Differential migration of passenger leukocytes and rapid deletion of naïve alloreactive CD8 T cells after mouse liver transplantation

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List of abbreviations:

B6	C57BL/6
LN	Lymph nodes
PL	Passenger leukocytes
TCR	T cell receptor
Tg	Transgenic
Rag	Recombinase activating gene

Abstract

Donor passenger leukocytes (PL) from transplanted livers migrate to recipient lymphoid tissues, where they are thought to induce deletion of donor-specific T cells and tolerance.

Difficulties in tracking alloreactive T cells and PL in rats and in performing this complex surgery in mice have limited progress in identifying the contribution of PL subsets and sites and kinetics of T cell deletion.

Here, we developed a mouse liver transplant model in which PL, recipient cells and a reporter population of transgenic CD8 T cells specific for the graft could be easily distinguished and quantified in allografts and recipient organs by flow cytometry.

All PL subsets circulated rapidly via the blood as soon as 1.5 hours post-transplantation.

By 24h, PL distributed differently in lymph nodes and spleen while donor NK and NKT cells remained in the liver. Reporter T cells were activated in both liver and lymphoid tissues, but their numbers dramatically decreased within the first 48h.

These results provide the first unequivocal demonstration of differential recirculation of liver PL subsets post-transplantation, and show that alloreactive CD8 T cells are deleted more rapidly than initially reported. This model will be useful for dissecting early events leading to spontaneous acceptance of liver transplants.

Introduction

Liver allografts possess unique tolerogenic properties compared to other solid organ transplants. In the clinical setting, HLA matching is not required, chronic rejection is uncommon, and up to 20% of recipients can be weaned off immunosuppression. (1) In animal models, liver transplants are spontaneously accepted in outbred pigs, most mouse and many rat strain combinations, and are further able to induce donor-specific tolerance and prevent rejection or prolong survival of other organs from the same donor, including kidney, heart, pancreas and skin. (1) Despite observation of this phenomenon for more than 40 years, the mechanisms of liver allograft tolerance are not fully understood.

Compared to other organs, the liver contains a large and diverse population of bone-marrow derived haematopoietic cells that includes lymphocytes, myeloid cells and progenitor cells. A syngeneic liver transplant is able to reconstitute the majority of the haematological lineages in a lethally irradiated host. (2) Collectively termed passenger leukocytes (PL), these donor liver leukocytes play important roles in liver transplant tolerance. Depleting PL by irradiation (3) or replacing donor PL(4), (5) prevents spontaneous liver graft acceptance in rats, which can be re-established by infusing purified donor leukocytes. (6)

Which PL induce liver transplant tolerance and how and where their effects are mediated are unclear. In rats, tolerance is associated with rapid migration of PL and immune activation in recipient draining lymph nodes (LN) and spleen. (7) High rates of apoptosis in the spleen (8) and liver (8, 9) are also associated with tolerance. It has been suggested

that the inappropriate timing and magnitude of allograft-reactive T cell activation in lymphoid organs, mediated by PL, is followed by their death-by-neglect in the liver due to insufficient cytokine signaling, resulting in depletion of donor-reactive T cells. (10) However, LN and spleen are not equivalent in priming of skin and heart transplant rejection. (11) Spleens are sometimes considered more tolerogenic (12), although most studies have not distinguished the contribution of these two organs. Studies using donor livers reconstituted with PL from the recipient strain have also shown that the liver parenchyma and/or liver-resident cells are key determinants of graft outcome (13), instead of PL. The liver parenchyma has been suggested to play an important role in tolerance by secreting soluble donor MHC class I molecules (4), or by inducing abortive activation of graft-reactive T cells. (13)

Although studies in rats have demonstrated early migration of PL in lymphoid tissues followed by apoptosis of alloreactive T cells, their conclusions were limited: they did not address whether all PL subsets migrated in lymphoid tissues and/or some remained in the liver. It is not also clear whether the spleen and LNs are seeded by distinct PL subsets. Finally tracking alloreactive T cells in rats is difficult, due to insufficient reagents for identifying specific lineages. Few studies have been performed in mice, where leukocyte lineages are better-defined and transgenic strains available, due to technical challenges in performing this surgery. A few studies in mice have indicated roles for apoptosis of recipient and donor cells (9, 14) and for NKT cells (15) that are enriched in the liver. However None of these studies have systematically investigated the migration of all PL subsets. Consistent with the clonal exhaustion/deletion hypotheses, mouse liver allografts

were shown to deplete a large population of donor-specific TCR-transgenic CD8 T cells in 5 days. (16)

