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ORIGINAL ARTICLE

Induction of long-term graft acceptance by a combination treatment of donor splenocytes and CTLA4Ig in a high responder rat liver transplantation model

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Summary

The CTLA4Ig has led to an improved survival rate in various allograft transplantation models. We investigated in a high responder rat model (Dark Agouti to Lewis) of orthotopic liver transplantation (ORLT), whether an additional adoptive cell transfer can enhance the effect of CTLA4Ig. After transplantation, recipients ($n = 13$/group) were treated with donor or third-party splenocytes alone or in combination with CTLA4Ig. Administration of splenocytes alone had no significant effect on survival (median 13 days, range 9–14) compared with untreated controls (median 10 days, range 8–12). CTLA4Ig monotherapy prolonged survival to a median of 30 days (range 11–150) but resulted in long-term graft rejection. The additional administration of third-party splenocytes showed no significant improvement over CTLA4Ig monotherapy. Only the combination of donor splenocytes with CTLA4Ig led to long-term graft acceptance (>150 days) without clinical and/or histological signs of rejection. A higher rate of apoptosis could be detected in livers at early time-points in long-term survivors receiving CTLA4Ig and donor splenocytes. Analysis of cytokine mRNA expression revealed a decrease of interleukin-2 at early time-points in all groups receiving CTLA4Ig; whereas, interferon-γ was increased in long-term survivors receiving CTLA4Ig and donor cells or donor cells alone. The combination of CTLA4Ig and donor derived splenocytes is potent to induce long-term survival and graft acceptance. The mechanisms appear to involve the induction of an early inflammatory impulse and apoptosis.

Introduction

The introduction of potent immunosuppressive drugs has improved long-term outcome in patients after solid organ transplantation. However, there are still many graft losses related to immunologic complications and other severe side-effects, such as infections or the development of malignancies. Therefore, a rising number of studies are concentrating on graft acceptance strategies independent of long-term administration of immunosuppressive drugs.

T-lymphocytes play a key role for the induction of immune responses. Therefore, mechanisms that suppress T-cell responses might help to increase graft acceptance of transplantation. Initiation of T-cell cascades against grafted tissues requires two distinct signals. The first essential signal is the engagement of a T-cell receptor (TCR) against antigen peptides, via the major histocompatibility complex (MHC) molecules on antigen presenting cells (APC). The presence of a second co-stimulatory signal determines whether responding T-cells are fully responsive or anergic [1]. CD28 has been identified as a major receptor for co-stimulatory ligands such as CD80 (B7.1) and CD86 (B7.2), which are required for stimulation of T-cells [2]. Only after activation of both signals are T-cells processed from a resting to a fully activated state [3], thereby producing various cytokines and growth factors which are required for further immune response [4–7].

The cytotoxic thymphocyte antigin 4-immunoglobulin (CTLA4Ig), a recombinant fusion protein consisting of the extracellular domain of CTLA-4 linked to the constant region of IgG1, binds B7 molecules with higher affinity than does CD28, and acts as a potent competitive inhibitor of B7:CD28-mediated T-cell stimulation [8]. Therefore, CTLA4-Ig blocks CD28-mediated co-stimulatory signals and inhibits *in vitro* and *in vivo* immune responses. This effect has been proven to prolong graft survival or enhance long-term graft acceptance in several animal models for allogenic islet cell, kidney and lung transplantation [3,9]. However, the duration of successful graft acceptance has been variable for different organs [10]. In a fully allogenic rat liver transplant model, repeated administration of CTLA-4Ig has resulted in prolonged graft survival up to 98 days but did not attain long-term graft acceptance [11]. Recent data suggest that these effects depend on the timing of CTLA-4Ig administration and may be improved by additional donor-specific cell administration [12].

Thus, the primary goal of our study was to evaluate the hypothesis that the additive administration of donor splenocytes and CTLA4Ig improves liver graft survival in comparison with CTLA4Ig alone. Our secondary goal was to assess time-dependent differences in the immune reaction against the graft under this treatment.

Material and methods

Animals

Inbred male rats of the strains Dark Agouti l (DA, $RT1^{av1}$), Lewis (LEW, $RT1^{l}$) and Brown Norway (BN, $RT1^{n}$) were purchased from Harlan–Winkelmann (Borchen, Germany). All animals were treated according to the National Guidelines of Animal Care. The project was approved by the regional ethics committee in accordance to the German law on the protection of animals.

Eight- to 14-week-old rats weighing 250–300 g were housed in cages in a temperature and light controlled environment, with access to tap water and food *ad libitum*, and were weighed and examined daily. After at least 1 week of acclimatization, liver transplantations were performed. Differences existed in the DA to Lewis combination in both major and minor histocompatibility complexes [13].

