Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

A novel model of allograft rejection: immune reconstitution of Rag-1 recipients with 2C transgenic T-cell receptor lymphocytes

Dora Ninova, Patrick G Dean, Michael Deeds and Mark D Stegall

The Transplant Immunology Laboratory, Division of Transplant Surgery, Mayo Clinic College of Medicine, Rochester, MN, USA

Keywords
2C transgenic mouse, adhesion molecules, allograft rejection, animal model, islet transplantation.

Correspondence
Mark D. Stegall MD, Mayo Foundation and Clinic, 200 First St SW, Rochester, MN 55905, USA. Tel.: 507 266 1774; fax: 507 266 2810; e-mail: stegall.mark@mayo.edu

Received: 29 November 2003
Revised: 15 July 2004 and
Accepted: 11 August 2004

doi:10.1111/j.1432-2277.2004.00012.x

Summary

The complexity of allorejection (cell activation, homing, and effector function) makes detailed studies difficult. We have developed a model of allograft rejection using purified monoclonal alloreactive effector cells. Immunodeficient C57Bl/6-Rag-1 (H-2^b) recipients of Balb/c (H-2^d) islet or skin grafts were reconstituted via adoptive transfer of splenocytes from 2C transgenic mice containing $CD8^+$ cytotoxic effector cells directed against L^d. Recipients were assessed for engraftment, activation and homing of effector cells, and ability to reject grafts. Both unpurified 2C splenocytes and purified 2C/$CD8^+$ cells durably reconstitute immunodeficient mice. Naïve 2C effector cells reject skin grafts, but not islet allografts. However, when effector cells are primed prior to reconstitution, islet allografts are rejected. Using this model, blockade of adhesion molecules LFA-1 and α4-integrin delayed infiltration of islet allografts and prolonged allograft survival. This model of allorejection may be useful to study the activation and homing of allospecific cells *in vivo*.

Introduction

Acute allograft rejection is a complex process involving multiple cell types. Numerous studies have shown that $CD8^+$ T cells are the primary effector cells in acute rejection [1], yet detailed study of the mechanisms involved in the various steps leading to graft destruction has been difficult [2]. A detailed study of allorejection is hampered in most animal models by the fact that not all the cells that appear at the graft site are allospecific. Lymphocytes are recruited to the graft during their recirculation as part of the immune surveillance process [3]. Naïve T cells recirculate randomly through secondary lymphoid sites until they are stimulated by antigen [4]. Activation of naïve cells can lead to the random acquisition of different cell adhesion molecules and chemokine receptors and to random homing of different sites in an antigen-independent mechanism and so activated T cells can be found in many organs [5]. However, recent studies have provided evidence for the importance of an antigen-dependent mechanism of lymphocyte recruitment to the allografts via antigen presentation by the major histocompatibility complex (MHC) on endothelial cells in vascularized grafts that enhances the rate of transendothelial migration [6,7] Thus, a model system utilizing a single cell population of alloreactive effector T cells might allow a more careful analysis of the stepwise progression of T cell activation, homing, and effector function. It might allow for the identification of alloreactive cell subsets with different functional characteristics involved in the mechanisms of induction of T-cell anergy or generation of immunoregulatory cells. Furthermore, a model system utilizing a single cell population of alloreactive effector T cells might allow for cell manipulation before reconstitution, which can modulate the expression of functional molecules involved in graft destruction. The aim of the current study was to develop a model of allograft rejection using a purified monoclonal population of alloreactive effector cells.

In pursuit of this goal, we used purified $CD8^+$ cells from 2C T-cell receptor (TCR) transgenic mice as effector cells and reconstituted immunodeficient C57BL/6-Rag-1 ($H\text{-}2^b$) recipients, which posses B and T-cell deficiency. The 2C TCR mice posses functionally rearranged TCR α- and β-chain genes from the cytotoxic T-cell clone that is derived from the allogeneic response of an $H\text{-}2^b$ mouse to $H\text{-}2^d$ cells. The 2C TCR cells respond specifically to cells that express L^d, one of the three class I MHC alloantigens encoded by $H\text{-}2^d$ haplotype [8–10]. The 2C mice have C57Bl/6 background similar to the immunodeficient Rag-1 mice. We found that both unpurified 2C splenocytes and purified 2C $CD8^+$ cells durably reconstitute immunodeficient C57BL/6-Rag-1 recipients. Naïve 2C effector T cells reject Balb/c ($H\text{-}2^d$) skin grafts, but not Balb/c islet allografts, efficiently. However, when the effector cells are primed prior to reconstitution, islet allografts are also rejected. After the initial characterization of the model we tested its usefulness to study the allograft rejection using immunosuppressive regimens directed against adhesion molecules. Our choice of a combination treatment with monoclonal antibodies directed against the adhesion molecules VLA-4 α-chain (α4 integrin) and LFA-1 was based on studies successfully targeting α4 integrins [11] and our previous experience [2,12]. The treatment delayed the infiltration of islet allografts and prolonged allograft survival. Thus, we believe that the 2C-Rag-1 TCR transgenic mouse model of allorejection may be a useful tool to study the mechanisms of activation and homing of allospecific T cells *in vivo*.

Materials and methods

Mice

2C transgenic mice were inbred at the Mayo Clinic Animal Facility. C57BL/6J-$Rag1^{tm1Mom}$ ($H\text{-}2^b$) and Balb/c ($H\text{-}2^d$) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Animals were handled in accordance with the regulations of the Institutional Animal Care and Use Committee of the Mayo Foundation and Clinic, Rochester, MN. After weaning, the mice were bled by the tail vein and their blood lymphocytes screened for 2C TCR transgene expression by FACS analysis.