In this study we established a mouse liver transplantation model in which donor and recipient leukocytes could be distinguished by allelic differences in CD45. To follow the fate of CD8 T cells activated via the direct allorecognition pathway, naïve Des CD8 T cells expressing a transgenic TCR specific for the donor MHC molecule H-2K^b were adoptively transferred into recipient mice before transplantation. Using multi-color flow cytometry, we were able to quantify PL, recipient leukocytes and directly allograft-specific CD8 T cells in the allograft and recipient organs post-transplant. This study confirms that PL migration occurs very early post-transplantation. However, by distinguishing systematically all main PL subsets, it demonstrates for the first time that all subsets did not migrate equally in the recipient. The composition of PL subsets in lymph nodes and spleen was different, suggesting that these compartments could induce distinct pathways of alloreactive T cell activation and play a different role in tolerance induction. We reported for the first time that although some NK and NKT cells recirculated in the blood, a significant proportion was retained in the liver. Finally, this study reveals that deletion of graft-reactive T cells is evident by 24h, an earlier time point than previously reported.

Experimental procedures

Animals

Male C57BL/6 and B10.BR mice were purchased from the Animal Resources Centre (Perth, Australia). B10.BR Ly5.1 (H-2^k, Ly5.1⁺/CD45.1⁺) mice were derived by crossing C57BL/6 Ptprc^a (H-2^b, Ly5.1⁺, CD45.1⁺) for more than 10 generations into the B10.BR background. Des and Des-RAG^{-/-} mice have been described previously. (17) Mice were housed at the Centenary Institute Animal Facility under specified pathogen-free conditions. All experimental procedures were approved by the Sydney University Animal Ethics Committee.

Adoptive transfer and orthotopic mouse liver transplantation

LN cells were isolated from Des-RAG^{-/-} mice and CFSE-labeled (5 μ M) as previously described. (18) 2.5-5.0 $\times 10^6$ cells were injected into recipients via the lateral tail vein 24h before liver transplantation. Donor livers were perfused and preserved in University of Wisconsin solution before orthotopic transplantation according to protocols described previously (19, 20), without hepatic re-arterialization. Infrahepatic vena cava, portal vein, and the common bile duct were anastomosed. This procedure results in indefinite survival for more than 90% of recipients of syngeneic grafts in the absence of immunosuppression and antibiotics (data not shown).

Preparation of leukocytes from different organs and flow cytometric analysis.

Liver, spleen, LN and blood were harvested and leukocytes prepared as previously described. (18) Antibodies, from BD Pharmingen unless otherwise stated, were: CD45.1

Horizon V450, CD45.2-Biotin, Streptavidin-Pacific Orange (Invitrogen), CD19-PE, CD3-PerCP-Cy5.5, NK1.1-APC, CD4-Alexa Fluor-700, CD8-APC-Cy7, F4/80-PE (AbD Serotec), B220-PerCP, CD11c-PECy7, CD11b-APC, Gr1-APC-Cy7, Des-biotin (in house), CD69-PE, CD25-APC, CD44-APC-Cy7 (Biolegend), CD8-Pacific Blue. Cells were stained with DAPI (Invitrogen) before acquisition on an LSR-II flow cytometer (BD Biosciences) and analyzed in FlowJo 9.4.11 (Tree Star Inc., Ashland, OR).

Gating strategy for leukocyte counting and identification.

Total leukocyte counts were calculated for each sample by flow cytometry using Sphero AccuCount particles as a reference population. Two panels of antibodies were used for analysis: For Panel 1, DAPI⁻ events were analyzed for lymphocytes: NK (CD3⁻ NK1.1⁺) and NKT cells (CD3⁺ NK1.1⁺) were first distinguished based on expression of CD3 and NK1.1. B cells were identified as CD19⁺CD3⁻NK1.1⁻ cells. T cells were further sub-gated into CD4⁺ and CD8⁺ populations. Panel 2 was used to identify myeloid cells following exclusion of CD3⁺ and B220⁺ cells. Eosinophils (CD3⁻B220⁻SSC-A^{high}), macrophages (CD3⁻B220⁻SSC-A^{low}F4/80⁺CD11b^{int}) and conventional dendritic cells (CD3⁻B220⁻SSC-A^{low}CD11c^{hi}CD11b^{hi}) were distinguished. F4/80⁺ monocytes were further sub-gated into Gr⁺ inflammatory monocytes (i-mono) and F4/80^{int} Gr1⁻ patrolling monocytes (p-mono). F4/80⁻Gr1⁺ were identified as neutrophils.

