Exhaustive differentiation of alloreactive CD8+ T cells : critical for determination of graft acceptance or rejection

Steger, Ulrich; Denecke, Christian; Sawitzki, Birgit; Karim, Mahzuz; Jones, Nick D; Wood, Kathryn J

[Link to publication on Research at Birmingham portal](#)

Exhaustive Differentiation of Alloreactive CD8^{+} T Cells: Critical for Determination of Graft Acceptance or Rejection[8]

Ulrich Steger[1,2,3], Christian Denecke[1,4], Birgit Sawitzki[1,4,5], Mahzuz Karim[1,5], Nick D Jones[1,6], Kathryn J Wood[1,7]

Transplantation Research Immunology Group, Nuffield Department of Surgery, John Radcliffe Hospital, University of Oxford, OX3 9DU, UK

Word Count: Abstract: 249

                  Main Body: 4102

Tables: 1

Figures: 5

Address for Correspondence:

Ulrich Steger M.D.
Department of Surgery I
University of Wuerzburg
97080 Wuerzburg
Germany
Tel.: 0049-931-2011
Fax.: 0049-931-20131049
ulrich.steger@mail.uni-wuerzburg.de

Footnotes:

Abbreviations:

AT: Adoptive Transfer; CFSE: Carboxyfluorescein diacetate succinimidyl ester; CTL: Cytotoxic T-Lymphocyte; mAbs: monoclonal Antibodies; MST: Median Survival Time; TCR: T Cell Receptor; tg: transgenic

Abstract

Background: The precise role that CD8$^+$ T cells play in the rejection and acceptance of different types of allograft is unclear and has been shown to vary between donor-recipient combinations.

Methods: The response of adoptively transferred CD8$^+$ T cells reactive to the donor alloantigen H2K^b was examined after transplantation of H2K^{b+} liver, kidney and heart grafts in mice.

Results: Following transfer of 6×10^6 alloreactive CD8$^+$ T cells to T-cell depleted syngeneic mice spontaneous long term acceptance of liver grafts was observed, whereas kidney and heart grafts were acutely rejected. Within five days of liver transplantation we found that the entire H2K^b-reactive T cell pool was stimulated to proliferate and differentiate into memory/effector cells that were detectable within lymphoid tissues as well as the liver graft itself. However, despite the generation of effector/memory T cells, liver allografts were accepted which correlated with the exhaustion/deletion of such cells. In contrast, although activation and proliferation of H2K^b-reactive CD8$^+$ T cells was observed after transplantation of heart or kidney grafts, unactivated, H2K^b-reactive CD8$^+$ T cells were still present in the spleen even long term. Interestingly, differences in the effector function of liver and kidney graft infiltrating donor-reactive CD8$^+$ T cells were not detected after adoptive transfer into immunodeficient mice, despite a reduction in Th1-type cytokines within liver grafts.

Conclusions: The rapid and extensive initial activation and differentiation of donor-reactive CD8$^+$ T cells that occurs after liver transplantation leads to clonal exhaustion/deletion of the alloreactive CD8$^+$ T cell repertoire resulting in spontaneous tolerance induction.

Introduction

CD8$^+$ T cells with either helper or cytotoxic activity can play an important role in graft rejection. Indeed, in some situations, CD8$^+$ T cells have been shown to reject vascularized and non vascularized allografts without help from CD4$^+$ T cells (1), as well as contributing to responses in collaboration with other leukocyte populations. In some mouse strain combinations it has been demonstrated that CD8$^+$ T cells can trigger costimulation blockade resistant allograft rejection (2;3). Administration of anti-CD154 mAbs led to prolongation of graft survival but not tolerance induction, unless the CD8 population was controlled independently by depletion or in some settings, tolerance induction (4). Recent studies have revealed that CD8$^+$ T cell–mediated immune responses, including rejection and memory responses, can be influenced by regulatory CD4$^+$ T cells (5-7). Whether CD8$^+$ T cells trigger tolerance or even play an active regulatory role in inducing unresponsiveness to donor alloantigens, independent of other cell populations, is not clear.

Deletion of activated T cells through the process known as activation induced cell death (AICD) has been shown to contribute to reducing the frequency of donor reactive T cells, a step on the path to tolerance induction (8). Deletion of cytotoxic T cells through AICD has been found to be one of the passive mechanisms involved in spontaneous graft acceptance and tolerance induction after liver transplantation (9;10). Contributing factors include apoptosis inducing soluble MHC class I, secreted by the liver, as well as deletion by overstimulation and subsequent clonal exhaustion of the alloreactive cytotoxic cell population (11;12). It has been suggested that the high alloantigen load delivered by the liver graft is responsible for the 'high dose' activation observed (13). This phenomenon was originally described for virus specific

cytotoxic effector T cells in the presence of persistent virus infection, and is therefore not restricted to host versus graft immunoresponses (14;15).

In organ transplantation the exclusive ability of fully MHC mismatched liver grafts to induce spontaneous tolerance in animal models (16;17), in comparison to heart and kidney, underlines the significant role of location and pattern of antigen presentation for tolerance induction, provided here by the graft itself. In addition, the finding in clinical liver transplantation that it is possible to wean a subgroup of liver transplant recipients off their immunosuppressive drug therapy confirms that the findings in animal models translates to the clinical setting (18;19). In order to transpose the naturally occurring tolerogenic effects witnessed after liver transplantation into therapeutic possibilities of tolerogenic immunomodulation, a better and more detailed understanding of the T cell responses, including the proliferation and activation pattern as well as the degree of cell elimination and survival, is needed.

