UNIVERSITY OF CATANIA

SCHOOL OF MEDICINE

Doctor of Philosophy in Advanced Multidisciplinary Research in Transplantatation - Cycle XXVI

(Director: Prof. Pierfrancesco Veroux)

Burcin Ekser, MD

THESIS OF DOCTOR OF PHILOSOPHY

Research Project I: Histopathology of livers and native organs of recipients following genetically-engineered pig-to-baboon liver xenotransplantation

Research Project II: Strategies to prevent adaptive immune response in xenotransplantation: MHC Class II knockdown pigs and the use of costimulation blockade regimens

Supervisor: Prof. Massimiliano Veroux, MD, PhD

Co-supervisor: Prof. David K.C. Cooper, MD, PhD

QUADRIENNIAL 2010-2014

UNIVERSITA' DEGLI STUDI DI CATANIA

FACOLTA' DI MEDICINA E CHIRURGIA

Dottorato di Ricerca in Ricerca Multidisciplinare Avanzata Nei Trapianti

Ciclo XXVI

(Direttore: Prof. Pierfrancesco Veroux)

Burcin Ekser, MD

TESI DI DOTTORATO DI RICERCA

<u>Progetto di Ricerca I</u>: Istopatologia di fegati e di organi nativi del babbuino ricevente dopo lo xenotrapianto di fegato da maiale geneticamente modificato

<u>Progetto di Ricerca II</u>: Strategie per prevenire la risposta immunitaria adattiva dello xenotrapianto: maiali MHC Classe II knockdown e l'uso di regime di immunosoppressione basato al blocco di costimolazione

Relatore: Chiar.mo Prof. Massimiliano Veroux

Correlatore: Chiar.mo Prof. David K.C. Cooper

QUADRIENNIO 2010-2014

…aileme…

…to my family…

…alla mia famiglia…

TABLE OF CONTENTS

CHAPTER 2 – MATERIALS AND METHODS – PROJECT 1 – HISTOPATHOLOGY OF LIVER XENOTRANSPLANTATION

CHAPTER 3 – RESULTS – PROJECT 1 – HISTOPATHOLOGY OF LIVER XENOTRANSPLANTATION

CHAPTER 6 – MATERIALS AND METHODS – PROJECT 2 – STRATEGIES TO PREVENT ADAPTIVE IMMUNE RESPONSE IN XENOTRANSPLANTATION

CHAPTER 7 – RESULTS – PROJECT 2 – STRATEGIES TO PREVENT ADAPTIVE IMMUNE RESPONSE IN XENOTRANSPLANTATION

ABSTRACT

Background: Xenotransplantation using pig organs could resolve the shortage of suitable donor organs. If pig organs could be transplanted successfully into human patients, the advantages would be numerous. The supply of organs would be prompt and unlimited, they would be available electively when needed, and the organ-source pig would be known to be free of specific microbes that might cause morbidity in the recipient. Genetically-engineered pigs could provide livers that might bridge the patient to allotransplantation. Although the rapid development of thrombocytopenia and its understanding are important to prolong liver xenografts, detailed histopathology of liver xenografts and native organs of recipients carries an important weight on the outcome.

Cellular-based adaptive immune response plays a critical role, which limits prolonged xenograft survival. Costimulatory blockade was shown to be effective on CD40/CD154 pathway to prevent adaptive immune response with anti-CD154mAb. However, it will likely not be available for clinical use due to its side effects, such as thromboembolic events. We evaluated whether CD154 pathway blockade could be replaced by CD28 pathway blockade (abatacept or belatacept) or by blockade of both pathways (using an anti-CD40mAb+belatacept). We also explored whether the transplantation of a patch from a pig with a mutant human MHC class II transactivator (CIITA-DN) reduced the T cell response.

Methods: In project 1, orthotopic liver transplantation was carried out in baboons using wild-type (WT, n = 1) or genetically-engineered pigs (α1,3-galactosyltransferase gene-knockout, GTKO), n = 1; GTKO pigs transgenic for human CD46, n = 7) and a clinically-acceptable immunosuppressive regimen. Biopsies were obtained from the WT pig liver

pre-transplantation and at 30 min, 1, 2, 3, 4 and 5 h post-transplantation. Biopsies of genetically-engineered livers were obtained pre-transplantation, 2 h after reperfusion and at necropsy (4–7 days after transplantation). The histopathology of livers and major native organs (i.e., heart, lung, kidneys, small intestine) and lymph nodes will be studied by direct light microscopy (H&E staining), immunohistology (immunofluorescence and immunohistochemistry staining), and electron microscopy to determine morphological changes in the liver and native organs;

(i) descriptive histopathology (light and electron microscopy)

(ii) baboon antibody deposition in the liver (IgG, IgM, and IgE) (immunohistology)

(iii) cellular infiltrates in the liver (T cells, B cells, macrophages, neutrophils) *(immunohistology)*

(iv) complement deposition in the liver (C3, C4d, C5b-9) *(immunohistology)*

(v) thrombotic microangiopathy in the liver and native organs (fibrin deposition and platelets) *(immunohistology)*

In project 2, with the knowledge of our previous experience in the pig-to-baboon artery patch model, we knew that with no immunosuppressive therapy, an artery graft from GTKO pig induces a significant adaptive immune response, which is effectively prevented by anti-CD154mAb-based immunosuppressive therapy. In the present project, twelve baboons (n=12) received carotid artery patch transplants (2x1cm into the wall of the abdominal aorta) from genetically-engineered pigs (either GTKO/CD46/CIITA-DN; Group 1 n=8 or GTKO/CD46; Group 2 n=4). One baboon in Group 1 received no immunosuppressive therapy. Group 1A (n=2) received CD28 pathway blockade only with abatacept-based regimen. Groups 1B (n=3) received CD28 pathway blockade only with belatacept-based regimen and Group 1C (n=2) received a regimen based on costimulation blockade of both the CD28/B7 and CD40/CD154 pathways, i.e., belatacept+anti-

CD40mAb (2C10R4), as in Group 2. Mixed lymphocyte reaction (MLR) to pig cells, and antibody responses to pig antigens (IgM and IgG) were monitored. After 28 or 48 days, the artery grafts were examined microscopically.

Results: In project 1, the WT pig liver underwent hyperacute rejection. After genetically engineered pig liver transplantation, hyperacute rejection did not occur. Survival was limited to 4–7 days due to repeated spontaneous bleeding in the liver and native organs (as a result of profound thrombocytopenia) which necessitated euthanasia. At 2 h, graft histology was largely normal. At necropsy, genetically-engineered pig livers showed hemorrhagic necrosis, platelet aggregation, platelet-fibrin thrombi, monocyte/macrophage margination mainly in liver sinusoids, and vascular endothelial cell hypertrophy, confirmed by confocal and electron microscopy. Immunohistochemistry showed minimal deposition of IgM, and almost absence of IgG, C3, C4d, C5b-9, and of a cellular infiltrate, suggesting that neither antibody- nor cell-mediated rejection played a major role.

In project 2, when GTKO/CD46/CIITA-DN grafts were transplanted (Group 1), but no immunosuppression was administered, an elicited anti-pig antibody response, a proliferative T cell response on MLR, and intense cell infiltration of the graft were documented. When CD28/B7 pathway blockade was administered (Group 1A), no proliferative response was seen in T cells on MLR, and the elicited antibody response was greatly attenuated. It was only when both pathways were blocked that no elicited antibody response was documented (Groups 1C and 2). In these cases, cell infiltration of the graft was minimal or absent. In recipients of CIITA-DN grafts with both CD28/B7 and CD40/CD154 costimulatory pathway blockades (Group 1C), there was no proliferative response on T cells post-transplantation, elicited antibody response was the lowest (almost none), and there was no cellular infiltration in the graft.

Only blockade of both pathways (Group 1C) prevented T cell proliferative and elicited antibody responses and cell infiltration of the graft. In order to determine whether the CIITA-DN mutation was playing a role, this regimen was administered to four baboons receiving grafts from GTKO/CD46 pigs (Group 2). No proliferative or elicited antibody responses were documented and there was no significant cell infiltration of the graft. However, when the results in Group 1A were compared with those of a historic group (GTKO only) in which CD28/B7 pathway blockade was administered, the elicited antibody response was significantly reduced when the graft expressed the CIITA mutation.

Conclusions: In project 1, after GTKO and GTKO/CD46 pig liver Tx in baboons, the rapid development of a profound thrombocytopenia was by far the major problem seen, and was suggested as the major causative factor in the hemorrhage that occurred, not only in the pig liver grafts, but also in several native organs and body cavities. The histopathologic features described can largely be explained on this basis. However, the factors influencing the activation and consumption of platelets, and therefore the development of coagulopathy, need to be studied in details. Since the features of rejection were minimal or absent, we believe that, if platelet activation and aggregation/phagocytosis can be prevented, possibly by further genetic modification of the pigs or by novel therapeutic agents, bridging by a pig liver to allotransplantation may become a feasible clinical option.

In project 2, blockade of both the CD40/CD154 and CD28/B7 pathways is required to prevent the baboon adaptive response to a pig artery patch graft. If the graft is taken from a CIITA-DN pig, then the adaptive response is reduced. The presence of a CIITA-DN graft reduced the baboon T cell-dependent anti-nonGal IgG response. This was particularly obvious and beneficial when the immunosuppressive therapy administered was inadequate to control the T cell response, e.g., when abatacept was administered.

SUMMARY (IN ITALIAN)

Introduzione: Se lo xenotrapianto dovesse diventare una realtà, potrebbe portare ad una fonte illimitata di organi di qualsiasi dimensioni e tipo per trapianti umani. Attualmente i maiali sono considerati la fonte preferita da specie animali per lo xenotrapianto clinico. La mancata disponibilità di fegati da donatori deceduti e la rapida evoluzione clinica dell'insufficienza epatica severa portano una elevata mortalità dei pazienti con insufficienza epatica fulminante. In queste condizioni, maiali geneticamente modificati resi piu' "simili" all'uomo potrebbero fungere da 'ponte' all'allotrapianto. Il trapianto di fegato ortotopico da maiali geneticamente modificati in babbuini è stato sperimentato per determinare se questi organi possano fungere da 'ponte'. Sebbene la comprensione dei motivi della trombocitopenia severa osservata subito dopo il trapianto e' importante, l'istopatologia dettagliata dei fegati di maiali e degli organi nativi porta un importante peso sull'outcome.

La risposta immunitaria adattiva cellulare e umorale ha un ruolo fondamentale per prolungare la sopravvivenza dello xenotrapianto. Il blocco di costimolazione ha dimostrato di essere efficace su CD40/CD154 pathway per prevenire la risposta immunitaria adattativa con l'anticorpo anti-CD154mAb. Tuttavia, l'anti-CD154mAb non e' disponibile per uso clinico a causa dei suoi significativi eventi tromboembolici. Abbiamo valutato se il blocco di CD154 pathway potrebbe essere sostituito dal blocco CD28 pathway (abatacept o belatacept) o dal blocco di entrambi i pathway (anti-CD40mAb+belatacept). Abbiamo anche esplorato se la risposta delle cellule T va ridotta con il trapianto del patch dell'arteria da un maiale con un mutante MHC di classe II umano transattivatore (CIITA-DN).