Calculation of the number of cell precursors using CFSE

The number of cells in each cell division CFSE peak was corrected for the increase due to division itself by summing the corrected numbers in each peak using the following

formula: Total number of cell precursors = $\sum n_i/2^i$ where n_i = the number of cells in the i th division peak. (21)

Histology and staining of liver sections

Frozen sections from spleen or LN collected 5 hours post-transplantation were stained with biotin conjugated anti-H-2K^b (BD biosciences), FITC-conjugated anti-CD4 (BD Biosciences) followed by Alexa Fluor 555-conjugated streptavidin (Invitrogen).

Results

Rapid migration of donor PL from the liver to lymphoid tissues after transplantation

To characterize PL migration from the liver after transplantation in the absence of alloreactivity, we first transplanted syngeneic livers from CD45.2⁺ B10.BR donors into CD45.1⁺ B10.BR recipients. CFSE-labeled H-2K^b-specific Des transgenic T cells (also CD45.2⁺) were adoptively transferred into recipient mice one day before the transplantation (Supplementary Fig.1). This experimental protocol allowed us to easily distinguish donor PL (CFSE⁻CD45.2⁺), directly graft-reactive CD8 Des T cells (CFSE⁺CD45.2⁺) and recipient leukocytes (CFSE⁻CD45.2⁻) by flow cytometry. Most PL (90%) exited the liver and were rapidly replaced by recipient leukocytes as early as 1.5h post-transplantation (Fig.1). PL were found in the blood at 1.5h, and seeded spleen and LN between 1.5h and 24h after completion of the transplant. The percentages of PL found in spleen and LN at 24h and 48h were similar (Fig.1). There was no statistically significant difference between the numbers of donor PL detected in recipient LN and spleen after syngeneic or allogeneic transplant (data not shown). These results demonstrated that PL migrated very rapidly into recipient lymphoid tissues following liver transplantation.

Leukocyte composition in the donor liver before transplantation

Before analyzing the migration of donor leukocytes following liver allograft, it was important to characterize intrahepatic leukocytes present in the donor organ, as this would determine the PL subsets migrating out of the graft post-transplantation. Flow cytometric analysis demonstrated that livers contained $1.7 \pm 0.4 \times 10^6$ total leukocytes

(mean $\pm$ SEM, n=9), which was fewer than LN ($2.8 \pm 0.1 \times 10^7$) and spleens ($3.5 \pm 0.4 \times 10^7$) (Figure 2A, legend). The blood contained $7.9 \pm 1.4 \times 10^6$ cells per 2 ml (estimated volume of blood per mouse; Supplementary Fig.2). Numbers of each leukocyte subset analyzed in various organs are shown in Fig.2a. NK and NKT cells were enriched within the liver compared with the other organs analyzed (Fig. 2A), consistent with previous reports. (22, 23) This composition was unique to the liver, as LN contained mostly T and B cells, and the spleen contained predominantly B cells with a smaller proportion of T cells. The spleen also contained more myeloid cells compared to LN, including a large proportion of macrophages and conventional dendritic cells, populations rarely detected in the blood, LN and liver (Fig. 2A and Supplementary Fig.3).

Specific retention and/or preferential homing of donor NKT cells to the liver graft

PL composition was then quantified in the liver graft, lymphoid tissues and blood of recipient animals following syngeneic and allogeneic transplantation. Donor PL counts at 24h and 48h post-transplantation are shown in the same graphs to highlight differences in their fate. Fig. 2B shows that with the exception of NKT cells, most PL subsets had exited the graft by 24h. Donor NKT cells represented >80% of intrahepatic donor PL at 24 and 48h (Supplementary Fig. 3). This was significantly increased compared to NKT representation (30%) pre-transplant (Fig. 2A and Supplementary Fig. 3), suggesting that donor NKT cells had either not left the graft, or, as their presence in the blood might suggest (Fig.2C), had preferentially returned to the liver. Donor NK, NKT and myeloid cells were also better represented in the blood compared to lymphocytes, suggesting that these cell types might not home to lymphoid tissues as efficiently as T and B cells.

