Journal of Zhejiang University SCIENCE B
ISSN 1673-1581
http://www.zju.edu.cn/jzus
E-mail: jzus@zju.edu.cn

Infusion of nonmyeloablative bone marrow alleviates acute rejection reaction in liver allotransplantation*

XIE Hai-yang (谢海洋), HUANG Dong-sheng (黄东胜),
JIA Chang-ku (贾长库), ZHENG Shu-sen (郑树森)†‡

(*Department of Hepatobiliary Pancreatic Surgery, Key Lab of Combined Multi-Organ Transplantation, Ministry of Public Health, First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310003, China*)

†E-mail: Zhengss@mail.hz.zj.cn

Received June 10, 2005; revision accepted Sept. 27, 2005

Abstract: Objective: To study the effect and implication of nonmyeloablative donor specific bone marrow (DSBM) infusion on the immunoreaction of liver allotransplantation. Methods: Orthotopic liver transplantation model was used in this study. Groups were set as follows: Group I, syngeneic control (Wistar-to-Wistar); Group II, acute rejection (SD-to-Wistar); Group III, acute rejection treated with cyclosporine A (CsA) by intramuscular injection (SD-to-Wistar+CsA); Group IV, bone marrow infusion at 7 d pretransplantation followed by short-term CsA treatment (SD-to-Wistar+DSBM); Another group of short-term CsA treatment preoperatively without bone marrow infusion was also set as control. General characteristics and survival time were observed. Histological grades of rejection were determined by pathological examination. IL-2 and IFN-γ level in peripheral blood and donor liver were detected respectively by Enzyme-Linked Immuno-Sorbent Assay (ELISA) and Western blot. Chimerism of donor cells was measured by PCR for a male-specific marker (Y-chromosome-specific sequence, Sry). Results: No signs of rejection were found in Group I. Acute rejection occurred in both Group II and the short-term CsA treated group. All the recipients died at (9~15) d posttransplantation with a median survival time of (10.7±0.5) d and (11.2±2.4) d, respectively. Only mild rejection could be seen in Group III. In Group IV, 4 out of 6 recipients had long-term survival (>100 d), the histological grade of rejection was significantly lower than that of Group II, so did the expression level of IL-2 and IFN-γ in both peripheral blood and grafted liver. Y-chromosome-specific sequence (Sry) of male SD rats could be detected in the bone marrow, spleen and thymus of female recipients at 15 d after bone marrow infusion. Conclusion: Mild preconditioning nonmyeloablative donor specific bone marrow infusion can enhance chimerism formation in recipients, alleviate the rejection of liver allotransplantation and prolong survival of liver allotransplantation.

Key words: Liver transplantation, Rejection, Bone marrow infusion, Chimerism
doi:10.1631/jzus.2005.B1188 **Document code:** A **CLC number:** R657.3

INTRODUCTION

Infusion of donor specific bone marrow (DSBM) is considered to enable enhancement of chimerism formation, and bone marrow infusion is considered to be a practical way to induce immunotolerance. Classically, the recipient undergoing transplant should receive sub-lethal doses of irradiation to partly reduce the number of leukocytes, followed by DSBM infusion. However, reluctance of clinicians to expose recipients to radiation has hampered its clinical application, although the liver is characterized by its specific immunity property and abundant for immunocytes, including kupffer's cells, immature DCs (dendritic cells), etc. and some particular structures such as double blood supply from both hepatic artery and portal vein, and Disse's space, which prevent leukocytes from adhering to the endothelium. As a

‡ Corresponding author
* Project (No. 2003CB515501) supported by the National Basic Research Program (973) of China

result, the donor liver avoids attack by the recipient immunity system and prevents rejection after transplantation. Clinical investigations have shown that some transplantation recipients would survive with normal liver function after withdrawal of immunosuppressants (Raimondo and Burroughs, 2002; Alberto and Terry, 2004). Therefore, it is acknowledged that the liver is prone to tolerate allografts. In this experiment, we investigated whether tolerance to liver allografts could be induced by short-term CsA administration and high doses of DSBM infusion pretransplantation in rat liver transplantation, and discussed the induction and maintenance of tolerance in liver allograft.