Animals and transplant procedures

Orthotopic rat liver transplantation (ORLT) was performed with hepatic artery revascularization, using DA rats as donors and Lewis rats as recipients. Bile duct reconstruction was accomplished by stent implantation according to the method of Zimmermann *et al.* [14]. Except for hepatic artery and bile duct, all anastomoses were completed with hand-sewn sutures. Portal clamping time in all transplantations was <18 min. Cold ischemic time in standard saline solution was below 60 min.

Separation of viable splenocytes

Dark Agouti or BN rats weighing between 250 and 300 g were used as donors for splenocytes. After sterile splenectomy, spleen tissue was purified and cells were mixed and filtrated through a 100-mesh nylon filter. Separation of mononuclear spleen cells was done over a Ficoll–Hypaque gradient (density 1.077) with 20 min of centrifugation. The separated population of mononuclear lymphocytes was diluted and washed twice in phosphate-buffered saline (PBS) buffer. This solution was centrifuged one more time at 300 *g* for 20 min. Splenocyte viability was consistently >95% as determined by Trypan Blue exclusion [15].

Experimental approach

Recipients (Lewis) were divided into six groups with each group receiving either intraperitoneal CTLA4Ig, intravenous splenocytes or a combination of both. The treatment started 3 days after transplantation. Group 1 ($n = 13$) received an ORLT with no further treatment. Group 2 ($n = 13$) received 2.5×10^8 donor-specific spleen cells (DA, $RT1^{av1}$) on day 4. Group 3 ($n = 13$) was treated with third party spleen cells (BN, $RT1^{n}$) on day 4. Group 4 ($n = 13$) was treated by an intraperitoneal injection of 0.5 mg CTLA4Ig on days 3 and 4 after transplantation. Group 5 ($n = 13$) received donor derived (DA) spleen cells (day 4) in addition to CTLA4Ig (days 3 and 4) administration; whereas, group 6 ($n = 13$) was treated with CTLA4Ig and non-specific third party (BN) splenocytes. Further immunosuppression was not used. In each group seven rats were used to obtain long-term survival (Fig. 1). All long-term survivors were harvested on day 150. To monitor the immunological reactions against the graft, in all groups rats were harvested on day 6 ($n = 3$) and 12 ($n = 3$). However, as we knew from our survival experiments, some rats in groups 1–3 did were not going to survive until day 12 and were harvested 1–2 days earlier to this, when signs of a deteriorating condition developed.

Native histology

A complete autopsy was performed on all rats except for those used to obtain survival figures that died prior to day 150. Harvesting was performed under general anesthesia. For hematoxylin and eosin (H&E) staining,

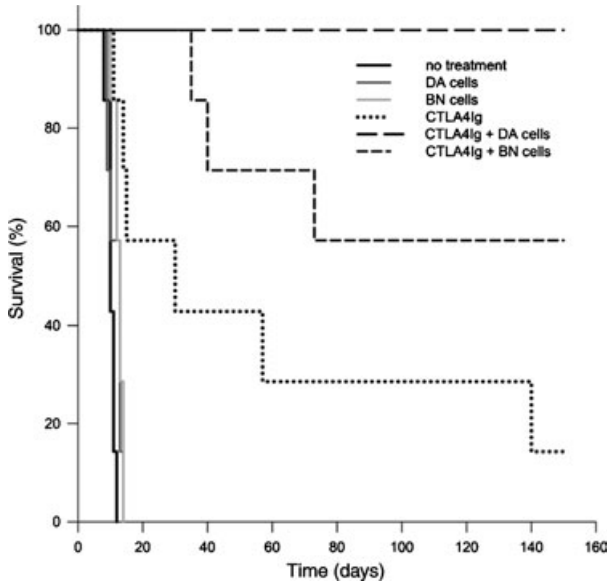

Figure 1 Survival figures after rat liver transplantation in the strain combination DA to Lewis with CTLA4Ig treatment and administration of splenocytes [DA or BN splenocytes given once on day 4; CTLA4Ig given twice a day (day 3 and 4); DA: Dark Agouti, BN: Brown Norway].

tissue samples from liver and spleen tissue were fixed in 10% buffered formaldehyde and embedded in paraffin. Samples were incubated for 5 min in Harris hematoxylin solution and for 60 s in eosin solution. In between, the sections were washed with plain water, and placed in counter stain (ammonia water). Finally, sections were rinsed in 70% ethyl alcohol and dehydrated in xylene solution. Blinded evaluation of the samples was done at random.

Immunohistological staining

The 5 μm frozen sections were used for immunohistological staining by 'alkaline-phosphatase-anti-alkaline-phosphatase' (APAAP) technique (Dako Diagnostika GmbH, Hamburg, Germany). Using this method, the Fuchsin substrate-chromogen yields a fuchsia-colored reaction product at the site of the target antigen. After incubation with a specific primary monoclonal antibody (see below), a biotinylated secondary antibody and the mixture was subsequently incubated with a streptavidin/alkaline phosphatase complex. The whole macromolecular complex was localized by incubation with the Fuchsin substrate-chromogen, to reach a final fuchsia-pink color for the positive antigen sections. Endogenous alkaline phosphatase activity was blocked by 0.1% levamisole.