Recirculating donor myeloid cells in the blood were largely Gr1⁺ monocytes (not shown), a phenotype characteristic of patrolling monocytes. Interestingly, the number of donor NK cells in the blood of allogeneic recipients at 48 hours post-transplant was significantly higher compared to syngeneic recipients. However, since the total number of NK cells retrieved from allogeneic recipients did not exceed pre-transplant numbers (not shown) it was unclear if selective expansion after allogeneic transplantation had occurred.

Differential migration patterns of donor PL to lymphoid tissues reflect their steady state distribution.

To address whether PL subsets distributed equally to spleen and LN, the total number and the composition of PL subsets were analyzed in these compartments. Donor PL migrating into LN and spleen at 24 and 48 hours post-transplant were almost exclusively T and B cells (Fig. 3 and Supplementary Fig. 3). However, the distribution of T and B cells in these tissues was different: in LN, more donor T cells than B cells were found, while in the spleen, donor B cells predominated (Fig.3 and Supplementary Fig. 3). In addition, while the LN contained very few donor myeloid cells (Fig. 3A, B), the spleen contained a significant number of donor NK, NKT cells and myeloid cells (comprised largely of patrolling Gr1⁺ monocytes, not shown). The differential distribution in LN and spleen reflected the original distribution of cells in these compartments (Fig.2A). Interestingly, a significant loss of donor T cells was observed at 48h in both LN and spleen of allograft recipients (Fig. 3A), raising the possibility that donor lymphocytes had been rejected between 24 and 48h. By contrast, NK and NKT cells persisted in the blood and liver at 48

hours (Fig. 2C) suggesting they did not undergo rejection as rapidly. There was a significant increase in donor B cell numbers in recipient spleen 24h after allogeneic transplantation (Fig. 3A).

Increased recipient T and B cell infiltrates in allografts compared to syngeneic grafts

After transplantation, recipient leukocytes infiltrated the liver, rapidly replacing donor PL by 1.5h (Fig. 1), although the infiltrating subsets did not necessarily reach pre-transplant numbers (Fig. 4). Recipient myeloid cells were significantly increased in the liver at 24h and 48h after transplant, regardless of whether donors were allogeneic or syngeneic, suggesting this was a nonspecific response to transplant surgery and associated reperfusion injury (Fig. 4). The myeloid subsets were predominantly neutrophils and Gr1⁺ inflammatory monocytes (not shown). The number of recipient T and B cells increased in the allograft and blood of allogeneic recipients between 24 and 48h (Fig. 4). However, only the increase of recipient B cells in the blood of allogeneic recipients reached statistical significance.

These data indicate that mobilization of some leukocyte subsets was associated with allograft-specific responses, and that they could be distinguished from non-specific responses.

Primary activation of transgenic allo-reactive CD8 T cells in the graft followed by rapid deletion

To analyze the site of activation and the fate of CD8 T cells specific for donor H-2K^b via the direct allo-recognition pathway, we assessed activation markers and cell division in

reporter Des T cells isolated from recipient mice. 5h after receiving an allogeneic donor liver, all Des T cells in liver, spleen, blood and LN had up-regulated the early activation marker CD69, indicative of parallel activation in these compartments (Fig. 5A). At 2 hours post transplantation, CD69⁺ CD8 Des cells were only found in the liver and blood but were mostly absent from lymphoid tissues (Fig. 5A) suggesting that intrahepatic activation has occurred independently from lymphoid tissues. Des T cell activation was a consequence of cognate recognition and not surgery, as activation was never observed in recipients of syngeneic B10.BR livers (Fig. 5B). Des T cells responding to C57BL/6 donor livers also increased in size, consistent with blast formation, and up-regulated CD25 and CD44 in all compartments by 24h (Fig. 5B). Together, these results suggest that naïve CD8 T cells were directly activated simultaneously by PL in lymphoid tissues and in the graft by liver resident cells and/or intrahepatic PL that did not exit the graft. To visualize where allograft reactive transgenic T cells interacted with donor cells and were activated in lymphoid tissues, Des T cells were labeled with CellTrace Violet before adoptive transfer into transplanted recipients. Spleen and LN were harvested at 5 hours post-T cell transfer. Immunostaining of liver sections revealed Des-TCR cells migration almost exclusively into the T cell zone in both lymphoid tissues (Fig. 5C), with interactions between allograft-reactive transgenic T cells and donor H-2K^b PL observed (Fig. 5C).