Materiali e Metodi: Progetto 1, sono stati eseguiti 9 xenotrapianti di fegato: 1 da maiale non-modificato (WT), 1 da maiali con α1,3-galactosyltransferase gene-knockout (GTKO), 7

da maiali con GTKO/CD46. L'istopatologia di fegati e dei principali organi nativi (ad. es, cuore, polmoni, reni, intestino tenue) e dei linfonodi e' stata esaminata dalla microscopia diretta (H&E colorazione), immunoistologia (immunofluorescenza ed immunoistochimica), e microscopia elettronica per determinare i cambiamenti morfologici nel fegato e negli organi nativi;

(i) istopatologia descrittiva *(microscopia diretta ed elettronica)*

(ii) deposizione dell'anticorpo del babbuino nel fegato di maiale (IgG, IgM, e IgE) *(immunoistologia)*

(iii) infiltrati cellulari nel fegato di maiale (cellule T, cellule B, macrofagi, neutrofili) *(immunoistologia)*

(iv) deposizione del complemento nel fegato di maiale (C3, C4d, C5b-9) *(immunoistologia)*

(v) microangiopatia trombotica nel fegato e negli organi native (deposizione della fibrin e delle piastrine) *(immunoistologia)*

Progetto 2, con la conoscenza della nostra esperienza precedente nel modello del trapianto del patch dell'arteria da maiale a babbuino, sapevamo che senza terapia immunosoppressiva, un innesto arteria da GTKO maiale induce una risposta immunitaria adattativa significativa, tutto ciò che è effettivamente impedita da immunosoppressivo a base di anti-CD154mAb terapia. Nel presente progetto, dodici babbuini hanno ricevuto trapianti di patch dell'arteria (2x1cm nella parete dell'aorta addominale) da maiali geneticamente modificati (GTKO/CD46/CIITA-DN; Gruppo 1, n=8 o GTKO/CD46; Gruppo 2, n=4). Un babbuino nel Gruppo 1 non ha ricevuto terapia immunosoppressiva. Il Gruppo 1A (n=2) ha ricevuto la terapia con abatacept (blocco di CD28 pathway). Il Gruppo 1B (n=3) ha ricevuto la terapia con belatacept (blocco di CD28 pathway) e il Gruppo 1C (n=2) ha ricevuto un regime basato sul blocco di costimolazione di 2 pathways CD28/B7 e

CD40/CD154 con belatacept+anti-CD40mAb (2C10R4), come nel Gruppo 2. La reazione linfocitaria mista (MLR) verso le cellule di maiale e le risposte anticorpali verso antigeni di maiale (IgM e IgG) sono stati monitorati. Gli innesti dell'arteria sono stati istologicamente esaminati dopo 28 o 48 giorni dallo xenotrapianto.

Risultati: Progetto 1, il fegato da maiale WT ha presentato un rigetto iperacuto, pero' il rigetto iperacuto non e' successo con il maiale geneticamente modificato. La sopravvivenza oltre 7 giorni è stata impedita da una profonda trombocitopenia che si e' sviluppata entro un'ora dopo la riperfusione del trapianto, risultando nell'emorragia spontanea in diversi organi. L'istopatologia dopo 2 ore era, in gran parte, normale, ma all'autopsia, i fegati di maiale geneticamente modificato hanno mostrato la necrosi emorragica, l'aggregazione piastrinica, i trombi formati da piastrine-fibrina, una marginazione dei monociti-macrofagi soprattutto nei sinusoidi epatici ed ipertrofia delle cellule endoteliali vascolari, confermata da microscopia confocale ed elettronica. L'immunoistochimica ha evidenziato la minima deposizione di IgM e quasi mancanza di IgG, C3, C4d, C5b-9, e di infiltrato cellulare, suggerendo che né il rigetto cellulare né il rigetto umorale gioca un ruolo importante.

Progetto 2, quando le arterie da maiali di GTKO/CD46/CIITA-DN sono state trapiantate (Gruppo 1), senza l'immunosoppressione, una risposta anticorpale e proliferativa delle cellule T, e l'infiltrazione cellulare nell'arteria e' stata documentata. Quando il blocco di CD28/B7 pathway è stato somministrato (Gruppo 1A), nessuna risposta proliferativa si e' osservata, ma la risposta umorale era presente. La risposta umorale era bloccato soltanto quando gli entrambi pathway sono state bloccate (Gruppi 1C e 2). In questi casi, l'infiltrazione cellulare nell'innesto arteriale era minima o mancante. Nei riceventi dell'arteria di CIITA-DN con il blocco di entrambi pathway di CD28/B7 e CD40/CD154

(Gruppo 1C), non vi era alcuna risposta proliferativa delle cellule T, ne' una risposta umorale, ne' l'infiltrazione cellulare nell'arteria. Per determinare se la mutazione CIITA-DN Il Gruppo 2 (GTKO/CD46) ha ricevuto lo stesso regime immunosuppressivo. Come il Gruppo 1C nessuna risposta significativa immunitaria adattiva e' stata vista. Tuttavia, quando i risultati del Gruppo 1A sono stati confrontati con quelli del gruppo storico (GTKO) con il blocco di CD28/B7 pathway, la risposta umorale è stata significativamente ridotta quando l'arteria veniva dal maiale CIITA-DN.

Conclusioni: Progetto 1, dopo il trapianto di fegato dai maiali GTKO e GTKO/CD46, lo sviluppo rapido di trombocitopenia profonda era il problema principale ed e' stato suggerito come il fattore principale dell'emorragia. Le caratteristiche istopatologiche descritte possono essere in gran parte spiegato su questa base. Tuttavia, i fattori che influenzano l'attivazione e il consumo di piastrine, e quindi lo sviluppo di coagulopatia, devono essere studiati in dettaglio. Poiché le caratteristiche del rigetto erano minime o assenti, crediamo che, se l'attivazione e l'aggregazione o la fagocitosi delle piastrine potesse essere impedita, possibilmente con una ulteriore modifica genetica o con gli agenti terapeutici, l'opzione del 'ponte' all'allotrapianto con il fegato di maiale potrebbe diventare una verita' clinica.

Progetto 2, ha dimostrato che il blocco di entrambi pathway CD40/CD154 e CD28/B7 è necessario per evitare la risposta immunitaria adattativa dello xenotrapianto. Il maiale CIITA-DN riduce la risposta proliferativa delle cellule T e la risposta umorale, percio' la risposta immunitaria adattativa. Questo era particolarmente evidente quando la terapia immunosoppressiva somministrata era insufficiente per controllare la risposta delle cellule T, ad esempio la terapia con abatacept.

ACKNOWLEDGEMENTS

I would like to thank Prof. Massimiliano Veroux and Prof. Pierfrancesco Veroux of the University of Catania for their guidance during my research years in doctor of philosophy. They definitely gave their full support to me while I was doing my research at the Thomas E. Starzl Transplantation Institute, University of Pittsburgh Medical Center. They fully understood my needs and supported my research and clinical positions.

I would like to specially thank Prof. David K.C. Cooper of the University of Pittsburgh Medical Center for his exceptional mentorship and fatherhood. Besides being an extraordinary teacher, he is an amazing person who supports his mentees in any area during their lives. I learned a lot from him. He gave me an instinct and desire. I am so honored to having worked with him. He is definitely a big part of what I present in this thesis. His knowledge in xenotransplantation is endless which saved me and amazed me in many situations.

I should also send special thanks to my other mentor in the research of liver xenotransplantation, Prof. Bruno Gridelli of the University of Pittsburgh Medical Center, Palermo (ISMETT – Istituto Mediterraneo per i Trapianti e Terapie ad Alta Specializzazione). He is a wonderful person with full of desire and ambition on liver surgery. Without his expertise, liver xenotransplantation would not be performed.

As two big studies they are, the research was done with the help of many other important colleagues of the Dr. Cooper's laboratory; Hayato Iwase, Hidetaka Hara, Mohamed Ezzelarab, Cassandra Long, Eefje M. Dons, Dirk J. van der Windt, Goutham Kumar, and

many other friends from the Starzl Transplantation Insitute. I cannot forget my hospital-free friend Guray S. Uner, PhD and all bike ridings, tennis games, and kayaking. He is definitely a big part of my healthy-being.

Lastly, I thank my family for their immense support to realize my dreams. They are the power behind my success.

Thank you very much all of you,

With my best wishes,

Burcin Ekser, MD

DECLARATION

- This thesis comprises my original work during the years of research in doctor of philosophy at the University of Catania and at the Thomas E. Starzl Transplantation Institute, University of Pittsburgh Medical Center, except where indicated.
- The research on xenotransplantation in the Thomas E. Starzl Transplantation Institute of the University of Pittsburgh was supported in part by the National Institute of Health (NIH) of the U.S.A grants # U01 AI068642 and # R21 A1074844 of Prof. David K.C. Cooper, by UPMC grant of Prof. Bruno Gridelli, and by Sponsored Research Agreements between the University of Pittsburgh and Revivicor, Inc., Blacksburg, VA. The baboons were provided by the Oklahoma University Health Sciences Center, Division of Animal Resources, which is supported in part by NIH P40 sponsored grant RR012317-09.
- I was a recipient of a National Institutes of Health (NIH), NIAID (National Institute of Allergy and Infectious Disease) Interdisciplinary Training Grant in Transplantation Biology Ruth L. Kirschstein National Research Service Award NIH NIAID T32 AI074490
- I was a recipient of a “Travel Grant” to attend 2011 Genzyme Annual Fellows Conference (Advances in Organ Transplantation), April 7-9, 2011, Cambridge, MA, USA. Oral abstract presentation ‘Genetically-modified pig liver xenotransplantation as a bridge to allotransplantation: in vivo and in vitro studies’.

PRESENTATIONS

Presentations performed at international congresses during the years of research in doctor of philosophy.

2011

1. **Ekser B**, Lin CC, Ezzelarab M, Long C, Echeverri GJ, Hara H, Klein K, Phelps C, Ayares D, Cooper DKC, Gridelli B. Genetically-modified pig liver xenotransplantation as a bridge to allotransplantation: in vivo and in vitro studies. 2011 Genzyme Annual Fellows Conference. April 7-9, 2011, Cambridge, MA, USA. **(Oral presentation).**

2. Silvestre C, Furian L, Baldan N, **Ekser B**, Marchini B, Rossi B, Bonfante L, Valente M, Rigotti P. Long term results of a large experience of double kidney transplantation (DKT) performed at a single center. XV European Society for Organ Transplantation Congress, 4-7 Sep 2011, Glasgow, Scotland, UK. (**Oral presentation**). Transplant Int 2011; S2: 1

3. **Ekser B,** Long C, Kumar G, Hara H, Dons EM, Thacker J, Toyoda Y, Veroux M, Ayares D, Cooper DKC, Ezzelarab M. Heart xenotransplantation using genetically-engineered pigs: can CTLA4-Ig replace anti-CD154mAb in the immunosuppressive regimen? Cell Transplantation Society (CTS) - International Xenotransplantation Association (IXA) Joint Congress, 23-26 Oct 2011, Miami, FL, USA. **(Oral Presentation**). Xenotransplantation 2011; 18: 284

4. Dons EM, Montoya C, Hara H, Long C, Echeverri G, **Ekser B**, Ezzelarab M, Roa D, Wolf RF, Ezzelarab M, West LJ, Cooper DKC. T cell-directed immunosuppressive therapy prevents development of natural anti-AB and anti-pig antibodies in infant baboons. Cell Transplantation Society (CTS) - International Xenotransplantation Association (IXA) Joint Congress, 23-26 Oct 2011, Miami, FL, USA. **(Oral**

Presentation). Xenotransplantation 2011; 18: 273

5. **Ekser B,** Lin CC, Long C, Echeverri GJ, Hara H, Ayares D, Cooper DKC, Gridelli B, Ezzelarab M. Where do platelets go after pig-to-baboon liver xenotransplantation? Evidence for platelet-WBC aggregation. Cell Transplantation Society (CTS) - International Xenotransplantation Association (IXA) Joint Congress, 23-26 Oct 2011, Miami, FL, USA. **(Oral Presentation**). Xenotransplantation 2011; 18: 288

6. Wijkstrom M, Bertera S, **Ekser B,** Long C, Hara H, Ezzelarab M, Dons E, Kumar G, van der Windt DJ, Votyakova T, Phelps C, Cooper DKC, Bottino R. Gal-knockout pigs transgenic for hCD46 and islet-specific TFPI/pCTL A4Ig have normal glucose metabolism and isolated islets exhibit normal glucose and arginine responses and should be considered as donors for islet xenotransplantation. Cell Transplantation Society (CTS) - International Xenotransplantation Association (IXA) Joint Congress, 23-26 Oct 2011, Miami, FL, USA. ***(Oral Presentation***) **(Abstract awarded with Travel Grant)**. Xenotransplantation 2011; 18: 275

7. Ezzelarab M, **Ekser B**, Isse K, Long C, Thacker J, Murase N, Shapiro R, Ayares D, Cooper DKC. Elevated C-reactive protein levels precede consumptive coagulopathy in GTKO pig organ xenograft recipients. Cell Transplantation Society (CTS) - International Xenotransplantation Association (IXA) Joint Congress, 23-26 Oct 2011, Miami, FL, USA. (**Oral Presentation**). Xenotransplantation 2011; 18: 287

8. Ezzelarab M, Ezzelarab C, Isse K, **Ekser B**, Echeverri GJ, Ayares D, Cooper DKC. T cell-directed immunosuppression is associated with downregulation of thrombin activation, but upregulation of inflammatory responses in xenograft recipients.Cell Transplantation Society (CTS) - International Xenotransplantation Association (IXA) Joint Congress, 23-26 Oct 2011, Miami, FL, USA. *(**Oral Presentation***). Xenotransplantation 2011; 18: 272

9. Iwase H, **Ekser B**, Zhou H, Dons EM, Cooper DKC, Ezzelarab M. Studies on platelet aggregation in human and nonhuman primates. Cell Transplantation Society (CTS) - International Xenotransplantation Association (IXA) Joint Congress, 23-26 Oct 2011,

Miami, FL, USA. (**Late Breaking**). Xenotransplantation 2011; (abstract book) (**Poster Presentation**)

2012

10. Can MF, Mejia JC, Urkan M, **Ekser B**, Humar A. A trend analysis of the UNOS data for liver transplantation and deceased donor liver utilization in the United States. ESSR 2012 47th Congress, Lille, France, June 6-9, 2012.

