodes of transplanted and naive mice by negative selection using commercially available T-cell purification columns according to the manufacturer's instructions (Accurate Chemical & Scientific, Westbury, NY, USA) (R&D Systems, Minneapolis, MN, USA). Purified T cells were washed in HBSS and used in ELISPOT assays.

Preparation of APC

Mitomycin C-treated splenocytes from donor and recipient naive mice were used as allogeneic stimulator cells or syngeneic APC, respectively. Single cell suspensions of splenocytes devoid of RBC were prepared in AIM-V medium containing 0.5% FBS and treated with mitomycin C (50 μg/mL) for 30 min at 37°C. The cells were washed once in HBSS, incubated for 10 min at 37°C and washed once again before resuspension in AIM-V, 0.5% FBS at 1–3 × 10^7 cells/mL.

Preparation of sonicates

Spleen cells were suspended at 3 × 10^7 cells/mL in AIM-V medium containing 0.5% FBS and sonicated with 10 pulses *per* 1 s each. The resulting suspension was frozen in a dry ice/ethanol bath, thawed at room temperature and centrifuged at 1200 rpm for 10 min to remove intact cells.

Anti-IL-4 mAb treatments

For anti-IL-4 mAb treatments, mice were given a single i.p. injection of 2 mg of rat mAb specific for mouse IL-4 cytokine (clone 11B11). Ab were purified from tissue culture supernatants using a protein G column. The hybridoma 11B11 was obtained from ATCC (Rockville, MD, USA).

Measurement of direct and indirect alloimmune T-cell responses

Spleen and lymph node T cells from naive and skin- or heart-transplanted mice were used as a source of responder cells to measure the total alloresponse as well as the direct and indirect responses. RBC were lyzed for 2 min in Tris-NH4Cl solution. T cells were then washed twice in AIM-V (Gibco BRL, Grand Island, NY, USA) medium containing 0.5% FBS and resuspended at 10^7 cells/mL with 0.5% FBS in AIM-V for use.

ELISPOT assays were performed as described elsewhere [39]. Briefly, ELISPOT plates (Polyfiltronics, Rockland, MA, USA) were coated with either 3 μg/mL of rat anti-mouse IL-2 (JES6-1A12) or 4 μg/mL of rat anti-mouse IFN-γ (R4-6A2) or 2 μg/mL of rat anti-mouse IL-4 (11B11) capturing mAbs. The plates were then blocked for 1.5 h with PBS containing 1% BSA and washed with sterile PBS. To measure the direct alloresponse, 0.3 × 10^6 T cells from transplanted or naive mice were cultured with 10^6 irradiated (2000 Rad) syngeneic or allogeneic splenocytes. To measure indirect responses, T cells from recipients were incubated with sonicates as previously described [39]. The frequency of T cells producing IL-2, IFN-γ and IL-4 was determined 24 h later. After removal of cells from the plates and washing, 2 μg/mL of biotin-ylated rat anti-mouse IL-2 mAb (JES6-5H4), rat anti-mouse IFN-γ mAb (XMG 1.2) or rat anti-mouse IL-4 mAb (BVD6-24G2) were used followed by incubation with streptavidin D horseradish peroxidase (Vector, Burlingame, CA, USA) diluted at 1:2000 in PBS/0.025% Tween. All mAbs were obtained from Pharmingen (San Diego, CA, USA). After washing, the plates were developed using 0.8 mL of 3-amino-9-ethylcarbazole (Pierce, Rockford, IL, USA; 10 μg dissolved in 1 mL dimethyl formamide) mixed with 24 mL of 0.1 M sodium acetate, pH 5.0, containing 12 mL H202. The resulting spots were counted using a computer-assisted enzyme-linked immunospot image analyzer (T Spot Image Analyzer, Cellular Technology, Cleveland, OH, USA).

Cytotoxic T-cell assays

Spleen T cells derived from BALB/c recipient mice were harvested 9–11 days after transplantation and tested for their ability to lyse peritoneal exudate cells from either donors (B6), syngeneic (BALB/c) or third party (CBA) origins as described elsewhere [35].

Morphology

Cardiac transplants were fixed in 10% buffered formalin, embedded in paraffin, coronally sectioned and stained with H&E for evaluation of cellular infiltrates and myocyte damage (acute rejection) by light microscopy. For assessment of chronic rejection, cardiac grafts were stained with Verhoeff's elastin (vessel arteriosclerosis scoring) or Mason's trichrome (evaluation of fibrosis). Arteriosclerosis was assessed by light microscopy and the percentage of luminal occlusion and intimal thickening was determined using a scoring system as previously described [59]. Only vessels that display a clear internal elastic lamina were included in morphometric analysis (5–7 vessels *per* section). All arteries were scored by at least two examiners in a blinded fashion.