MATERIALS AND METHODS

Animals

Inbred male Wistar and SD rats weighing 200 g to 250 g were purchased from Shanghai Experimental Animal Center (China). Animals were raised in a special pathogen free environment.

Isolation of bone marrow cells

Isolation of bone marrow cells from the femurs of SD rats. Red cells were lysed with ammonia chloride solution. Typan Blue dye exclusion experiment suggested the survival percentage of bone marrow cells in the suspension was more than 98%.

Surgical procedure, experimental groups and sample harvesting

The rats were anesthetized by inhalation of ether. Orthotopic rat liver transplantation was performed according to Kamada's two-cuff technique (Kobayashi *et al*., 1993). Wistar rats serving as recipients were randomly divided into five groups. Group I: syngeneic control, donor livers of Wistar rats were transplanted into Wistar recipients (Wistar-to-Wistar); Group II: acute rejection, donor livers of SD rats were transplanted into Wistar recipients (SD-to-Wistar); Group III: CsA treated group, donor livers of SD rats were transplanted into Wistar recipients (SD-to-Wistar+CsA), and received CsA 3 mg/(kg·d), im. from 0 d to 12 d posttransplantation; Group IV: bone marrow infusion group (SD-to-Wistar+DSBM), Wistar recipients were infused with bone marrow from SD rats (2×10^8) via tail vein at 7 d pretransplantation, and intramuscularly injection of CsA was given at the day before and first three days after bone marrow infusion at dose of 3 mg/(kg·d). Each of the above groups was divided into six subgroups, namely 1 d, 3 d, 5 d, 7 d, 12 d posttransplantation (n=3 for each time point) and another subgroup for investigation of survival (n=6). At each time point, recipients were sacrificed for sample harvesting. Liver allografts were taken for histology assessment and cytokine determination. In addition, a group (SD-to-Wistar) treated with CsA for 3 d preoperatively without bone marrow infusion was also set as control group for survival investigation.

Histopathological examination

Grafted liver samples were fixed in 10% buffered formalin and embedded in paraffin. Five micrometers thick sections were affixed to slides, deparaffinized, and stained with hematoxylin and eosin to assess morphological changes and severity of acute rejection according to Kemnitz's standard (Kemnitz *et al*., 1989).

ELISA analysis of IL-2 and IFN-γ level in peripheral blood

Serum samples were stored frozen at −80 °C until time for analysis. Commercially available enzyme-linked immunosorbent assay kits (Cytoscreen^{TM}, BioSource, USA) were used to evaluate serum levels of IL-2 and IFN-γ proteins according to the manufacturers' instructions.

Western blot analysis of IL-2 and IFN-γ level in grafted liver

The grafted liver was homogenized with lysate buffer (50 mmol/L Tris-Cl, 150 mmol/L NaCl, 1% NP40, 0.25% deoxycholate, 2 mmol/L EDTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 mmol/L PMSF). After centrifugation, the concentration of protein in the supernatant was determined by Bio-Rad kit (BCA). Forty μg of protein sample was boiled for denaturization, then separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to a nitro-cellulose membrane (Amersham Pharmacia, USA). The membrane was blocked in blocking buffered solution for 2 h at room temperature, and then

treated with mouse anti-rat IL-2, IFN-γ monoclonal antibody (RnD, 1:500 dilution) overnight at 4 °C. After being washed, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibodies (Chemicon, 1:2000) and then was visualized with an enhanced chemiluminescence detection system (Kodak, USA).

Detection of chimerism

Chimerism in female Wistar rats after bone infusion from male SD rats was confirmed by polymerase chain reaction (PCR) analysis with primers specific for the sex-determinant Y chromosome. Genomic DNA was prepared from BM, spleen and thymus taken at the time of liver harvesting by a standard procedure (Joseph and David, 2000). PCR was carried out with Sry-specific oligonucleotide primers (5′-GCCTCCTGGAAAAAGGGCC-3′ and 5′-GAGAGAGGCACAAGTTGGC-3′). The reaction products were analyzed by electrophoresis in 2% agarose gel, followed by ethidium-bromide staining.

Statistics

All data were expressed as mean±*SD* and analyzed by one-way repeated measures analysis of variance (ANOVA) using SPSS software (version 11.0 for Windows). $P<0.05$ was considered as statistically significant.