The model system

The model system involves the use of immunodeficient Rag mice as recipients of either islet or skin grafts. Seven days after transplantation the immune system of these graft recipients is reconstituted with alloreactive 2C splenocytes of various purity. The various steps toward allograft rejection are assessed as follows: (i) Engraftment is determined using immunohistochemical analysis for either 2C or CD8. (ii) Activation of alloreactive cells is determined by expression of the IL-2 receptor on adoptively transferred 2C cells in lymph nodes, spleen, or in the graft either immunohistologically or by FACS analysis. (iii) Homing is assessed by graft infiltration of $2C^+$ cells. The allografts were performed prior to the immune reconstitution in order to avoid the homeostatic proliferation reported in Rag-$1^{-}/^{-}$ mouse [13].

Islet isolation and transplantation

Six hundred islets from Balb/c donor mice were isolated by collagenase digestion, density-gradient separation, hand-picked and transplanted under the left renal capsule in streptozotocin-induced (250 mg/kg) C57BL/6-Rag-1 diabetic recipients [2]. Rejection was defined as persistent tail vein blood glucose levels >250 mg/dl.

Skin grafts

Skin grafts were performed by first removing the donor skin graft from the dorsum of the tail of Balb/c donors using a scalpel. The recipient site was prepared using a similar technique and the skin grafts were placed on the recipient site, manually compressed and allowed to adhere. Two allografts and one isograft control were placed on each recipient. A protective dressing was placed for 4 days and the grafts were inspected daily for viability. Rejection was defined as loss of the normal hair pattern and sloughing of the skin graft.

Reconstitution of C57BL6/Rag-1 mice with 2C cells

Allograft recipients were reconstituted with intravenous (i.v.) or intraperitoneal (i.p.) injection of either: (i) naïve unfractioned splenocytes; (ii) naïve purified T cells or 2C/$CD8^+$ splenocytes; (iii) Balb/c-primed purified 2C/$CD8^+$ cells (5×10^7, 2×10^7, 10^7, 5×10^6, and 10^6 cells). The 2C mice were primed by i.p. injection of 10^7 Balb/c splenocytes. Two weeks after priming spleens were harvested, splenocytes isolated and injected into recipients. Immune reconstitution was verified by weekly FACS analysis of peripheral blood lymphocytes. At the time of rejection or after 50 days of follow-up the recipients were killed and their grafts, spleens, and lymph nodes examined by immunohistochemical analysis.

2C splenocyte subfraction isolation

All of the $CD8^+$ cells in the native 2C mouse were found to be positive for the 2C marker using 1B2-H6 monoclonal antibody by FACS analysis. Therefore, we

used the anti-CD8 antibody to isolate purified, monoclonal alloreactive 2C TCR transgenic cells. Purified 2C T cells and $CD8^+$ 2C splenocytes were isolated by magnetic cell sorting using MicroBeads CD90 (Thy 1.2) and CD8a (Ly-2), respectively, MACS LS^+ Separation Columns and MidiMACS magnetic cell separators (Miltenyi Biotec, Auburn, CA, USA). Briefly, 10 μl of CD 90 or CD8a MicroBeads per 10^7 total cells were added to the cell suspension and incubated for 15 min at 6–12 °C. After washing, the cells were resuspended in PBS containing 0.5% bovine serum albumin and 2 mM EDTA and loaded onto the separation columns for positive selection separation. After washing the column was removed from the separator and the positive fraction flushed out using a plunger.

FACS analysis

The 2C TCR transgene expression was verified by the clonotypic antibody 1B2 as culture supernatant from mouse hybridoma 1B2-H6, which was a gift from Dr L. Pease, Mayo Clinic. The cell innoculum was examined by FACS analysis for 2C, CD3, CD8, CD4, B220, and IL-2R expression. We used primary antibodies labeled with FITC or R-Phycoerythrin, anti-CD3 (clone 145-2C11), CD4 (L3T4, clone GK 1.5), CD8a (Ly-2, clone 53-6.7); CD45R/B220 (clone RA3-76B20) CD25 (IL-2R α chain, clone 7D4 (PharMingen, San Diego, CA, USA), and secondary antibodies anti-mouse IgG $F(ab)_2$ FITC (Sigma, St Louis, MO, USA) and anti-mouse IgG $F(ab)_2$–PE (Accurate Chemical Corp., Westbury, NY, USA).

Immunohistochemical analysis

Cryostat sections were cut at 4 μm, fixed in acetone and sequentially incubated with 5% blocking serum, appropriately diluted primary biotinylated antibody, avidin-biotin-peroxidase complex (Vectastain ABC Elite; Vector Laboratories, Burlingame, CA, USA), 3,3′-diaminobenzidine substrate and counterstained with hematoxylin. 2C expression was revealed using a nonlabeled mouse monoclonal antibody (1B2-H6) and anti-mouse IgG $F(ab)_2$ FITC. For control sections, the primary antibodies were substituted with normal immunoglobulins of the appropriate class and isotype. Antibodies to the following antigens were used: 2C (clone 1B2-H6, hybridoma supernatant produced in the laboratory); insulin (Dako Corporation, Carpinteria, CA, USA), CD3 (clone 145-2C11); CD4 (clone GK 1.5); CD8a (clone 53-6.7); CD45R/B220 (clone RA3-76B20), CD25 (IL-2R α chain, clone 7D4); CD11a (LFA-1 α_L chain, clone 2D7) and CD49d (VLA-4 α4 integrin chain, clone R1-2) (all from PharMingen).

Treatment with antibodies against adhesion molecules

Anti-α4 integrin antibody (clone PS/2; American Type Culture Collection, Manassas, VA, USA) and anti-LFA-1 antibody (clone KBA, gift from Dr H. Yagita from Juntendo University School of Medicine, Tokyo, Japan) were isolated as hybridoma ascites from Balb/c- scid mice. Ascites immunoglobulin content was quantified using an ELISA assay. Rat immunoglobulin (PharMingen) was used as a treatment control. The reconstituted islet allograft recipients received a combination treatment with anti-LFA-1 and anti-α4 integrin antibodies by daily i.p. injections at a dose of 100 μg each for 10 consecutive days.