11. Ezzelarab M, Isse K, **Ekser B**, Ayares D, Cooper DKC. Does soluble (s)CD154 play a role in promoting the adaptive immune response in xenograft recipients? American Transplant Congress, 2-6 June 2012, Boston, MA, USA. **(Late breaking Poster presentation).** Am J Transplant 2012; 12 (suppl 3): 360 (Abstract LB23)

12. Ezzelarab M, Isse K, **Ekser B**, Ayares D, Cooper DKC. Systemic inflammation in xenograft recipients (SIXR) precedes activation of coagulation in the presence of T cell-directed immunosuppression. XXIV Congress of The Transplantation Society, Berlin, Germany, July 15-20, 2012 ***(Oral Presentation).*** Transplantation 2012; 94 (suppl 10S): 69 (abstract 2292)

13. Can MF, Meija JC, Urkan M, **Ekser B,** Humar A. Amerika Birlesik Devletleri'nde karaciger nakli ve kadavra donor karaciger kullanimi icin OPTN (organ procurement and transplantation network) verilerinin trend analizi. 18. Ulusal Cerrahi Kongresi, 23-27 May 2012, Izmir, Turkey. (Turkish) (**Poster presentation).**

14. Iwase H, **Ekser B**, Satyananda V, Hara H, Ezzelarab M, Ayares D, Cooper DKC. Human platelet activation/aggregation induced by pig vascular endothelial cells. Thomas E. Starzl Transplantation Institute Fall Scientific Retreat 2012 (**Poster presentation**).

2013

15. Iwase H, Satyananda V, **Ekser B,** Bhama JK, Hara H, Ezzelarab M, Bajona P, Wijkstrom M, Phelps C, Ayares D, Cooper DKC. Human platelet aggregation and thrombotic microangiopathy in a pig cardiac xenograft is reduced by the expression of human thrombomodulin. International Society of Heart and Lung Transplantation, April 24-27, 2013, Montreal, Canada. **(Oral presentation)**. J Heart Lung Transplant 2013; Suppl 4: S137

16. **Ekser B**, Goggins WC, Mangus RS, Shah AP, Beduschi T, Kubal CA, Fridell JA, Powelson JA, Vianna RM, Tector AJ. Combined liver-kidney transplantation: simultaneous versus delayed transplantation of the kidney graft. American Transplant Congress, 18-22 May 2013, Seattle, WA, USA. **(Poster presentation).** Am J Transplant 2013; 13 (Suppl 5): 526 (Abstract #D1679).

17. Iwase H, Satyananda V, **Ekser B,** Hara H, Ezzelarab M, Phelps C, Ayares D, Cooper DKC. Expression of human coagulation-regulatory proteins on pig vascular endothelial cells reduces in vitro human platelet aggregation. International Xenotransplantation Association (IXA), 10-13 Nov 2013, Osaka, Japan. **(Oral presentation)**. Xenotransplantation 2013; 20: 344-345 (Abstract 286)

18. Iwase H*, **Ekser B*,** Satyananda V, Ezzelarab M, Cooper DKC. Plasma free triiodothyronine (fT3) levels in baboons undergoing pig organ transplantation; relevance to early recovery of organ function (*contributed equally). International Xenotransplantation Association (IXA), 10-13 Nov 2013, Osaka, Japan. **(Oral presentation)**. Xenotransplantation 2013; 20: 339-340 (Abstract 267)

19. Iwase H, **Ekser B,** Bhama JK, Satyananda V, Hara H, Ezzelarab M, Bajona P, Wijkstrom M, Long C, Phelps C, Klymiuk N, Wolf E, Ayares D, Cooper DKC. Expression of human thrombomodulin delays the development of thrombotic microangiopathy and consumptive coagulopathy in pig-to-baboon heart transplantation. International Xenotransplantation Association (IXA), 10-13 Nov 2013, Osaka, Japan. **(Oral presentation)**. Xenotransplantation 2013; 20: 329-330 (Abstract

235)

20. Iwase H, Wolfe R, **Ekser B**, Ezzelarab M, Hara H, White G, Cooper DKC. Are there advantages in the use of specific-pathogen-free baboons in pig organ transplantation models? International Xenotransplantation Association (IXA), 10-13 Nov 2013, Osaka, Japan. **(Oral presentation)**. Xenotransplantation 2013; 20: 330-331 (Abstract 240)

21. Iwase H, Ezzelarab M, Satyananda V, **Ekser B**, Bhama JK, Bajona P, Wijkstrom M, Hara H, Phelps C, Dai Y, Ayares D, Cooper DKC. Sensitization to nonGal antigens is significantly reduced in baboons with artery patch grafts from GTKO pigs transgenic for a mutant human MHC class II transactivator (CIITA). International Xenotransplantation Association (IXA), 10-13 Nov 2013, Osaka, Japan. **(Oral presentation)**. Xenotransplantation 2013; 20: 343 (Abstract 279)

22. Satyananda V, **Ekser B**, van der Windt DJ, Hara H, Ezzelarab M, Cooper DKC. What progress has been made in pig-to-nonhuman primate transplantation models during the past 15 years? A retrospective review of the literature. International Xenotransplantation Association (IXA), 10-13 Nov 2013, Osaka, Japan. **(Oral presentation)**. Xenotransplantation 2013; 20: 326 (Abstract 225)

23. Iwase H, Humar R, Humar P, Ezzelarab M, Satyananda V, **Ekser B**, Bhama JK, Hara H, Bajona P, Wijkstrom M, Phelps C, Ayares D, Cooper DKC. Do genetically-engineered pig-to-baboon artery patch transplants reflect the coagulation dysregulation/inflammation seen after pig-to-baboon heart transplants? International Xenotransplantation Association (IXA), 10-13 Nov 2013, Osaka, Japan. **(Oral presentation)**. Xenotransplantation 2013; 20: 357 (Abstract 357)

24. Iwase H, Satyananda V, **Ekser B,** Bhama BK, Hara H, Ezzelarab M, Bajona P, Wijkstrom M, Phelps C, Ayares D, Cooper DKC. Combined CD40-CD154/CD28-B7 costimulation blockade successfully replaces anti-CD154mAb in preventing a T cell response to genetically-engineered pig artery patch transplants in baboons International Xenotransplantation Association (IXA), 10-13 Nov 2013, Osaka, Japan.

(Oral presentation). Xenotransplantation 2013; 20: 326-327 (Abstract 226)

25. Kumar G, Satyananda V, Fang J, Zhou H, Fujita M, **Ekser B,** Long C, Dons E, Sun Q, Ayares D, Hara H, Cooper DKC. Correlation between anti-pig antibody levels in human and geographic location during childhood: implications for clinical xenotransplantation. International Xenotransplantation Association (IXA), 10-13 Nov 2013, Osaka, Japan. **(Oral presentation)**. Xenotransplantation 2013; 20: 331 (Abstract 242)

26. Ezzelarab M, Isse K, **Ekser B,** Iwase H, Morelli A, Ayares D, Cooper DKC. Does soluble CD154 (CD40 ligand) promote elicited anti-pig IgG antibodies in xenograft recipients? International Xenotransplantation Association (IXA), 10-13 Nov 2013, Osaka, Japan. **(Oral presentation)**. Xenotransplantation 2013; 20: 340-341 (Abstract 272)

PUBLICATIONS

Manuscripts already published, in press, submitted, or in preparation generated during the years of research in doctor of philosophy.

2011

1. **Ekser B**, Furian L, Rigotti P. Optimizing allocation of kidneys from older donors: between resources and results. ***American Journal of Transplantation 2011;*** *11: 183 (IF= 6.192)*

2. **Ekser B,** Kumar G, Veroux M, Cooper DKC. Therapeutic issues in the treatment of vascularized xenotransplants using Gal-Knockout donors in nonhuman primates. ***Current Opinion in Organ Transplantation 2011;*** *16: 222-230 (IF= 2.818)*

3. De Pasquale C, Pistorio ML, Veroux P, Giuffrida G, Sinagra N, **Ekser B**, Zerbo D, Corona D, Giaquinta A, Veroux M. Quality of life in kidney transplantation from marginal donors. ***Transplantation Proceedings 2011;*** *43: 1045-1047 (IF= 0.993)*

4. Veroux M, Corona D, **Ekser B**, Giaquinta A, Tallarita A, De Martino C, Gentile F, Virgilio C, Gagliano M, Fiamingo P, Veroux P. Kidney transplantation from Hepatatis B virus core antibody-positive donors: prophylaxis with Hepatitis B immunoglobulin. ***Transplantation Proceedings 2011;*** *43: 967-970 (IF= 0.993)*

5. Buizza V, Feltrin A, Moretti M, Pasquato S, Furian L, **Ekser B**, Bonfante L, Rossi B, Marchini F, Rigotti P. Quality of life in aged kidney transplant recipients. ***Organs, Tissues & Cells 2011***; 14: 189-196 *(IF= 0)*

6. **Ekser B**, Gridelli B, Veroux M, Cooper DKC. Clinical pig liver xenotransplantation: how far do we have to go? ***Xenotransplantation 2011;*** *18: 158-167 (IF= 2.565)*

7. Ezzelarab M*, **Ekser B***, Gridelli B, Iwase H, Ayares D, Cooper DKC. Thrombocytopenia after pig-to-baboon liver xenotransplantation: Where do platelets go? (*shared first authors). ***Xenotransplantation 2011;*** *18: 320-327 (IF= 2.565)*

2012

8. **Ekser B**, Klein E, He J, Stolz DB, Echeverri GJ, Long C, Lin CC, Ezzelarab M, Hara H, Veroux M, Ayares D, Cooper DKC, Gridelli B. Genetically-engineered pig-to-baboon liver xenotransplantation: histopathology of xenografts and native organs. ***PLoS ONE 2012;*** *7 (1): e29720 (IF= 4.411)*

9. **Ekser B,** Ezzelarab M, Hara H, van der Windt DJ, Wijkstrom M, Bottino R, Trucco M, Cooper DKC. Clinical xenotransplantation: the next great medical revolution? ***Lancet 2012;*** *379 (9816): 672-683 (IF= 33.633)*

10. **Ekser B**, Gridelli B, Cooper DKC. Porcine alanine transaminase after liver allo- and xeno-transplantation. ***Xenotransplantation 2012;*** *19: 52-55 (IF= 2.565)*

11. Manji RA, Menkis AH, **Ekser B,** Cooper DKC. The future of bioprosthetic heart valves. ***Indian Journal of Medical Research 2012;*** *135: 150-151 (IF= 1.520)*

12. Dons EM, Montoya C, Long CE, Hara H, Echeverri GJ, **Ekser B**, Ezzelarab C, Medellin DR, van der Windt DJ, Murase N, Rigatti LH, Wagner R, Wolf RF, Ezzelarab M, West LJ, Ijzermans JNM, Cooper DKC. T cell-based immunosuppressive therapy inhibits the development of natural antibodies in infant baboons. ***Transplantation 2012;*** 93: 769-776 *(IF = 3.676)*

13. Veroux M, Grosso G, **Ekser B**, Corona D, Veroux P. Impact of conversion to a once daily tacrolimus-based regimen in kidney transplant recipients with gastrointestinal complications. ***Transplantation 2012;*** *93: 895-899 (IF = 3.676)*