To study the behaviour of alloreactive CD8$^+$ T cells in a model of allograft acceptance and tolerance induction, we visualized the fate and function of adoptively transferred CFSE labelled alloantigen-reactive CD8$^+$ T cells (BM3) after liver transplantation in a mouse model. The changes observed were compared with those taking place after heart and kidney transplantation under the same conditions that have been shown previously to lead to rejection of a H2K^{b+} heart graft (20).

Materials and Methods

Animals

CBA/Ca (CBA;H2^k) and C57BL/10 (B10;H2^b) were originally purchased from Harlan Olac (Bicester, U.K.). CBA/RAG-1 knockout mice (mutation in the recombination

activating gene-1; $H2^k$), which are totally deficient in mature T and B cells (21), were a kind gift from Dr. D.Kioussis (Mill Hill, London, U.K.), and BM3 TCR- transgenic mice (BM3; $H2^k$) from Prof. A.L.Mellor (IMMG, Augusta, USA) (22). All mice are now bred and housed in the SPF facilities of the Biomedical Services Unit, John Radcliffe Hospital (Oxford, U.K.). All donor and recipient mice were sex and age matched between 8 and 12 weeks at the time of transplantation and were treated in strict accordance with the Animals (Scientific Procedures) Act of 1986.

Monoclonal Antibodies (mAbs) and Hybridomas

YTA3.1 and YTS169 hybridomas were a gift from Professor H.Waldmann of the Sir William Dunn School of Pathology, Oxford University (23). The Ti98 hybridoma was kindly provided by A.L.Mellor and described previously (24). All hybridomas and cell lines were grown in either RPMI 1640 or α-MEM (Life Technologies, U.K.), supplemented with 2 mM glutamine, antibiotics, and 10% (v/v) fetal calf serum (FCS; Life Technologies). Anti-CD4 (YTA3.1) and anti-CD8 (YTS169) mAbs were purified and dialyzed into phosphate buffered saline (PBS) before being used in vivo. Anti-CD8-PerCp (53-6.7), anti-clonotypic TCR-biotin (Ti98), anti-CD44-PE (IM7), anti-CD69-PE (A1.2F3) and anti-CD25-PE (PC61) mAbs were used in vitro for fluorescent activated cell sorter (FACS) analysis. Isotype-matched mAb R3-34-PE (rat IgG1) was used as control. All mAbs were obtained from Pharmingen (Becton Dickinson, UK) unless stated otherwise.

Surgical Procedures

Orthotopic mouse liver transplantation was performed as previously described (25). Hepatic arterialization was not reconstructed. Kidney grafts were transplanted orthotopically as described by Han et al. (26). The remaining right recipient kidney was removed seven days after transplantation. Heterotopic heart transplantation was performed as documented by Corry et al. (27). For skin transplantation full thickness

tail skin grafts were prepared to fit the graft bed on the lateral thorax of anesthetized recipients. The grafts were inspected regularly until they were completely destroyed, at which time the grafts were considered to have been rejected. Thymectomies were performed as previously described (20).

Cell Purification and Labelling

A single cell suspension was made from spleens and mesenteric lymph nodes harvested from BM3 TCR-transgenic mice. CD8^{+} T cells were purified by positive selection using anti-CD8 MACS beads (Miltenyi Biotec, Germany). Typically, CD8^{+} T cells were isolated to >95% purity. More than 90% of the CD8^{+} T cells expressed the H2K^b-specific Tg-TCR. The isolated cells were incubated for 10 minutes at 37°C with 5μM CFSE (Molecular Probes, The Netherlands) washed twice, and resuspended in PBS ready for i.v. injection.

Standard experimental protocol

CBA/Ca mice were thymectomized (day −24) and treated with depleting anti-CD4 (YTA3.1; 100μg/dose) and anti-CD8 (YTS169; 100μg/dose) mAbs on day −17 and day −16. For optimal depletion of the majority of T cells (95% T cell depletion on day −2) and clearance of the mAbs, the mice were rested for a further 14 days. These "empty" immunocompromised mice were then reconstituted with an i.v. injection of the CFSE-labelled purified CD8^{+}tg-TCR^{+} (BM3) T cells (day −2). On day 0 mice received an H2K^{b+} (C57BL/10) liver, kidney or heart allograft.

Flow cytometric analysis

Graft infiltrating leukocytes were isolated from liver, kidney and heart grafts by squeezing the organs through a sieve followed by enzymatic digestion for 30 min in 37°C PBS with 0,02% collagenase IV and 0,002% DNase I (Sigma). Before cell isolation, the organs were washed out with saline to remove all circulating blood cells. Lymphoid cells were separated from the tissue suspension with a density gradient

centrifugation in 40% Percoll on a layer with 80% Percoll (Amersham, Uppsala, Sweden). The interphase, enriched with lymhocytes, was finally washed in PBS. Then graft infiltrating cells, splenocytes and lymph node cells were stained with anti-CD8-PerCp and Ti98-biotin mAbs. After washing, a streptavidin-conjugated fluorochrome was added (streptavidin-APC (Pharmingen)). The samples and fluorochrome were then incubated at 4°C before being washed twice. Finally, the samples were stained with PE-labelled mAbs for detection of activation markers. All samples were immediately acquired on a FACSort (Becton Dickinson), and analyzed using the Cellquest software package (Becton Dickinson).