Statistical analyses

All statistical analyses were performed using STATView software (Abacus Concepts, Berkeley, CA, USA). *p*-values were calculated using paired *t*-test. *p*-value <0.05 was considered statistically significant.

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Abbreviations

HEL	hen eggwhite lysozyme
IFA	incomplete Freund's adjuvant

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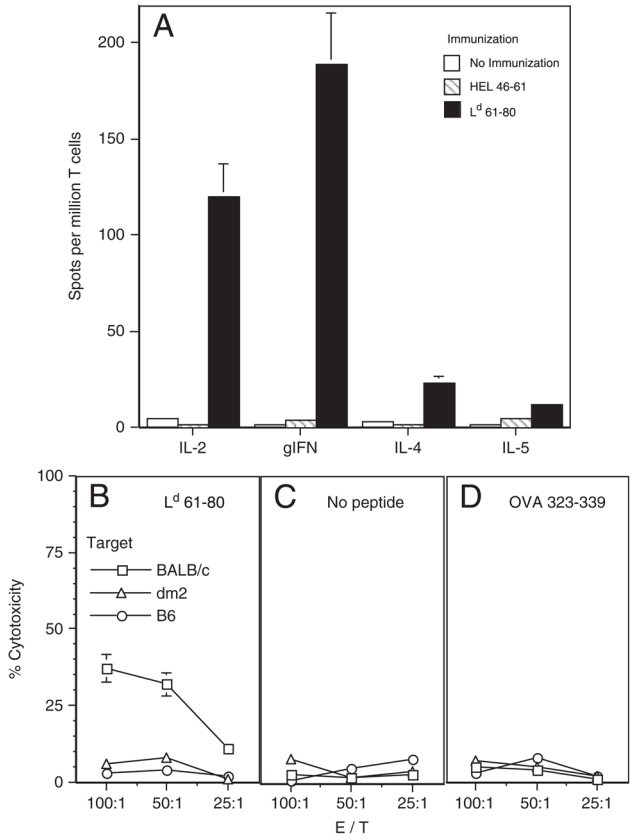

Figure 1.

T-cell response of dm2 mice immunized with L^d 61–80 peptide emulsified in CFA. dm2 mice were injected subcutaneously in the hind foootpad with L^d 61–80 peptide (100 ug) emulsified in CFA. Ten days later, draining popliteal LN T cells were collected and tested for cytokine production by ELISPOT and for cytotoxic response against various targets. (A) T cells were cultured *in vitro* with the immunizing Ag (L^d 61–80, solid bars) or with a control Ag (HEL 46–61, hatched bars). Control non-immunized mice are shown as white bars. The results are presented as spots *per* million T cells + SD for each cytokine. The results are representative of five mice tested individually. CTL responses of mice immunized with L^d 61–80 peptide (B), no peptide (C), and control OVA 323–339 peptide (D). The ability of T cells to kill allogeneic L^{d+} (BALB/c, squares), L^d (B6, circles) and control syngeneic (dm2, triangles) are shown. Spontaneous release ranged from 10 to 15% of maximal release. Data are representative of three independent experiments including 4–5 mice tested individually.