RESULTS

General characteristics and survival posttransplantation

Fig.1 shows that high dose of bone marrow infusion combined with short-term CsA treatment prolonged the survival time of recipients. Four out of six recipients in Group IV survived long-term (>100 d), In Group II, the median survival time of recipient was (10.7±0.5) d (Fig.1). Besides, all the recipients in short-term CsA-treated group without bone marrow infusion died at (9~15) d with median survival time of (11.2±2.4) d.

Histopathological manifestation

No signs of rejection were found at any time point in Group I except for mild portal inflammatory infiltration. In Group II, lymphocytes infiltration in portal area with mild vein endothelialitis were found at 1 d posttransplantation but without evidence of rejection. With time going on, portal lymphocyte infiltration became significant, and degeneration of hepatic parenchyma was found in some cases at 3 d and 5 d post transplantation with average histological grades of rejection being 1.83 and 2.67, respectively. Marked mononuclear leucocytes infiltration, severe vein subendothelialitis with bridging hepatocellular necrosis could be found at both 7 d and 12 d with grades of 2.87 and 3, respectively. Owing to the immunosuppressive effect of CsA, the rejection reaction was significantly inhibited in Group III. No evidence of rejection was found at the first three time points. Mixed cell infiltration accompanied with vein endothelialitis was found only at 7 d and 12 d with pathological degree of 1. In Group IV, rejection of grafts was alleviated to some degree, with histological grade being 1.25 at 3 d and 5 d posttransplantation ($P<0.05$, vs Group II). The pathological degree was 1.5 at both 7 d and 12 d in Group IV, with no significant difference from Group III (Fig.2).

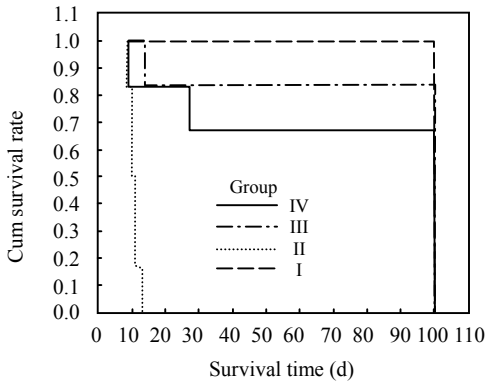

Fig.1 Survival time of different groups posttransplantation

I: Wistar-to-Wistar group; II: SD-to-Wistar group; III: SD-to-Wistar+CsA group; IV: SD-to-Wistar+DSBM group

Expression of IL-2 and IFN-γ in peripheral blood

Due to the rejection reaction, the IL-2 protein was anticipatively highly expressed in peripheral blood in Group II from day 3 to day 12 after transplantation. Compared with the rejection group, Group IV showed significantly lower expression of IL-2 at 3-, 5- and 7-d posttransplantation, similar to the case of syngeneic and CsA treated groups (Fig.3).

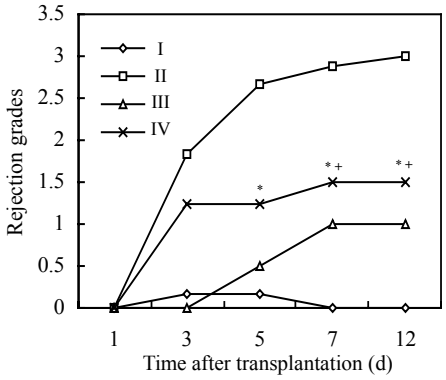

Fig.2 Rejection grades of grafted liver in different groups
I: Wistar-to-Wistar group; II: SD-Wistar group; III: SD-to-Wistar+CsA group; IV: SD-to-Wistar+DSBM group; Rejection grades of Group IV are significantly lower than those of Group II at all but day 1 time point; $^{*}P<0.05$ Group IV vs Group II; $^{+}P>0.05$ Group IV vs Group III