Cytokine mRNA expression

Grafted livers, kidneys and hearts as well as the spleens of recipients were analysed for the expression of mRNA encoding CD3, IFN-γ, IL-2, IL-4, IL-10, perforin and Fas Ligand (FasL) by employing quantitative real-time RT-PCR (TaqMan; PE Biosystems, CA). RNA isolation and cDNA synthesis were performed as previously described (28). The cDNA was then analysed for target gene expression by TaqMan PCR, using HPRT as a house keeping gene as described (28). The sequences of the oligonucleotides used are as follows:

HPRT-sense5´-ATCATTATGCCGAGGATTTGGAA-3´,

HPRT-antisense5´-TTGAGCACACAGAGGGCA-3´,

HPRT-probe5´-TGGACAGGACTGAAAGACTTGCTCGAGATG-3´,

CD3-sense5'-ATTGCGGGACAGGATGGAG-3',

CD3-antisense5'-CTTGGAGATGGCTGTACTGGTCA-3',

CD3-probe5'-TCGCCAGTCAAGAGCTTCAGACAAGCA-3',

IFN-γ-sense5´-AGCAACAGCAAGGCGAAAAA-3´,

IFN-γ-antisense5´-AGCTCATTGAATGCTTGGCG-3´,

IFN-γ-probe5´-ATTGCCAAGTTTGAGGTCAACAACCCACA-3´,

Steger et al   9

IL-2-sense5'-CCCAGGATGCTCACCTTCAA-3',

IL-2-antisense5'-CATGCCGCAGAGGTCCAA-3',

IL-2-probe5'-CAATTCTGTGGCCTGCTTGGGCAA-3',

IL-4-sense5'-ATCGGCATTTTGAACGAGGTC-3',

IL-4-antisense5'-TCGTTGCTGTGAGGACGTTTG-3',

IL-4-probe5'-AGAAGGGACGCCATGCACGGAGA-3',

IL-10-sense5'-GAAGACCCTCAGGATGCGG-3',

IL-10-antisense5'-CCTGCTCCACTGCCTTGCT-3',

IL-10-probe5'-CGCTGTCATCGATTTCTCCCCTGTGA-3',

Perforin-sense5'-CAAGGTGGAGTGGAGGTTTTTGT-3',

Perforin-antisense5'-TTTTGCAGCTGAGAAGACCTATCA-3',

Perforin-probe5'-CCAGGCGAAAACTGTACATGCGACACT-3',

FasL-sense5'-GAAGGAACTGGCAGAACTCCG-3',

FasL-antisense5'-CCCTGTTAAATGGGCCACACT-3',

FasL-probe5'-AAAGCAAATAGCCAACCCCAGCACACC-3'.

Sense and antisense oligonucleotides were purchased from MWG (Germany). The probes were labelled with the reporter FAM (6-carboxyfluorescin) at the 5´ end and with the quencher TAMRA (6-carboxy-tetramethyl-rhodamine) at the 3´end and were purchased from Oswel (UK).

Transfer of graft infiltrating cells and in vivo effector assay

Graft infiltrating cells of liver and kidney grafts, as well as leucocytes from naive BM3 transgenic mice were isolated as described above. CD8$^+$ T cells were purified from the suspension by positive selection using anti-CD8 MACS beads (Miltenyi Biotec). Purity was >95%. CFSE negative cells were isolated out of this population on a FACStar cell sorter (BD Biosciences, CA) with a purity of >99%. CD8$^+$ graft infiltrating cells and naive CD8$^+$BM3$^+$ cells each were then adoptively transferred into

immunodeficient CBA/RAG1$^{-/-}$ mice. Donor B10 skin grafts were transplanted on the following day, and the survival rate of these grafts was measured as an indicator of rejection potency of the transferred CD8^{+} cells.

Immunohistochemistry

Cryostat sections (6μm) were air-dried and then fixed in acetone for 10 min. After inhibition of endogenous peroxidase activity and blockade of endogenous biotin sections were incubated with an anti-CD8-biotin mAb (BD-Biosciences) for 1h at room temperature. Binding was detected by an avidin biotin-peroxidase complex (ABC, Vector Laboratories) and visualized using diaminobenzidene (Sigma-Aldrich) and Gill's hematoxylin (BDH).

Statistics

Results are given as mean $\pm$ SD. Data were compared and analysed using an unpaired two-tailed Student's t test. The Wilcoxon test was used for comparison of survival data. A p-value of <0.05 was considered significant.

Results

Survival of allogeneic mouse liver, kidney and heart transplants.

To compare the response of non immunocompromised naive mice possessing the full repertoire of CD4^{+} and CD8^{+} T cells to three different fully allogeneic vascularized organ grafts, the survival of B10 (H2^{b}) liver, kidney or heart grafts transplanted into CBA/Ca (H2^{k}) recipients was examined. 95% of liver grafts were accepted for more then 100 days, while in marked contrast, kidney or heart grafts from the same donor strain were rejected acutely, within 10 days after transplantation **(Figure 1)**.

Susceptibility of solid organ grafts to rejection mediated by alloreactive CD8^{+} T cells.

In order to be able to dissect the impact of transplantation of different types of graft on a defined population of T cells, we next investigated whether the same differences in graft survival were observed when the T cell repertoire comprised only CD8$^+$ T cells reactive to a donor MHC class I alloantigen H2K^b. T cell deficient CBA/Ca mice, i.e. depleted of CD8$^+$ and CD4$^+$ T cells that accept liver, kidney and heart grafts in the absence of T cell reconstitution (1), were adoptively transferred with 6x10^6 H2K^b-reactive BM3 T cells two days before transplantation of a H2K^{b+} liver or kidney graft **(Table 1)**. 4 out of 5 liver recipients survived long term, whereas all recipients of an allogeneic kidney graft rejected the kidney within 37 days. Decreasing the number of H2K^b-reactive T cells transferred revealed that the susceptibility of kidney grafts to rejection was related to the number of CD8$^+$ T cell transferred; transfer of 4x10^6 cells led to a long term survival of 3 out of 4 recipients after transplantation of kidney grafts. A similar threshold between graft rejection and acceptance seen after allogeneic mouse heart transplantation has also been described (1).

