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Analysis of cellular events in hepatic allografts: Donor progenitors induce intragraft chimerism

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Abstract Long-term graft acceptance and tolerance induction after allogeneic rat liver transplantation are well described. However, the underlying mechanisms remain unclear. In this study we investigated the cellular events within the liver graft during initial immunosuppression and long-term acceptance. Orthotopic liver transplantation was performed in the Dark Agouti (DA)-to-Lewis (LEW) and LEW-to-DA rat strain combination. In order to achieve long-term acceptance, LEW recipients of DA livers were treated with two different short-term therapies. Non-parenchymal cells (NPC) were isolated from liver allografts on days + 10 and + 100 after transplantation and donor-specific leukocytes were immunophenotyped by flow cytometry. Both the monotherapy and triple therapy prolonged graft survival (> 100 days). Liver allografts from LEW donors into DA recipients were spontaneously accepted across a complete MHC mismatch without immunosuppression. Liver allograft rejection was induced by infiltrating alloreactive immunocompetent cells. But the intensities of cell infiltration in the early and late phases after transplantation did not correlate with eventual outcome. Donor-specific NPC decreased to 18–25% on day + 10 in both therapeutic groups, but had rebounded to up to 40% by day + 100. Recurrence of donor-specific cells was caused almost exclusively by rising T cell counts. The persistence of dendritic cells in the late phase after transplantation could be clearly demonstrated. Repopulation by donor-specific T lymphocytes was observed in long-term accepted liver grafts. This recurrence may be based on the differentiation of liver-derived progenitor cells. The persistent coexistence of donor and recipient cells within the liver allograft (intrahepatic chimerism) appears to be characteristic and may be important for long-term acceptance.

Key words Rat liver transplantation · Intrahepatic leukocytes · Dendritic cells · T lymphocytes · Chimerism

Introduction

Although the liver is known to be the most tolerogenic vascularized organ graft [1], the underlying basic immunological mechanisms involved in the acceptance of liver grafts are poorly understood and remain the subject of controversy [2, 3]. Various experimental studies in rats have demonstrated the importance of liver-resident leukocytes or passenger leukocytes in inducing donor-specific tolerance [4, 5]. This observation and the potential of the adult liver for multilineage hematopoietic reconstruction [6] led us to investigate the cellular events within the liver allograft itself. We therefore examined the phenotype of liver-resident leukocytes during the

phase of rejection, under immunosuppression, and after long-term acceptance.

Materials and methods

Animals

Inbred male rats from the Dark Agouti (DA, $RT1^{av1}$) and Lewis (LEW, $RT1^{l}$) strains were purchased from Charles River (Sulzfeld, Germany). The rats weighed 180–220 g and were maintained under controlled conditions. All animals received care according to the national guidelines for animal care (German law on the protection of animals).

Immunosuppression and experimental groups

The following immunosuppressants were used: (1) cyclosporine A (CsA; Novartis Pharma, Basel, Switzerland) given by intramuscular injections, (2) the monoclonal antibody (mAb) NDS-61 against IL-2R (anti-CD25), and (3) the mAb 1A29 against ICAM-1 (anti-CD54). The two purified antibodies were both of the mouse IgG1 subtype and were purchased from Serotec, Oxford, UK (NDS-61) and R + D Systems, Abingdon, UK (1A29). They were administered intravenously via the tail vein. Immunosuppression (CsA and mAbs) was administered daily from day 0 to day + 13 postoperative (p.op.). The DA-to-LEW combination was divided into the following groups: group 1; CsA monotherapy (3.0 mg/kg per day); group 2; CsA triple therapy [CsA (0.25 mg/kg per day) + NDS-61 (600 μg/kg per day) + 1A29 (30 μg/kg per day); group 3; no immunosuppression (rejection model); and group 4; the LEW-to-DA combination where DA recipients spontaneously accepted LEW liver allografts across a complete MHC mismatch with no requirement for immunosuppression (spontaneous liver tolerance model). All animals were killed on day + 10 (early phase) and day + 100 (late phase) after transplantation (five rats per time point and group).

