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• A Model to Study the Liver's Role in Peripheral Tolerance



## The Goal of Transplantation

**L**iver transplantation has progressed remarkably since the first successful human liver transplant was performed in 1963. The surgical technique for the operation was quickly mastered, but understanding how to avoid rejection of the transplanted organ has been more difficult. With the discovery of cyclosporine and other immunosuppressive drugs, patient survival has risen to a high enough level that liver transplantation has long ceased to be considered experimental. Nevertheless, the ultimate goal of transplantation has yet to be achieved, which is acceptance of the transplanted organ without compromising the patient's overall immune system. This ideal state is referred to as "tolerance," in which the body accepts the grafted organ while yet defending itself against all other "foreign" substances.

There are two types of tolerance: "central" and "peripheral." Central tolerance occurs when immature lymphocytes encounter antigens and are deleted (the process of "negative selection," also called "clonal deletion," "programmed death," or "apoptosis.")

## The Influence of the Liver on Peripheral Tolerance

The liver has long been known to have a positive effect on the induction of peripheral tolerance. Patients who receive a combined liver-kidney transplant experience significantly less rejection of the kidney than patients receiving a kidney transplant alone. In both animals and humans, certain vascularized allografts have improved survival with the venous drainage via the portal vein into the liver. In mice, liver allografts (unlike heart or kidney allografts) are accepted spontaneously without the need for immunosuppression. The tolerance induced by liver allografts in these animals subsequently protects future donor hearts or skin grafts from acute and chronic rejection.

The liver is a major hematopoietic organ which gives birth to all leukocyte lineages, including extrathymic T cells, natural killer (NK) cells, natural killer T (NKT) cells, dendritic cells, and granulocytes. This unique combination of leukocytes in the liver may be the major cause of liver tolerogenicity. Extrathymic T cells during their development in the liver undergo incomplete negative selection. It is unknown whether the

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Peripheral tolerance occurs in peripheral lymph organs, such as the lymph nodes and spleen, where mature lymphocytes encounter antigens under particular conditions. Three principle mechanisms contributing to peripheral tolerance are: 1) clonal deletion, 2) clonal anergy (functional inactivation of lymphocytes without cell death), and 3) immune regulation (suppression of lymphocyte activity by regulatory T cells). These three mechanisms are not mutually exclusive.

mechanisms in the liver for clonal deletion for selecting naïve extrathymic T cells and for removing antigen-specific T cells to develop peripheral tolerance are linked. Studies have revealed that apoptotic cells adhere to liver sinusoidal endothelial cells (LSEC) in the periportal region. LSEC have been demonstrated to trap and induce apoptotic cells by an active receptor-mediated binding process. NKT cells have also been suggested as necessary for the formation of tolerance

induction by portal vein injection of antigens and necessary for the induction of oral tolerance. The exact mechanism of NKT cell tolerance is unknown. Therefore, several mechanisms of peripheral tolerance may be active in the hepatic immune system.

Antigen given via a mucosal route favors the induction of peripheral tolerance. This type of induced peripheral tolerance is commonly called "oral tolerance." In a dog model, the liver has been shown to play a critical role in oral tolerance induction. The mechanisms of the liver's role in oral tolerance induction are not clear.

### The Murine Transplant Model

Over the past year, we have used a murine transplant model to study the liver's role in inducing and maintaining peripheral tolerance induced via oral antigens (Fig. 1). We chose OVA (chicken albumin) in a low dose and a high dose as an agent to induce oral tolerance. Our unique model has allowed for removal and insertion of various liver combinations to facilitate study of the liver's role in the different mechanisms of peripheral tolerance.

For our study, we utilized male BALB/c mice, 8–12 weeks of age. The mice were divided into six groups according to how they received liver transplants: 1) and 2) OVA fed donor livers (low or high doses) to non-fed recipients; 3) and 4) non-fed donor livers to fed recipients (low or high doses); 5) Non-fed donor livers to non-fed recipients; and 6) non-transplanted, non-fed groups (the controls). Two days after the last feeding or after liver transplantation, all mice were immunized with 50 µg OVA emulsified in complete Freund's adjuvant (CFA) in a total of 50 µl injected at the base of the tail. Seven days after the immunization, 50 µg OVA in 30 µl dH<sub>2</sub>O was injected intradermally into the footpad. The footpad thickness was measured 24 or 48 hours later using digital calipers. The increase in the footpad thickness was determined by subtracting the naïve footpad thickness from the OVA-injected footpad thickness. Additionally, liver non-parenchymal cells (NPC) and spleen cells (SC) were isolated from OVA-fed BALB/c mice and adoptively transferred to naïve syngeneic mice through tail vein injection. The NPC and SC from non-fed mice were used as controls. Further OVA immunization was performed one day after adoptive transfer, and the delayed-type-hypersensitivity (DTH) response was examined 7 days after the immunization.