14. Veroux M, Giuffrida G, **Ekser B**, Sinagra N, Giaquinta A, Veroux P. Vascular reconstruction in monolateral dual kidney transplantation with multiple arteries. ***Clinical Transplantation 2012;*** *93: 895-899 (IF = 1.751)*

15. Cooper DKC, **Ekser B**, Burlak C, Ezzelarab M, Hara H, Paris L, Phelps C, Azimzadeh A, Tector AJ, Ayares D, Robson SC, Pierson RN. Clinical lung xenotransplantation – what genetic modifications to the organ-source pig may be necessary? ***Xenotransplantation 2012;*** *19: 144-158 (IF= 2.565)*

16. **Ekser B***, Lin CC*, Long C, Echeverri GJ, Hara H, Ezzelarab M, Bogdanov VY, Stolz DB, Enjyoji K, Robson SC, Ayares D, DorlingA, Cooper DKC, Gridelli B. Potential factors influencing thrombocytopenia and consumptive coagulopathy after genetically-modified pig liver xenotransplantation (*shared first authors). ***Transplant International 2012;*** *25: 882-896* ***(highlighted on the cover - cover figure – August 2012).*** *(IF= 3.155)*

17. Manji RA, Menkis AH, **Ekser B,** Cooper DKC. Porcine bioprosthetic heart valves: the next generation. ***American Heart Journal 2012;*** *164: 177-185 (IF= 5.052)*

18. Iwase H, **Ekser B**, Zhou H, Dons EM, Cooper DKC, Ezzelarab M. Platelet aggregation in humans and nonhuman primates: relevance to xenotransplantation. ***Xenotransplantation 2012;*** *19: 233-243 (IF= 2.565)*

19. Ezzelarab M, **Ekser B**, Echeverri GJ, Hara H, Ezzelarab C, Long C, Isse K, Bajona P, Garcia B, Murase N, Ayares D, Cooper DKC. Costimulation blockade in pig artery patch xenotransplantation – A simple model to monitor the adaptive immune response in nonhuman primates. ***Xenotransplantation 2012;*** *19: 221-232 (IF= 2.565)*

20. Grosso G, Corona D, Mistretta A, Zerbo D, Sinagra A, Giaquinta A, Tallarita T, **Ekser B**, Leonardi A, Gula R, Veroux P, Veroux M. Predictive value of the Charlson comorbidity index in kidney transplantation. ***Transplantation Proceedings 2012;*** *44(7):1859-63 (IF= 0.993)*

21. Grosso G, Corona D, Mistretta A, Zerbo D, Sinagra A, Giaquinta A, Di Martino , **Ekser B**, Giuffrida G, Leonardi A, Gula R, Veroux P, Veroux M. Delayed graft function and long term outcome in kidney transplantation. ***Transplantation Proceedings 2012;*** *44:1879-83 (IF= 0.993)*

22. De Pasquale C, Pistorio ML, Corona D, Mistretta A, Zerbo D, Sinagra N, Giaquinta A, Tallarita A, **Ekser B**, Mociskyte D, Leonardi A, Gula R, Veroux P, Veroux M. Correlational study between psychic symptoms and quality of life among hemodialysis patients older than 55 years of age. ***Transplantation Proceedings 2012;*** *44:1876-1878 (IF= 0.993)*

23. **Ekser B,** Burlak C, Waldman JP, Lutz AJ, Paris LL, Veroux M, Robson SC, Rees MA, Ayares D, Gridelli B, Tector AJ, Cooper DKC. Immunobiology of liver xenotransplantation. ***Expert Reviews in Clinical Immunology 2012,*** *8: 621-634 (IF= 2.890)*

24. van der Windt DJ, Marigliano M, He J, Votyakova T, Echeverri GJ, **Ekser B**, Ayares D, Lakkis FG, Cooper DKC, Trucco M, Bottino B. Early islet damage after direct exposure of pig islets to blood – has humoral immunity been underestimated? ***Cell Transplantation 2012;*** *21: 1791-1802 (IF= 6.204)*

25. van der Windt DJ, Bottino R, Kumar G, Wijkstrom M, Hara H, Ezzelarab M, **Ekser B,** Phelps C, Murase N, Casu A, Ayares D, Lakkis FG, Trucco M, Cooper DKC. Clinical islet xenotransplantation – how close are we? ***Diabetes 2012;*** *61: 3046-3055 (IF= 8.3)*

26. **Ekser B**, Bianchi J, Ball S, Iwase H, Walters A, Ezzelarab M, Veroux M, Gridelli B, Wagner R, Ayares D, Cooper DKC. Comparison of hematologic, biochemical, and coagulation parameters in α1,3-galactosyltransferase gene-knockout pigs, wild-type pigs and 4 primate species. ***Xenotransplantation 2012;*** *19: 342-354 (IF= 2.565)*

2013

27. Kumar G, Satyananda V, Fang G, Zhou H, Fujita M, **Ekser B**, Long C, Dons E, Sun Q, Ayares D, Hara H, Cooper DKC. Is there a correlation between anti-pig antibody levels in humans and geographic location during childhood? ***Transplantation 2013;*** *96: 387-393. (IF = 3.676)*

28. Byrne GW, Azimzadeh AM, Ezzelarab M, Tazelaar HD, **Ekser B**, Pierson RN, Robson SC, Cooper DKC, McGregor CGA. Histopathological insights into the mechanism of anti-nonGal antibody-mediated pig cardiac xenograft rejection. ***Xenotransplantation 2013:*** *20: 292-307 (IF= 2.565)*

In Press

29. Fujita M, Dons EM, Kumar G, Echeverri GJ, Long C, **Ekser B**, Cooper DKC, Hara H. Technique of endoscopic biopsy of islet allografts transplanted into the gastric submucosal space in pigs. ***Cell Transplantation 2013, in press*** *(IF= 6.204)*

30. Veroux M, Corona D, Sinagra N, Tallarita T, **Ekser B,** Giaquinta A, Zerbo D, Veroux P. Nutrition in kidney transplantation. ***International Journal of Artificial Organs 2003, in press*** *(IF= 1.759)*

31. Iwase H, **Ekser B,** Hara H, Phelps C, Ayares D, Cooper DKC, Ezzelarab MB. Regulation of human platelet aggregation by genetically modified pig endothelial cells and thrombin inhibition. ***Xenotransplantation 2013, in press*** *(IF= 2.565)*

32. Manji RA, **Ekser B**, Menkis AH, Cooper DKC. The future of bioprosthetic heart valves. ***Xenotransplantation 2013, in press*** *(IF= 2.565)*

33. Iwase H, Ezzelarab M, Ekser B, Cooper DKC. The role of platelets in coagulation dysfunction in xenotransplantation, and therapeutic options. ***Xenotransplantation 2013, in press*** *(IF= 2.565)*

Submitted

34. Ezzelarab MB, **Ekser B**, Isse K, Iwase H, Morelli AE, Ayares D, Cooper DKC. Does soluble CD154 (CD40 ligand) promote elicited anti-pig IgG antibodies in xenograft recipients? ***Transplantation 2013, submitted***

35. **Ekser B**, Mangus RS, Powelson JA, Goble ML, Mujtaba MA, Taber TE, Fridell JA. Impact of years of diabetes on outcome following pancreas transplantation. ***American Journal of Transplantion, submitted***

In preparation

36. **Ekser B**, Walters E, Nottle MB, Iwase H, Bhama JK, Hara H, Salvaris E, Fisicaro N, d'Apice AJF, Cooper DKC, Cowan PJ, Robson SC. Initial experience of xenotransplantation with hearts from pigs expressing coagulation-regulatory genes. ***In preparation***

37. **Ekser B**, Goggins WC, Mangus RS, Shah AP, Kubal CA, Kinsella SB, Fridell JA, Powelson JA, Tector AJ. Combined liver-kidney transplantation: simultaneous versus delayed transplantation of the kidney graft. ***In preparation***

38. Mangus RS, **Ekser B**, Farmer D, Grant D, Tector AJ. Current status of intestinal transplantation. ***In preparation***

39. Satyananda V, **Ekser B**, van der Windt DJ, Hara H, Ezzelarab M, Schuurman HJ, Cooper DKC. Progress in pig-to-nonhuman primate transplantation models (1998-2012). ***In preparation***

40. Iwase H, **Ekser B**, Satyananda V, Bhama JK, Hara H, Bajona P, Wijkstrom M, Veroux M, Phelps C, Ayares D, Ezzelarab MB, Cooper DKC. Pig artery patch transplantation in baboons using costimulation blocaked and mutant MHC (CIITA-DN) pigs. ***In preparation***

41. Iwase H*, **Ekser B*,** Satyananda V, Bhama JK, Hara H, Ezzelarab M, Klein E, Wagner R, Long C, Thacker J, Li J, Zhou H, Jiang M, Zhou H, Veroux M, Bajona P, Wijkstrom M, Reimann KA, Phelps C, Klymiuk N, Wolf E, Ayares D, Cooper DKC. Pig-to-baboon heart transplantation – first experience with pigs transgenic for human thrombomodulin andc omparison of three costimulation blockade-based regimens (*contributed equally). ***In preparation***

ABBREVIATIONS

Abbreviations	Full name
Ab	Antibody
ACK	Ammonium chloride-potassium
ADP	Adenosine diphosphate
AHXR	Acute humoral xenograft rejection
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AVR	Acute vascular rejection
cAMP	Cyclic adenine monophosphate
CIITA-DN	Dominant negative class II transactivator
CMV	Cytomegalovirus
CRP	Complement regulatory protein
CVF	Cobra venom factor
DAF	Decay-accelerating factor (DAF, CD55)
DAPI	4',6-diamidino-2-phenylindole
DIC	Disseminated intravascular coagulation
ECs	Endothelial cells

Abbreviations	**Full name**
ecto-NAPDases	Ecto-nucleoside ATP diphosphohydrolases
F	Factor
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
Gal	Gal α 1,3 gal β 1,4 GlcNac
GTKO	α 1,3-galactosyltransferese gene knock-out
HAR	Hyperacute rejection
HI	Heat-inactivated
ICAM 1	Inter-cellular adhesion molecule 1
Ig	Immunoglobulin
iGb3	Isoglobotrihexosylceramide
LDH	Lactate dehydrogenase
MAC	Membrane attack complex
MCP	Membrane cofactor protein (CD46)
MFI	Mean fluorescence intensity
NF-KB	Nuclear factor-K-B
PAECs	Porcine aortic endothelial cells
PAI-1	Plasminogen activator inhibitor-1
PAR	Protease-activated receptor
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline

Abbreviations	Full name
PE	Phycoerythrin
PI	Propidium iodide
TBS	Tris buffered saline
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TM	Thrombotic microangiopathy
TNF	Tumor necrosis factor
TRITC	Tetramethyl rhodamine isothiocyanate
VCAM-1	Vascular cellular adhesion molecule 1
vWF	von Willebrand factor
WT	Wild-type
XNA	Xenoreactive antibodies
α1,3 GTα	1,3-galactosyltransferese

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1 - INTRODUCTION

1.1 ORGAN SHORTAGE

1.1.1 Progress in organ transplantation

Since the first kidney transplantation was performed in 1954 between identical twins [**Merrill, 1956**], organ transplantation has emerged as a definite treatment for many end-stage organ diseases. Starzl introduced liver transplantation as a treatment for end-stage liver disease in 1967 [**Starzl, 1967**]. Since that time, refinements in surgical technique, organ preservation, immunosuppression, and management of complications (surgical and infectious) have resulted in improved patient and graft survival in liver transplantation (now 80% and 72% at 5 years, respectively) [**UNOS**].

1.1.2 Organ shortage and its results

The success of organ transplantation has dramatically increased the discrepancy between organ demand and supply. Indeed, there is a well-known shortage of organs and tissues from deceased human donors for the purposes of clinical organ and cell transplantation. Although there are >100,000 patients waiting for an human organ of one sort or another in the USA today, the number of organs that will become available during the current year will be <30,000 [**UNOS**]. The discrepancy between available transplantable organs and patients on the waiting list grows each year. The shortage continues despite the fact that surgeons have liberalized their acceptance criteria for suitable deceased donor kidney and livers, have exploited the use of ABO-incompatible and marginal deceased donors, and have also used living-related donors for kidney and liver transplantation. It is clear that an alternative source of organs must be pursued if we are ever going to offer liver transplantation to all patients who could benefit from this form of therapy. It remains,

however, exceedingly unlikely that human organs will fulfill the needs of all of those who require organ transplantation.