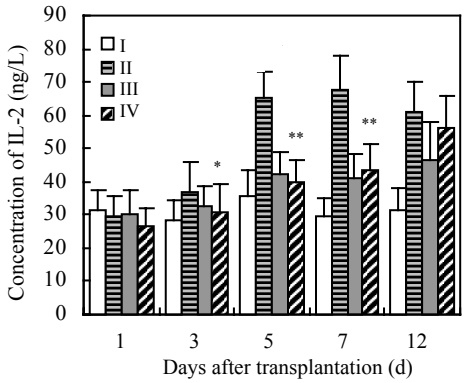

Fig.3 Expression of IL-2 in peripheral blood at different time points posttransplantation
I: Wistar-to-Wistar group; II: SD-to-Wistar group; III: SD-to-Wistar+CsA group; IV: SD-to-Wistar+DSBM group; $^{*}P<0.05$ Group IV vs Group II; $^{**}P<0.01$ Group IV vs Group III

The IFN-γ protein expression showed trend similar to that of IL-2. No statistical differences were seen among Groups at 1 d posttransplantation; and apparently decreased IFN-γ levels were detected in Group IV at 3-, 5- and 7-d posttransplantation compared with those of Group II (vs Group II, $P<0.05$), although there was a slightly increased level at 12 d (Fig.4).

Expression of IL-2 and IFN-γ in grafted liver

No or low expression of IL-2 protein was detected at any time point in Groups I, III and IV, whereas high expression was detected in Group II 5 d after transplantation.

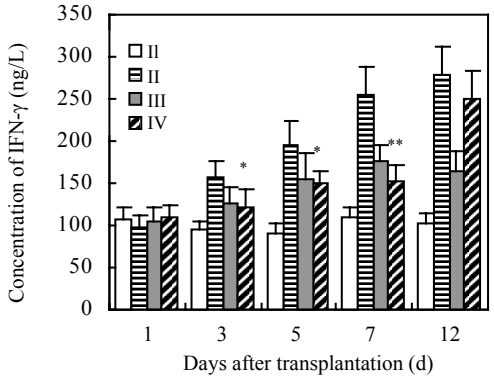

Fig.4 Expression of IFN-γ in peripheral blood at different time points posttransplantation
I: Wistar-to-Wistar group; II: SD-to-Wistar group; III: SD-to-Wistar+CsA group; IV: SD-to-Wistar+DSBM group; $^{*}P<0.05$ Group IV vs Group II; $^{**}P<0.01$ Group IV vs Group III

IFN-γ protein was highly expressed in Group II from day 3 after transplantation. As for Group IV, the expression of IFN-γ protein obviously decreased, although there was slight increase with prolongation of survival time (Fig.5 and Fig.6).

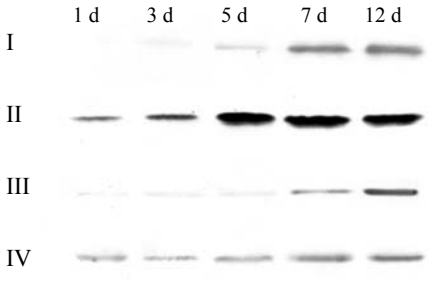

Fig.5 Expression of IL-2 in grafted livers at different time points posttransplantation
I: Wistar-to-Wistar group; II: SD-to-Wistar group; III: SD-to-Wistar+CsA group; IV: SD-to-Wistar+DSBM group

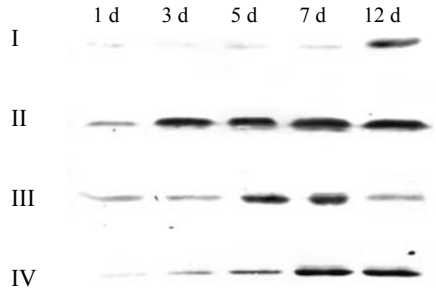

Fig.6 Expression of IFN-γ in grafted livers at different time points posttransplantation
I: Wistar-to-Wistar group; II: SD-to-Wistar group; III: SD-to-Wistar+CsA group; IV: SD-to-Wistar+DSBM group

Detection of chimerism

SD rat donor-specific Y-chromosome-specific sequence could be detected in both bone marrow and spleen in 5 out of 6 female Wistar recipients at 15 d after bone marrow infusion, and were detected in thymus in 3 out of 6 female Wistar recipients at the same time point (Fig.7).