Orthotopic liver transplantation

Orthotopic rat liver transplantation (ORLT) was performed in an arterialized model with hepatic artery revascularization as originally described by Lee in 1975 [7] and used here in the modification by Engemann (for details see [8]). The death of an animal before day + 3 was attributed to technical errors and it was excluded from the study.

Postoperative monitoring

After transplantation, all animals were examined daily for signs of graft failure as previously described [9].

Isolation of non-parenchymal cells (NPC)

Livers were perfused via the portal vein, excised, cut into small pieces, and forced through a fine tea strainer in PBS. These mechanically homogenized suspensions were incubated with 50 U/ml collagenase IV (Sigma, Deisenhofen, Germany) in 20 ml PBS for 30 min at 37 °C. The cell suspensions were centrifuged twice at 30 g for 2 min to remove hepatocytes and cell clumps and were then filtered through a nylon mesh with 100 μm pore size. The cell pellet was resuspendend in 10 ml PBS, then layered onto 15 ml 30 % (w/v) metrizamide (Cederlane, Ontario, Canada) in 50-ml conical tubes and centrifuged at 1500 g and 4 °C for 30 min. The NPC at the interphase were collected, washed twice with PBS, counted, and resuspended at 10^7 cells/ml in PBS for further analysis.

Flow cytometry

Flow cytometric analysis was performed as previously described [10]. Liver-derived immunocompetent cells were detected by the mAb MN4–91–6 ($RT1^{av1}$-specific). The following additional antibodies were used: R73 ($\alpha\beta$ T cells), OX-62 (dendritic cells), OX-6 (MHC class II), 10/78 [natural killer (NK) cells], OX-33 (B cells), W3/25 (CD4 + T cells), OX-8 (CD8 + T cells), and ED2 (macophages). All of these mAbs were purchased from Serotec.

Results

Survival and histology of liver allografts

ORLT was performed in the DA-to-LEW and the LEW-to-DA combination. Untreated LEW recipients of DA liver allografts died of acute liver rejection within 14 d p.op. (group 3). They showed a progressive increase in serum total bilirubin levels (up to 70 mg/ml) from day + 5 p.op onward (not shown). In contrast, long-term allograft acceptance (> 100 days) in more than 94 % of the grafted animals was induced by initial CsA monotherapy (group 1: 3 mg/kg) and by low-dose CsA (group 2: 0.25 mg/kg) in combination with mAbs (triple therapy). The long-term accepted liver allografts of group 2 exhibited normal architectures with very few cellular infiltrates, while livers of group 1 were found to have a persisting mononuclear cell infiltrate. Furthermore, donor-specific tolerance was demonstrated in animals of group 2 [11]. All DA recipients of LEW livers, the spontaneous liver tolerance combination (group 4), survived long term.

Number of isolated NPC reflects early and late allograft infiltration

The number of isolated NPC in syngeneically grafted DA livers was usually 3–4 × 10^7 cells. During acute rejection (group 3: untreated controls) the number of NPC increased dramatically to 16 × 10^7 cells in the early phase after transplantation (up to day + 10). Triple therapy caused a moderate infiltration by day + 10 of 5 × 10^7 NPC (total number of NPC was 8 × 10^7) that declined to normal counts on day + 100. After monotherapy a very mild early infiltration (total number of NPC: 3–4 × 10^7) increased to 8 × 10^7 NPC in the late period (Fig. 1). The NPC in the early phase after transplantation were com-