Chimerism

To detect DA cells in the spleen tissue by immunofluorescence, a rat-specific biotin-conjugated rat monoclonal antibody (anti-RTA1^{a,b}, Pharmingen, Heidelberg, Germany) was used that reacts with epitope S of the rat MHC class I antigen (haplotype RTA1^{a}, which is specific for DA) and does not react with RT1^{l} (Lewis).

CD25 (α-chain of IL2 receptor) detection

To account for the presence of CD25 positive cells in liver tissue a monoclonal mouse antibody was used (anti-CD25 (IgG1). Serotec, Duesseldorf, Germany). All stained tissue sections were examined under a high power magnification field at 250×. The results were graded semi-quantitatively in a blinded fashion by three examiners: (−) no expression, (±) partial expression, (+) low expression, (++) moderate expression, (+++) strong expression.

Non-competitive semiquantitative RT-PCR

Expression of RNA in liver and tissue samples was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). We used a TRIZOL© reaction kit (Invitrogen, Karlsruhe, Germany) for RNA isolation by phenol extraction as first described by Chomczynski and Saachi [16]. All samples were incubated with deoxyribonuclease I (DNAse) for 15 min to exclude DNA contamination. After photometric determination of RNA purity and concentration, it was stored in aliquots of 10 μl at −80 °C. RNA was amplified using a RT-PCR kit (Titan One-Tube RT-PCR System, Boehringer, Ingelheim, Germany), which combines reverse transcriptase and taq polymerase activity in a final reaction volume of 50 μl. Controls using no RNA or only taq polymerase without reverse transcriptase activity were performed. Amplification products were analyzed for size and quantity using gel electrophoresis (agarose 2% with ethidium bromide). In addition to cytokines, β-actin was also amplified for each sample to normalize cytokine expression to a standardized RNA control, expected to be constant through all samples. Primers for β-actin were designed to anneal in two different exons to further control against possible DNA contamination, which would result in a bigger size PCR product, including the intron in between. Finally, to suggest correct amplification products, samples were digested with restriction enzymes, and the fragmentation patterns were compared with theoretical patterns of digestions in a sample with the correct sequence.

Sequences used for primers were as follows: Interleukin-2: reverse 5′-TGG CTC ATC ATC GAA TTG GC-3′,

forward 5′-CAG CTG TTG CTG GAC TTA C-3′, 291 bp; Interleukin-4: reverse 5′-CAA GTA TTT CCC TCG TAG GAT GC-3′, forward 5′-GCA CCG AGA TGT TTG TAC CAG-3′, 309 bp; Interferon-γ: reverse 5′GAG TGC TAC ACG CCG CGT CTT 3′, forward 5′TCA TTG ACA GCT TTG TGC TGG 3′, 338 bp; β-actin: reverse 5′CGG AAC CGC TCA TTG CC-5′, forward 5′CTG AAC CCT AAG GCC AAC-3′, 440 bp.

Western-blot analysis for apoptosis detection in liver tissue

Protein was isolated from liver tissue samples that were homogenized in lysis buffer (100 mM EDTA, 50 mM Tris, 100 mM NaCl, 0.5% Triton X-100) that contained a protease inhibitor cocktail (Complete Tabs, Roche, Penzberg, Germany). Total protein concentration was determined using the colorimetric method of Bradford, which is based on the fact that Coomassie® Brilliant Blue G-250 dye reacts with proteins in the presence of acidic alcohol solution (all chemicals by Bio-Rad Corp., Muenchen, Germany). The extent of blue staining was determined by photometry (595 nm), which is directly proportional to the protein concentration. Proteins were separated by a 4–14% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Finally, protein bands were transferred ('blotted') from the separating gel to a polyvinylidene fluoride (PVDF) membrane, which was preincubated in 60% methanol for 5 min. To prevent non-specific binding, proteins on the PVDF membrane were blocked in tris buffered saline (TBS) buffer and milk 5% and washed with TBS-Tween (0.5%) afterwards. Proteins were incubated with primary antibodies at 4 °C for 24 h thereafter, and washed again with TBS buffer and Tween (0.1%), before a secondary HRP-conjugated (horseradish peroxidase) rabbit anti-mouse antibody was added (incubation for 2 h at room temperature). After another washing step (TBS buffer, Tween 0.1%), the protein-antibody complex was visualized using the Amersham ECL detection kit (Amersham Biosciences, Piscataway, NJ, USA). Detection of apoptosis was analyzed using a specific monoclonal anti-poly-adenosine-diphosphate-ribose-polymerase (anti-PARP) antibodies [PARP (Ab-2), anti-mouse IgG_1; Oncogene Science, Cambridge, MA, USA], which recognizes the 85–90 kDa apoptosis related cleavage fragment of PARP.

