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# Mechanisms of tolerance induction in second renal allografts of a chronic rejection model

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**Abstract** In a previous experiment we demonstrated the induction of tolerance by the allograft itself. In this model of weak histoincompatibility, second grafts of donor origin replacing chronically rejected first renal allografts were accepted long term. Additionally grafted donor-specific hearts functioned indefinitely while adoptive transfer experiments demonstrated the development of donor-specific transferable tolerance. In the current experiment we compared intragraft gene expression of chronically rejected first and tolerant second grafts by RT-PCR. Second renal allografts of donor origin (F-344) replaced first grafts 2, 4, 8, 12, and 16 weeks after the initial engraftment. No immunosuppression was used during second engraftment. Grafts were followed by serial proteinuria; morphological and immunohistological studies (APAAP/infiltrating cells, ICAM-1, MHC II expression) and competitive RT-PCR analyses (expressed as arbitary units AU/cDNA) for relevant cells and cytokines (CD-3, IFN$\gamma$, IL-10, and IL-4) were assessed by the end of the observation period (16 weeks). Macrophages/monocytes (ED-1 + ) and T-cells (CD-5 and CD-4 + ) infiltrated first allografts in high numbers by 12 weeks associated with strong structural signs of chronic graft rejection (ca. 30% arterio- and glomerulosclerosis, tubular atrophy and interstitial fibrosis). Cellular infiltrates in second grafts were prominent, however significantly reduced, while histological changes were minor. At cDNA levels, CD-3 transcripts were elevated in second renal allografts performed 2, 4, and 8 weeks after the initial engraftment while comparable levels were observed when second engraftment was performed after 12 and 16 weeks. Analyses of relevant cytokines demonstrated a TH1/TH2 shift independent from the time interval between first and second engraftment. These results emphasize the role of alloresponsiveness for the development of chronic graft dysfunction. Mechanisms of tolerance induction in our model are associated with a distinct alloresponsive pattern. A crucial role for regulatory T-cells is suggested.

**Key words** Tolerance introduction · Chronic graft dysfunction · Second renal allograft · TH1/TH2 shift

## Introduction

Chronic graft dysfunction represents the leading cause of the still unsatisfactory long-term results after organ transplantation. Clinical and experimental studies showed the effects of alloantigen-dependent and -independent risk factors. The predominance of the process in allografts compared to isografts supports the role of

specific immunological events for the process. In addition, experimental studies demonstrated an abrogation and in some experiments the absence of chronic graft rejection following tolerance induction [1–3].

In a previous experiment we demonstrated the development of allograft-induced donor-specific transferable tolerance in a rat model using a donor/recipient combination of weak histoincompatibility [F-344-to-Lewis (LEW)]. Sequential replacement (as early as 2 weeks after the initial engraftment) of chronically rejected renal allografts by second native renal allografts of donor origin resulted in long-term graft acceptance in the absence of immunosuppressive treatment. Additionally grafted donor-specific hearts were accepted indefinitely while third party hearts were rejected in a similar fashion to those in untreated controls. Adoptive transfer experiments from recipients of second renal allografts into native animals demonstrated the development of a donor-specific, transferable tolerance [4]. In the current study we followed the mechanisms associated with tolerance induction analyzing intragraft gene expression in rejected vs tolerant grafts.

## Materials and methods

Fischer 344 (F-344, $RT1^{lv1}$) allografts were transplanted into LEW ($RT1^{l}$) recipients using standard microsurgical techniques ($n = 25$). One native kidney was removed during transplantation, the remaining kidney 10 days later, and cyclosporine treatment was given for 10 days at a dosage of 1.5 mg/kg per day.

After 2, 4, 8, 12, and 16 weeks, allografts were replaced by a second renal graft of donor origin and followed for 16 weeks ($n = 5$/group). Operative details were similar in first and second grafts; no immunosuppression was used during the second engraftment. Principles of laboratory animal care including the German law on the protection of animals and a permission by the local authorities (Reg G 0087/96) were observed.

