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Induction of oral tolerance and the effect of interleukin-4 on murine skin allograft rejection

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Abstract

We studied the effect of oral and portal vein administration of alloantigens on mouse skin allograft survival. Graft receptor BALB/c mice received spleen cells (30, 90, 150 or 375 x 10^6) from donor C57BL/6 mice intragastrically on three successive days, starting seven days before the skin graft. Allograft survival was significantly increased with the feeding of 150 x 10^6 allogeneic spleen cells by one gavage (median survival of 12 *vs* 14 days, $P \leq 0.005$) or when 300 x 10^6 cells were given in six gavage (12 *vs* 14 days, $P < 0.04$). A similar effect was observed when 150 x 10^6 spleen cells were injected into the portal vein (12 *vs* 14 days, $P \leq 0.03$). Furthermore, prolonged allograft survival was observed with subcutaneous (12 *vs* 16 days, $P \leq 0.002$) or systemic (12 *vs* 15 days, $P \leq 0.016$) application of murine interleukin-4 (IL-4), alone or in combination with spleen cell injection into the portal vein (12 *vs* 18 days, $P \leq 0.0018$). Taken together, these results showed that tolerance induction with spleen cells expressing fully incompatible antigens by oral administration or intraportal injection partially downmodulates skin allograft rejection. Furthermore, these findings demonstrated for the first time the effect of subcutaneous or systemic IL-4 application on allograft skin survival suggesting its use as a beneficial support therapy in combination with a tolerance induction protocol.

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Key words
- Mouse oral tolerance
- Intraportal route
- Skin transplantation
- Interleukin-4
- Alloantigen

Introduction

Oral administration of antigens induces peripheral immune tolerance and can suppress subsequent humoral and cellular responses to these same antigens (1). Induction of tolerance has good potential for treatment due to its specificity and to the reduction of the risks of undesirable side effects. It can be used to reduce allograft rejection as well as autoimmune and allergic experimental diseases (2,3).

Several factors can influence oral tolerance, including age, genetically determined susceptibility, dose and time between oral antigen administrations (4,5). The primary mechanisms involved in oral tolerance are clonal deletion, anergy and suppression (6-10). The antigen may stimulate cells from the gastrointestinal lymphoid tissue system to produce regulatory cytokines, such as transforming growth factor-ß (TGF-ß), interleukin-10 (IL-10) and IL-4 (3). Induction of tolerance has also been observed when donor cells are injected into the portal vein, with prolongation of renal allograft survival

(11). The effect of portal vein antigen injection on allogeneic graft acceptance in mice may involve γ/δTCR+ producers of Th2 cytokines (12).

An important cell-mediated host response is produced during allograft rejection, involving macrophages, polymorphonuclear cells, CD8+ cells, and Th1 subtype CD4+ cells. These cells contribute to the inflammatory response by secreting cytokines. Since IL-4, a Th2 cytokine, has an important regulatory role, it could be involved in the modulation of skin graft rejection. However, no investigations have been carried out on how the local application of IL-4 affects skin allograft survival.

We determined the effect of the oral administration of various doses of spleen cells from fully incompatible donors and of donor cell injection into the portal vein on skin graft rejection in mice. Additionally, the effect of subcutaneous (*sc*) and intraperitoneal (*ip*) administration of IL-4 on skin graft rejection was measured, associated or not with the injection of allogeneic spleen cells via the portal vein.

Material and Methods

Animals

Seven- to twenty-week-old male C57BL/6 mice served as donors and male BALB/c mice of the same age as recipients of the skin grafts. The animals were provided by the animal facility of the University of São Paulo Medical School and kept under controlled light and temperature in our own facilities. The animals received standard laboratory diet (Purina, Campinas, SP, Brazil) and water *ad libitum*.

Skin graft

Full-thickness skin grafts were harvested from the dorsum of C57BL/6 mice and grafted onto the backs of BALB/c mice using the technique of Billingham and Medawar (13). Each graft was sutured into place using 4-0 Mononylon® thread and protected with Brown's dressing for five days. The grafts were evaluated daily and scored as having been rejected when more than 90% of the grafted skin was visibly inviable. Groups of mice receiving isografts were also assessed daily.

Spleen cell harvest

Spleens from C57BL/6 mice were collected aseptically pressed through a stainless steel wire screen in RPMI-1640 culture medium (Gibco BRL, Gaithersburg, MD, USA), and erythrocytes were removed by hypotonic shock. The cells were washed twice and their concentration and viability determined.