Statistical analysis

The time to rejection in experimental groups was compared using the two-tailed *t*-test. $P < 0.05$ was considered statistically significant.

Results

Characterization of 2C TCR effector cells

Before every cell transfer the innoculum was examined by FACS analysis. Unfractionated splenocytes in 2C mice are composed primarily of $1B2^+$ $2C/CD8^+$ TCR transgenic T cells (approximately 50%), $CD19^+$ B cells (approximately 40%), nontransgenic, mostly $CD4^+$ T cells (approximately 5%) and other unidentified cell types (approximately 5%). After magnetic bead purification, the $2C/CD8^+$ population was increased to >90%. Figure 1 shows a typical FACS analysis after positive selection using anti-CD8 antibody of primed 2C cells (see above for details of priming). Cells are $CD3^+$, 2C/CD8 double positive and CD25 (IL-2R) positive. Unprimed, purified cells showed a similar pattern, but do not generally express the IL-2R.

Time course of immune reconstitution of the C57BL/6-Rag-1 mice

The time course of the engraftment of the lymphoid organs of C57Bl/6-Rag-1 mice injected with 10^7 2C/CD8 splenocytes was studied at day 5, 10, 20, and 30 after the adoptive transfer. As early as 5 days after the cell transfer, immunohistochemical analysis of the spleen shows cells positive for CD3, CD8, IL-2R, and 2C (Fig. 2a–d). At day 10 the spleen was populated with $CD3^+$ lymphocytes; however, they were relatively homogeneously distributed and not organized in distinct areas (Fig. 2e). The typical spleen architecture was seen at day 20 (Fig. 2f). At day 30 $CD3^+$ areas were well formed and the $2C^+$ cells were

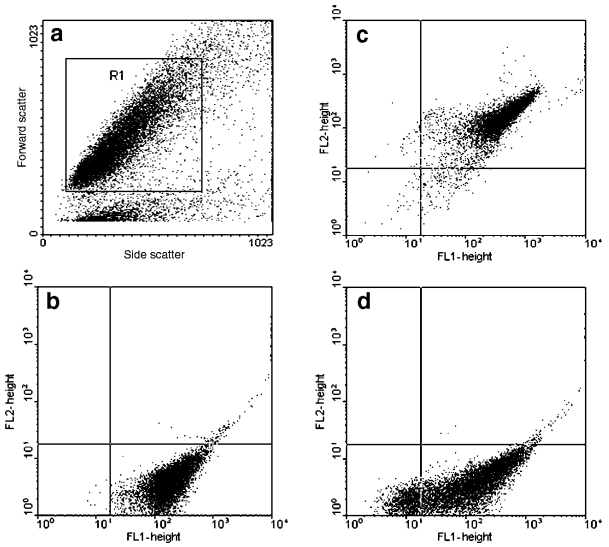

Figure 1 FACS analysis of the 2C CD8 cell innoculum administered for reconstitution of C57Bl/6-Rag-1 immunodeficient mice. 2C mice were primed intraperitoneally with 10^7 Balb/c splenocytes and after 2 weeks their splenocytes were isolated and fractionated using CD8 positive selection magnetic cell sorting. The positive fraction was examined by FACS analysis for purity and $2C^+/CD8^+$ cells were administered to the Rag-1 recipients. A representative experiment is shown demonstrating (a) Light scatter of the cell population; (b) CD3-positive cells; (c) double staining for CD8 and 2C markers; (d) CD25 (IL-2R)-positive cells.

abundant (Fig. 2h,g). At the same time points the lymph nodes showed similar reconstitution course.

Naïve 2C effector cells do not cause islet allograft rejection but reject skin grafts

We performed reconstitution with various numbers of splenocytes or purified T cells or $CD8^+$ cells from 2C mice to C57BL/6-Rag-1 recipients of Balb/c grafts in order to find the optimal conditions for the model – which cell fraction, what cell dose and what application way would lead to an acute allograft rejection. After reconstitution with splenocytes from naïve 2C mice the islet allograft recipients generally did not reject with only one recipient of 5×10^6 splenocytes developing hyperglycemia (Table 1). Similarly, recipients of 5×10^6 purified naïve 2C T cells remained normoglycemic for over 50 days: one animal rejected at day 55, one rejected at day 88 and four survived for over 100 days. We further purified the 2C effector cells to $2C/CD8^+$ cell fraction. Similarly, recipients of 5×10^6 purified naïve $2C/CD8^+$ effector cells also remained normoglycemic for over 100 days even when 2×10^7 $2C/CD8^+$ cells were injected. In contrast, mice reconstituted with even relatively few (5×10^6) naïve purified 2C T cells reproducibly rejected skin allografts (21.0 ± 1.1 days; $n = 4$) (Table 1).

Following adoptive transfer, the spleens and lymph nodes of the C57BL/6-Rag-1 mice (both islet and skin graft recipients) showed numerous cells that stained positive for both 2C and CD8 suggesting that the lack of rejection in islet allografts was not due to the lack of reconstitution. Interestingly, functioning islet allografts (Fig. 3a) in mice reconstituted with purified cells showed peri-islet infiltration with $2C^+/CD8^+$ cells (Fig. 3b,c). These cells also appeared to be activated in that they express IL-2 receptor (Fig. 3d). Thus, in the case of the islet allograft model, the naïve purified $2C/CD8^+$ cells were able to become activated *in vivo* and home to the graft site, but were unable to cause islet allograft rejection.