By 48h, all Des T cells transferred into recipients of allografts had proliferated as seen by CFSE dilution (Fig. 5B, bottom panel). Despite the similar extent of Des T cell proliferation observed in lymphoid tissues and liver, the total number of Des T cells retrieved from allograft recipients was significantly lower than that retrieved from

recipients of syngeneic grafts or non-transplanted controls, in which proliferation was not observed. To account for cell division, we calculated the number of precursors that the daughter Des T cells at 48h were derived from. These results indicate a profound loss of transferred Des T cells even before division (within the first 24 hours, Fig. 5D), suggesting early deletion or redistribution to other tissues.

Discussion

Since the first report of spontaneous acceptance of liver transplants by Sir Roy Calne in 1969 (24), research on this phenomenon has been hampered by the lack of transgenic tools and reagents for phenotyping rat leukocytes, the difficulty in transplanting mouse livers and the problem faced in tracking complex polyclonal responses, limiting progress in identifying how alloreactive T cells are tolerized. Using mouse strains with congenic markers in combination with multi-color flow cytometry we have identified and tracked multiple PL subsets and monitored the fate of a reporter population of directly allograft reactive CD8 T cells showing for the first time that i) most liver PL but NKT cells exited the graft rapidly, migrating to recipient lymphoid organs as early as 1.5h post-transplantation; ii) PL subsets did not distribute equally to the spleen and LN, but rather reflected steady state leukocyte populations frequencies in these organs; iii) donor-specific naïve CD8 T cells were activated within both graft and lymphoid tissues within 2h but were deleted within 24h; and iv) recipient cell subsets infiltrating the liver graft differed in response to allo- and syngeneic transplants.

The early migration of PL to recipient lymphoid tissues is thought to be a critical event in the spontaneous acceptance of liver allografts. (7) Most studies investigating alloreactive T cell fate have focused on lymphoid tissues, where activation by PL presenting donor MHC on the surface of donor APC (direct pathway) or by allograft-derived peptides associated with recipient MHC (indirect pathway) occurs. The presence of donor PL in recipient draining LN and spleen post-transplant has been shown to be associated with

high levels of IL-2 and IFN γ mRNA production and with increased apoptosis, presumably of recipient T cells, (10) suggesting that PL induce abortive activation or exhaustion of alloreactive T cells, reducing the repertoire that mediates rejection. (16) Although this is an attractive hypothesis, it remains unclear why liver PL induced tolerance when PL from kidney, skin and heart grafts primed recipient T cells for rejection. (1) It has been hypothesized that the large number of PL in liver compared to other organs could be responsible for this liver property. However, the inability of large numbers of donor splenocytes to induce long-term acceptance of fully-mismatched heart and kidney grafts (1) argues against this possibility. Alternatively, it has been proposed that intrahepatic PL might differ qualitatively from splenocytes, containing subsets more potent in inducing tolerance. While this cannot be discounted, donor splenocytes were equivalent to purified donor liver leukocytes in their ability to reconstitute acceptance of PL-depleted transplanted livers. (6) Finally, it is possible that the combination of both PL and liver parenchyma contributes to the induction of tolerance by liver allografts. Characterizing the type of PL seeding lymphoid tissues and the site of recipient T cell activation were thus among the aims of this study.

We confirm here results in rat models showing that donor PL exited the graft rapidly, and some migrated to recipient LN and spleen. However, by systematically analyzing all the main liver PL subsets, we demonstrate here that all PL subsets did not migrate equally in the recipients. First, PL migration to LN and spleen was not equivalent, as myeloid cells were hardly recovered from the LN. By 24h post-transplantation, the majority of PL in LN were T and B cells, whereas in the spleen, B cells predominated, followed by T cells,

donor NK and myeloid cells. This differential migration reflects the distribution of the various cellular subsets in these organs, and suggests that allo-reactive T cells might encounter different types of APC at these two sites, potentially leading to different outcomes. Antigen presentation by B cells has been shown to “turn off” naïve T cells. (25; 26) In a fully-MHC mismatched kidney transplant model, transfer of donor B cells, but not T cells, led to prolongation of graft survival, suggesting there are inherent differences in their ability to induce tolerance. (27) Interestingly, at 48h there was significant loss of donor T cells in the LN and spleen of allograft recipients, suggesting they had recirculated or had been rejected. We could not detect any significant difference between the numbers of recipient CD4 and CD8 T cells in allogeneic vs syngeneic transplantation recipients (data not shown). This is expected given the low percentage of alloreactive cells in the recipient polyclonal T cell population (1-10%). Deletion by apoptosis of recipient allogeneic T cells after liver transplantation has been suggested in other studies (8, 9), although it was not clear whether apoptosis occurred in recipient T cell subsets.