Statistical analysis

Comparison of survival data was by log-rank analysis of the product-limit estimate of Kaplan and Meier. Differences between groups were considered to be significant if $P < 0.05$. To obtain these figures SigmaPlot/SigmaStat software (Systat Software, Point Richmond, CA, USA) was used.

Results

Survival

Recipients who were treated with BN or DA splenocytes both showed a median survival rate of 13 days (range 9–14 days), which was not significantly different to untreated controls (median 10, range 8–12 days). Recipients who were treated with CTLA4Ig alone showed a significant prolongation of survival to a median of 30 days (range 11–150). Finally, the combined treatment with CTLA4Ig and donor-derived DA cells led to a significant extension of survival. All rats in this group survived more than 150 days. Improved survival rates were also observed in the combined treatment of BN cells and CTLA4Ig, with a median survival of 150 days (range 35–150). However, this was not significant compared with group 4, which was treated with CTLA4Ig alone (Fig. 1).

Histological changes

In our survival groups, all recipients of groups 1–3 died within 13 days, which suggested that administration of splenocytes alone had no effect compared with untreated controls. The liver transplants in groups 1–3 showed severe signs of periportal and sinusoid lymphocyte infiltration on day 6 (Fig. 2a). Eventually, this led to a confluent hepatocyte necrosis and bile duct proliferation. In contrast, we only found mild histological signs of inflammation in groups 4–6 (CTLA4Ig mono-therapy or in combination with adoptive cell transfer) in the early course. In these groups however, only the long-term surviving recipients in the group treated with DA splenocytes and CTLA4Ig showed normal liver tissue with no histological signs of chronic rejection after 150 days (Fig. 2b), whereas long-term survivors which had been treated with CTLA alone (group 4) or in combination with BN cells (group 6) also showed signs of ongoing rejection with chronic bile duct alteration, liver cell necrosis and mononuclear cell infiltration (Fig. 2c,d).

Immunohistological staining

Expression of CD25 (α-chain of IL2 receptor) in liver tissue

In all groups that had not received CTLA4Ig, there was a marked increase of CD25 positive cell infiltration in the portal fields and sinusoids of the liver during the early course on day 6 (Table 1; Fig. 3a). CD25 expression was significantly reduced after administration of CTLA4Ig,