Primed, purified 2C effector cells cause islet allograft rejection

While the purified T cells from naïve 2C mice were efficient in rejecting skin allografts, neither they nor further purified $CD8^+$ cells could cause islet destruction. One possible explanation for the lack of rejection of islet allografts by naïve 2C cells is the lack of appropriate activation *in vivo* in immunodeficient mice. To test this hypothesis, we primed the native 2C donors using an i.p. injection of 10^7 Balb/c splenocytes 2 weeks prior to cell isolation. The primed 2C effector cells were purified and adoptively transferred into C57BL/6-Rag-1 islet or skin allograft recipients as before and the time of rejection was compared with the rejection time of the unprimed model. While low doses (10^6) of primed $2C/CD8^+$ cells did not show islet rejection, higher doses did cause islet destruction (Table 2). Two of three mice injected with 5×10^6 cells rejected their grafts at days 22 and 34. The dose of 10^7 $2C^+/CD8^+$ cells administered i.v. was well tolerated and the mice developed rejection at a mean of 17.8 ± 4.7 days ($n = 7$) (Table 2). The time to rejection was significantly shorter than in the other groups and closer to the rejection time of native 2C recipients of BALB/c islets which rejected at 10.2 ± 3.4 days ($n = 5$). Similar to islet allografts, the primed 2C effector cells were more efficient than naïve cells in rejecting skin grafts leading to rejection in 8.0 ± 0.4 days ($n = 4$), which was significantly faster than the unstimulated group ($P < 0.0001$) (Table 2). The i.p. injection of 5×10^7 primed $2C/CD8^+$ cells caused rejection in three of 5 recipients at 10 and 41 days after the cell transfer. The dose of 2×10^7 $2C^+/CD8^+$ cells administered i.v. resulted in fast rejection at 5 and 14 days in two mice, but it was not well tolerated by the animals (data not shown).

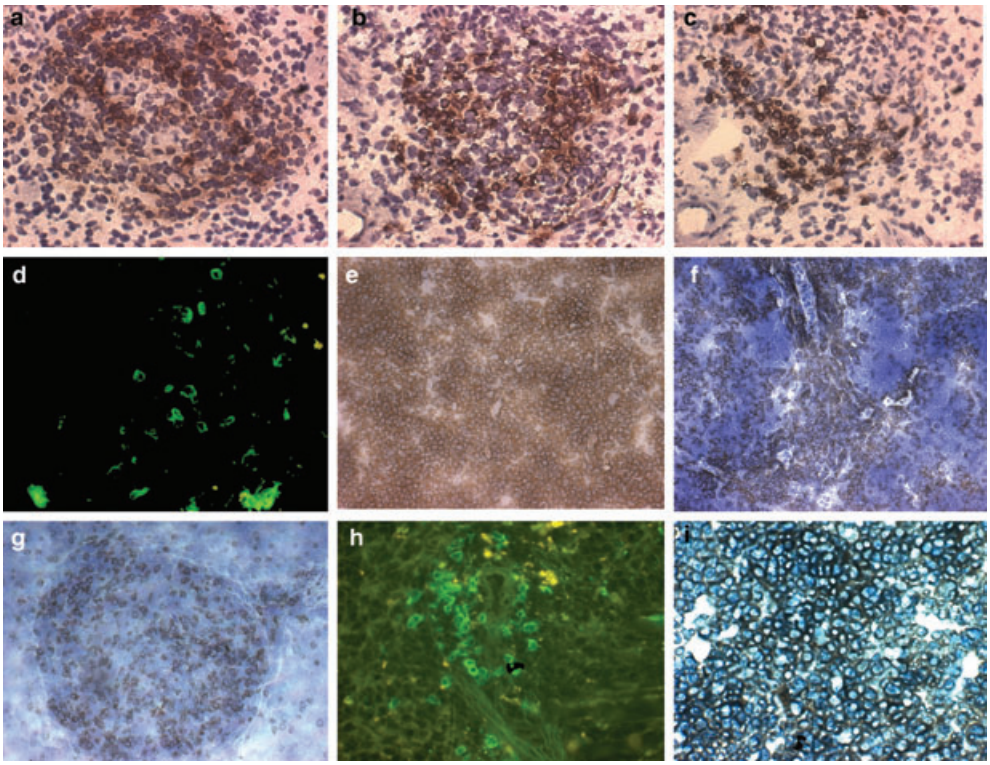

Figure 2 Engraftment of the spleen and lymph nodes of 2C/Rag-1 mice. Five days after the cell transfer immunohistochemical analysis of the spleen of the C57Bl/6-Rag-1 mice injected with 10^7 2C/CD8$^+$ splenocytes shows cells positive for CD3 (a), CD8 (b), IL-2R (c) and 2C (d). At day 10 the spleen is populated with CD3$^+$ lymphocytes; however, they are relatively homogeneously distributed and not organized in distinct areas (e). The typical spleen architecture can be seen at day 20 (f). At day 30 CD3$^+$ areas are well formed (g) and the 2C$^+$ cells (h) are abundant. At the same time points the lymph nodes showed similar reconstitution course; CD3$^+$ lymphocytes in a mesenteric lymph node, day 30 (I). (a – c, e – g and I) Direct ABC-peroxidase staining; (d, h) indirect FITC staining; original magnification: (a – d, h, i) 200×; (e–g) 100×.

Table 1. Rejection of Balb/c allografts in C57BL/6-Rag-1 recipients injected with naïve 2C cells.

Graft	Cells transferred	Cell number	Number of animals	Graft survival (days)
Islet	None	0	6	>100 × 6
Islet	Splenocytes	5×10^6	3	35, >100 × 2
Islet	Splenocytes	2.5×10^7	3	>100 × 3
Islet	Purified T cells	5×10^6	6	55, 88, >100 × 4
Islet	Purified CD8	5×10^6	3	>100 × 3
Islet	Purified CD8	2×10^7	3	>100 × 3
Skin	Purified T cells	5×10^6	4	19, 20, 21, 24*

*Mean ± SD = 21.0 ± 1.1.