The ability of myeloid cells to home preferentially to the spleen suggests that liver myeloid cells, in particular dendritic cells, could exert their immunomodulatory properties (Th2/tolerogenic induction (28) or ‘veto’ function (29)) at this site. The role of liver DC in liver transplantation is controversial - when infused, purified liver DC prolonged allograft survival, but livers containing Flt3L-mobilised DC were rapidly rejected. (30, 31) As DC mature upon culture and recirculation, infusion or mobilization approaches might not recapitulate the endogenous response. There are also different subsets of DCs that might exert different functions in transplantation tolerance. Higher

incidences of circulating plasmacytoid (pDC) relative to myeloid or conventional (mDC) have indeed been reported in operationally tolerant pediatric liver allograft recipients and in patients on low dose immunosuppressive therapy undergoing prospective drug weaning, and were correlated with increased frequencies of CD4+CD25^{hi}Foxp3+ Treg (32). pDC are also known to promote tolerance in several animal models of transplantation (reviewed by (33)). Donor DC have been detected in recipients after liver transplantation in the rat and human (34, 35) although their role in the induction of liver transplant tolerance has never been directly demonstrated. Unfortunately, we had not included the pDC markers mPDCA1 and B220 in our panels to distinguish pDCs. More in depth experiments that include proper enzymatic digestion protocol, are thus required to address the phenotype and migration of the different DC subsets. Our study did not also address whether donor regulatory CD4 or CD8 T cells Tregs migrated differently in the different compartments following liver transplantation, resulting in a different effector cell/Treg ratio. Although Tregs might play a role in liver transplantation, we excluded analysis of this cell subset from our panels, as we were investigating events of tolerance induction occurring within the first 48h after transplantation. Tregs take several days to differentiate from naive T cells, and their involvement in the spontaneous acceptance of liver transplants, is in long-term maintenance rather than induction of tolerance (reviewed in (1)).

The second important finding of this study was the enrichment of donor NK and NKT cells in the liver and blood post-transplantation, suggesting that these cells failed to migrate to lymphoid organs, and/or homed preferentially back to the liver. It is also

possible that some donor NKT cells never left the graft; residence of NKT cells in the liver would be consistent with recent observations in parabiotic mice that these cells failed to recirculate despite their location in the sinusoids, and hence direct access to the circulation. (36)

In response to liver transplantation, the number of non-transgenic recipient cells also increased in lymphoid tissues. An increase in recipient myeloid cells (mainly neutrophils and inflammatory monocytes) was observed after both allo- and syngeneic transplantation, indicating that non-antigen specific responses related to the inflammation, and reperfusion injury had occurred.

A significant advantage of the mouse liver transplant model over previous studies was that it allowed the fate of a reporter population of naïve donor-specific transgenic CD8 T cells to be monitored following transplantation, in contrast to studies analyzing the polyclonal T cell response in rats, which have not revealed significant difference in infiltrates between tolerant and rejecting livers and did not distinguish between naïve and activated/memory T cells. We were able to demonstrate that naïve CD8 T cell activation occurred within hours in both recipient lymphoid tissues and hepatic allograft. Activation in LN and spleen seems to have occurred in the T cell zones, where the transgenic T cells were predominantly located and contacted PL (Fig. 5c). In the liver, primary activation occurred in the sinusoids (data not shown) as we have previously reported. (18) We also found that Des T cells start to be deleted within the first 24h and most were deleted within 48h. This suggests that alloreactive T cell deletion occurs at a significantly earlier

time point than the one described by Steger and colleagues' who reported deletion of a large cohort of donor-specific naïve CD8 T cells by day 5 following mouse liver transplantation (16) following T cell proliferation. This suggests that deletion would occur even before T cells start to divide. This type of rapid allo-T cell deletion is consistent with recent studies performed in a non-transplant setting, showing that a large proportion of naïve CD8 T cells activated within livers expressing high levels of antigen underwent deletion by a non-apoptotic process, due to invasion and degradation inside hepatocytes within hours (37), and the residual cells were further deleted due to subsequent Bim-dependent apoptosis. (38) This is in contrast to a previous study using mouse liver transplantation, where it was reported that naïve CD8 T cells develop cytotoxic function 7 days after intrahepatic activation in the liver graft. (39) Importantly however, in that particular study the cognate antigen was only expressed transiently, in contradistinction to continuous expression of donor antigen occurring in allografts as reflected in the current work.