Oral and intraportal administration of spleen cells

Groups of anesthetized mice were fed intragastrically using a urethral tube 30, 90, 150 or 375 x 10^6 C57BL/6 spleen cells on three consecutive days, starting seven days before the allograft. Other groups of mice also received intragastrically 300 x 10^6 cells divided into six doses on the 7th, 6th and 5th days before the graft and on the 7th, 8th and 9th days after the graft, or were treated with 300 x 10^6 cells divided into six doses on the 7th, 6th and 5th days before the graft and on the 3rd, 4th and 5th days after the graft. Other graft recipient mice were injected once with 150 x 10^6 spleen cells into the portal vein seven days before the allograft.

Recombinant IL-4 treatment protocol

Allografted mice were injected *sc* with 300 ng murine recombinant IL-4 (rIL-4; Pharmingen, San Diego, CA, USA) on the day of the skin graft (0) and on the 3rd, 5th and 7th

days after grafting or by *ip* injections of 300 ng rIL-4 on days 0, 3, 5 and 7 after grafting. Other groups of mice received *ip* injections of 300 ng rIL-4 and 150 x 10^6 donor spleen cells via the portal vein.

Statistical analysis

The overall survival curves were evaluated by the Kaplan Maier test and comparison between survival curves was performed by the Log rank test. Data are reported as median survival.

Results

Effect of oral alloantigen administration on skin graft rejection

Varying doses of splenocytes from skin graft donor C57BL/6 mice were administered orally to BALB/c mice. Recipient mice were fed intragastrically a total of 30, 90 or 150 x 10^6 spleen cells from C57BL/6 mice, divided evenly among three successive days, seven days before the skin graft. Figure 1 shows that feeding 150 x 10^6 splenocytes significantly increased allograft survival (median survival, 14 days) compared to the allograft control group (12 days, $P \leq 0.005$).

To investigate the influence of dose and time of antigen delivery on allograft rejection, 300 x 10^6 spleen cells were administered intragastrically, 50 x 10^6 cells per day, 7, 6 and 5 days before the graft and 50 x 10^6 cells per day, 7, 8 and 9 days after graft (Figure 2). This procedure did not modify the course of graft rejection. However, when the second series was administered 3, 4 and 5 days after the graft, there was a significant ($P \leq 0.04$) increase in graft survival (Figure 2). In contrast, a similar total dose of 375 x 10^6 cells administered as a single dose did not produce a significant change in the rate of graft rejection (8.5 days) compared to control (12 days).

Effect of the administration of donor splenocytes via the portal vein on skin graft rejection

Another group of allografted mice received 150 x 10^6 splenocytes via the portal

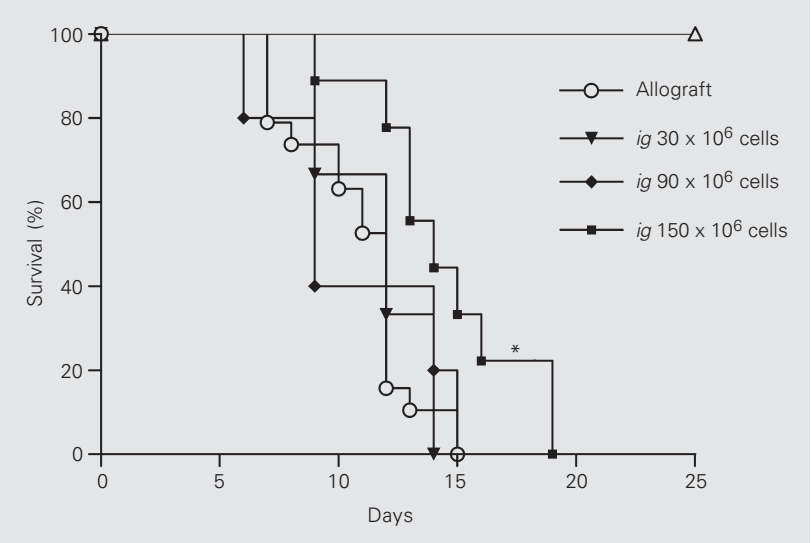

Figure 1. Effect of oral administration of donor splenocytes on skin allograft rejection. Splenocytes from skin graft donor C57BL/6 mice were administered by the intragastric (*ig*) route with 30 x 10^6 (N = 6), 90 x 10^6 (N = 5) or 150 x 10^6 cells (N = 9). The control allograft alone with no allogeneic cells (N = 21) and isograft (N = 5) groups are shown. *$P \leq 0.005$ compared to the survival curve of the allograft group (Log rank test).