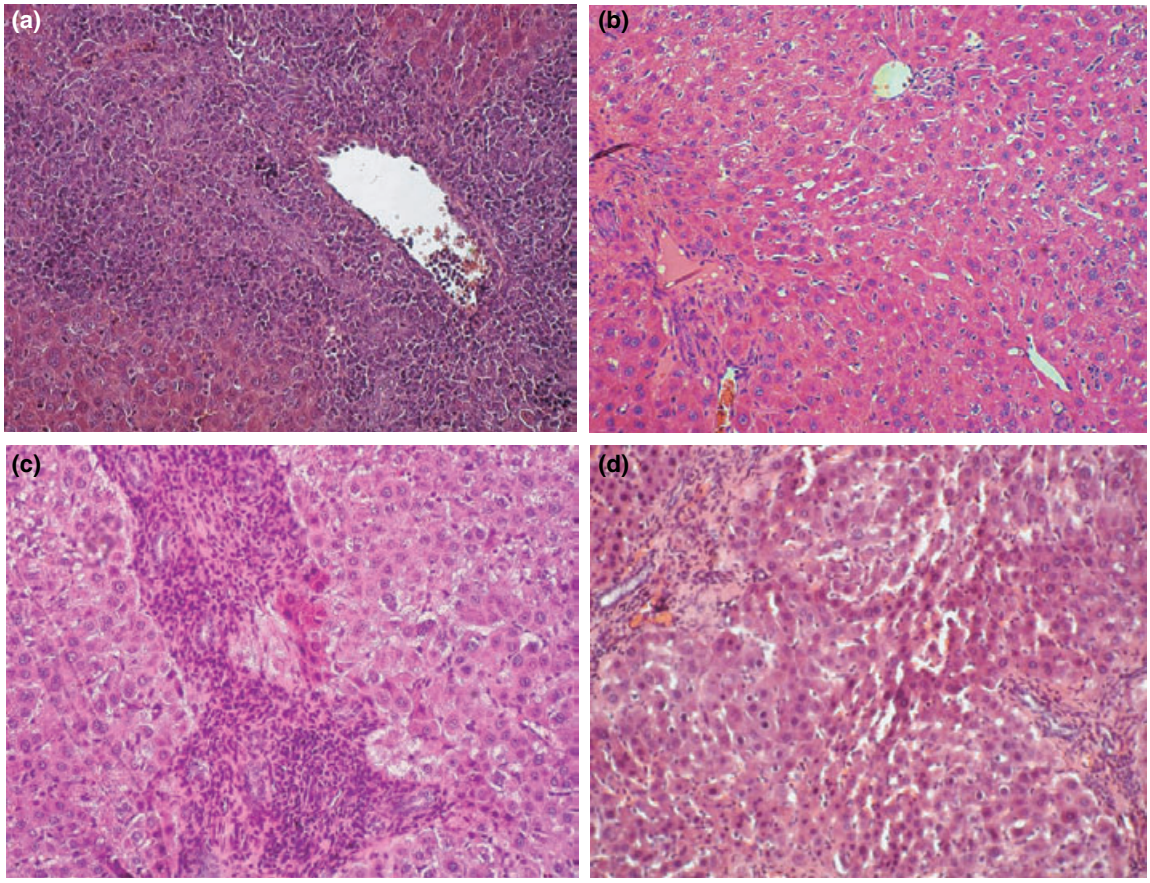

Figure 2 (a) Histological staining, day 6 after transplantation, no treatment group (controls), severe liver cell necrosis, bile duct lesions, mononuclear cell infiltration. (b) Histological staining (H&E), group 5 (CTLA4Ig and donor specific DA cells) 150 days after transplantation, long-term survivor, normal liver tissue, no signs of inflammatory cell infiltration, normal bile duct texture. (c) Histological staining (H&E), group 4 (CTLA 4Ig on day 3 and 4) 150 days after transplantation, mononuclear cell infiltration, bile duct alterations and reduction of hepatocytes. (d) Histological staining (H&E), group 6 (CTLA4Ig and donor unspecific BN cells) 150 days after transplantation, long-term survivor, severe lymphocyte infiltration and bile duct proliferation.

and even in the long-term course of graft accepting rats only single CD25 positive cells could be obtained (Fig. 3b).

Chimerism (anti-RT1 $A^{a,b}$)

The MHC-I detection to identify DA cells in recipient spleen tissue, a condition defined as microchimerism, was highest in group 5 at early time-points (day 6 and 12), which declined to only a few detectable cells in the long-term survivors of this group. All other groups (1–4 and 6) had only low numbers of detectable MHC-I positive DA cells on day 6, and no specific donor cells could be detected in the spleen tissue on day 12 (Table 1).

Western blot detection of apoptosis

On day 6, quantification of apoptosis in liver tissue by protein detection techniques (PARP) showed a marked increase in rats treated with CTLA4Ig alone or in combination with either donor-derived or third party cells. In those groups with donor specific (DA) cell or no additional cell application, degraded DNA products were still detectable on day 12, indicating a sustained incidence of apoptotic cells in the liver tissue. In contrast, the two groups that had received an adoptive cell transfer without CTLA4Ig showed only small numbers of apoptotic cells, without a peak on either day 6 or 12, throughout the whole post-transplant course. In controls (no treatment), we found DNA degradation products after 12 days in coincidence with the development of liver rejection (Table 1).

RT-PCR for interleukins

Interleukin-2 mRNA expression in liver tissue

Semi-quantitative analysis of mRNA expression for interleukin-2 (IL-2) on day 6 and 12 was low in all groups trea-

Group	1	2	3	4	5	6
Treatment	None	DA cells	BN cells	CTLA4Ig	CTLA4Ig + DA cells	CTLA4Ig + BN cells
α-chain of IL-2 receptor (CD25)/liver						
Day 6	++	+	++	–	+	–
Day 12	++	++	++	+	+	+
Long-term	n.a.	n.a.	n.a.	++	+	+
Chimerism (anti-MHC-I)/spleen						
Day 6	+	+	+/–	+	++	+
Day 12	–	–	–	+/–	++	–
Long-term	n.a.	n.a.	n.a.	–	+/–	–
Apoptosis [PARP (AB-2)]/liver						
Day 6	–	+	–	++	++	++
Day 12	++	+	+	+	+	–
Long-term	n.a.	n.a.	n.a.	+	+	–
Cytokine expression IFN-γ/liver						
Day 6	+/–	++	–	+	+	+
Day 12	++	++	–	+	++	+
Long-term	n.a.	n.a.	n.a.	++	+/–	–
Cytokine expression IL-2/liver						
Day 6	+++	++	++	–	+	–
Day 12	++	+	++	+	+	+
Long-term	n.a.	n.a.	n.a.	++	–	+
Cytokine expression IL-4/liver						
Day 6	++	++	+++	+++	+++	++
Day 12	++	++	+++	++	+++	+
Long-term	n.a.	n.a.	n.a.	+	++	+

Table 1. Expression of CD25 positive cells and apoptosis [anti-PARP (Poly-adenosine-diphosphate-ribose-polymerase] in liver tissue. DA-specific MHC-I antigen (anti-RT1 $A^{a,b}$) in spleen tissue, cytokine mRNA expression of liver tissue in different treatment groups at time points described previously (long-term corresponds to a harvesting at day 150).