DISCUSSION

Infusion of DSBM and other cells including splenocytes and lymphocytes is helps to induce specific tolerance or low immunoresponse, decreases the immunosuppressants dosage and improve allograft survival. This phenomenon has recently become the focus of study on transplantation immunology. Sublethal dose of thymic or whole-body irradiation-induced cytoreduction/ablation followed by infusion of DSBM has been the most consistently successful approach to experimental tolerance induction. The infusion of DSBM post irradiation induces complete or mix allogeneic chimerism in recipients because of inhibition of the immune response. Unfortunately, the toxicity to man and destruction to recipient bone marrow system of such regimens and the incidence of graft-vs-host disease thereafter has limited their clinical research and application. Meanwhile the effect of non-radiation preconditioning DSBM on immunotolerance induction in heart and skin transplantation is not satisfactory yet. Some successful studies are also the results of infusion of DSBM combined with blocking of the co-stimulation molecular pathway (CTLA4Ig) or selective deletion of recipient T cell subgroups by anti-CD3, anti-CD4, anti-CD8 and anti-CD25 antibodies (Li *et al*., 2001; Shirasugi *et al*., 2002; Hale *et al*., 2000). It is well known that the difficulty of tolerance induction depends on different organs transplantation. Because the liver has proved to be an immunologically privileged organ, capable in several animal models to be accepted as an allograft without any intervention by the immune system of the recipient, grafted liver survived longer than other grafted organs (Raimondo and Burroughs, 2002; Alberto and Terry, 2004). Bone marrow combined with solid organ transplantation was often used in research on induction of chimerism and immunotolerance, based on cardiac, skin and kidney transplantations. But this protocol, especially mild preconditioning of nonmyeloablative bone marrow transplantation, has not been reported in liver transplantation. Thus we studied the effect of short-term CsA administration and high dose of DSBM infusion without irradiation on the immune reaction of allograft in rat liver transplantation. CsA, as a common immunosuppressant in clinics, can suppress the activation of T lymphocyte and subsequent release of cytokines such as IL-2. Moreover, CsA itself was reported to inhibit the differentiation of immature thymocytes into mature T lymphocytes and destroy DC in thymus medulla (Jenkins *et al*., 1988; Gao *et al*., 1988; Urdahl *et al*., 1994; Liu, 1993). It could also promote DC immigration from donor bone marrow to thymus of recipient (Beschorner *et al*., 1995). Consequently, we speculated that short-term administration of CsA would create "space" for bone marrow engraftment, and facilitate chimerism induction. A study by Hale *et al*.(2000) showed that sirolimus with antilymphocyte serum, combined with infusion of DSBM, induced tolerance and chimerism

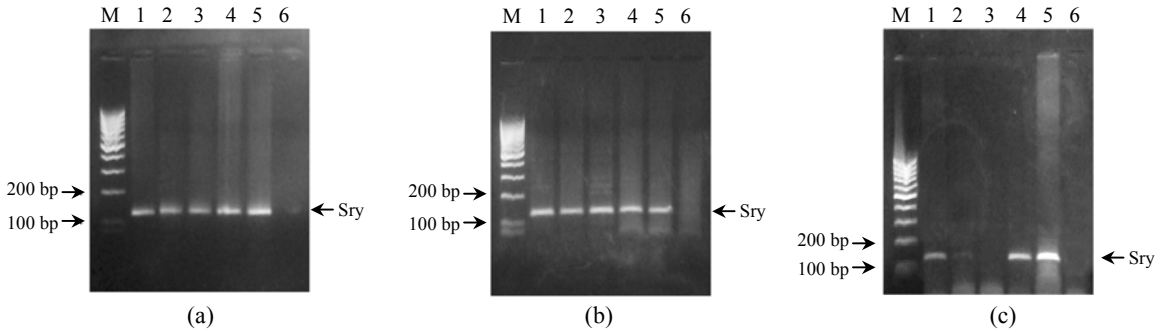

Fig.7 Detection of chimerism 15 d after donor specific bone infusion (a) Bone marrow; (b) Spleen; (c) Thymus
M: GeneRuler^{TM} 100 bp DNA ladder; 1, 2, 3, 4, 5, 6: Different individuals

in completely mismatched murine model. Meanwhile the level and duration of induced-chimerism were directly related to the administration dose of DSBM. High dose (>1.5×10^8 cells) of DSBM was considered to be associated with permanent tolerance of allografts, while low dose may prime the recipient to accelerate the process of transplant rejection.