The situation is even worse for patients in need for cell transplantation, such as islet for those affected by diabetes mellitus. Many of the 2-3 million patients with Type 1 diabetes in the USA would benefit from pancreatic islet transplantation, but clearly the number of deceased human donors available each year (<7,000) will not resolve this problem. Indeed, the potential supply of islets from human donors will never be sufficient to treat the millions of patients with diabetes [**Ekser, 2010a**].

However, organ transplantation using healthy living donors is appropriated with considerable risks to these altruistic donors, especially in the case of partial liver donation. The mortality rate of hepatic lobe donation for liver transplantation is around 0.15-0.2% and the overall complication rate is up to 38% although the reasons are not definitely related to donor surgery [**Olthoff, 2005; Trotter, 2006**]. The overall percentage of live donor liver transplants peaked at 10% of the total number of liver transplants performed in 2001. Since then, the percentages have reduced to 5% of the total, about 320 each year in the USA [**ustransplant**].

1.1.3 The necessity of expanding the donor pool

The lack of sufficient numbers of livers resulted in the deaths of 1,560 patients on the waiting list only in 2012 and 30,171 patients during the past 18 years. In 2012, 785 patients were removed from the liver transplantation waiting list due to severe deterioration of the clinical conditions prior to undergoing even transplantation; since 1995 this number has totaled 9,454. In summary, it is estimated that, during the past 18 years ~56,000 patients have died waiting for a liver transplantation [**UNOS**] and a total of ~ 227,000

patients has died waiting for a solid organ transplantation (kidney, liver, pancreas, intestine, heart, lung) [**UNOS**].

1.2 XENOTRANSPLANTATION

1.2.1 What is xenotransplantation?

Xenotransplantation (*xeno*- from the Greek meaning "foreign") (cross-species transplantation) is the transplantation of living cells, tissues or organs from one species to another, such as from pigs to humans. Such cells, tissues or organs are called xenografts or xenotransplants. The term allotransplantation refers to a same-species transplant. Human xenotransplantation offers a potential treatment for end-stage organ failure, a significant health problem in parts of the industrialized world. Xenotransplantation may provide a potential solution to the lack of human organs available for transplantation. However, for xenotransplantation to become a clinical reality, the immunological mechanisms that underlie the rejection of xenografts must be understood and controlled [**Auchincloss, 1988; Platt, 1996; Bach, 1994**]. In addition, concerns have also been raised about the cross-species transfer of porcine zoonotic agents into xenograft recipients [**Patience, 1997; Gustavsson, 1998; Bach, 1995; Weiss, 1999**]. However, the recent publication of data showing the lack of transmission of porcine endogenous retroviruses to humans exposed to live porcine tissues has generated cautious optimism with respect to the potential clinical application of xenotransplantation [**Fishman, 2004**].

1.2.2 The advantages of xenotransplantation

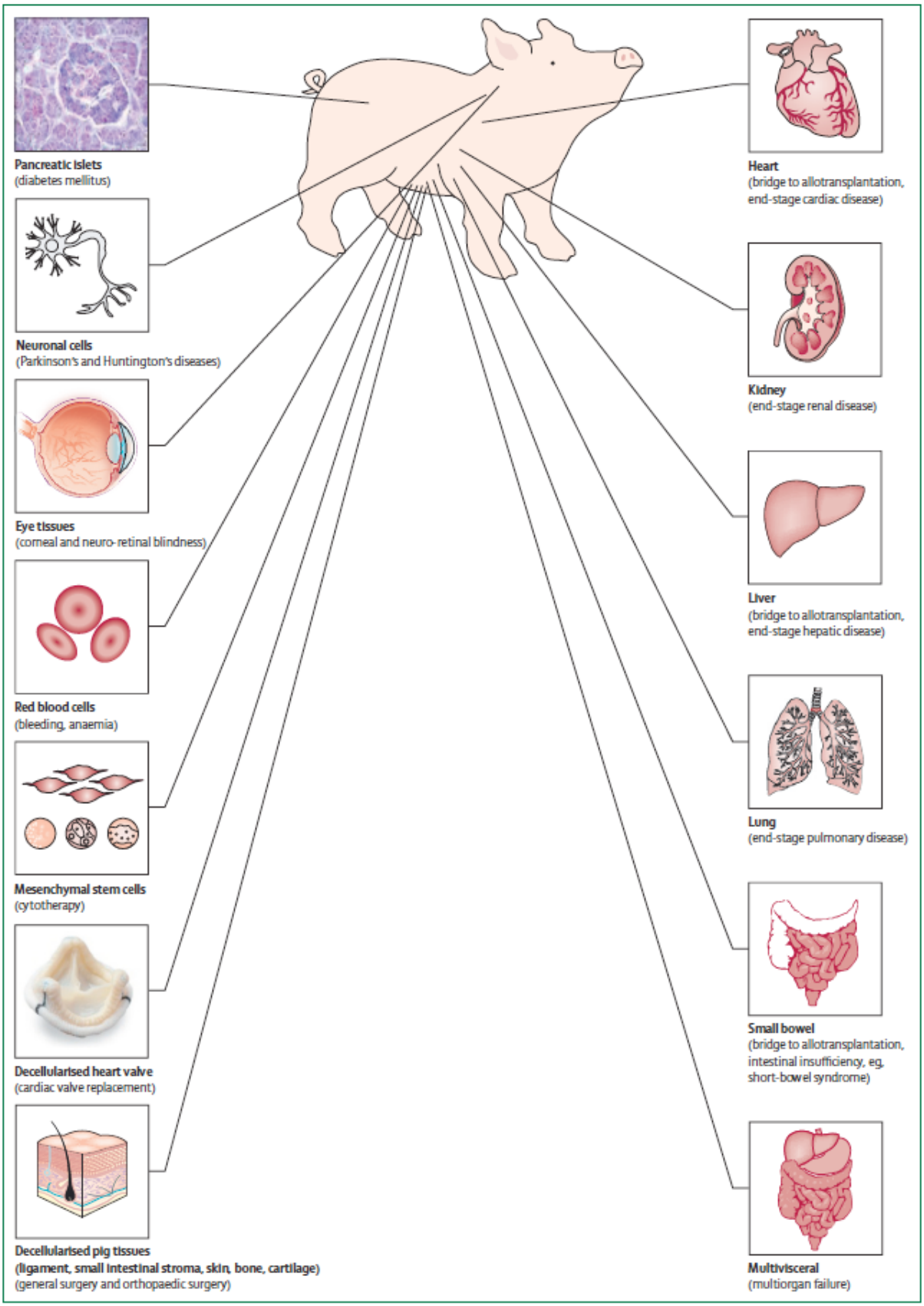
If xenotransplantation were to become a reality it could lead to an unlimited supply of organs of any type or size, for human transplantation (Table 1). The availability of such organs would minimise the time spent on the waiting list, avoid the progressive clinical

deterioration which usually occurs while waiting for an organ and allow elective and programmed surgery in ideal clinical conditions. The reduced time spent on the waiting list would considerably reduce the costs related to the treatment of patients with terminal organ failure such as those in dialysis. It would also allow the large number of patients, for whom organs are not currently available, to be transplanted [**Cooper, 1993**]. Xenotransplantation would allow a better organisation of the activities in the transplant units. Ischemia time, which is considered an important factor for the long-term survival of an allograft, would be reduced substantially. Finally, the large availability of organs would hopefully eliminate the repeatedly denounced and illegal trading of human organs [**Daar, 1997**]. Many disorders could be treated with clinical xenotransplantation [**Ekser, 2012a**] (Figure1).

Table 1: The advantages of xenotransplantation.

a)	Unlimited and prompt supply of organs of choice
b)	Organs of any size
c)	Reduction of the time spent on the waiting list
d)	Avoidance of clinical deterioration while waiting for the organ
e)	Reduction of the costs related to terminal organ failure
f)	Improved organisation of the activities in the transplant units
g)	Substantial reduction of ischemia time
h)	Disappearance of illegal trading of human organs

Figure 1: Disorders for which xenotransplantation is a potential therapy



(Obtained from Ekser et al. Lancet 2012;379:672-683)

1.2.3 Classification of xenografts

In 1970, having observed that the fate of xenografts between some animal species combinations was more favourable than others, Calne proposed a novel classification for two distinct categories of xenografts [**Calne, 1970**]. In order to simplify the definitions and make this classification easily accessible to the transplant community, Calne tried to draw a parallel with what scientists had already learnt from their experience performing transplants between individuals from the same species. In this context, the fundamental bulk of data used to build this classification was originally derived from the extensive work on transplantation by Medawar and colleagues, who had convincingly shown that graft rejection was an immunologically driven phenomenon [**Gibson, 1943; Medawar, 1948**]. In his set of experiments, Medawar had transplanted skin grafts between different strains of mice. His results had shown that allotransplantation between naïve animals lead to graft loss between day 7 and 10 by a process called “first-set” rejection. A subsequent skin graft transplanted from the same donor was rejected more rapidly, after only 2 or 3 days by what he called “second-set” rejection. First-set, rather than second set-rejection occurred if the second graft was derived from a different strain of mice, indicating the donor-strain specificity of this phenomenon.

Medawar’s work constituted a real breakthrough in the understanding of the rejection process. However, as it was done with skin grafts, the findings were only in part representative of the rejection mechanisms occurring in the context of immediately vascularized organ allografts. Despite this, the general principles were valid and formed the basis of Calne’s xenograft classification.

"…Because we need to base the classification on the severity of organ xenograft rejection…. " Calne writes, … "it is logical to define the categories according to the immunological response. Thus the first-set cross-species grafts which are rejected at a tempo and with morphological characteristics similar to first-set allografts would be one class: e. g., chimpanzee-to-man, sheep-to-goat, while first-set cross–species grafts which are hyperacutely rejected with vascular lesions similar to those observed in second-set allografts in sensitised animals would belong to the other category: e. g., pig-to-dog. …. I would suggest "concordant" and "discordant". These established adjectives would be used quite simply, in the context of xenografting, according to the above definitions as follows: chimpanzee-to-man organ grafts would be concordant xenografts; pig-to-dog would be discordant xenografts".

Calne drew attention to the fact that, whilst general rules are applicable to most individuals within a species combination, exceptions may exist. In his paper, Calne himself was the first scientist to illustrate, in a convincing manner, the existence of such exceptions by showing a picture of a baboon recipient of a porcine liver whose xenograft had not been hyperacutely rejected. He defined the fate of that particular xenograft as "concordant".

Almost fifty years went by since the publication of this article and, although the general principles of the Calne classification still hold, advances in this field have allowed the identification of a third pattern of rejection, not reported at that time. Specifically, Calne defined any category of xenograft rejection on the basis of both a temporal and a histological element. He defined as concordant the xenografts which were rejected at a tempo and with morphological characteristics similar to first-set allografts. However, there was no mention in his classification of concordant xenografts with a tempo of rejection

similar to first-set allografts but with histological features primarily of vascular lesions, similar to second-set allografts in sensitised animals.

This subset of xenograft rejection is not uncommon, as it is also observed in models such as baboon-to-human [**Bailey, 1985; Starzl, 1993**] or hamster-to-rat [**van den Bogaerde, 1991; Hasan, 1992**]. In these combinations the presence of high titres of anti-donor antibodies is very prominent and seems to be the key immunological element in the onset of xenograft rejection. To introduce this specific subset of xenografts into the original classification of Calne, White and colleagues proposed to subdivide the concordant xenografts into "easy", i.e. where rejection is principally T-cell mediated and "difficult", where rejection does not involve T cells and humorally mediated damage is prevalent (Table 2) [**White, 1992**].

Table 2: Classification of the xenografts.