In addition to the above *in vivo* study measuring footpad thickness, we performed *in vitro* studies to measure the cytokine levels of IL-2, IFN-γ, and IL-10

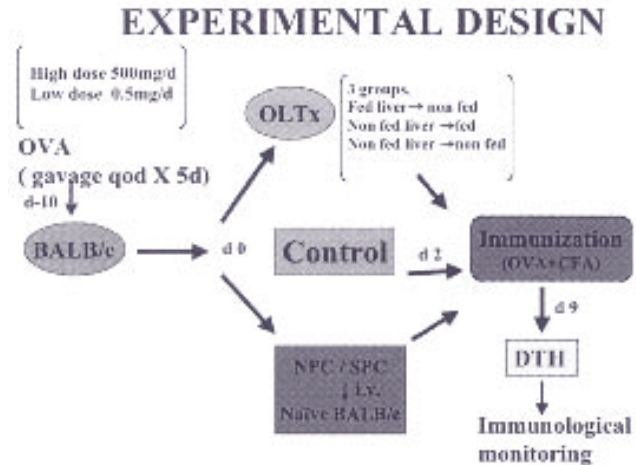


FIGURE 1: A model to study the liver's role in peripheral tolerance.

from mixed lymphocyte reaction (MLR) culture detected by ELISA.

### How the Model Demonstrated Induction and Transfer of Tolerance

To date, the results in our model can be summarized as follows:

- OVA feeding induced tolerance to OVA.
- The transplanted murine livers transferred tolerance from OVA fed mice to naïve mice.
- OVA feeding inhibited T cell proliferative activities of liver graft NPC and recipient spleen cells.
- OVA feeding inhibited IL-2 and increased IL-4 production of liver NPC and SC.
- Liver NPC from OVA-fed mice were capable of transferring tolerance to OVA to naïve mice.
- Removal of the liver from tolerant mice could not break the established tolerance.

Our experiments demonstrated that several sites, including the intestinal epithelial cells and gut-associated lymphoid tissue, are involved in peripheral tolerance induction to orally administered antigens. Furthermore, our results suggest that different mechanisms of tolerance are influenced differently by the liver depending on the dose of the antigen. Oral tolerance can be adoptively transferred by the NPC of the liver from either the low dose or high dose groups; however, the SC only from the low dose group can transfer tolerance. The high dose group is more tolerizing since the DTH response and the proliferative responses are significantly less than with the low dose group. Possibly with a lower dose, less antigen reaches the liver via the portal vein, and the gut lymph dominates the tolerance

mechanisms. With the higher dose more native antigen gets to the liver, and the liver with its relatively large size in proportion to body weight has an increased role in tolerance induction. This could help explain some controversies regarding the liver's role in peripheral tolerance.

Our results of the proliferative response and the cytokine profiles also suggest that the mechanisms of tolerance induction for the high dose and low dose fed livers are different. IL-10 is increased in both the NPC and SC in the low dose fed antigen group, but is not increased in the NPC of the high dose group. This indicates that the tolerance in the low dose fed group is more suggestive of a TH2 response, while that of the high dose fed group is not. Other reports have also indicated that IL-10 is enhanced in oral tolerance. Another mechanism involved with the immunologically diverse hepatic immune system is NKT cells. Our data is consistent with NKT cells being involved in the induction of oral tolerance, specifically for the high dose of antigen. Since our proliferative assay did not produce increased amounts of IFN- $\gamma$  production, this suggests that different lineages of NKT cells contribute to the induction of tolerance.

### Future Investigations and Remaining Questions

In addition to the work we have done so far, our model would also be useful in exploring several other proposed mechanisms of liver tolerance, including the role of Kupffer cells, liver sinusoidal endothelial cells,  $\gamma/\delta$  cells, and immature dendritic cells. The model could be manipulated to include transplanting livers between different genders, transplanting irradiated livers and other variations, and it could be used to study specific cell types previously unable to be evaluated.

We have shown that the liver is sufficient to transfer tolerance, but several other questions remain. Is the liver's role in peripheral tolerance unique to itself? Are the mechanisms of inducing tolerance operational in all peripheral nodal tissues? Does the liver's perceived influence come only from its large relative size? Are there clinical applications to the liver's role in peripheral immune tolerance, such as lowering the rejection of organ allografts, and preventing autoimmune disease, chronic viral infections of the liver, and cancer metastases to the liver? We look forward to using our model in further studies to elucidate the mechanisms of this tolerance induction. The end goal is to offer new therapeutic approaches for unsolved problems.

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