Additional B10 liver grafts were transplanted into native BM3 TCR-transgenic mice i.e. where the whole T cell repertoire comprises of H2K^b-reactive CD8$^+$ T cells and as such far exceeding the 6x10^6 cells adoptively transferred above. Even under these conditions long term liver graft acceptance was observed. In contrast, primary B10 kidney grafts were acutely rejected under the same conditions within 10 days **(Table.1)**.

Activation and proliferation of alloreactive CD8$^+$ T cells after liver, kidney and heart transplantation.

To monitor the proliferation and activation pattern of the adoptively transferred alloreactive CD8$^+$ T cells after transplantation we used the model described above, utilising the adoptive transfer of BM3 CD8$^+$ T cells labelled with CFSE before transfer.

Analysis of H2K^b-specific T cells 5 days after liver transplantation revealed that 93.4% of BM3 T cells in the spleen had divided more than 4 times. In contrast, after kidney and heart transplantation only 27.3% and 16.3% of the H2K^b-reactive CD8$^+$ population in the spleen had divided more than four times respectively. Following transplantation of syngeneic grafts no proliferation of alloreactive BM3 T cells was observed after either liver or kidney transplantation (data not shown). In each setting, after 2-3 divisions, the proliferating cells differentiated into a CD44^{high}-phenotype, a marker associated with antigen experience and effector function. Thus, most of the H2K^b-reactive T cells isolated from spleen five days after liver transplantation had already acquired an activated phenotype **(Figure 2a)**. Expression of CD69 and CD25 was used to identify activated cells independent of their pattern of proliferation, however, no significant upregulation of either marker on H2K^b-specific BM3 T cells was detectable in the spleen of liver, kidney or heart recipients (data not shown). Similar proliferation and differentiation patterns were also observed in the mesenteric lymph nodes 5 days after transplantation (data not shown).

To obtain more information about the role and behaviour of donor-reactive CD8$^+$ T cells at arguably the most important location i.e. the graft itself, graft infiltrating lymphocytes were isolated from allogeneic liver, kidney and heart grafts 5 days after transplantation (**Figure 2b**). In contrast to the proliferation pattern observed in the peripheral lymphoid organs, the percentage of BM3 T cells that had undergone 5 or more divisions was much higher amongst the graft infiltrating cells isolated from all three different types of grafts. While no undivided donor-reactive CD8$^+$ T cells were detectable in liver grafts, a higher percentage of H2K^b-specific T cells showing fewer than five divisions were present in kidney and heart allografts. These undivided, or less divided cells, predominantly expressed a CD44^{low}CD69^{high}-phenotype, which

linked to recent activation, whereas T cells with more than four divisions were characterized by the expression of a CD44^{high}CD69^{low}-phenotype **(Figure 2b)**.

Expression of CD3 mRNA compared to the house keeping gene HPRT was used as a surrogate marker to quantitate T cell infiltration into the graft. Interestingly, the relationship between T cells and the remaining resident organ cells present in kidney and heart grafts was found to be higher that present in allogeneic liver grafts 5 days after transplantation (**Figure 3a**), despite a 3 fold increase in the absolute cell number of infiltrating alloreactive CD8+ T cells in liver grafts in comparison to kidney grafts (**Figure 3b**). This observation was confirmed by staining liver, kidney and heart allografts for expression of CD8 5 days after transplantation. In liver grafts, an intensive infiltration by CD8^{+} T cells was confined to portal areas without further damage to hepatocytes or endothelium inside the lobuli, whereas the infiltration of kidney and heart grafts was diffuse and widespreaded throughout the transplanted organ (**Figure 3c**). This discrepancy in the pattern of T cell infiltration may be explained by a lack of adhesion molecules inside the liver sinusoids (29).

To clarify whether the rapid proliferation of donor reactive CD8^{+} T cells triggered by a liver transplant led to elimination of the donor reactive T cell pool, the number of graft infiltrating and splenic H2K^{b}-specific T cells was determined 5 and 15 days after liver transplantation and compared to the absolute cell numbers of alloreactive T cells in heart and kidney recipients. We found that 5 days after transplantation, 1.4×10^{6} donor reactive graft infiltrating CD8^{+} T cells were detected after liver transplantation, whereas only 0.42×10^{6} and 0.09×10^{6} graft infiltrating BM3 T cells were isolated following kidney and heart transplantation, respectively confirming the more rapid activation and expansion of BM3 T cells following liver transplantation. In clear contrast, analysis of liver graft recipients 15 days after transplantation revealed that

rather than increasing the number of infiltrating BM3 T cells had fallen to 0.66×10^6 which was mirrored in the peripheral lymphoid tissues where the number of BM3 T cells decreased 6 fold (**Figure 3b**).

Cytokine expression in spleen and graft

We hypothesised that a shift from initial hyperresponsiveness to unresponsiveness may also be necessary for graft acceptance after liver transplantation. To test this hypothesis, cytokine mRNA expression of Th1 and Th2 associated cytokines as well as, the cytotoxic molecules perforin and FasL were measured in the graft and spleen five days after transplantation (Figure 4).