–, no expression; ±, partial expression; +, low expression; ++, moderate expression; +++, strong expression; n.a., not applicable

ted with CTLA4Ig. In contrast, untreated controls (group 1) showed the highest expression of IL-2 on day 6 and 12. However, recipients treated with donor derived or third party BN cells (groups 2 and 3) also had a high IL-2 expression, whereas long-term survivors of group 5 (DA and CTLA4Ig) showed no mRNA expression of IL-2 (Table 1).

Interleukin-4 mRNA expression in liver tissue

As IL-4 was evidently expressed in all groups on day 6 and 12, there were no differences at these time-points. The expression of IL-4 decreased in the long-term survivors of groups treated with CTLA alone in combination with unspecific cells (groups 3 and 4). In contrast, long-term survivors of group 5 who had no signs of rejection showed a marked increase of IL-4 expression 150 days after ORLT.

Interferon-γ mRNA expression in liver tissue

Graft acceptance of long-term survivors with donor-derived adoptive cell transfer and CTLA4Ig treatment was associated with a marked expression of interferon-γ (IFN-γ) on day 6, which further increased on day 12. However, in the long-term course, it declined below detectable levels in five rats (71%). In contrast, in long-term survivors with CTLA4Ig mono-therapy, IFN-γ expression showed a further increase. In liver tissue of controls and after treatment with donor derived DA cells alone, IFN-γ expression was significantly increased on day 12 with the incidence of rejection.

Discussion

Administration of CTLA4Ig has been used to prolong graft survival in several solid organ transplant models such as small bowel, cardiac or pancreas [1,3,9,17,18]. Although most of the studies showed that inhibition of the co-stimulatory signal resulted in a significant prolongation of graft acceptance, only a few were able to achieve long-term tolerance [11]. Therefore, modifications of treatment strategies have been performed to improve these results, including blockage of other co-stimulatory signals (e.g. anti-CD40) and adoptive cell transfer [19–22].

Our study of rat liver transplantation could confirm that postoperative treatment with only CTLA4Ig may lead to a prolongation of survival but fails to induce long-term graft acceptance and eventually leads to rejection. However, with this study, we were the first to demonstrate that a single treatment of CTLA4Ig in combination with

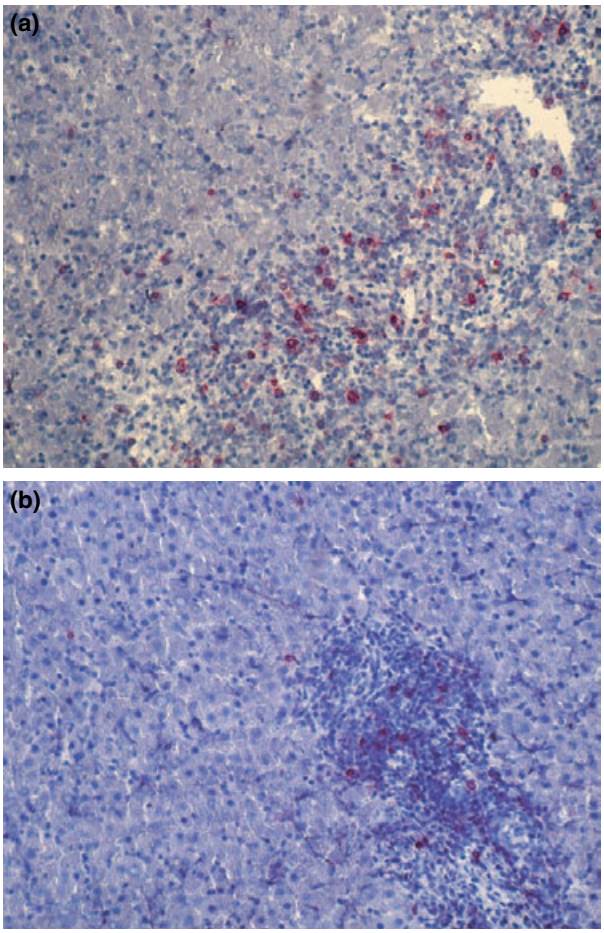

Figure 3 Immunohistology for CD25 in liver tissue. (a) Group 1 (no treatment), day 6, significant expression of CD25 throughout the liver tissue. (b) Group 6 (CTLA4Ig and DA splenocytes), day 150, low CD25 expression.

donor-derived splenocytes induces long-term acceptance of liver allografts in a high-responder model using Lewis rats as recipients and DA rats as donors. Although we did not apply any further testing to confirm tolerance, as recently published by Murase *et al.* [23], a tolerant state can not be identified by any current tests, including cell-mediated lymphocytotoxicity (CML), any more accurately in humans than in rodents. In his study, he had always found an intact mixed-lymphocyte reaction (MLR) in bone marrow conditioned rat recipients, which had accepted heart and liver allografts. Therefore, to describe the dichotomy between the *in vitro* and *in vivo* results, he introduced the term 'split tolerance' according to a previous published study [24].