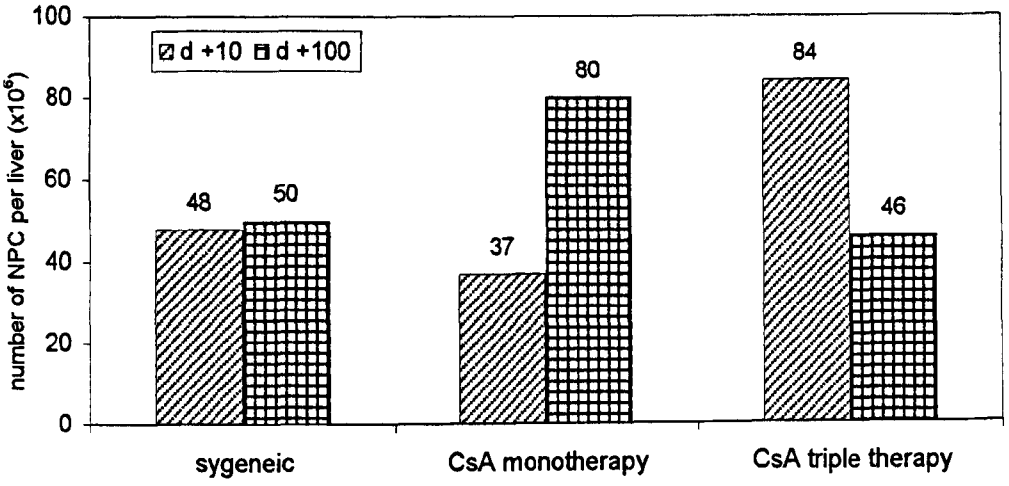

Fig. 1 Comparison of syngeneically and allogeneically grafted animals treated with cyclosporine A (CsA) monotherapy and CsA triple therapy for the number of non-parenchymal cells (*NPC*) in the early [day (*d*) + 10] and late phases (day + 100) after liver transplantation. No correlation was found between long-term outcome and the intensities of cellular infiltration on day + 10 and day + 100

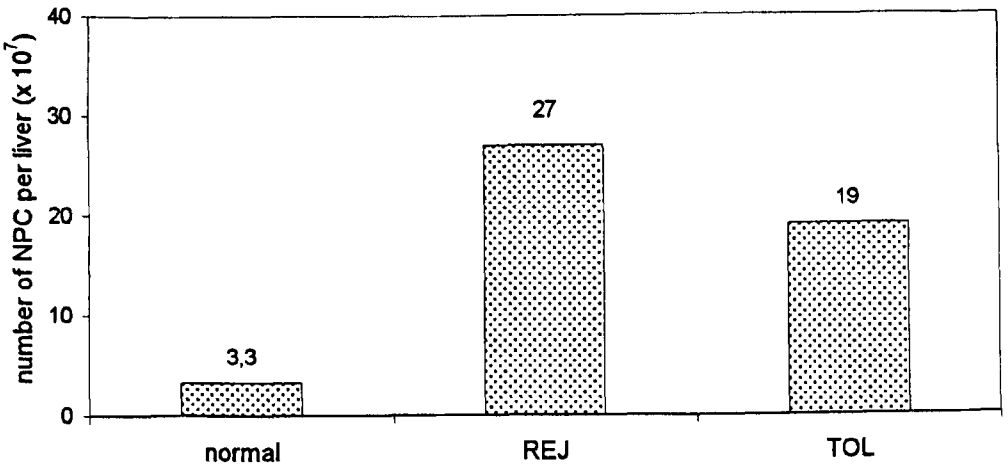

Fig. 2 Comparison of non-grafted (*normal*) livers and allogeneically grafted livers for NPC counts in the early phase after transplantation (day + 6). During this phase, intense infiltration was evident in both the rejection (*REJ*) model DA-to-LEW (no immunosuppression) and the spontaneous liver tolerance (*TOL*) model LEW-to-DA

posed of 75–80% recipient-derived cells. In spontaneously accepted LEW livers a total number of 19×10^7 isolated NPC on day + 10 indicated an early cell infiltration. In contrast to the infiltration in the rejection model, this infiltration resolved over the following 2 weeks (not shown). In the context of the later outcome, no correlation existed between the intensities of cellular infiltration in the early phase versus the late phase after transplantation (Fig. 2). The increasing number of infiltrating CD4 + T cells in the rejection model may indicate that liver allograft rejection is mediated by these cells in response to alloantigen (Fig. 3).

Recurrence of donor-specific leukocytes within long-term accepted liver allograft

In a next step we investigated the fate of donor-specific leukocytes within liver allografts during the early and late phases after transplantation. Recurrence of donor-specific cells was caused almost exclusively by increasing T cell counts (Fig. 4). This phenomenon may be attributable to the fact that the fetal liver was a site of hematopoiesis and that the repopulation was brought about by resident stem cells. At 1–3%, donor-specific NK cells and B cells represented minor populations among the NPC in the early and late phases.