Type of Xenograft	**Examples**
Concordant easy	Mouse to rat Rat to mouse Baboon to cynomolgus monkey Chimpanzee to man
Concordant difficult	Hamster to rat Baboon to man
Discordant	Guinea pig to rat Pig to dog Pig to baboon Pig to cynomolgus monkey Pig to man New World primate to Old world primate

1.2.4 Pig donors

From the immunologic perspective, nonhuman primates would be preferable organ donors. However, many of them are endangered species, and the time and expense of breeding these animals are also prohibitive. Therefore, the UK Department of Health Advisory Group on the Ethics of Xenotransplantation and the International Xenotransplantation Association (IXA) recommended, “it would be ethically unacceptable to use primates as source animals for xenotransplantation”. Secondly, concerns about the transmission of infectious agents from nonhuman primates to humans have been raised, particularly since most nonhuman primates are either wild-caught or have been housed under colony conditions for relatively few generations. Furthermore, there is no experience in generating genetically-modified primate animals. Instead of primates, pigs are thought the most suitable animals as a source of xenografts for human [**Cooper, 1991, Cooper 2007**].

1.2.5 Physiology of pig and human

Pigs emerge as the most suitable source of xenograft organs. They are easily bred with sexual maturity within 9 months, gestation of 3.5 months, and litters of 6-16 piglets. Furthermore, the physiology and anatomy of organs in adult pigs are similar to adult human with life expectancy of approximate 30 years. The hematological and chemistry profiles are somehow compatible with those in adult humans and nonhuman primates (Figure 2) [**Ekser, 2012b**]. Genetically-engineered pigs which are grown in specific pathogen free environments have less WBC counts than wild-type (WT) pigs. Figure 2 shows differences in GTKO pigs’ hematological and biochemistry normal values in comparison to WT pigs and four primate species [**Ekser, 2012b**]. Relatively few data are available with regard to the function of a pig liver in a primate and the data available have been reviewed by Ibrahim [**Ibrahim, 2006**]. However, although the follow-up was limited to

7 days, a recent study of Ekser et al indicated a surprisingly good hepatic function, including parameters of coagulation, after the orthotopic transplantation of livers from genetically-engineered pigs into baboons [**Ekser, 2010b, 2010c**]. These data would suggest that, from this perspective, a pig liver would be able to support a patient adequately until an allograft could be obtained.

Figure 2: Comparison of normal values for biochemistry and hematology in GTKO and wT pigs and four primate species.

	Test	Unit	GTKO[a]	WT[a]	Baboon	Cyno[a]	Rhesus[a]	Human
Hematology	WBC	/mm^3	**15.5**	18.6	10.0	11.9	8.0	3.8–10.6
	RBC	$\times10^6$/mm^3	6.3	6.9	5.1	5.8	5.6	3.73–4.89
	Hemoglobin	g/dl	12.2	10.0	12.7	13.0	13.2	11.6–14.6
	Hematocrit	%	37.9	37.9	39.9	43.1	40.9	34.1–43.3
	Platelets	$\times10^3$/mm^3	425.0	506.6	419.2	431.7	359.0	156–369
	MCV	fl	**61.3**	58.7	77.2	74.6	74.4	82.6–97.4
	MCH	pg	**19.7**	17.4	25.1	22.7	23.7	27.8–33.4
	MCHC	g/dl	32.3	25.5	32.6	30.7	32.0	32.7–35.5
	RDW	%	**21.4**	19.0	13.1	13.7	13.0	11.8–15.2
	MPV	fl	9.1	n.a.	8.5	9.0	n.a.	6.8–10.4
	Neutrophil	%	41.8	45.6	52.0	33.2	54.3	44–77
	Lymphocyte	%	49.1	45.5	44.4	58.4	38.2	13–44
	Monocyte	%	4.9	4.7	3.2	4.4	3.5	4–13[a]
	Eosinophil	%	2.3	1.9	0.9	2.6	3.1	0–6
	Basophil	%	0.1	0.4	0.3	0.7	0.2	0–1
Renal function and electrolytes	Sodium	mm	144.0	142.9	146.1	158.4	147.3	136–146
	Potassium	mm	**5.3**	5.3	3.8	5.8	4.0	3.5–5.0
	Chloride	mm	103.0	104.8	108.0	113.4	112.6	95–110
	Calcium	mg/dl	**10.8**	11.0	9.5	11.1	9.4	8.4–10.2
	Phosphorus	mg/dl	8.8	8.6	4.3	5.6	4.1	2.5–4.5
	CO 2	mm	28.1	29.0	22.9	n.a.	13.9	21–32
	Urea	mg/dl	12.8	12.4	14.3	23.1	17.3	5.0–20.0
	Creatinine	mg/dl	1.1	1.0	0.7	0.8	1.0	0.6–1.1
Liver function	AST	IU/l	37.0	40.1	36.6	33.1	33.6	<40
	ALT	IU/l	42.0	58.5	39.2	47.1	44.6	<40
	ALP	IU/l	**272.0**	263.1	637.7	1138.6	183.7	38–126
	LDH	IU/l	**472.0**	825.9	284.2	598.8	311.0	< 170
	GGT	IU/l	**74.0**	43.0	39.0	124.1	61.0	<40
	Tot Bilirubin	mg/dl	0.2	0.5	0.1	0.3	0.4	0.3–1.5
	Dir Bilirubin	mg/dl	0.1	0.1	0.1	0.1	0.7	0.1–0.4
	Indir Bilirubin	mg/dl	0.1	0.1	0.1	n.a.	2.1	0.2–1.1
	Tot Protein	g/dl	5.9	5.2	6.8	7.9	7.1	6.3–7.7
	Albumin	g/dl	3.4	2.6	4.1	4.3	4.0	3.4–5.0
	Cholesterol	mg/dl	81.0	100.6	103.2	131.5	141.7	<200
	Triglyceride	mg/dl	31.0	57.5	60.1	44.8	75.3	<150
	Glucose	mg/dl	89.0	92.1	96.4	81.0	67.1	70–99
Coagulation profile	PT	s	13.6	11.7	14.3	9.4	14.2	11.3–14.5
	PTT	s	34.1	15.5	32.6	21.6	43.0	22.7–35.6
	INR		1.1	n.a.	1.3	n.a.	n.a.	0.8–1.2
	D-Dimer	µg/ml	0.2	n.a.	0.7	n.a.	n.a.	<0.45
	FDP	µg/ml	5–20	n.a.	<5	n.a.	n.a.	<5 neg, >20 pos
	Fibrinogen	mg/dl	250.0	n.a.	189.5	235.8	n.a.	200–400
Others	Amylase	IU/l	**1622.0**	n.a.	185.7	440.0	n.a.	35–118
	Lipase	IU/l	13.1	n.a.	29.8	n.a.	n.a.	22–51
	Iron	µg/dl	104.0	175.2	158.9	156.3	156.0	28–170
	Total CPK	IU/l	**1166.0**	n.a.	n.a.	n.a.	n.a.	60–400
	CPK-MB isoenzyme	ng/ml	4.4	n.a.	n.a.	n.a.	n.a.	0–3
	CPK-MB relative index		0.7	n.a.	n.a.	n.a.	n.a.	0–3
	Troponin I	ng/ml	0.1	n.a.	n.a.	n.a.	n.a.	<0.4

Legend - Figure 2: *ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; INR, international normalized ratio; LDH, lactate dehydrogenase; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PT, prothrombin time; PTT, partial thromboplastin time; RDW, red blood cell distribution width.*

[a]*GTKO, α1,3-galactosyltransferase gene-knockout pigs; WT, wild-type pigs; Cyno, cynomolgus monkeys; Rhesus, rhesus monkeys. n.a., not available. GTKO values indicated in bold are statistically different from human values. (Obtained from Ekser et al, Xenotransplantation 2012;19:342-354)*

1.2.6 Concern of zoonosis

The potential for the development of a xenozoonosis in the recipient of a pig graft, i.e., the potential for a porcine microorganism to cause infection in the recipient, has been of concern for a number of years [**Onions, 2000; Fishman, 2004; Patience, 1998**]. These potential risks, particularly with regard to porcine endogenous retroviruses (PERV), are now considered to be much less significant than they were a few years ago [**Fishman, 2004; Paradis, 1999; Fishman, 2005**], and a clinical trial would be deemed justified if there were a realistic possibility that the graft would be life-saving for the patient. Furthermore, activation of PERV could now be prevented by siRNA technology [**Dieckhoff, 2008; Ramsoondar, 2009**], although this is unlikely to be necessary. Nevertheless, largely because of the possibility of transferring a porcine infectious microorganism, xenotransplantation is highly-regulated by national regulatory authorities, such as the Food and Drug Administration in the USA. The regulatory requirements have recently been reviewed by Schuurman and Cozzi [**Schuurman, 2008; Cozzi, 2009**].

A major advantage of pig xenotransplantation may be the potential resistance of the pig liver to infection by certain human pathogens, including HIV, HTLV, hepatitis viruses (HBV, HCV), and herpes viruses (including CMV) [**Fishman, 2004; Zhou, 1999**]. For example, porcine cytomegalovirus does not appear to infect baboon tissues *in vivo* [**Mueller, 2000**]. This `species specificity` may reflect the absence of receptors or of cellular 'machinery' necessary for viral replication in human cells [**Fishman, 2004**].

1.2.7 Medical ethics of xenotransplantation

Non-human primates, such as chimpanzees, have intellectual and social natures, similar to humans. They are considered as endangered species as well. Hence, using nonhuman primates as donors raises several ethical concerns. In contrast, from the point of view of

ethics, pigs as xenograft donors raise fewer concerns. There are still some other ethical issues associated with xenotransplantation. For example, whether the transfer of organs or tissues from animals is natural and whether to kill animals to benefit human beings is justified remain controversial points. Nevertheless, the shortage of organs leaves patients desperate. This demand stimulates progress in xenotransplantation from the scientific point of view. Taken together, the aforementioned issues indicate that pigs are currently considered the most suitable sources of xenografts [**Cooper, 1993; Heneine, 1998; Elliott, 2000**] although clinical application of this technique still requires ethical, regulatory, and legal frameworks to provide guidelines and to establish a consensus.

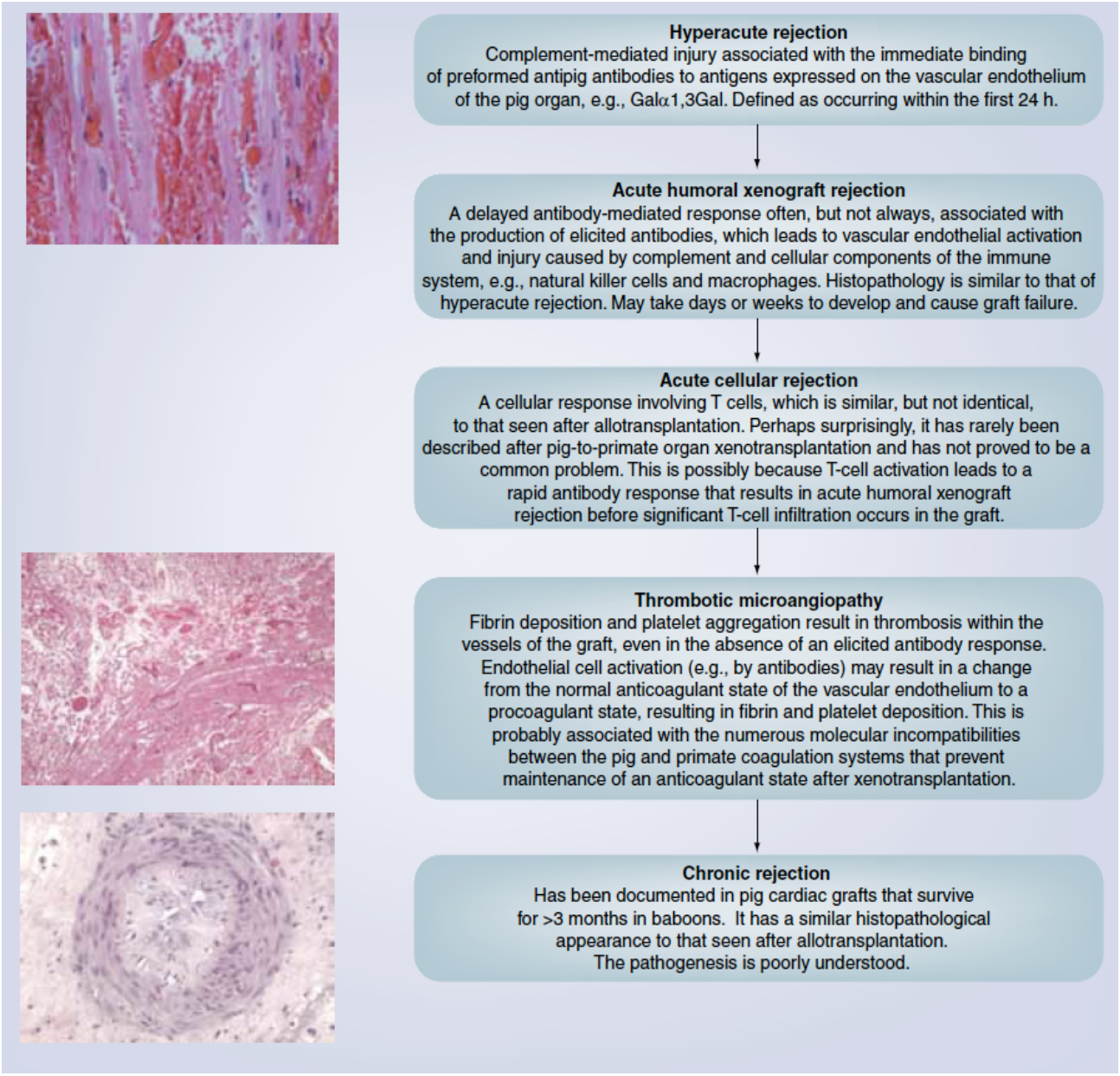
1.3 HURDLES IN XENOTRANSPLANTATION

This section will summarise the current knowledge in this field, concentrating on the immunological challenges in xenotransplantation. Where possible, particular attention will be placed on the pig-to-primate model, since this species combination currently provides the most relevant model for clinical xenotransplantation.