In an experimental setup similar to ours, Yan *et al.* [25] were able to find a tolerance inducing effect of donor derived cells alone. They applied donor leukocytes at the time of kidney transplantation in a mismatched rat strain combination and prevented the incidence of rejection effectively. However, although this study mentions that this effect was also found in high-responder rat strain combinations (PVG to Lewis), the data was only shown using a low-responder model (PVG to DA) in which livers showed no signs of rejection when transplanted without any additional treatment. In this low-responder combination, an early increase in IL-2 and IFN-γ expression was observed on day 1. There was also a high incidence of apoptotic cells and activated T-cells in spleen tissue from day 1 until day 5 after transplantation. As a possible mechanism, the activation of donor cells might lead into an exhaustion of the immune response which is described as activation-induced-cell-death (AICD) [25]. However, this mechanism is questioned by the fact that passenger leukocytes that are present in the interstitium of allografts and migrate to the lymphoid organs of the recipient after implantation may also contribute to the immunogenic effect of allografts. Animal studies have demonstrated that elimination of graft-resident donor-derived leukocytes by gamma irradiation may lead to a prolongation of allograft survival in heart and kidney transplantation [26,27], an effect that could not be confirmed for rat liver transplantation, indicating its exceptional position in transplant immunology [28].

In our study postoperative administration of neither donor derived (DA) nor third party (BN) splenocytes alone significantly improved graft survival compared with untreated controls. The ineffectiveness of an isolated adoptive cell transfer to improve graft survival in our transplantation model might be explained by the fact that cell administration was performed at a later time-point after transplantation and the usage of a different, more mismatched rat strain combination as described by Yan *et al.* [25]. However, we were able to demonstrate that a postoperative combination therapy of CTLA4Ig and donor derived splenocytes is a potent means to induce long-term allograft survival which was our study goal.

We know from other publications [e.g. 29] that CTLA4 signals are required for the induction of allograft acceptance. The cell surface molecule CTLA4 (CD152) is homologous to CD28 and shares the same ligands, B7-1 and B7-2. Unlike CD28, CTLA4 is expressed in a significant number only on activated T-cells, has a much higher affinity for B7 proteins and delivers a negative signal which turns off the proliferation of activated T-cells [30,31]. Thus, early in the immune response B7 proteins interact with CD28 receptors on naïve T-cells to effect co-stimulation whereas later on the high affinity interaction between B7 proteins and CTLA4 on activated T-cells leads to inhibition of cell cycling. This inhibitory mechanism is essential for the optimal induction of peripheral

tolerance to protein antigen or to transplanted organs [32]. Moreover, the inhibitory actions of CTLA4 on T-cells are independent of CD28-mediated signals as blockade of CTLA4 accelerates acute rejection of cardiac allografts in CD28-deficient mice [33]. However, in contrast to these findings, several publications have shown that blocking the B7-CD28 pathway with CTLA4-Ig may effectively delay rejection in rodent transplantation models [20,22,34–36].

As a basis for the immunosuppressive effect of postoperative donor-cell infusion several mechanisms can be postulated. One potential explanation is high-dose antigen-exposure [37] resulting in subsequent clonal deletion or exhaustion of mature host's T lymphocytes specific for that antigen [38]. Although the details of this mechanism are poorly understood, antigen overloading seems to be involved [39]. Considering the obvious relevance of T-cells for this immunoregulatory effect, one mechanism that must be taken into account as an explanation is graft-versus-host (GvH) reactivity. Although there were no clinical signs of GvH disease in our study, subclinical GvH reactions may have occurred that suppress alloresponsive host cells either by directly attacking the host's hematopoietic cells or by indirect effects (e.g. via cytokines). The transition from a subclinical to a definitive state of GvH disease is probably dependent on the number of T cells applied. The importance of dosage with all cell suspensions was already demonstrated by Billingham and Silvers in 1961 [40] who showed that blood leukocytes in sufficient quantity are easily engrafted, and can be either tolerogenic or cause graft-versus-host disease.

A temporary state of immunological hyporesponsiveness toward the graft after cell administration was not only demonstrated by prolonged graft survival but also by a decrease of CD25 expression in allografts. Therefore, the improved graft survival in group 5 may result from this additional immune-compromising effect that may be the neutralization of the recipients host-versus-graft response by the GvH reactivity of the infused cells, as already speculated by Tsui *et al.* [41].