Persistence of dentritic cells (DC) in long-term surviving liver allografts

DC are a minor part of the normal resident leukocyte population within the liver. They are characterized by a strong MHC class I and MHC class II expression and are highly effective activators of allogeneic T cell proliferation in vitro (not shown). In pharmacologically induced long-term acceptance (groups 1 and 2), the persistence of DC was clearly demonstrated in the late phase (nearly 2–4%) by the mAb OX-62 and strong MHC class II expression. Further, we demonstrated that the number of DC in long-term accepted liver allografts at-

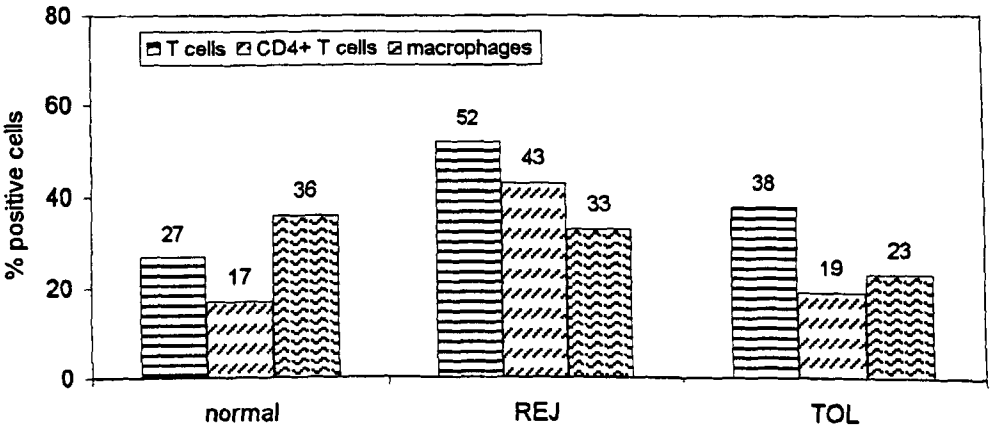

Fig. 3 Comparison of non-grafted (*normal*) and spontaneously accepted livers (*TOL*) with livers of the rejection model (*REJ*) for the quantity and quality of NPC. CD4 + T cells dominate the T cell fraction in the REJ model

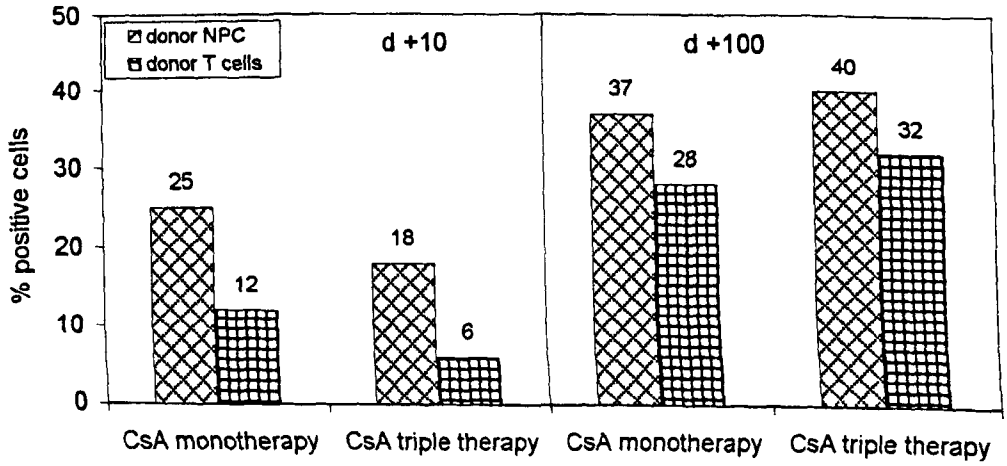

Fig. 4 Donor-specific cells repopulate liver allografts in the late phase (day + 100) after transplantation in the pharmacologically induced long-term acceptance models: group 1, CsA monotherapy, and group 2, CsA triple therapy. The recurrence was caused almost exclusively by T cells