Immune reconstitution of C57Bl/6-Rag-1 allograft recipients at the time of rejection

In recipients of primed, purified cells, peripheral blood lymphocytes and splenocytes of the recipients showed differing number of 2C$^+$ cells. The percentage of the 2C cells in the blood or in the spleen did not correlate with the presence or absence of rejection. For example, two mice injected with 5×10^6 cells, which rejected at days 22, and 34 showed 77.6% and 37.4% 2C cells in the blood and 10.0% and 18.3% in the spleen, respectively. Three mice injected with 5×10^7 primed, purified cells i.p. which rejected at days 10, 11, and 41 showed 27.0%, 30.6%, and 77.8% of 2C cells in the peripheral lymphocytes, and 7.5%, 7.2%, and 95.9% in the spleen, respectively. Recipients injected with 10^7 2C$^+$/CD8$^+$ cells consistently demonstrated higher levels of 2C$^+$, CD8$^+$, and CD25$^+$ cells as shown in Table 3.

The immunohistochemical analysis of the spleen and mesenteric lymph nodes of the recipient mice reconstituted with primed cells that developed rejection confirmed the engraftment with cells positive for CD8, 2C, and IL-2R (Fig. 4). Immunohistochemical analysis of islet graft infiltrate in all animals showed that the infiltrating cells were 2C transgenic (1B2$^+$) (Fig. 5a). The infiltrating cells expressed markers of T-cell activation as CD25 (IL-2R)

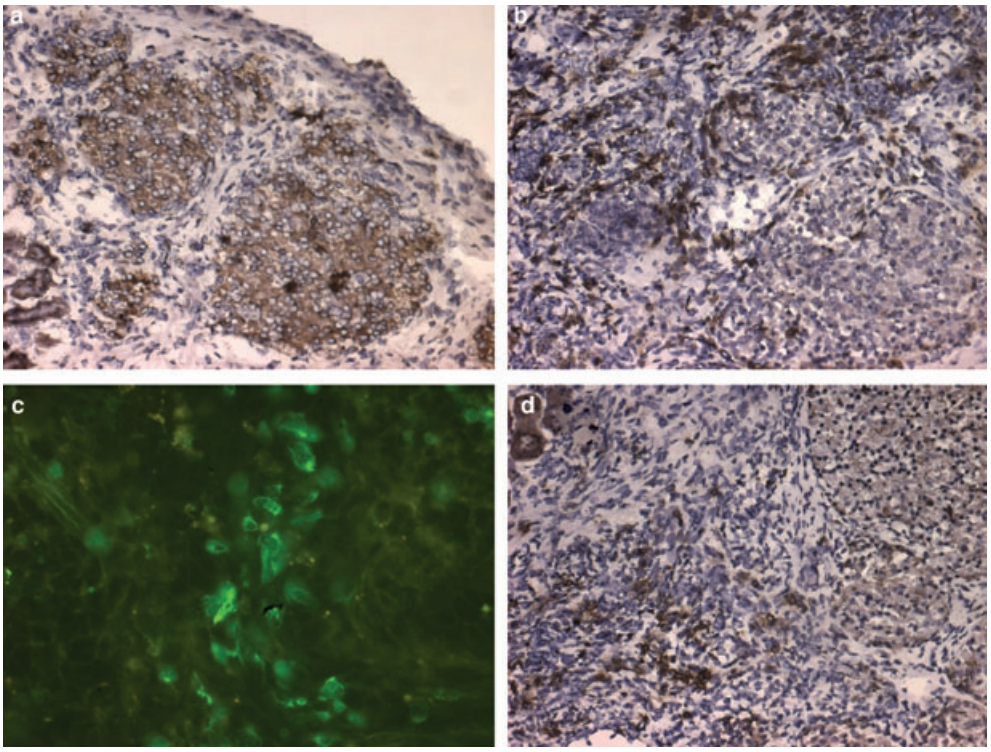

Figure 3 Functioning islet allograft in a mouse reconstituted with naïve purified 2C/CD8$^+$ cells demonstrating peri-islet infiltration. The islets show positive insulin staining (a). The infiltration shows cells positive for CD8 (b), 2C (c), and CD25 (d). (a, b and d) ABC-peroxidase staining; (c) indirect FITC staining; original magnification: (a, b and d) 100×, (c) 200×.

Table 2. Rejection of Balb/c allografts in C57BL/6-Rag-1 recipients injected with primed 2C cells.

Graft	Cells transferred	Cell number	Number of animals	Graft survival (days)
Islet	Splenocytes	2.5×10^7	5	7, >100 × 4
Islet	Purified CD8	10^6	3	>100 × 3
Islet	Purified CD8	5×10^6	3	22, 34, >100
Islet	Purified CD8	10^7	7	11, 14, 15, 16, 21, 21, 25*
Skin	Purified T cells	5×10^6	4	7, 8, 8, 9**

Mean ± SD: *17.8 ± 4.7; **8.0 ± 0.4.

Table 3. Immune reconstitution at the time of rejection of C57BL/6-Rag-1 allograft recipients which rejected Balb/c islets after inoculation with 10^7 2C$^+$/CD8$^+$ cells: FACS analysis of the peripheral blood lymphocytes and splenocytes ($n = 7$).

	% of positive cells (mean ± SD)	
Cell marker	Blood	Spleen
2C$^+$ cells	60.8 ± 23.0	31.7 ± 13.3
CD8$^+$ cells	58.6 ± 23.5	28.1 ± 16.0
CD25$^+$ cells	52.8 ± 11.3	28.4 ± 24.5

(Fig. 5d). It is also important, with respect to homing, to note that these graft-infiltrating cells express the adhesion molecules, LFA-1 and VLA-4 α4-integrin (data not shown).