Expression of IFN-γ mRNA in liver grafts was found to be significantly reduced compared to that found in either heart and kidney grafts 5 days after transplantation. In addition, IL-2 mRNA expression showed a trend towards downregulation in the liver grafts, but the differences were not significant. Surprisingly, there was a marked downregulation of IL-10 mRNA expression in kidney grafts, in comparison with hearts and livers. Differences in IL-4 mRNA expression were not significant, but showed higher expression in liver grafts **(Figure 4a)**. Interestingly, the lowest expression of perforin and FasL mRNA was detectable in liver grafts 5 days after transplantation with the highest expression being detectable in heart grafts. No significant differences were found in the spleen for all of these molecules 5 days after transplantation (**Figure 4b**).

Effector potential of graft infiltrating cells

We postulated that the low expression of IFN-γ, perforin and FasL mRNA in liver allografts, as well as the persistence of donor-reactive T cells, was due to liver infiltrating donor-reactive T cells being in a state of unresponsiveness, whereas

donor-reactive T cells infiltrating kidney or heart grafts 5 days after transplantation had full effector function. To test this hypothesis, graft infiltrating cells were isolated from liver and kidney transplants and transferred into immunodeficient CBA/RAG1$^{-/-}$ mice whereupon their ability to mediate rejection of donor B10 skin grafts was assessed. We first confirmed the number of naïve H2K^b-specific CD8$^+$ T cells required to reject a B10 H2K^{b+} skin allograft. To this end, 0.2 to 5x10^5 naive BM3 T cells were transferred to CBA/RAG$^{-/-}$ recipients that were subsequently transplanted with donor-type B10 skin grafts. As few as 2x10^4 naïve H2K^b-reactive CD8$^+$ T cells were found to be sufficient to reject B10 skin grafts in less than 20 days **(Figure 5a)**. Transfer of 2x10^5 graft infiltrating CD8$^+$ T cells from liver allografts, i.e. 10 times the number of naïve T cells required to trigger rejection, resulted in the prolonged survival of B10 skin grafts, although all grafts were rejected by 72 days after transplantation. Surprisingly, adoptive transfer of 2x10^5 CD8$^+$ graft infiltrating cells isolated from kidney allografts 5 days after transplantation also resulted in extended graft survival **(Figure 5b)**. The alloreactivity of graft infiltrating T cells from liver allografts seems to be reduced compared to cells isolated from kidney grafts, but significance could not be proved. To rule out the potential contribution of freshly activated and proliferating T cells (CD44^{low}CD69^{high} and CFSE positive), 2x10^5 CFSE negative graft infiltrating T cells from kidney allografts (ie >5 cell divisions) were isolated by cell sorting and adoptively transferred into CBA/RAG1$^{-/-}$ mice. In these mice H2K^{b+} skin grafts were rejected with a MST of 47.5 days (n=4). Thus, independent of their activation or differentiation status, graft infiltrating T cells, isolated from spontaneously accepted liver or rejected kidney grafts retain the potential to reject allogeneic tissue.

Discussion

Transplantation of a fully allogeneic liver graft can lead to spontaneous graft acceptance in naive immunocompetent recipients (17). In contrast to previous studies (30), where spontaneous kidney graft acceptance has also been described (31), B10 renal allografts were acutely rejected when transplanted to CBA/Ca recipients in the study reported here without any exceptions with similar kinetics to that of heart allografts. Moreover, the threshold for the susceptibility of heart and kidney allografts to rejection driven by donor reactive CD8$^+$ T cells was very similar (see Table1 and Jones et al. 2001 (1)), whereas liver grafts were not found to be susceptible to rejection under the same conditions. Previous work from our group demonstrated a decreasing susceptibility of islet, skin and heart grafts to rejection mediated by donor reactive CD8$^+$ T cells (1). Kidney grafts, that have an organ mass approximately comparable to that of heart grafts, were also unable to resist destruction mediated by 6×10^6 donor-reactive T cells, whereas liver grafts with a much larger mass were accepted under the same conditions (Table 1). This relationship between the mass of donor tissue transplanted and rejection or acceptance has previously been demonstrated in studies showing that simultaneous transplantation of two kidneys and two hearts resulted in long term graft acceptance, whereas single allogeneic heart and kidney graft were rejected acutely (13). One explanation that has been proposed to account for these findings is that the antigen load provided by a large amount of transplantated donor tissue, causes hyper- or overstimulation of donor reactive leukocytes present in the recipient (32). Our findings support this hypothesis as, visualization of the proliferative pattern of donor-reactive T cells at day 5 after transplantation demonstrated that liver allografts triggered early hyperstimulation of donor-reactive T cells with a much higher proportion of the donor-reactive T cell pool undergoing more then four divisions

compared to that observed after transplantation of a kidney or heart allograft (Figure 2).

Deletion of donor-reactive T cells has been postulated to be a significant factor in the induction of tolerance leading to the spontaneous graft acceptance as high rates of T cell apoptosis have been observed after liver transplantation in mouse and rat models (9;33). The liver has been reported to have special properties in this regard (34;35), despite recent findings that intrahepatic activation by local antigen presentation to CD8$^+$ T cells resulted not in abortive interactions, failed maturation or premature T cell death (36). However, a rapid deletion of the entire activated alloreactive T cell pool inside the liver graft seems to be not the case, since 5 days after transplantation more than one million donor reactive CD8$^+$ T cells remained inside the graft, and even after 15 days approximately half a million donor reactive cells were still detectable inside the liver grafts. Nevertheless we found that the absolute number of alloreactive CD8$^+$ T cells, especially in the spleen, decreased after liver transplantation in comparison to recipients of heart grafts over the time ((1), Figure 3).