Protein excretion (mg/24 h) was measured at serial intervals in both groups by precipitation with 20% $CCl_3COOH$. Turbidity was assessed at a wavelength of 415 nm using a Hitachi 911 analyzer. Morphological and immunohistological evaluations were performed at the end of the observation period. Hematoxylin and eosin and periodic acid-Schiff stains were assessed by light microscopy. Specimen were quantified by the extent of glomerulo- and arteriosclerosis (arterio- and glomerulosclerosis index: % of sclerosis); cellular infiltrates, tubular atrophy, and interstitial fibrosis were quantified on a 0–4 + scale (4 + = strongest structural deterioration).

For immunohistological studies using the APAAP method mAbs to CD5 + T-cells (OX-19), CD4 + T-helper cells (W3/25), CD8 + T-cytotoxic/suppressor cells (OX-8), monocytes/macrophages (ED-1), and MHC class II (OX3; all mAbs from Serotec, Wiesbaden, Germany) were used. MHC class II was quantified on a 0–4 + scale (4 + = dense); positive cell counts were expressed as mean ± SD of cells/field of view (c/FV) More than 20 FV/section were evaluated at 400 ×.

Competitive RT-PCR analyses (expressed as arbitary units AU/cDNA) were performed serially at 2, 4, 8, 12, and 16 weeks for relevant cells and cytokines (CD-3, IFN$\gamma$, IL-10, and IL-4) in first (chronically rejected) and second (tolerant) renal allografts, respectively.

## Results

While urinary protein excretion increased progressively in first renal allografts (130 ± 12 mg/24 h by 12 weeks), second renal allografts demonstrated only a minor increase in protein excretion by 12 weeks (42 ± 10 mg/24 h; $P < 0.01$). First renal allografts demonstrated characteristic signs of chronic graft rejection by 12 and 16 weeks while second grafts of donor origin showed only insignificant histological changes including minor glomerulo- and arteriosclerosis (ca. 15%) independent of the time of second engraftment. Macrophages/monocytes (ED-1 + ) and T-cells (CD-5 and CD-4 + ) infiltrated first allografts in high numbers by 12 and 16 weeks, while second renal allografts demonstrated fewer ($P < 0.05$), however, prominent cellular infiltrates. CD-8 + T-cells, MHC II, and ICAM-1 were expressed moderately in both rejected and tolerant grafts. At cDNA levels, CD-3 transcripts were significantly elevated in second allografts vs first renal allografts when the second engraftment was performed 2, 4, and 8 weeks after the initial engraftment. Second grafts replacing first grafts after 12 and 16 weeks showed comparable CD-3 levels. Levels for IFN$\gamma$, a TH1-associated cytokine, were significantly elevated in first (chronically rejected) vs second (tolerant) renal allografts independent of the time interval between first and second engraftment. The TH2-related cytokines, IL-4 and IL-10, were significantly elevated in second (tolerant) kidney allografts vs first (chronically rejected) grafts throughout the observation period (Fig. 1).

## Discussion

The role of alloantigen-dependent and -independent effects for the initiation of chronic graft deterioration remains unknown. In a recent experiment we could show the induction of tolerance by the allograft itself in a model of weak histoincompatibility [4]. Those results demonstrated the role of alloresponsiveness for the process. In the current experiment we followed the events of intragraft gene expression in chronically rejected vs tolerant animals. A distinct alloresponsive pattern including a TH1/TH2 shift was associated with the development of tolerance in second renal allografts replacing chronically rejected first grafts. Interestingly, high intragraft levels of CD-3 expression were found in tolerant grafts. While transcripts for IFN-$\gamma$ were significantly reduced, elevated levels for IL-4 and IL-10 were found in second grafts. These results show a type 1/type 2 immune deviation. In addition a role for regulatory T-cells for the development of tolerance in second grafts is suggested.

The role of TH1- and TH2-related cytokines for the development of either rejection or tolerance is currently