Antibodies directed against the adhesion molecules LFA-1 and α4 integrin prolong allograft survival in the 2C-Rag-1 TCR model

Using the primed islet allograft model, we examined the effect of adhesion molecule blockade with monoclonal antibodies directed against the adhesion molecules, LFA-1 and α4 integrin on graft survival. C57BL/6-Rag-1 recipients of Balb/c islets received 10^7 primed 2C/CD8$^+$ splenocytes i.v. On the day of cell transfer the mice started receiving a combination treatment with both anti-LFA-1 and anti-α4 integrin antibodies (100 μg of each i.p.) that continued daily for 10 days. This combination anti-adhesion molecule treatment prolonged islet allograft survival (31.3 ± 9.5 days, $n = 3$) when compared with the control IgG-treated group (16.8 ± 5.2 days, $n = 6$) ($P < 0.05$) (Fig. 6). Immunohistochemical analysis of the grafts showed islet infiltration; however, the treatment delayed infiltration. The infiltrating cells in treated animals showed expression of LFA-1 (CD11a) and α4-integrin (Fig. 7).

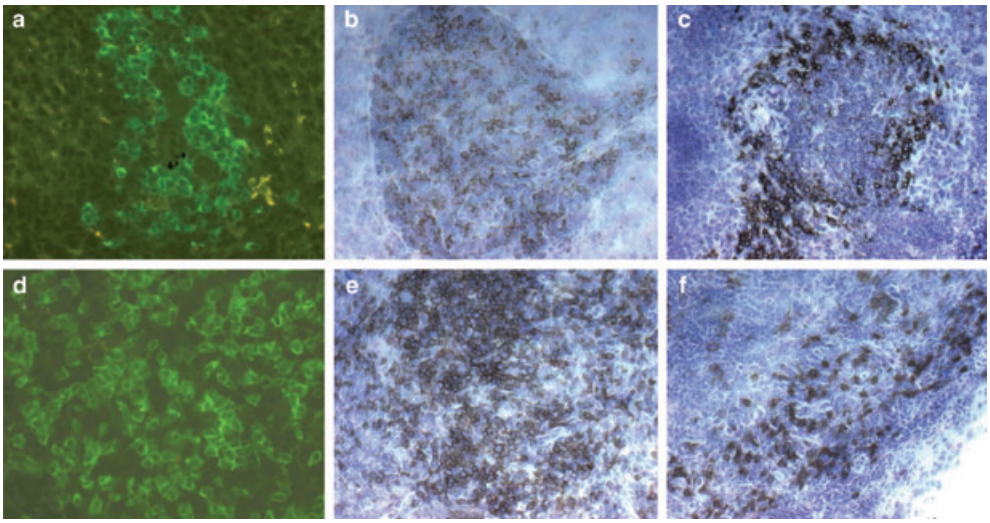

Figure 4 During the allograft rejection the immunohistochemical analysis of the spleen (a, b, c) and the lymph nodes (d, e, f) showed full engraftment with cells positive for 2C (a, d), CD8 (b, e) and CD25 (c, f). (a and d) Indirect FITC staining, 200×; (b, c, e, and f) Direct ABC-Peroxidase staining, 100×).

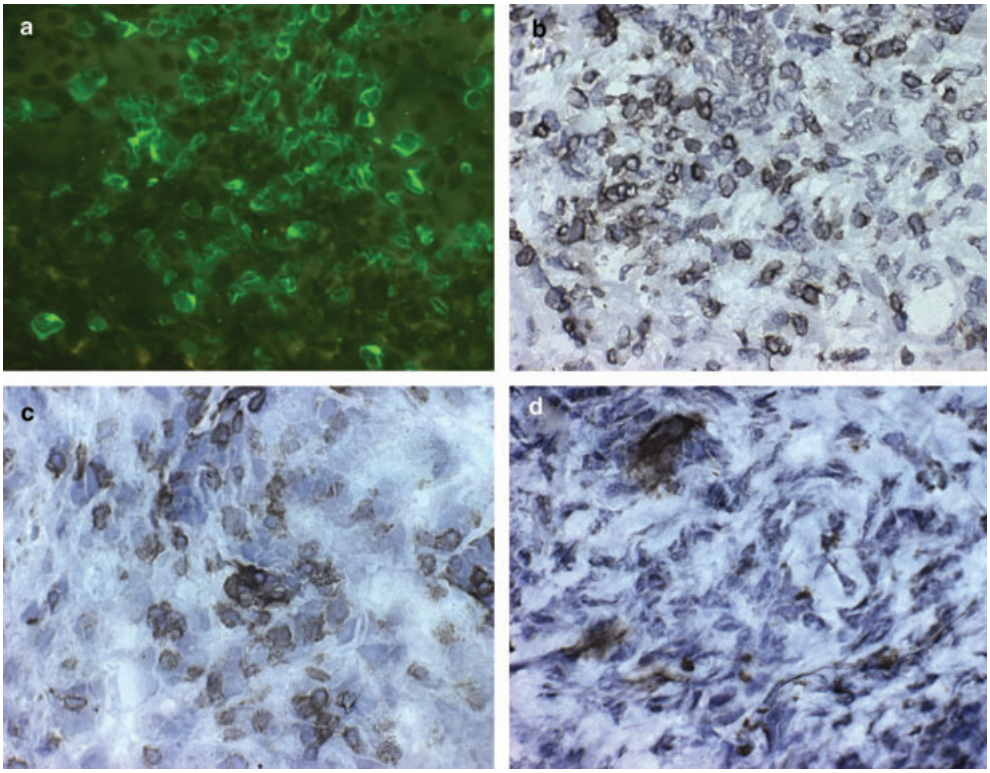

Figure 5 Immunohistochemical examination of islet allograft at the time of rejection. 2C positive cells (a) in a rejected islet graft of a C57Bl/6-Rag-1 mouse 11 days after intravenous reconstitution with 10^7 2C/$CD8^+$ splenocytes. Islets are heavily infiltrated and only remnant islet cells can be seen; the intraislet infiltration showed $CD3^+$ (b), $CD8^+$ (c), and $CD25^+$ cells (d). (a) Indirect FITC staining; (b–d) Direct ABC-peroxidase staining; original magnification 200×.