1.3.1 Hyperacute rejection

In initial experiments, when wild-type pig organs were transplanted into nonhuman primates, the binding of natural (preformed) antibodies to the pig vascular endothelium initiated activation of the complement cascade [**Lexer, 1986; Cooper, 1988; Rose, 1991; Rose, 1996; Rose, 2000; Ekser, 2010a**] (Figure 3). The endothelial cells of the graft responded to the immune activation by converting from an anticoagulant to a procoagulant phenotype [**Bach, 1994; Saidi, 1999**]. The result of activation of the complement and coagulation systems was hyperacute rejection.

Figure 3: Immunologic barriers to xenotransplantation



Legend – Figure 3: Summary of the major known immunologic barriers to pig-to-primate organ transplantation, as exemplified in the transplanted pig heart. In hyperacute rejection (HAR), the graft develops microvascular thrombi, beginning in the venules. Occlusion of the vessels leads to rupture with interstitial hemorrhage and edema. Some cells of the innate immune system, e.g., neutrophils, macrophages, may be present. The appearances in acute humoral xenograft rejection (AHXR) are similar though infiltration by cells of the innate immune system is more pronounced. Isolated acute cellular rejection (ACR) is very rare, but T cells can be seen when thrombotic microangiopathy (TM develops). The role of T cells in the development of TM is uncertain and controversial. (Obtained from Ekser et al. Expert Rev Clin Immunol 2010;6:219-230)

1.3.2 Identification of Gal-antigen and depletion of xenoreactive antibodies

The main cause of HAR results from the existence of natural xenoreactive antibodies. The most important one is the antibody against the Galα1–3Galβ1–4GlcNAc (Gal) epitope, which accounts for over 85% [**Galili, 1984; Cooper, 1993**]. The Gal epitope is a common carbohydrate structure found in many mammals, and this epitope has been reported to be expressed on glycoproteins and glycolipids of different species. In 1988, Galili investigated Gal epitope expression in mammals by immunostaining with anti-Gal antibodies, and correlated this with binding of the lectin *Griffonia (Bandeiraea) simplicifolia*-IB4 (GS-IB4), which interacts with Gal residues. Many cells of nonprimate mammals, prosimians and New World monkeys are readily bound by both anti-Gal antibodies and the GS-IB4 lectin. The Gal epitope is synthesized by the enzyme α1,3-galactosyltransferase (α1,3GT) using *N*-acetyllactosamine as the sugar acceptor and uridine diphosphate-Gal as the sugar donor. However, humans, apes and Old World monkeys (monkeys of Asia and Africa) only possess the pseudogene for this enzyme; therefore, they carry anti-Gal antibodies that are raised in response to antigenic stimulation by Gal epitopes expressed in gastrointenstinal tract bacteria [**Galili, 1987a; Galili, 1987b; Galili, 1993**]. The gene for α1,3-galactosyltransferase enables this enzyme to add Gal oligosaccharides to various underlying glycoproteins and glycolipids in the pig [**Galili, 1988; Oriol, 1993**].

Since Gal antibodies (Abs) play a pivotal role in the pathogenesis of HAR, the activation of xenoreactive antibody interaction is critical to avert HAR. Removal of the natural Abs by plasmapheresis is effective in preventing HAR but entails some disadvantages, such as marked depletion of complement and coagulation proteins. Its use in the peri-transplant period therefore is limited [**Fischel, 1990**]. Nonspecific Ig-binding columns were designed to result in 90.5 and 86.0% reduction in total IgG and IgM, with the preservation of albumin and coagulation proteins. They resulted in prolonged xenograft function in ex-vivo

perfusion systems [**Kroshus, 1995**] and averted HAR in kidney xenotransplantation models [**Leventhal, 1995**]. Gal-specific affinity columns also effectively removed anti-Gal Abs and minimized hypogammaglobulinemia in spite of the requirement of multiple absorptions [**Kozlowski, 1998; Watts, 2000**]. However, these modalities can only be used temporarily because they require vascular access, real-time separation of blood and plasma. Importantly, the production of anti-Gal Abs continues, and near normal levels of Abs return rapidly after cessation of immunoabsorption, which leads to graft loss. Another approach is neutralizing anti-Gal Abs by continuous intravenous infusion of large amounts of soluble antigens, such as Gal sugar (e.g. GAS914 and TPC) [**Teranishi, 2002; Katopodis, 2002; Schirmer, 2004**]. Nevertheless, despite the delayed return, anti-Gal Abs are never eliminated and graft rejection is delayed but not abrogated.

1.3.3 Genetically-engineered pigs

In order to minimize the immune response between different species, the modification of donors that would supply xenografts to human offers a unique way to solve the problems which are encountered in xenotransplantation. Advances in transgenic manipulation and cloning in swine has fulfilled the reality of genetically-modified organs from pigs. Pigs over-expressing human complement regulatory proteins (hCRP) [**Dalmasso, 1991; Oglesby, 1991**] such as CD59 [**Byrne, 1997**], membrane cofactor protein (CD46) [**Loveland, 2004**], or human decay-accelerating factor (DAF, CD55) [**Cozzi, 1995**] have suggested that the modification of swine is a practical way to overcome the barriers between swine and primates.

1.3.4 α1,3-galactosyltransferase gene-knockout (GTKO) pigs

The development of nuclear transfer technology has provided a means for locus-specific modification of large animals. It allowed for the development of pigs in which the gene of α

α1,3GT is deleted. By cloning fetal fibroblast cells, which are used as nuclear donors for embryos reconstructed with enucleated pig oocytes, heterogenous GTKO pigs have been generated in the early period of this decade [**Dai, 2002; Lai, 2002**]. Currently, homozygous GTKO pigs are available for pig-to-primate animal studies by several groups [**Phelps, 2003; Sharma, 2003**]. It is worth noting that the deletion of Gal from donor organs has been recognized as a major step toward the clinical application of xenotransplantation. By transplanting hearts from GTKO pigs into baboons, the medium survival of xenografts was extended up to over 70 days with a co-stimulatory pathway blockade-based immunosuppressive regimen [**Kuwaki, 2005**]. Meanwhile, in the kidney model, the longest survival with GTKO pigs was extended up to 83 days with the tolerance-inducing regimens by co-transplantation of vasculized thymic tissue [**Yamada, 2005**]. The production of homozygous pigs is based on a disruption in the α1,3GT gene, which encodes Gal epitope. The concerns have arisen regarding GTKO pigs as to whether the Gal epitope is exclusively produced by the α1,3GT gene. Isoglobotrihexosylceramide (iGb3) synthase was shown to be another candidate that synthesizes Gal epitope [**Taylor, 2003; Milland, 2006**]. Low levels of Gal are still expressed in GTKO mice in the form of a lipid (iGb3), which is produced by iGb3 synthase. Similarly, GTKO cells in pigs have been demonstrated to synthesize the Gal antigen at 1 to 2% of the level of control heterozygous cells by flow cytometry [**Sharma, 2003**]. Although GTKO pigs do avert HAR and prolong graft survival, the presence of secondary enzymes, such as iGb3 synthase may possibly have implications for long term graft survival in pig-to primate xenotransplantation using GTKO organs. Other genetically engineered pigs available for xenotransplantation research are listed in Table 3 [**Ekser, 2012a**].

Table 3: Genetic modifications of pigs produced for xenotransplantation research

	Purpose and indications of modification
Human CD59[6]	Complement regulation
Human CD55[7]	Complement regulation
Human H-transferase[8]	Reduction of Gal antigen expression
Human CD46[9]	Complement regulation
GTKO[10]	Deletion of Gal antigen expression
Endo-β-galactosidase C[11]	Reduction (but not deletion) of Gal antigen expression
Human TFPI[12]	Antagonise the function of tissue factor
Human TRAIL[13]	Control mechanisms of rejection mediated by cellular components of immune system
vWF-deficient[14]	Inhibit platelet activation
PERV siRNA[15]	Prevention of PERV activation
Porcine CTLA4-Ig[16]	Local co-stimulation blockade; T-cell suppression
Human thrombomodulin[17]	Anticoagulation (activates protein C)
HLA-E/human beta-2-microglobulin[18]	Protection against cytotoxicity of human natural killer cells
Human A20[19]	Anti-inflammatory; antiapoptosis
CIITA-DN[20]	Suppression of T-cell activation
Human Fas ligand[21]	Protection against cytotoxicity of human CD8+ and natural killer cells
Human GnT-III[22]	Downregulation of antigenicity to human natural antibodies
Human heme oxygenase 1[23]	Antiapoptosis; cytoprotection; anti-inflammatory
Human ENTPD1 (CD39)*	Anticoagulation and anti-inflammatory; conversion of ATP to ADP and AMP

Pigs with multiple gene modifications exist (eg, GTKO/hCD55/hCD46). Gal=galactose-α-1,3-galactose. GTKO=pigs with knockout of gene for α-1,3-galactosyltransferase. TFPI=tissue factor pathway inhibitor. TRAIL=tumour necrosis factor-related apoptosis-inducing ligand. vWF=von Willebrand factor. PERV=porcine endogenous retrovirus. CIITA DN=MHC class II transactivator knockdown (SLA class II knockdown). GnT-III=human β-d-mannoside β-1,4-*N*-acetylglucosaminyltransferase III gene. *Robson SC, Harvard Medical School, and Cowan PJ, University of Melbourne, personal communication.