The anergic environment inside the accepted liver grafts - despite a significant amount of remaining alloreactive T cells in the graft - was mirrored by the low mRNA expression of IFN-γ, IL-2, and especially the cytotoxic-molecules perforin and FasL inside the grafts 5 days after transplantation in comparison to kidney and heart allografts undergoing acute rejection. The changes of interleukin-expression could be explained in part by the complete and extensive proliferation of donor reactive T cells inside liver grafts and the attendant changes in cytokine expression by progressive cell division, but the low level of IFN-γ is uncharacteristic and not

observed even after 8 rounds of division after in vitro stimulation of naive T cells (37). This discrepancy may be explained by the differences between intracellular staining, where cells are restimulated *ex vivo* prior to the staining process (revealing what cytokines may be produced following activation), and the technique used here of mRNA-expression (detecting the potential cytokines that are being produced *in situ* in the graft (albeit at the gene and not protein level). In addition a limited application of intracellular staining of cytokines for the differentiation between graft acceptance and rejection was shown by our group before (38).

Unresponsiveness of the liver graft infiltrating CD8$^+$ T cells seems to be unlikely due to the remaining ability of these cells to reject a skin graft after adoptive transfer into a secondary host (Figure 5). However it could be possible that initial anergic T cells were partially reactivated in the presence of inflammatory signals created by the transplantation of the challenge skin graft. The phenomenon of split anergy, describing receptor-dependent cytolysis in the absence of IL-2 production (39) is most likely responsible for a distinct difference between intragraft cytokine expression and functional behaviour after adoptive transfer to a fresh host in vivo. A phenomenon similar to this, called split tolerance, has already been observed after mouse liver transplantation, where long term surviving grafts still possessed CTLs with donor specific cytotoxic potential in vitro (40).

The rapid and extensive activation of the donor reactive CD8$^+$ T cells in the initial period after transplantation followed by deletion of the alloreactive T cell repertoire inside the peripheral lymphatic tissues was the major difference in the response of donor reactive cells after liver transplantation compared to the response of the same population of T cells after heart and kidney transplantation. These findings support

the hypothesis that initial hyperstimulation by infusion of additional donor alloantigen (41-43) might create a beneficial window for immunological engagement facilitating the induction of unresponsiveness (44). However the perfect time frame and load of alloantigen required has still to be defined.

References

1. Jones ND, Turvey SE, Van Maurik A et al. Differential susceptibility of heart, skin, and islet allografts to T cell-mediated rejection. J Immunol 2001:166: 2824-2830.

2. Jones ND, Van Maurik A, Hara M et al. CD40-CD40 ligand-independent activation of CD8+ T cells can trigger allograft rejection. J Immunol 2000:165: 1111-1118.

3. Trambley J, Bingaman AW, Lin A et al. Asialo GM1(+) CD8(+) T cells play a critical role in costimulation blockade-resistant allograft rejection. J Clin Invest 1999:104: 1715-1722.

4. Honey K, Cobbold SP, Waldmann H. CD40 ligand blockade induces CD4+ T cell tolerance and linked suppression. J Immunol 1999:163: 4805-4810.

5. Piccirillo CA, Shevach EM. Cutting edge: control of CD8+ T cell activation by CD4+CD25+ immunoregulatory cells. J Immunol 2001:167: 1137-1140.

6. Haeryfar SM, DiPaolo RJ, Tscharke DC, Bennink JR, Yewdell JW. Regulatory T cells suppress CD8+ T cell responses induced by direct priming and cross-priming and moderate immunodominance disparities. J Immunol 2005:174: 3344-3351.

7. Maurik Av, Herber M, Wood KJ, Jones ND. Cutting Edge: CD4+CD25+ Alloantigen-Specific Immunoregulatory Cells That Can Prevent CD8+ T Cell-Mediated Graft Rejection: Implications for Anti-CD154 Immunotherapy. J Immunol 2002:169: 5401-5404.

8. Wells AD, Li XC, Li Y et al. Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. Nat Med 1999:5: 1303-1307.

9. Qian S, Lu L, Fu F et al. Apoptosis within spontaneously accepted mouse liver allografts: evidence for deletion of cytotoxic T cells and implications for tolerance induction. J Immunol 1997:158: 4654-4661.

10. Kamada N. The immunology of experimental liver transplantation in the rat. Immunology 1985:55: 369-389.

11. Zavazava N, Kronke M. Soluble HLA class I molecules induce apoptosis in alloreactive cytotoxic T lymphocytes. Nat Med 1996:2(9).

12. Bishop GA, Sun J, DeCruz DJ et al. Tolerance to rat liver allografts. III. Donor cell migration and tolerance-associated cytokine production in peripheral lymphoid tissues. J Immunol 1996:156: 4925-4931.

13. Sun J, Sheil AG, Wang C et al. Tolerance to rat liver allografts: IV. Acceptance depends on the quantity of donor tissue and on donor leukocytes. Transplantation 1996:62: 1725-1730.

14. Moskophidis D, Lechner F, Pircher H, Zinkernagel RM. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. Nature 1993:362: 758-761.

15. Lang KS, Recher M, Navarini AA et al. Inverse correlation between IL-7 receptor expression and CD8 T cell exhaustion during persistent antigen stimulation. Eur J Immunol 2005:35: 738-745.