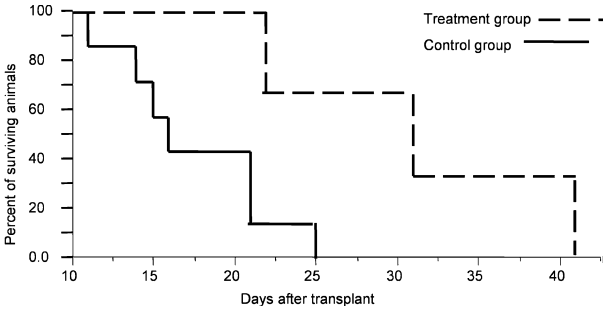

Figure 6 Combination treatment with monoclonal antibodies against LFA-1 and α-4 integrin delays islet allograft rejection in the primed model system. C57BL/6-Rag-1 recipients of Balb/c islets received 10^7 primed 2C/CD8$^+$ splenocytes intravenously. On the day of cell transfer the mice started receiving a combination treatment with both anti-LFA-1 and anti-VLA-4 antibodies (100 μg of each intraperitoneally) that continued daily for 10 days. This treatment prolonged islet allograft survival (31.3 ± 9.5 days, $n = 3$) when compared with the control IgG-treated group (16.8 ± 5.2 days, $n = 6$) ($P < 0.05$).

Discussion

The development of transgenic T-cell strains has provided useful tools to study antigen-specific T-cell interactions both *in vivo* and *in vitro* [14]. The 2C CD8$^+$ TCR transgenic mouse has been used for a variety of T-cell studies including T-cell development [15], anti-tumor reactivity [16], peripheral T-cell expansion and apoptosis [17], and for identifying differences between naïve and memory T cells *in vivo* [11,13,18].

We developed a model of allorejection using purified 2C/CD8$^+$ TCR transgenic effector cells in reconstituted immunodeficient C57BL/6-Rag-1 mice. In our initial characterization of this model system, we found the unpurified 2C splenocytes containing a significant number of nontransgenic 1B2$^-$ T cells, including many B cells and even a few nontransgenic T cells. Thus, we recommend that future studies that utilize the 2C TCR transgenic model should include a purification step to avoid contamination by nontransgenic T cells.

In further studies, we found that both unpurified and purified 2C/CD8$^+$ T cells reconstitute and endure in immunodeficient mice and cause skin graft rejection. However, both unpurified and purified naïve 2C TCR transgenic cells are poor effector cells *in vivo* in the islet allograft model. These cells appear to be capable of becoming activated in that they express the IL-2 receptor and even are capable of homing to islet allografts *in vivo*. However, these naïve cells do not reject islet allografts efficiently. The reason for this is unclear and could be due to either a qualitative or quantitative phenomenon. It could be that these cells are not completely functional effector cells despite the expression of this activation marker. The finding that primed 2C/CD8$^+$ cells reject islets supports this hypothesis. Similarly, skin grafts, known to be much more immunogenic than islet allografts, appear to stimulate rejection by 2C/CD8$^+$ cells even when naïve cells are used for reconstitution. The finding that naïve 2C effector cells do not reject islet allografts demonstrates that this model system may require further enhancements to completely mimic the usual allorejection process (for example, the addition of antigen-presenting cells). Furthermore, the lack of rejection may be due to the lack of proper Fas-FasL signaling and insufficient expression of Fas-FasL on the islets and 2C T cells as these interactions are critical for beta cell destruction in mouse models of spontaneous autoimmune diabetes [19,20].

Another limitation of the model system as currently described is that the transplants are performed prior to reconstitution. Thus, the nonspecific inflammatory response that might contribute to the early events of activation and allograft rejection is not present when the effector cells are introduced [21]. Alternatively, the

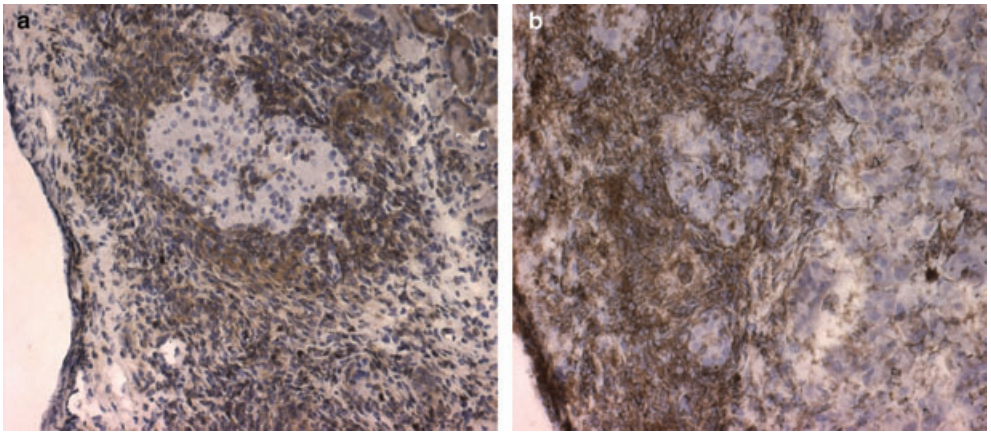

Figure 7 LFA-1 (a) and α-4 integrin (b) expression on the infiltrating cells in an LFA-1/α-4 integrin monoclonal antibody-treated islet allograft with prolonged graft survival; ABC-peroxidase staining, original magnification 100×.

presence of antigen at the time of introducing the immune reconstitution may favor antigen-induced T-cell differentiation rather than homeostasis-induced cell differentiation. The latter differentiation may occur upon adoptive transfer into Rag-1 mice as lymphopenia-induced spontaneous proliferation of the naïve T cells without administration of exogenous antigen [13]. Therefore, it would be of interest to examine whether naïve 2C T cells would be able to reject the allogeneic islets when transferred immediately following transplantation.