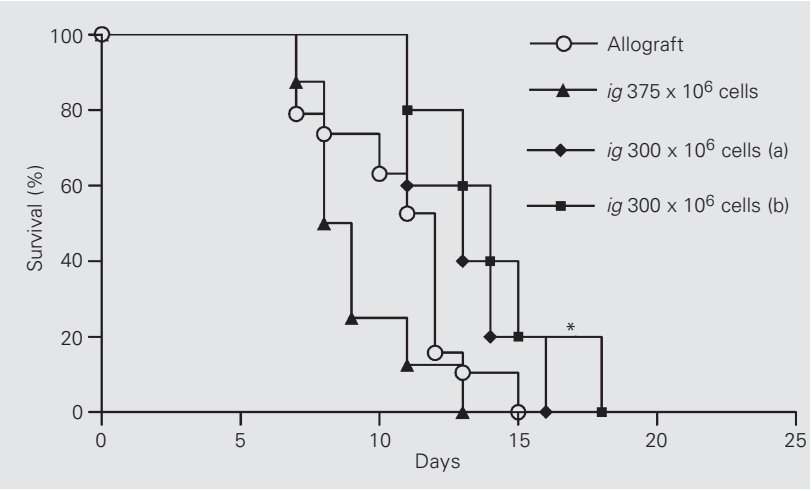

Figure 2. Effect of dose and antigen timing on skin allograft rejection. Recipient mice received by the intragastric (*ig*) route a total of 375 x 10^6 donor cells (N = 7) or a) 300 x 10^6 cells divided into six doses (7, 6 and 5 days before the graft and 7, 8 and 9 days after the graft, N = 5), or b) 300 x 10^6 cells graft divided into six doses (7, 6 and 5 days before the graft and 3, 4 and 5 days after the graft, N = 5). *$P \leq 0.04$ compared to the survival curve of the allograft group (Log rank test).

vein. Graft rejection in this group began later (on day 13) than in the control group (day 7) and graft survival increased significantly ($P \leq 0.03$, Figure 3).

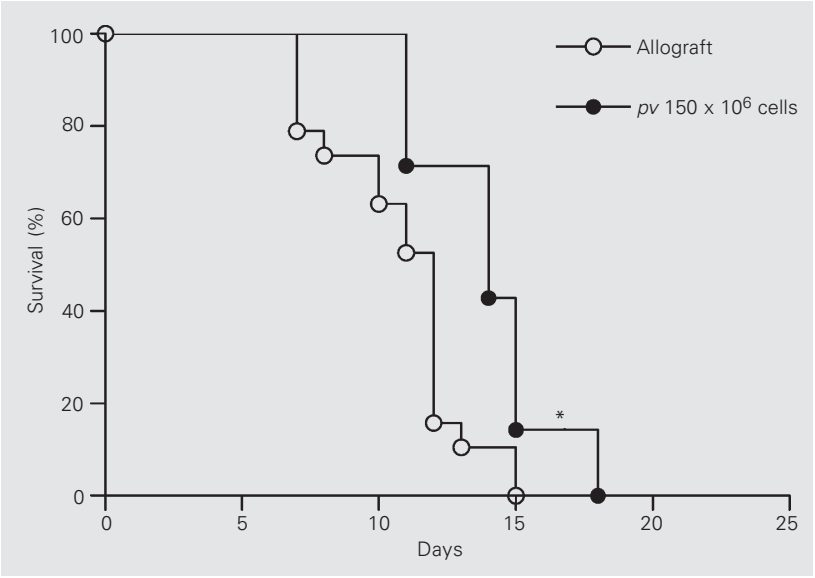

Figure 3. Effect of donor splenocyte injection via the portal vein on skin allograft rejection. Recipients received an injection of 150 x 10^6 spleen cells via the portal vein (*pv*) seven days before allografting (N = 5). *P ≤ 0.03 compared to the survival curve of the allograft group (Log rank test).

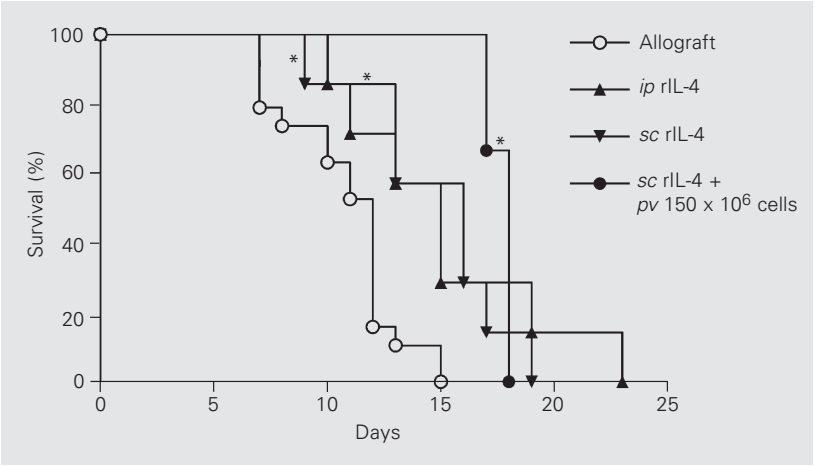

Figure 4. Effect of local or systemic application of rIL-4 on skin graft rejection. Allografted mice injected with rIL-4 subcutaneously (*sc*, N = 7, **P ≤ 0.02) or intraperitoneally (*ip*, N = 7, *P ≤ 0.002) had increased graft survival compared to the allograft group (Log rank test). Other skin graft recipients (N = 3) were injected with 150 x 10^6 spleen cells into the portal vein (*pv*) and *sc* with rIL-4. *P ≤ 0.002 compared to the survival curve of the allograft group (Log rank test).