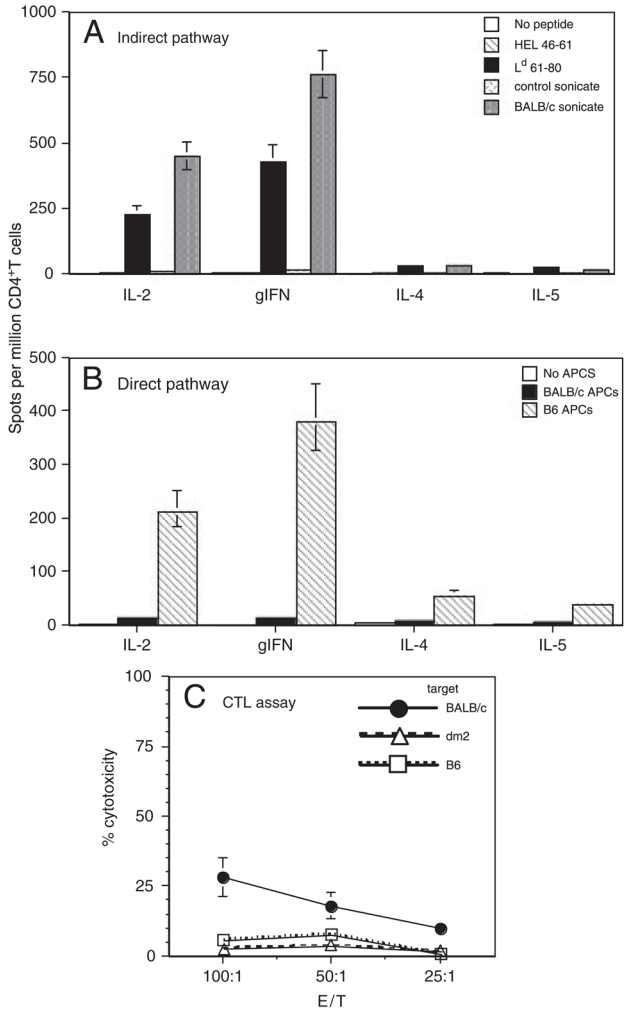

Figure 2.

T-cell response of dm2 mice transplanted with a BALB/c skin. dm2 mice (L^{d-}) were transplanted with a skin from single MHC class I-mismatched BALB/c donors (L^{d+}). At the time of rejection (10–12 days post-transplantation), $CD4^+$ and $CD8^+$ T cells were isolated from recipients' spleens and tested for their alloreactivity. (A) $CD4^+$ T cells were tested by ELISPOT for their indirect alloresponse to BALB/c donor. T cells were incubated with recipient APC along with donor sonicates or the dominant donor allopeptide L^d 61–80. T cells cultured with medium alone or control B6 sonicates and irrelevant peptide HEL 46–61 were used as controls. The results are presented as spots *per* million T cells ± SD for each cytokine. Data are representative of two experiments each including 3–5 mice tested individually. The number of spots obtained with non-transplanted mice ranged from 5–15 spots/million T cells. (B) $CD4^+$ T cells were tested by ELISPOT for their direct alloresponse (MLR) to BALB/c donor. T cells were incubated with donor irradiated MHC class I-mismatched BALB/c APC (solid bars) or fully mismatched B6 APC (hatched bars) or medium (white bars). The results are presented as spots *per* million T cells ± SD for each cytokine. Data are representative of five experiments each including 2–3 mice tested individually. (C) Cytotoxic response of $CD8^+$ T cells from transplanted dm2 mice against donor BALB/c targets (solid dots). Syngeneic targets (triangles) and third-party B6 targets (squares) were used as controls. The spontaneous release ranged from 10 to 15% of maximal release. The data shown here are representative of two independent experiments including

three mice tested individually. The percentages of cytotoxicity recorded with T cells from non-transplanted mice ranged from 2 to 8%.

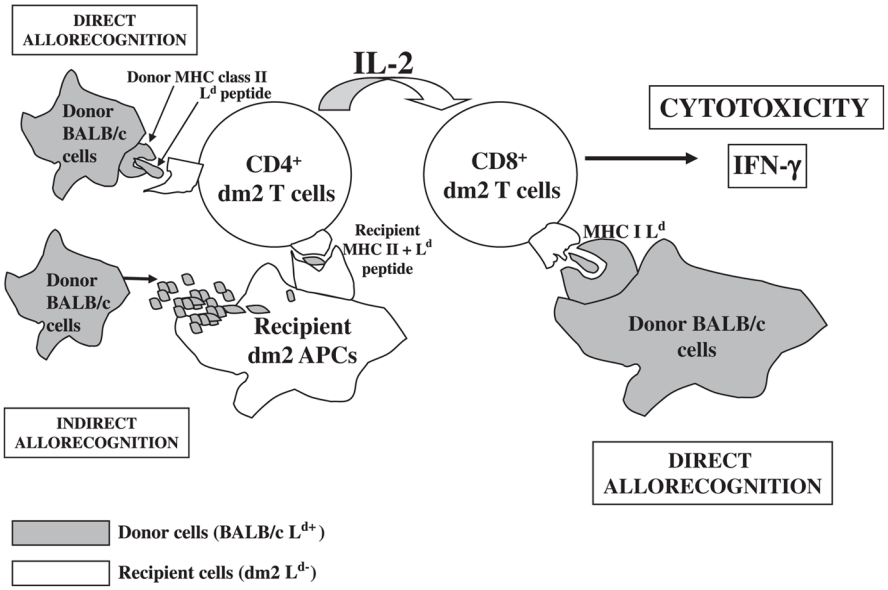

Figure 3.