Despite these limitations, we believe that the model as is currently used does provide a useful method for studying activated alloreactive T cells *in vivo*. We plan to use this model to study homing *in vivo*. Most prior studies of homing have involved *in vitro* systems such as flow chambers that do not fully mimic the entire dynamic process [22]. Conversely, the study of homing *in vivo* also has met with difficulties. For example, in attempts to study the effect of adhesion molecule blockade *in vivo*, it has always been unclear whether the mechanism of graft prolongation has been via the inhibition of T-cell activation (LFA-1 and VLA-4 has been implicated in T cell-antigen presenting cell interactions) or via homing or both. Our data show that adhesion molecule blockade delays infiltration by primed $2C^+$ cells; however, at the time of rejection, LFA-1 and α4-integrin are expressed on infiltrating cells. The possibility of staining of bound antibody was avoided by using different antibody clones for treatment and for immunohistochemical analysis. The finding that adhesion molecule blockade delays infiltration and islet allograft rejection suggests that this model might allow for the isolated study of homing *in vivo*.

The antigen-induced 2C differentiation in Rag-1 recipients has been useful in studying the memory cell development in a similar model as a substantial number of memory cells can be generated in the recipient, which can be easily purified for functional and biochemical analysis [13]. Our study suggests that the 2C-Rag-1 TCR transgenic mouse model of allorejection may be a useful tool to study the mechanisms of activation and homing process.

Acknowledgement

Supported by RO1 DK54429-01A1 (MDS).

References

1. Yamada A, Laufer TM, Gerth AJ, *et al.* Further analysis of the T-cell subsets and pathways of murine cardiac allograft rejection. *Am J Transplant* 2003; **3**: 23.
2. Stegall M, Dean P, Ninova D, *et al.* a4 integrin in islet allograft rejection. *Transplantation* 2001; **71**: 1549.
3. Butcher EC, Picker LJ. Lymphocyte homing and homeostasis. *Science* 1996; **272**: 60.
4. Bradley LM, Watson SR. Lymphocyte migration into tissue: the paradigm derived from CD4 subsets. *Curr Opin Immunol* 1996; **8**: 312.
5. Davenport MP, Grimm MC, Lloyd AR. A homing selection hypothesis for T cell trafficking. *Immunol Today* 2000; **21**: 315.
6. Denton MD, Geehan CS, Alexander SI, Sayegh MH, Briscoe DM. Endothelial cells modify the costimulatory capacity of transmigrating leukocytes and promote CD28-mediated CD(+) T cell alloactivation. *J Exp Med* 1999; **190**: 555.
7. Marelli-Berg FM, Frasca L, Weng L, Lombardi G, Lechler RI. Antigen recognition influences transendothelial migration of CD4+ T cells. *J Immunol* 1999; **162**: 696.
8. Kranz DM, Sherman DH, Sitkovsky MV, Pasternack MS, Eisen HN. Immunoprecipitation of cell surface structures of cloned cytotoxic T lymphocytes by clone-specific antisera. *Proc Natl Acad Sci USA* 1984; **81**: 573.
9. Kranz DM, Tonegawa S, Eisen HN. Attachment of an anti-receptor antibody to non-target cells renders them susceptible to lysis by a clone of cytotoxic T lymphocytes. *Proc Natl Acad Sci USA* 1984; **81**: 7922.
10. Sha WC, Nelson CA, Newberry RD, Kranz DM, Russell JH, Loh DY. Selective expression of an antigen receptor on CD8-bearing T lymphocytes in transgenic mice. *Nature* 1988; **335**: 271.
11. Jackson DY. Alpha 4 integrin antagonists. *Curr Pharm Des* 2002; **8**: 1229.
12. Ninova D, Dean PG, Stegall MD. Immunomodulation through inhibition of multiple adhesion molecules generates resistance to autoimmune diabetes in NOD mice. *J Autoimmun* 2004; **23**: 201.
13. Chen J, Eisen HN, Kranz D. A model T-cell receptor system for studying memory T-cell development. *Microbes Infect* 2003; **5**: 233.
14. Mondino A, Khoruts A, Jenkins MK. The anatomy of T-cell activation and tolerance. *Proc Natl Acad Sci USA* 1996; **93**: 2245.
15. Sha WC, Nelson CA, Newberry RD, Kranz DM, Russell JH, Loh DY. Positive and negative selection of an antigen receptor on T cells in transgenic mice. *Nature* 1988; **336**: 73.
16. Kedl RM, Mescher MF. Migration and activation of antigen-specific CD8+ T cells upon in vivo stimulation with allogeneic tumor. *J Immunol* 1997; **159**: 650.
17. Yu XZ, Martin PG, Anasetti C. CD28 signal enhances apoptosis of CD8 T cells after strong TCR ligation. *J Immunol* 2003; **170**: 3002.
18. Kedl RM, Mescher MF. Qualitative differences between naive and memory T cells make a major contribution to the more rapid and efficient memory CD8+ T cell responses. *J Immunol* 1998; **161**: 674.
19. Chervonsky AV, Wang Y, Wong FS, *et al.* The role of Fas in autoimmune diabetes. *Cell* 1997; **89**: 17.

20. Silva DG, Petrovsky N, Socha L, Slaterry R, Gatenby P, Charlron B. Mechanisms of accelerated immune-mediated diabetes resulting from islet beta cell expression of a Fas ligand transgene. *J Immunol* 2003; **170**: 4996.
21. Matzinger P. The danger model: a renewed sense of self. *Science* 2002; **29**: 301.
22. Reinhardt PH, Kubes P. Differential leukocyte recruitment from whole blood via endothelial adhesion molecules under shear conditions. *Blood* 1998; **92**: 4691.