Effect of local or systemic application of rIL-4 on skin graft rejection

Since Th1 response inflammatory cytokines are known to be secreted during the graft rejection process (10), we determined the effect of 300 ng rIL-4 on the allograft by systemic or local administration. Groups of skin-grafted mice that received 400 ng rIL-4 *sc* in the skin graft (median survival, 16 days, $P \leq 0.02$), or 300 ng *ip* showed significantly increased graft survival (15 days, $P \leq 0.002$) when compared to the saline-injected allograft control group (12 days) (Figure 4).

The *sc* application of rIL-4 to the skin graft in combination with 150 x 10^6 splenocytes injected via the intraportal vein resulted in an increased allograft survival similar to that obtained with the application of rIL-4 alone (Figure 4).

Discussion

We investigated the effect of oral administration and portal vein injection of donor splenocytes on murine skin allograft rejection. We also evaluated the effect of local or systemic injection of rIL-4 on receptor allograft survival.

The efficiency of oral administration of donor splenocytes in prolonging graft survival was dose dependent. Feeding 150 x 10^6 cells increased allograft survival, while 375 x 10^6 cells enhanced graft rejection. The partial modulation of graft survival could occur because fully mismatched allografts were employed; it is possible that longer graft survival would be obtained if there were only minor MHC disparities between the mouse strains. The alloantigen dose, the timing and the number of antigen administrations appear to be important factors in oral tolerance induction. This was evident and clearly shown by the fact that 375 x 10^6 cells administered on three consecutive days shortened graft survival, whereas a similar dose of 300 x 10^6 cells administered in six

equal doses over a period of six days was able to prolong graft survival. Oral alloantigen administered continuously just after grafting was more effective than when administered one week after the graft. In another murine model of oral tolerance induction such as type I hypersensitivity to dust mite (14,15), a dose-dependent effect on the modulation of the IgE response was observed in sensitized mice. An additional increase in the allergen dose enhanced the hypersensitivity response, worsening the allergic response (14,15).

Similar to the effect obtained with antigen delivery to the mucosal site, injection of donor splenocytes into the portal vein prolonged skin allograft survival. The liver plays a critical role in oral tolerance, which may involve a loss of antigen-specific T cells after primary antigen injection, or hyporesponsiveness on reexposure to the antigen (16), due to the absence of co-stimulatory signals (17) or to defective antigen presentation by liver nonparenchymal cells (18). Prolonged allograft survival in mice by an impaired Th1 cytokine response, with a shift towards Th2 cytokine production has been described in mice injected with ovalbumin into the portal vein before transplantation (18). Antigen administration through the portal vein in an experimental colitis model stimulated liver-associated T NK1.1 lymphocytes with high serum IL-4 and TGF-ß 1 and low IFN-γ levels (19). Furthermore, IL-12 in combination with anti-IL-10 reverses graft prolongation after portal venous immunization (3).

During the allogenic response the generation of IFN-γ-producing CD8+ T cells may skew both direct and indirect alloreactive responses towards the Th1 type. The modulation of allograft survival through tolerance could be mediated by down-regulation of pro-inflammatory Th1 cytokines, since tolerance to antigens induces Th2 cytokine production. The regulatory role of IL-4 in favoring a Th2 response by directly down-regulating the transcription factors promoting IFN-γ synthesis is well known (20). Our results demonstrated that rIL-4 injected *sc* around the skin graft or even *ip* increased allograft survival, supporting the idea that IL-4 has a beneficial role in the initial allograft response. Injection of alloantigen through the portal vein associated with IL-4 treatment delayed the initial process of graft rejection, prolonged graft survival, but did not interfere with the final graft rejection process. It is possible that IL-4 regulates the initial alloresponse by modulating IFN-γ activity in mismatched allografts, whereas it could have a different role in chronic rejection or in vascular grafts. Chronically rejected grafts have a marked accumulation of both IL-4 and IL-5 mRNA (21), and the dominance of Th2 type cells apparently does not prevent cardiac allograft vasculopathy (22).

Oral administration or portal vein injection of splenic cells from fully incompatible donors retarded skin graft rejection. Local and systemic administration of rIL-4 to the skin graft receptor seems to be beneficial as a combined therapy in tolerance induction protocol.

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