Schematic representation of the alloresponse in dm2 mice transplanted with BALB/c skin. Donor MHC class I, including L^d 61–80 peptide, are processed and presented in an MHC class II context by recipient APC (exogenous processing, indirect pathway) or donor APC (endogenous processing, direct pathway) to $CD4^+$ T cells. Activated donor-specific $CD4^+$ T cells produce IL-2 thus promoting the differentiation of cytotoxic $CD8^+$ T cells recognizing donor MHC class I at the surface of donor cells (direct allorecognition).

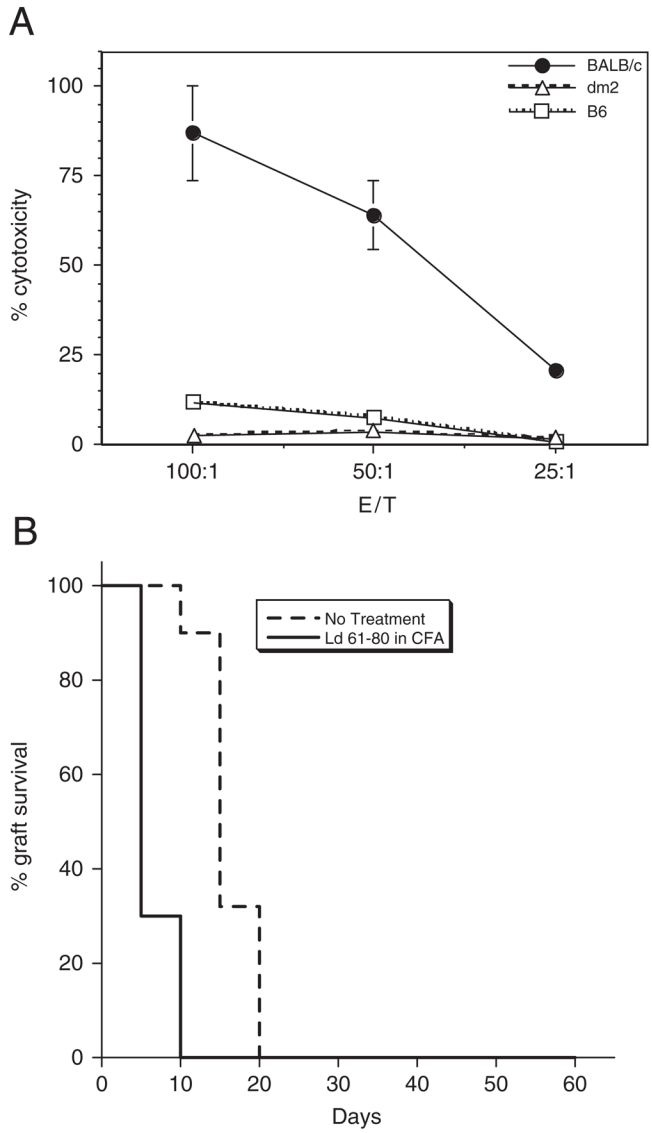

Figure 4.

Effects of L^d 61–80 immunization in CFA on allocytotoxicity and skin graft rejection. dm2 mice were immunized subcutaneously with L^d 61–80 MHC class I peptide emulsified in CFA. Ten days later, these mice received a skin graft from a BALB/c donor. (A) Cytotoxic response of $CD8^+$ T cells against donor BALB/c targets (solid circles). Syngeneic targets (triangles) and third-party B6 targets (squares) were used as controls. Spontaneous release ranged from 10 to 15% of maximal release. The percentages of cytotoxicity recorded with T cells from non-transplanted mice ranged from 5 to 10%. (B) Survival of BALB/c skin grafts in L^d 61–80-CFA-immunized dm2 mice (solid line) and control non-immunized mice (dotted line). Data are representative of three independent experiments each including four mice tested individually. The Student's *t*-test was used to assess statistical significance between two groups, and one-way ANOVA was used to assess statistical significance between the scores. Graft survival was analyzed using the Kaplan–Meier method, and survival curves were compared using the log-rank test.