It is unclear from the current study which of the 3 compartments supporting naïve CD8 T cell activation was also essential for their early deletion. We recently observed Des T cell deletion in splenectomized recipients (S. Tay, manuscript in preparation) suggesting the spleen was not essential for this process. The nature of the APC leading to activation and deletion of Des T cells is as yet undefined, but given the preferential migration patterns of the different PL subsets, activation in the separate compartments could involve distinct APCs. In the liver, the ability to tolerize CD8 T cell responses has largely been attributed to hepatocytes expressing cognate antigen. However, the unique hepatic architecture and slow blood flow would also permit prolonged interactions in the sinusoids with Kupffer

cells and liver sinusoidal endothelial cells (LSEC), and as discussed above, NK and NKT cells, that could act as APCs. Kupffer cells and LSEC have reported abilities to tolerize CD8 and CD4 T cells, and in the transplant setting (40) might play key roles in modulating both subsets.

In summary, we have developed a mouse model of liver transplantation to elucidate a question that has fascinated transplant immunologists for four decades. This new approach has yielded new insights into the general mechanisms leading to the spontaneous acceptance of liver grafts, and will be further developed in the future to properly address the sequence of events leading to this intriguing phenomenon.

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Figure legends:

Figure 1: *Rapid exit of donor PL from the liver and migration to lymphoid organs after syngeneic transplantation.* B10.BR Ly5.2⁺ livers were transplanted into B10.BR Ly5.1⁺ recipients. Livers, spleens, LN and blood were harvested at 1.5h, 24h and 48h from syngeneic transplanted recipients. Lymphocytes were stained and analyzed for CD45.1 and CD45.2 expression after gating out transgenic CFSE⁺ Des T cells.

Figure 2: *Migration of different liver leukocyte and myeloid cell subsets in the blood.*

(a) Normal distribution and numbers of leukocyte subsets in livers and blood, LN and spleens of B10.BR mice before transplantation. Leukocytes are represented in leftmost panels while myeloid subsets are shown in middle and rightmost panels. To better demonstrate the distribution of the different myeloid subsets, the y axis of the middle panels has been magnified and is shown in the right panels. Counts were collected from 9 separate experiments.

(b) and **(c)** Donor PL in liver grafts **(b)** and blood **(c)** were analyzed at 24h and 48h after grafting of liver from Ly5.2⁺ B10.BR syngeneic donors (B10) or Ly5.2⁺ C57BL/6 allogeneic donor (B6) into Ly5.1⁺ B10.BR recipients. The absolute number of the various PL subsets in each compartment was quantified as described. Bar shows mean of three samples. Statistical analysis performed by ANOVA followed by Bonferroni post-hoc tests between groups showed significant differences ($p < 0.05$).

Figure 3: *Differential migration of donor PL to recipient spleen and LN.*

(a) Absolute number of the various PL subsets in LN and spleen at 24h and 48h after grafting of liver from Ly5.2⁺ B10.BR syngeneic donors (B10) or Ly5.2⁺ C57BL/6 allogeneic donor (B6) into Ly5.1⁺ B10.BR recipients. Bar shows mean of three samples. Statistical analysis performed by ANOVA followed by Bonferroni post-hoc tests between groups showed significant differences ($p < 0.05$). (b) Proportion of the different PL subsets in LN and spleen of syngeneic and allogeneic at 24 post-transplantation showing the over-representation of T cells and B cells in the LN and spleen respectively.

Figure 4: *Recipient cell subsets in different compartments at 24h and 48h after grafting of liver from Ly5.2⁺ B10.BR syngeneic donors (B10) or Ly5.2⁺ C57BL/6 allogeneic donor (B6) into Ly5.1⁺ B10.BR recipients.* The absolute number of the various leukocyte subsets in each compartment was quantified. Bar shows mean of three samples. Pre-transplant values of leukocyte subsets were collected from 9 separate experiments.