The results of this study showed that high dose DSBM (>1.5×10^8 cells) combined with short-term use of CsA did induce low immunoresponse and prolong recipient survival in rat liver transplantation model. IL-2 and IFN-γ are representative cytokines excreted by Th1 cells (Kita *et al*., 1999) and play important roles in the rejection reaction of allograft, and their expression increased during rejection. The results of ELISA and Western blot indicated that the expression of IL-2 and IFN-γ in Group IV were significantly lower than that in Group II, though expression of IFN-γ increased slightly with prolongation of survival time. These results suggested that infusion of DSBM alleviated Th1 cytokine-induced acute rejection of liver transplantation (Lee *et al*., 2000). The mechanism of Th1 to Th2 deviation may be involved and be worthy of further study.

The relationship between chimerism and immunotolerance has become the hotspot of study on transplantation immunology. There are many immature DCs and hematopoietic stem cells in bone marrow, so infusion of DSBM can possibly induce mixed chimerism and improve allograft survival in animal models and clinical trial, although the mechanisms involved still remain unclear. Clonal deletion of thymocytes is a major goal aimed for in the establishment of tolerance. In the thymus, to which donor bone marrow-derived DC or hematopoietic stem cells immigrate, recipient T cell clone reacting to allogeneic graft antigen being eliminated during its developing process, which is similar to that of self-reacting T cells being deleted by the process of negative selection during the development of immune system (Manilay *et al*., 1998; Jia *et al*., 2003).

In this study the Y-chromosome-specific sequence of male SD rats could be detected not only in the bone marrow and spleen but also in the thymus of 3 out of 6 female Wistar recipients at day 15 after bone marrow infusion, which suggested that mix chimerism existed in the bone marrow. However central clone deletion hypothesis cannot completely explain this phenomenon. Apart from immunological hypothesis, the differentiation of bone marrow stem cells to hepatocytes is also possible to prolong the recipient survival posttransplantation. Many researches proved that stem cells in bone marrow have potential to differentiate towards hepatocytes. Petersen *et al*.(1999) identified liver cells of donor bone marrow origin in cross-sex or cross-strain bone marrow and whole liver transplantation models by in situ hybridization. In model of bone marrow transplant from age-matched male donors to female mice, Theise *et al*.(2000) also found that hepatocytes could derive in vivo from bone marrow cells by simultaneous fluorescence in situ hybridization (FISH) for the Y-chromosome and albumin mRNA, which confirmed male-derived cells were mature hepatocytes. Thus, we postulate that when the liver is damaged by rejection, some bone marrow cells from the donor may differentiate towards hepatocytes and move into the liver to substitute or replenish damaged hepatic tissue and help to resume liver function. This is probably one of reasons why bone marrow infusion can lead to long-term survival of recipient rats.

Due to the difference of tolerance induction among different organs transplantation, different nonmyeloablative preconditioning regime should be taken. In this study, we found that nonmyeloablative bone marrow infusion combined with short-term CsA administration before liver transplantation can alleviate the acute rejection of SD to Wistar rat liver allograft and promote its long-term survival with chimerism formation and maintenance, thus providing reasonable suggestion of clinical application of DSBM to liver transplantation immunotolerance.

References

Alberto, S.F., Terry, B.S., 2004. Immunological tolerance and liver transplantation. *Journal of Hepatology*, **41**(5):698-705. doi:10.1016/j.jhep.2004.09.013.

Beschorner, W.E., Yao, X., Divic, J., 1995. Recruitment of semiallogeneic dendritic cells to the thymus during post-cyclosporine thymic regeneration. *Transplantation*, **60**(11):1326-1330.

Gao, E.K., Lo, D., Cheney, R., Kanagawa, O., Sprent, J., 1988. Abnormal differentiation of thymocytes in mice treated with cyclosporine A. *Nature*, **336**(6195):176-179. doi:10.1038/336176a0.

Hale, D.A., Gottschalk, R., Umemura, A., Maki, T., Monaco, A.P., 2000. Establishment of stable multilineage hematopoietic chimerism and donor-specific tolerance without

irradiation. *Transplantation*, **69**(7):1242-1251. doi:10.1097/00007890-200004150-00008.