tained values approaching those prior to transplantation. The NPC to DC proportion in non-grafted livers was approximately 23:1, increased to 500:1 in the early phase, and decreased to 50:1 in the late phase after transplantation. The absolute yield of DC per liver ranged between 6.6×10^5 and 10^6 DC and was nearly equal in non-grafted livers and liver allografts in the early and late phases after transplantation. This observation may indicate that DC counts remain nearly constant in the early and late phases after transplantation and might indirectly indicate the potential of DC stem cells to differentiate to mature DC under the influence of the liver microenvironment.

Discussion

In the present paper we demonstrate the repopulation of long-term accepted liver allografts by donor-derived leukocytes on day + 100 after transplantation. This recurrence was caused almost exclusively by increasing T cell counts and may be based on the differentiation of liver-derived T cell progenitors. The adult liver has the potential to reconstitute all hematopoietic cell lines and supralethally irradiated recipients can be rescued if they receive a liver allograft [6]. Furthermore, there is evidence of extrathymic T cell development within the liver [12, 13]. The repopulation of liver allografts by DC may also be based on the differentiation of DC progenitors. If NPC depleted of mature DC through density centrifugation are cultured in vitro in the presence of the growth factor granulocyte macrophage colony stimulating factor, mature DC can be identified after 5–7 days by their strong MHC class II expression (unpublished data).

Liver allograft rejection is induced by the infiltration of allogeneic immunocompetent cells. The intensities of cell infiltration in the early and late phases after transplantation do not correlate with eventual outcome, as we have shown for two different immunosuppressive protocols. In the early phase, the rejection and spontaneous liver tolerance groups showed similar intensities of graft infiltration. For the LEW-to-DA combination this infiltration resolved over the following 2 weeks.

The induction of donor-specific tolerance of liver allografts under temporary immunosuppression has been

described in several experimental species [14]. Acceptance is more readily available for liver allografts than for other leukocyte-rich solid organs, for example, the small bowel. It seems that the tolerogenicity of liver allografts may be based on the quality and quantity of their resident leukocyte population. The role of the resident liver leukocyte population in the process of liver allograft rejection and tolerance induction has been thoroughly investigated, but details of the underlying mechanisms remain unclear [15]. A number of mechanisms have been postulated to explain the phenomenon of tolerance of liver allografts [2]. Recent reports show that liver-resident leukocytes are required to induce tolerance [4]. Depletion of these donor leukocytes by low dose (10 Gy) total-body irradiation of the LEW liver donor prevents spontaneous tolerance by the DA recipient. However, tolerance can be restored by parking the irradiated LEW liver into a second LEW host for 48 h before retransplantation into a new DA recipient. Furthermore, treatment of DA recipients with endogenous IL-2 abrogated the spontaneous acceptance of LEW livers and resulted in acute rejection [5]. Starzl et al. postulate that the migration of donor passenger leukocytes into recipient lymphoid tissue influences the transplantation outcome [3]. We detected very low but significant numbers of donor cells in spleen (0.4%) and mesenteric lymph nodes (0.2%) on day + 100 p.op. in group 2 (not shown). At the same time we identified definite cellular processes, such as an up to 40% increase in donor-specific T cells and persistence of DC within liver allografts. The importance of these phenomena for tolerance induction is poorly understood; it cannot even be ruled out that they represent mere epiphenomena. Recent studies indicate a possible role for donor-derived DC in the induction of liver tolerance [16].

In summary, distinct cellular events, i.e., recurrence of donor T cells and persistence of donor DC, occur in the liver graft itself and might play a role in tolerance induction. Further investigations are necessary to clarify whether intrahepatic chimerism and the cellular processes involved are as important in inducing tolerance as the cellular events taking place within the recipient, for example, microchimerism and high-dose/activation-associated tolerance [2]. The role of donor-derived T cells and DC in the phenomenon of liver-induced tolerance requires further clarification. The capacity of resident DC to deliver tolerogenic signals to recipient T cells in long-term accepted liver grafts is currently under investigation.

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