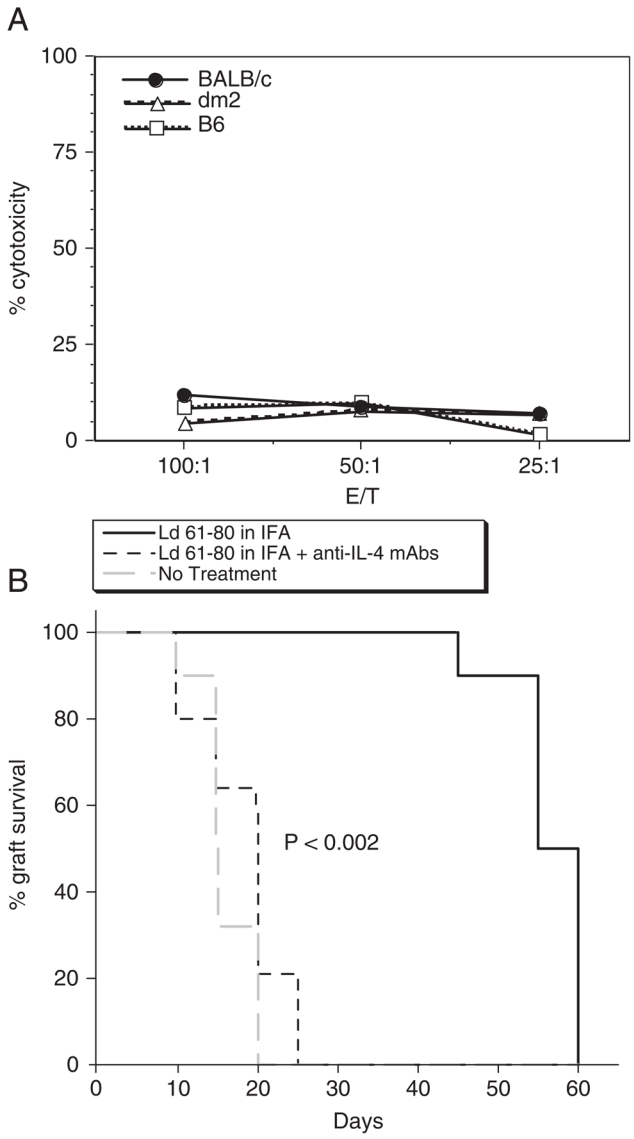

Figure 5.

Effects of L^d 61–80 immunization in IFA on allocytotoxicity and skin graft rejection. dm2 mice were immunized i.p. with L^d 61–80 MHC class I peptide emulsified in IFA. Ten days later, these mice received a skin graft from a BALB/c donor. (A) Cytotoxic response of $CD8^+$ T cells against donor BALB/c targets (solid circles). Syngeneic targets (triangles) and third-party B6 targets (squares) were used as controls. Spontaneous release ranged from 10 to 15% of maximal release. The percentages of cytotoxicity recorded with T cells from non-transplanted mice ranged from 3 to 7%. (B) Survival of BALB/c skin grafts in L^d 61–80-CFA-immunized dm2 mice (black, solid and dotted lines) and control non-immunized mice (grey doted line). Data are representative of three independent experiments each including three mice tested individually. The Student's *t*-test was used to assess statistical significance between two groups, and one-way ANOVA was used to assess statistical significance between the scores. Graft survival was analyzed using the Kaplan–Meier method, and survival curves were compared using the log-rank test.