This hypothesis is supported by the finding of a marked expression of CD25 (the α-chain of the IL2 receptor) positive cells in rapidly rejecting rats (groups 1–3) on day 6 that remained high constantly up to day 12. Thereafter, rats died following rejection. In contrast, on day 6 in graft accepting long-term survivors (group 5), we found a reduced number of CD25 positive cells. Accordingly, a CD25(+) T cell reduction was already described by Guo *et al.* [42] in lymph nodes of liver allograft recipients after administration of an adenovirus vector containing the CTLA4Ig gene.

Although, CD25 can be expressed on activated T-, B-cells and monocytes, and a subset of regulatory T-cells exists that expresses both CD4 and CD25, we consider the major proportion in our rejecting groups to be activated T-cells corresponding with the clinical findings of rejection. However, with the finding of a small number of these cells even in long-term survivors, we assume these cells to be CD4+CD25+ (regulatory T-cells) that suppress an immune response in these rats.

These mechanisms would also explain the presence of chimerism at early time-points (day 6 of 12) that was found to the highest extent in spleens of graft-accepting animals (group 5). At the same time, as also found in long-term survivors of group 5, the presence of a stable chimerism seems to be vital for the maintenance of long-term graft acceptance as shown by other studies [43–45]. However, even in combination with CTLA4Ig, the dosage of splenocytes given correlates with the extent of chimerism and graft acceptance where a low dose of splenocytes (5×10^7) failed to induce graft survival in a heart allograft model although (micro-)chimerism was induced [15].

Interestingly, we found a high IL-4 expression at early time-points (day 6) in graft accepting rats, which was similar to that of rejecting ones. This finding is in accordance to a recently published study on tolerance induction that demonstrates that rejecting recipient rats can be identified by IL-4 mRNA expression during the early course until day 5 after transplantation. However, after 7 days no difference for IL4 expression could be found in any group [46].

In our study, long-term survivors (150 days) receiving CTLA4Ig monotherapy (group 4) showed an increase of IL-2 and IFN-γ expression, whereas rats treated with a combination of donor derived cells (DA) and CTLA4Ig (group 5) showed no expression of IL-2 mRNA and only partial expression of IFN-γ but an increase of IL-4 mRNA expression 150 days after transplantation.

The role of cytokines in the induction of immune tolerance has been largely studied in the context of the Th1/Th2 paradigm, supposing that Th2 lymphocytes favor tolerance by IL-4 and IL-10 secretion, whereas Th1 lymphocytes hinder tolerance by producing IL-2 and IFN-γ [47]. However, this has to be questioned, as long-term pancreatic-islet-cell, cardiac and skin allograft survival can be achieved in IL-4 −/− recipients treated with agents that block T cell co-stimulation – despite Th1 cytokine expression in these mice [17,48]. It is also postulated that IFN-γ has important properties in tolerance induction, as co-stimulatory blockage of the CD28 and/or CD40 ligand in IFN-γ-deficient mice fails to induce long-term tolerance in cardiac and skin allografts [49]. In IL-2 deficient recipients, administration of CTLA4Ig fails to induce long-term cardiac allograft acceptance presumably because IL-2 functions as an important factor to induce apoptosis in T-cells. In IL-2 −/− mice the inability to trigger apop-

tosis leads to an accumulation of allostimulated T lymphocytes and failure of allograft acceptance [47].

Although true tolerance was not confirmed in our experimental setting, our data indicates that early expression of IL-4 seems not to be crucial for long-term graft acceptance but as IL-4 expression can be found in long-term survivors of group 5, it might play a role in the maintenance of graft acceptance.

The different results of Th1/Th2 cytokine expression patterns might be due to the different time-points in which it was obtained. Nevertheless, the data suggest that after an early active phase, which requires T-cell activation and the involvement of Th1 cytokines, a second phase with the dominance of Th2 cytokines that eventually leads into apoptosis is required. This is underlined by our results, which show a higher rate of apoptotic cells in the early course of long-term survivors with combined treatment compared with non-treated controls. Possibly, the repeated transfer of cells to the recipient might amplify AICD, which is independent of co-stimulatory signaling. This possible mechanism is supported by a study of Wells *et al.* [50] who found that inhibition of immune activation by application of Tacrolimus or corticosteroids, which prevents the development of apoptosis, consecutively hinders tolerance induction, whereas immunosuppressive drugs that promote apoptosis (Rapamycin) may induce tolerance.

In contrast to pretreatment studies, the results of our post-transplant treatment protocol may contribute as an important first step for possible clinical applications. However, the dividend of stable chimerism, apoptosis and its corollary of drug-free graft acceptance are expected to take years of further investigation as most of the underlying pathophysiological mechanisms are still unsolved.

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