Jenkins, M.K., Schwartz, R.H., Pardoll, D.M., 1988. Effects of cyclosporine A on T cell development and clonal deletion. *Science*, **241**(4873):1655-1658.

Jia, C.K., Zheng, S.S., Zhang, A.B., 2003. Intrathymic inoculation of donor liver specific antigen alleviates rejection of liver allotransplantation. *Journal of Zhejiang University Science*, **4**(4):485-490.

Joseph, S., David, W.R., 2000. Molecular Cloning: A Laboratory Manual on the Web, 3rd Ed. Cold Spring Harbor Laboratory (CSHL) Press, New York, p.127-128.

Kemnitz, J., Ringe, B., Cohnert, T.R., Gubernatis, G., Choritz, H., Georgii, A., 1989. Bile duct injury as a part of diagnostic criteria for liver allograft rejection. *Hum. Pathol.*, **20**(2):132-143. doi:10.1016/0046-8177(89)90177-9.

Kita, Y., Li, X.K., Ohba, M., Funeshima, N., Enosawa, S., Tamura, A., Suzuki, K., Amemiya, H., Hayashi, S., Kazui, T., Suzuki, S., 1999. Prolonged cardiac allograft survival in rats systemically injected adenoviral vectors containing CTLA4Ig-gene. *Transplantation*, **68**(6):758-766. doi:10.1097/00007890-199909270-00007.

Kobayashi, E., Kamada, N., Goto, S., Miyata, M., 1993. Protocol for the technique of orthotopic liver transplantation in the rat. *Microsurgery*, **14**(8):541-546.

Lee, W.C., Jeng, L.B., Chiang, Y.J., Wang, H.C., Huang, C.C., 2000. Dendritic cell progenitors prolong allograft survival through T-helper 2 deviation of the Th1/Th2 paradigm. *Transplant Proc.*, **32**(7):2076-2077.

Li, S., Thanikachalam, M., Pang, M., Kawaharada, N., Aitouche, A., Pham, S.M., 2001. A clinically relevant CTLA4-Ig-based regimen induces chimerism and tolerance to heart grafts. *Ann. Thorac. Surg.*, **72**(4):1306-1310. doi:10.1016/S0003-4975(01)03066-1.

Liu, J., 1993. FK506 and cyclosporine, molecular probes for studying intracellular signal transduction. *Immunol. Today*, **14**(6):290-295. doi:10.1016/0167-5699(93)90048-P.

Manilay, J.O., Pearson, D.A., Sergio, J.J., Swenson, K.G., Sykes, M., 1998. Intrathymic deletion of alloreactive T cells in mixed bone marrow chimeras prepared with a nonmyeloablative conditioning regimen. *Transplantation*, **66**(1):96-102. doi:10.1097/00007890-199807150-00015.

Petersen, B.E., Bowen, W.C., Patrene, K.D., Mars, W.M., Sullivan, A.K., Murase, N., Boggs, S.S., Greenberger, J.S., Goff, J.P., 1999. Bone marrow as a potential source of hepatic oval cells. *Science*, **284**(5417):1168-1170. doi:10.1126/science.284.5417.1168.

Raimondo, M.L., Burroughs, A.K., 2002. Single-agent immunosuppression after liver transplantation: what is possible? *Drugs*, **62**(11):1587-1597.

Shirasugi, N., Adams, A.B., Durham, M.M., Lukacher, A.E., Xu, H., Rees, P., Cowan, S.R., Williams, M.A., Pearson, T.C., Larsen, C.P., 2002. Prevention of chronic rejection in murine cardiac allografts: a comparison of chimerism- and nonchimerism-inducing costimulation blockade-based tolerance induction regimens. *J. Immunol.*, **169**(5):2677-2684.

Theise, N.D., Badve, S., Saxena, R., Henegariu, O., Sell, S., Crawford, J.M., Krause, D.S., 2000. Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloahlation. *Hepatology*, **31**(1):235-240. doi:10.1002/hep.510310135.

Urdahl, K.B., Pardoll, D.M., Jenkins, M.K., 1994. Cyclosporin A inhibits positive selection and delays negative selection in αβ TCR transgenic mice. *J. Immunol.*, **152**(6):2853-2859.