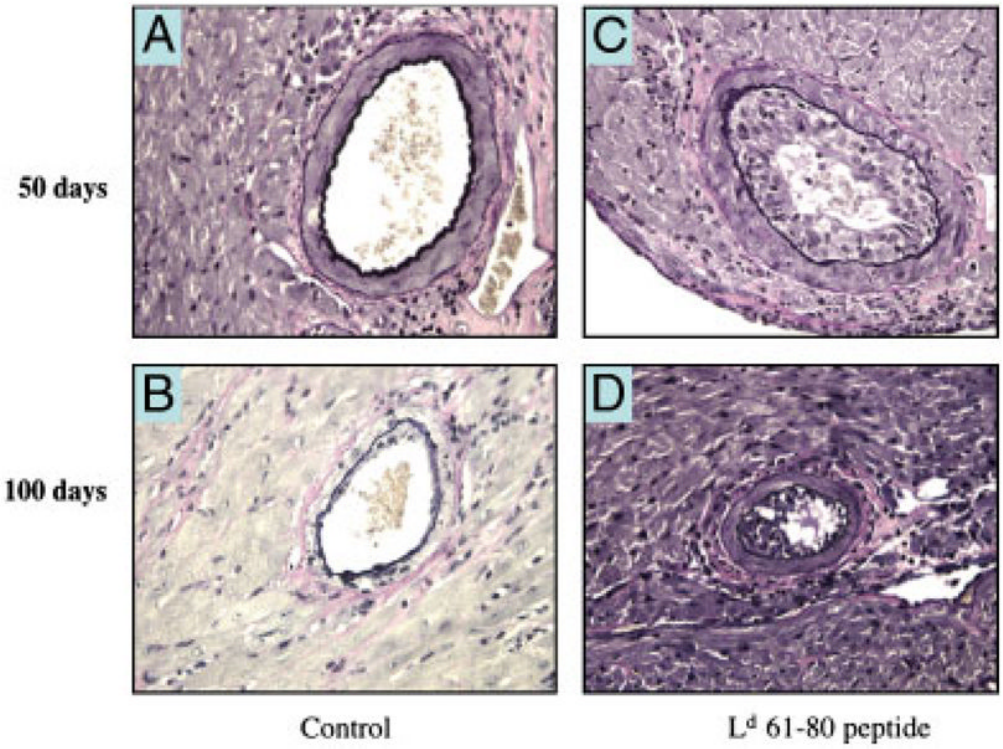

Figure 6.

Histopathology of BALB/c heart transplants from dm2 mice sensitized with L^d 61–80 peptide emulsified in IFA. dm2 mice were immunized i.p. with L^d 61–80 MHC class I peptide emulsified in IFA. Ten days later, these mice received a heart transplant from a BALB/c donor. (A, B) Histology of BALB/c heart transplants from control unimmunized dm2 mice. (C, D) Photomicrographs (40×) of allogeneic hearts harvested 50 and 100days post-transplantation from a L^d 61–80-sensitized recipient mouse display a lymphocytic inflammatory cell infiltrate and vessel obstruction. Data are representative of eight mice tested individually.

Table 1

Adjuvant-driven T-cell responses of L^d 61–80 peptide in dm2 mice

	Immunization			
	none	**L^d 61–80 CFA**	**L^d 61–80 IFA**	**L^d 61–80 soluble**
IL-2	8	**188**	12	15
gIFN	5	**224**	8	12
IL-4	2	5	**162**	3
IL-5	2	2	**98**	4

dm2 mice were immunized subcutaneously with Ld 61–80 MHC class I allopeptide given with CFA, IFA or without adjuvant. Ten days later, $CD4^+$ T cells were isolated from draining lymph nodes and cultured *in vitro* for 40 h with L^d 61–80 peptide. The frequencies of $CD4^+$ T cells producing type 1 cytokines (IL-2 and IFN-γ) and type 2 cytokines (IL-4 and IL-5) were measured by ELISPOT. The results represent the number of spots per million $CD4^+$ T cells. Bold underlined numbers represent values that are significantly higher than the background ($p<0.001$). The number of spots obtained with T cells from non-immunized mice and mice immunized with HEL 46–61 control peptide ranged from 0 to 4 spots per million T cells. The number of spots obtained with T cells from L^d 61–80 peptide-immunized mice cultured in the absence of peptide ranged from 2 to 5 spots per million T cells. The results are representative of three experiments performed on 2–4 mice tested individually.

Table 2

Chronic rejection scores of BALB/c heart transplants in dm2 mice immunized with L^d 61–80

Immunization	Graft survival (days)	Vessel occlusion (%)	Fibrosis (1–5)	Interstitial inflammation (1–5)
None	>100	5±0.5	0	1±0.3
IFA	>100	4±0.6	1±0.2	0
HEL 46–61-IFA	>100	7±1.7	1±0.3	1±0.2
L^d 61–80 IFA	52±10	85±10	4±0.5	4±0.7

dm2 mice were immunized i.p. with L^d 61–80 alloMHC class I peptide emulsified in IFA. Ten days later, these mice were transplanted with a BALB/c heart. One hundred days later, heart transplants were collected and examined for chronic rejection using histological techniques. The vascular cross-sections were graded using morphometric scale (0–5) of luminal occlusion. In this scale 0 represents a normal vessel, whereas a score of 5 indicates more than 80% occlusion of the lumen. Second, from this scale, we determined the percentage of vessels elaborating disease (*i.e.* morphometric score >0). Non-immunized mice as well as mice injected with control HEL peptide 46–61 administered in IFA or with IFA alone were used as controls. The results represent mean values ± SD obtained from 5–8 mice tested in each group.