16. Calne RY, Sells RA, Pena JR et al. Induction of immunological tolerance by porcine liver allografts. Nature 1969:223: 472-476.

17. Qian S, Demetris AJ, Murase N, Rao AS, Fung JJ, Starzl TE. Murine liver allograft transplantation: tolerance and donor cell chimerism. Hepatology 1994:19: 916-924.

18. Ramos HC, Reyes J, Abu-Elmagd K et al. Weaning of immunosuppression in long-term liver transplant recipients. Transplantation 1995:59: 212-217.

19. Takatsuki M, Uemoto S, Inomata Y et al. Weaning of immunosuppression in living donor liver transplant recipients. Transplantation 2001:72: 449-454.

20. Jones ND, Van Maurik A, Hara M, Gilot BJ, Morris PJ, Wood KJ. T-cell activation, proliferation, and memory after cardiac transplantation in vivo. Ann Surg 1999:229: 570-578.

21. Mombaerts P. Lymphocyte development and function in T-cell receptor and RAG-1 mutant mice. Int Rev Immunol 1995:13: 43-63.

22. Auphan N, Curnow J, Guimezanes A et al. The degree of CD8 dependence of cytolytic T cell precursors is determined by the nature of the T cell receptor (TCR) and influences negative selection in TCR-transgenic mice. Eur J Immunol 1994:24: 1572-1577.

23. Cobbold SP, Jayasuriya A, Nash A, Prospero TD, waldmann H. Therapy with monoclonal antibodies by elimination of T-cell subsets in vivo. Nature 1984:312: 548-551.

24. Hua C, Boyer C, Guimezanes A, Albert F, Schmitt-Verhulst AM. Analysis of T cell activation requirements with the use of alloantigens or an anti-clonotypic monoclonal antibody. J Immunol 1986:136: 1927-1936.

25. Steger U, Sawitzki B, Gassel AM, Gassel HJ, Wood KJ. Impact of hepatic rearterialization on reperfusion injury and outcome after mouse liver transplantation. Transplantation 2003:76: 327-332.

26. Han WR, Murray-Segal LJ, Mottram PL. Modified technique for kidney transplantation in mice. Microsurgery 1999:19: 272-274.

27. Corry RJ, Winn HJ, Russell PS. Primarily vascularized allografts of hearts in mice. The role of H-2D, H-2K, and non-H-2 antigens in rejection. Transplantation 1973:16: 343-350.

28. Sawitzki B, Amersi F, Ritter T et al. Upregulation of bag-1 by ex vivo gene transfer protects rat livers from ischemia/reperfusion injury. Hum Gene Ther 2002:13: 1495-1504.

29. Lalor PF, Edwards S, McNab G, Salmi M, Jalkanen S, Adams DH. Vascular adhesion protein-1 mediates adhesion and transmigration of lymphocytes on human hepatic endothelial cells. J Immunol 2002:169: 983-992.

30. Bickerstaff AA, Wang JJ, Pelletier RP, Orosz CG. Murine renal allografts: spontaneous acceptance is associated with regulated T cell-mediated immunity. J Immunol 2001:167: 4821-4827.

31. Zhang Z, Zhu L, Quan D et al. Pattern of liver, kidney, heart, and intestine allograft rejection in different mouse strain combinations. Transplantation 1996:62: 1267-1272.

32. Bishop GA, Sun J, Sheil AG, McCaughan GW. High-dose/activation-associated tolerance: a mechanism for allograft tolerance. Transplantation 1997:64: 1377-1382.

33. Sharland A, Yan Y, Wang C et al. Evidence that apoptosis of activated T cells occurs in spontaneous tolerance of liver allografts and is blocked by manipulations which break tolerance. Transplantation 1999:68: 1736-1745.

34. Crispe IN, Dao T, Klugewitz K, Mehal WZ, Metz DP. The liver as a site of T-cell apoptosis: graveyard, or killing field? Immunol Rev 2000:174: 47-62.

35. Uchiyama H, Kishihara K, Minagawa R, Hashimoto K, Sugimachi K, Nomoto K. Crucial Fas-Fas ligand interaction in spontaneous acceptance of hepatic allografts in mice. Immunology 2002:105: 450-457.

36. Klein I, Crispe IN. Complete differentiation of CD8+ T cells activated locally within the transplanted liver. J Exp Med 2006:203: 437-447.

37. Gett AV, Hodgkin PD. Cell division regulates the T cell cytokine repertoire, revealing a mechanism underlying immune class regulation. Proc Natl Acad Sci U S A 1998:95: 9488-9493.

38. Spriewald BM, Hara M, Bushell A, Jenkins S, Morris PJ, Wood KJ. Differential role for competitive reverse transcriptase-polymerase chain reaction and intracellular cytokine staining as diagnostic tools for the assessment of intragraft cytokine profiles in rejecting and nonrejecting heart allografts. Am J Pathol 2000:157: 1453-1458.

39. Otten GR, Germain RN. Split anergy in a CD8+ T cell: receptor-dependent cytolysis in the absence of interleukin-2 production. Science 1991:251: 1228-1231.

40. Dahmen U, Qian S, Rao AS et al. Split tolerance induced by orthotopic liver transplantation in mice. Transplantation 1994:58: 1-8.

41. Barber WH, Mankin JA, Laskow DA et al. Long-term results of a controlled prospective study with transfusion of donor-specific bone marrow in 57 cadaveric renal allograft recipients. Transplantation 1991:51: 70-75.