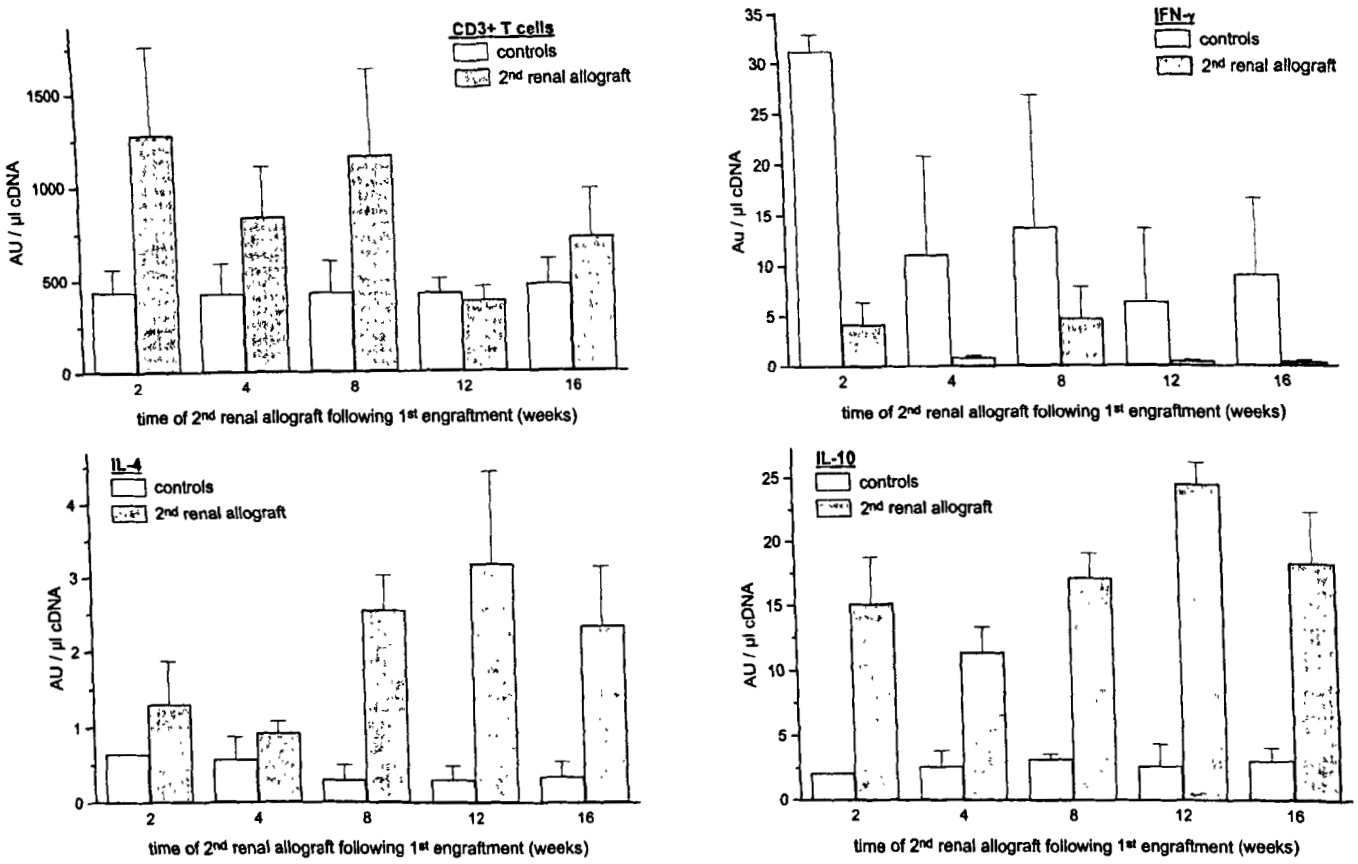


**Fig. 1** Intragraft gene expression in first (chronically rejected) and second (tolerant) grafts

discussed controversially. It has been suggested that the increase of neither population is strictly associated with the development of rejection or tolerance [5]. While pancreatic islets from transgenic mice with constitutive expression of IL-10, a TH2-related cytokine, were rejected acutely, acute rejection, albeit delayed, also occurred in IL-2 and IFNγ knock-out mice [6, 7]. On the other hand, there are contradicting reports on the importance of IL-4 for tolerance induction. High levels of IFNγ produced by graft infiltrating cells at early stages may be responsible for the development of graft vessel sclerosis [8]. The role of type 2 cytokines in the pathogenesis of chronic rejection is not clear although evidence exists that chronic rejection is associated with high intragraft type 2 cytokine expression [9]. However, our data demonstrate that high intragraft IL-4 expression does not necessarily lead to chronic rejection.

In summary, our results demonstrate the role of alloresponsiveness for the development of chronic graft rejection. Tolerance induction by the allograft itself is associated with a TH1/TH2 shift while the role of regulatory T-cells is suggested.

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