Figure 5: *Early activation of allo-reactive reporter Des T cells by donor PL followed by their rapid loss from analyzed organs.* Des-RAG^{-/-} T cells were adoptively transferred into B10.BR mice transplanted with a C57BL/6 or B10.BR liver. Livers, LN and spleens were harvested at 2 or 5 hours post-transplant, and lymphocytes recovered and analyzed. (a) Representative flow histograms of CD69 expression by Des transgenic T cells in recipients of allogeneic C57BL/6 grafts (black lines, top panel) compared to untreated B10.BR controls (shaded, top panel) or compared to recipients of syngeneic B10.BR

grafts (grey, shaded, bottom panel, 5h). **(b)** Representative histograms of FSC, CD69, CD25 and CD44 expression (at 24 hours) and CFSE fluorescence intensity (at 48 hours), of CD8⁺Des⁺ T cells in the spleen, LN and liver of recipients that received allogenic C57BL/6 (black lines, right panel) or syngeneic B10.BR (black line, left panel) grafts, compared to Des T cells transferred into un-transplanted B10.BR controls (shaded, both panels) within the same experiment. **(c)** 10 x 10⁶ LN cells were isolated from Des-RAG^{-/-} mice, labeled with CellTrace Violet (5μM) and transferred into B10.BR recipients 24h before grafting a C57BL/6 liver. Frozen sections from spleen (top panel) or LN (bottom panel) collected 5 hours post-transplantation were stained for H-2K^b to label PL (red), and CD4 (green) to reveal T cell zones. Bars: 50μm. Arrowheads indicate interactions between donor H-2K^bPL (red) and CellTrace Violet⁺ Des T cells (blue). **(d)** Calculated numbers of Des T cell precursors from those retrieved from the LN, spleen, blood and liver at 24 and 48 hours post-transplant. Bars represent mean ± SD of n=4 mice per group. Statistically significant differences between groups (p<0.05) found by ANOVA and Bonferroni post-hoc tests are indicated.

Legends for Supplementary Figures:

Supplementary Figure 1: *Experimental mouse liver transplantation system used in this study to identify donor CD45.2⁺ PL, recipient CD45.1⁺ cells and reporter Des-RAG^{-/-} TCR transgenic T cells after liver transplants (LT) performed using C57BL/6 (B6) or B10.BR (B10) donors*

Supplementary Figure 2: *Total number of leukocytes and myeloid cells in liver, blood (2ml), LN and spleen. Leukocytes were harvested from n=9 non-transplanted B10.BR mice to establish the numbers of different leukocyte and myeloid cell subsets present using antibody panels 1 and 2.*

Supplementary Figure 3: *Proportions of leukocytes and myeloid cells in liver, blood, LN and spleen. Leukocytes were harvested from non-transplanted B10.BR mice (top panel) and from recipient B10.BR mice pre-injected with transgenic Des T cells and transplanted with a syngeneic B10.BR (B10) or allogeneic C57BL6 (B6) liver at 24 and 48 hours post-transplantation (Tx). Passenger leukocytes were identified as CD45.1⁻ CD45.2⁺ following exclusion of the CFSE⁺Des⁺ T cells. The different leukocyte and myeloid cell subsets were distinguished using antibody panels 1 and 2. The proportion of NKT cells in the liver (red) was higher than that compared to that in the blood, spleen and LN (p<0.05, ANOVA and Bonferroni post-hoc analysis).*

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Fig. 1

Rapid exit of donor PL from the liver and migration to lymphoid organs after syngeneic transplantation.
69x92mm (300 x 300 DPI)

Fig. 2

Migration of different liver leukocyte and myeloid cell subsets in the blood.
117x146mm (300 x 300 DPI)

Fig. 3

Differential migration of donor PL to recipient spleen and LN.
108x112mm (300 x 300 DPI)

Fig. 4

Recipient cell subsets in different compartments at 24h and 48h after grafting of liver from Ly5.2+ B10.BR syngeneic donors (B10) or Ly5.2+ C57BL/6 allogeneic donor (B6) into Ly5.1+ B10.BR recipients.
77x123mm (300 x 300 DPI)

Fig. 5

Early activation of allo-reactive reporter Des T cells by donor PL followed by their rapid loss.
86x178mm (300 x 300 DPI)

Supplementary Fig. 1

Experimental mouse liver transplantation system used in this study to identify donor CD45.2+ PL, recipient CD45.1+ cells and reporter Des-RAG-/- TCR transgenic T cells after liver transplants (LT) performed using C57BL/6 (B6) or B10.BR (B10) donors
108x62mm (300 x 300 DPI)

Accepted

Supplementary Fig. 2

Total number of leukocytes and myeloid cells in liver, blood (2ml), LN and spleen.
81x72mm (300 x 300 DPI)

Accel

Leukocytes Pre-transplantation

Passenger Leukocytes post-transplantation

Supplementary Fig. 3

Proportions of leukocytes and myeloid cells in liver, blood, LN and spleen.
123x133mm (300 x 300 DPI)