42. Opelz G, Vanrenterghem Y, Kirste G et al. Prospective evaluation of pretransplant blood transfusions in cadaver kidney recipients. Transplantation 1997:63: 964-967.

43. Wood KJ, Jones ND, Bushell AR, Morris PJ. Alloantigen-induced specific immunological unresponsiveness. Philos Trans R Soc Lond B Biol Sci 2001:356: 665-680.

44. Calne R. WOFIE hypothesis: some thoughts on an approach toward allograft tolerance. Transplant Proc 1996:28: 1152.

45. Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry. J Immunol Methods 1994:171: 131-137.

Tables

Table 1:   Survival time of solid organ allografts

	Liver Transplantation	Kidney Transplantation
B10→BM3	5 x >60 d	8, 8, 9, 10 d (MST:8,5d)
B10→CBA (**6x10^6** BM3)	5, 4 x >60 d	10, 27, 33, 37 d (MST: 30d)
B10→CBA (**4x10^6** BM3)	- - -	38, 3 x >60d
B10→CBA (No BM3)	4 x >60 d	8, 4 x >60 d

Table 1: Survival time of different solid C57BL/10 organ grafts in T-cell depleted CBA mice that have received either 4 or 6x10^6 TCR-transgenic BM3 CD8$^+$ T cells, or unmanipulated BM3 mice. (MST=Median Survival Time).

Figure Legends

Figure 1: Survival rate after mouse liver (LTx), kidney (KTx) and heart (HTx) transplantation of C57BL/10 organ grafts (H-2^b) in naive unmanipulated CBA/Ca-recipients (H-2^k). LTx: MST>100 days, n=20, KTx: MST=9 days, n=8; HTx: MST=7 days, n=12).

Figure 2: To characterize the division kinetics, H2K^b-specific T cells were labelled with CFSE. Cells stained with the fluorescein dye CFSE have been shown to lose half of their intensity with each cell division, which can be monitored by flow cytometry (45). By using four colour flow cytometry and gating on CD8$^+$ tg-TCR$^+$ (TI98) cells, it was possible to visualize this single alloreactive cell population independently of other cells, as described recently in more detail (20). **a)** Proliferation of alloreactive CD8+T cells, isolated seven days after adoptive transfer (AT only) or five days after liver (LTx), kidney (KTx) and heart (HTx) transplantation from spleens of immunocompromised CBA recipients, transferred with 6x10^6 H2K^b-specific T cells at day –2. Percentage of cells with more then 4 cell divisions (n=3/group) and the CD44 expression dependent on their proliferation status. **b)** Proliferation and CD44 / CD69 expression of graft infiltrating alloreactive CD8+T cells (GIC) labelled with CFSE and transferred to immunocompromised CBA recipients at day -2 and isolated 5 days after liver, kidney and heart transplantation. Percentages of CD8+T cells with more than 4 cell divisions (n=3/group.). Dotplots are representative for 3 different recipients per organ (GICs of hearts were pooled for phenotype analysis).

Figure 3: a) Expression of CD3 mRNA, as an indicator of T cell infiltrating intensity in graft and spleen, five days after liver, kidney and heart transplantation, was measured by real-time RT-PCR (n=3). Results are expressed as mRNA (units/HPRT) $\pm$ SD. **b)** The absolute cell numbers of alloreactive T cells inside grafts and spleens were measured at day 5 and 15 after transplantation by counting the total leukocyte number of each organ using trypan blue exclusion. The leukocytes were then stained for expression of CD8 and the tg-TCR and the percentage of these cells was determined by FACS analysis. The percentage and total leukocyte count were then used to calculate the absolute cell number of CD8$^+$tg-TCR$^+$ cells per organ. Cell numbers are demonstrated in million ($\times 10^6$) as mean $\pm$ SD. Three mice were used per group (except day 15 liver: n=2, GIC of heart grafts were pooled). **c)** Histology of heart, kidney and liver allografts five days after transplantation and 7 days after adoptive transfer of alloreactive CD8+ T. Sections were stained with anti-CD8 mAb to reveal the infiltration of BM3 T cells into the grafts (magnification 100x liver, 200x kidney and heart graft)

Figure 4: mRNA expression of T cell related cytokines and cytolytic molecules in allografts and spleens 7 days after transfer of 6×10^6 alloreactive CD8+T-cells into immunocompromised CBA mice and 5 days after allogeneic liver, kidney and heart transplantation, measured by quantitative real-time RT-PCR. **a)** mRNA of Th1 and Th2 related cytokines IFN-γ, IL-2, IL-4 and IL-10 were measured in tissues of infiltrated allografts and recipient spleens relative to CD3 expression. **b)** Expression of cytolytic effector protein perforin and the cytolytic ligand of the Fas receptor (FasL), both complementary for the killing activity of cytotoxic T cells, in grafts and spleens five days after liver, kidney and heart transplantation. 3 different recipients were used per organ. Significant differences are marked with * ($p < 0.05$).

Figure 5: Survival rate of C57BL/10 skin grafts after transfer of **a)** 0.2 - $5x10^5$ naive or **b)** $2x10^5$ liver / kidney graft infiltrating Ti98+CD8+ T cells (GIC; postoperative day 5) at day -1 into immunodeficient CBA/RAG1-/- mice. MST: 59.5 days with cells from liver grafts; n=4 vs 41.5 days; n=4 with cells from kidney allografts (Difference is not significant; 100% survival rate of B10 skin grafts on CBA/RAG1-/- mice without T cell transfer, n=4, data not shown)