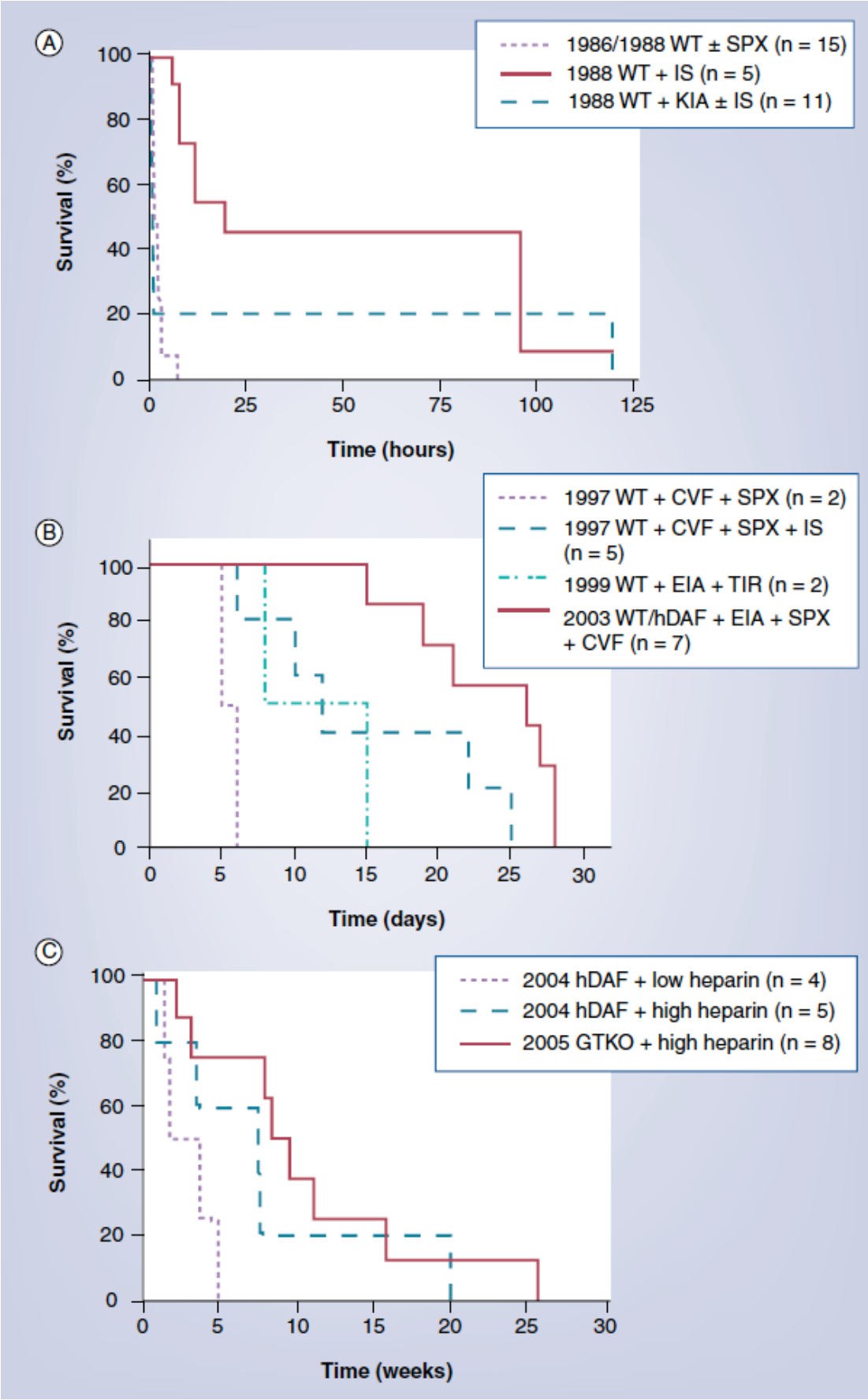
(Obtained from Ekser et al. Lancet 2012;379:672-683)

1.3.5 Overcoming hyperacute rejection

Gal is the major target for human and nonhuman primate anti-pig antibodies (reviewed in Kobayashi and Cooper [**Kobayashi, 1999**]), and its deletion from pigs has greatly reduced

the incidence of HAR of pig grafts in nonhuman primates as indicated above. GTKO pigs additionally transgenic for a human complement-regulatory protein (Table 3) provide increased benefit over either modification alone [**Hara, 2008**]. IgM, IgG, and complement deposition in grafts is absent or less marked, and innate cellular infiltration has also been minimized. Progress during the past 20 years or so can be illustrated by the prolongation of survival or pig hearts transplanted heterotopically into baboons using wild-type or genetically-modified pigs and various immunosuppressive protocols (Figure 4A, 4B, and 4C) [**Ekser, 2010a**]. Although the availability of GTKO pigs has been a major step forward, there are well-documented natural antibodies to nonGal antigens in humans and nonhuman primates [**Ezzelarab, 2006; Baumann, 2007**], the nature of which remains still partially unknown [**Bryne, 2013**].

Figure 4: Progress in the results of pig heterotopic heart transplantation in baboons (1986-2005).



Legend – Figure 4: ***(A)*** *Survival (in hours) of selected pig heterotopic heart grafts in baboons (1986–1996).* ***(B)*** *Survival (in days) of selected pig heterotopic heart grafts in baboons (1997–1999).* ***(C)*** *Survival (in weeks) of selected pig heterotopic heart grafts in baboons (2000–2005).*

CVF: Cobra venom factor; EIA: Extracorporeal immunoadsorption; GTKO: a1,3-galactosyltransferase gene-knockout; hDAF: Pig transgenic for human decay-accelerating factor; IS: Pharmacologic immunosuppressive therapy; KIA: Prior pig kidney perfusion to deplete antipig antibodies; SPX: Splenectomy; TIR: Tolerance-inducing regimen; WT: Wild-type. (Obtained from Ekser et al. Expert Rev Clin Immunol 2010;6:219-230)

1.3.6 Acute humoral xenograft rejection

When steps were taken to prevent HAR, e.g., by the depletion of anti-pig antibodies or complement from the nonhuman primate serum [**Cooper, 1988; Alexandre, 1989; Buhler, 2001**], a delayed form of antibody-mediated rejection occurred, known variously as acute humoral xenograft rejection (AHXR), acute vascular rejection, or delayed xenograft rejection (Figure 3) [**Gollackner, 2004**]. Natural antibody binding and complement activation resulted in vascular endothelial cell activation and injury caused by the complement and cellular components of the innate immune system. There is increasing evidence that primate neutrophils may be involved in pig endothelial cell activation [**Cardozo, 2004; Gilli, 2005; Al-Mohanna, 2005**], a topic that has been discussed recently by Ezzelarab et al [**Ezzelarab, 2009**]. Natural killer (NK) cells play a role in AHXR [**Inverardi, 1997; Baumann, 2004; Rieben, 2005**] as do macrophages [**Fox, 2001**], but their exact importance remains unclear. AHXR may occur despite the administration of pharmacologic immunosuppressive agents, and is seen particularly following the development of a T cell-dependent elicited antibody response.

1.3.7 Cellular and chronic rejection

Potent pharmacologic agents can largely prevent acute cellular rejection (i.e., T and B cell infiltration of the graft and T cell activation) and a T cell-dependent elicited antibody response, even though the T cell response is believed to be stronger than the allo

response [**Yamada, 1995; Dorling, 1998; Buhler, 2005; Lin, 2008**]. This is possibly because T cell activation leads to a rapid antibody response that results in AHXR before significant T cell infiltration occurs in the graft. Acute cellular rejection is therefore typically not seen with intense immunosuppressive drug regimens [**McCurry, 1997; Cozzi, 2000; Cozzi, 2003; Chen, 2005; Byrne, 2006**]. Costimulation blockade agents, such as an anti-human CD154 monoclonal antibody, have been found particularly effective in preventing T cell activation in the xenotransplant setting [**Buhler, 2000**].

In grafts that survive for more than a few weeks, features of chronic vasculopathy develop, similar to the chronic rejection seen in long-surviving allografts (Figure 3). Its causative factors remain poorly understood.

1.3.8 Coagulation dysregulation

Despite considerable attention in recent years, the exact mechanisms by which coagulation disorders develop after xenotransplantation remain elusive. Previous reports suggested that consumptive coagulopathy is initiated by the expression of tissue factor in the porcine graft [**Blakely, 1994; Gollackner, 2003; Ekser, 2012a**]. In response to the binding of xenoreactive antibody and/or activation by complement, endothelial cells in the graft are activated to increase tissue factor activity and initiate intragraft thrombosis and consumptive coagulopathy [**Bach, 1994; Gollackner, 2004, Lin, 2009a; Ekser, 2012a**].

During inflammation, type I activation of endothelial cells induces P-selectin and vascular leakiness of plasma proteins; this process takes 10-20min. Type II activation of endothelial cells is triggered by stimulation of tumor necrosis factor-α and interleukin-1, induces more effective leukocyte recruitment by synthesis of adhesion proteins, such as E-selectin and CD106 (vascular cell adhesion molecule-1,VCAM-1), and is sustained for 6-24h after

cytokine-mediated activation. Type I and type II activations are believed to be associated with HAR and AHXR, respectively [**Bach, 1994**]. The activated endothelial cells and the generated thrombin subsequently activate platelets, leukocytes, and other inflammatory cells in the recipient, initiating a vicious cycle.

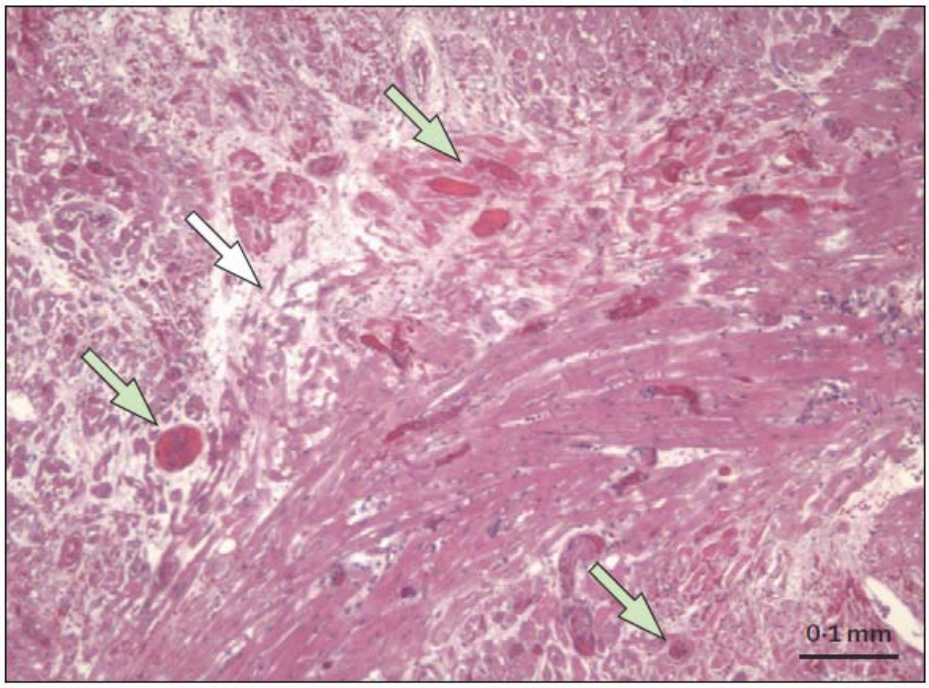
Recent *in vitro* studies at our center by Lin et al [**Lin, 2008**] have indicated that porcine aortic endothelial cells (PAECs) are able to induce human tissue factor exposure on human platelets and monocytes through an immune response-*independent* pathway. This problem has been investigated *in vivo* in pig-to-baboon kidney [**Lin, 2009b**] or liver [**Lin, 2009c; Ekser, 2012d**] transplantation models.

For example, the rapid development of the consumptive coagulopathy in a pig-to-baboon liver xenotransplantation model has been studied [**Lin, 2009c; Ekser, 2012d**]. Using genetically-modified pig liver transplantation into baboons, we observed that there is a massive loss of platelets from the circulation within minutes after reperfusion [**Ekser, 2010b**]. The development of thrombocytopenia was accompanied by thrombin formation. Circulating platelets and PBMCs expressed functional TF and aggregated in the graft without the documented activation of donor endothelial cells (confirmed by negativity for P- and E-selectin, CD106, and tissue factor expression on the porcine endothelial cells by immunofluorescence staining) [**Lin, 2009c; Ekser, 2012c, 2012d**]. Although there was a minimal measurable immune response (indicated by a lack of antibody and complement activity), consumptive coagulopathy still occurred. The severity and rapidity of thrombocytopenia were not alleviated by manipulation of the immune response, e.g., by prior depletion of complement by administration of cobra venom factor. Therefore, it has been tentatively concluded that recipient tissue factor initiated the consumptive

coagulopathy by a mechanism that is independent of the immune response [**Ekser, 2012a**].

These observations suggest that further manipulation of the immune response (with the increased risks of infection and other complications) will not completely overcome consumptive coagulopathy after xenotransplantation. Determination of the exact mechanism by which thrombotic microangiopathy (Figure 5) [**Ekser, 2012a**] and consumptive coagulopathy are initiated after xenotransplantation is important because it may enable further genetic modification of the pig or suggest the therapy that might prevent them. The introduction of genes for human thrombomodulin [**Iwase, 2013a; Ekser, 2013a**], tissue factor pathway inhibitor [**Chen, 2004**], or CD39 [**Dwyer, 2004**] have been suggested to overcome the coagulation incompatibilities between pig and primate.

Figure 5: Typical features of thrombotic microangiopathy in a pig heart 6 months after heterotopic transplantation into an immunosuppressed baboon

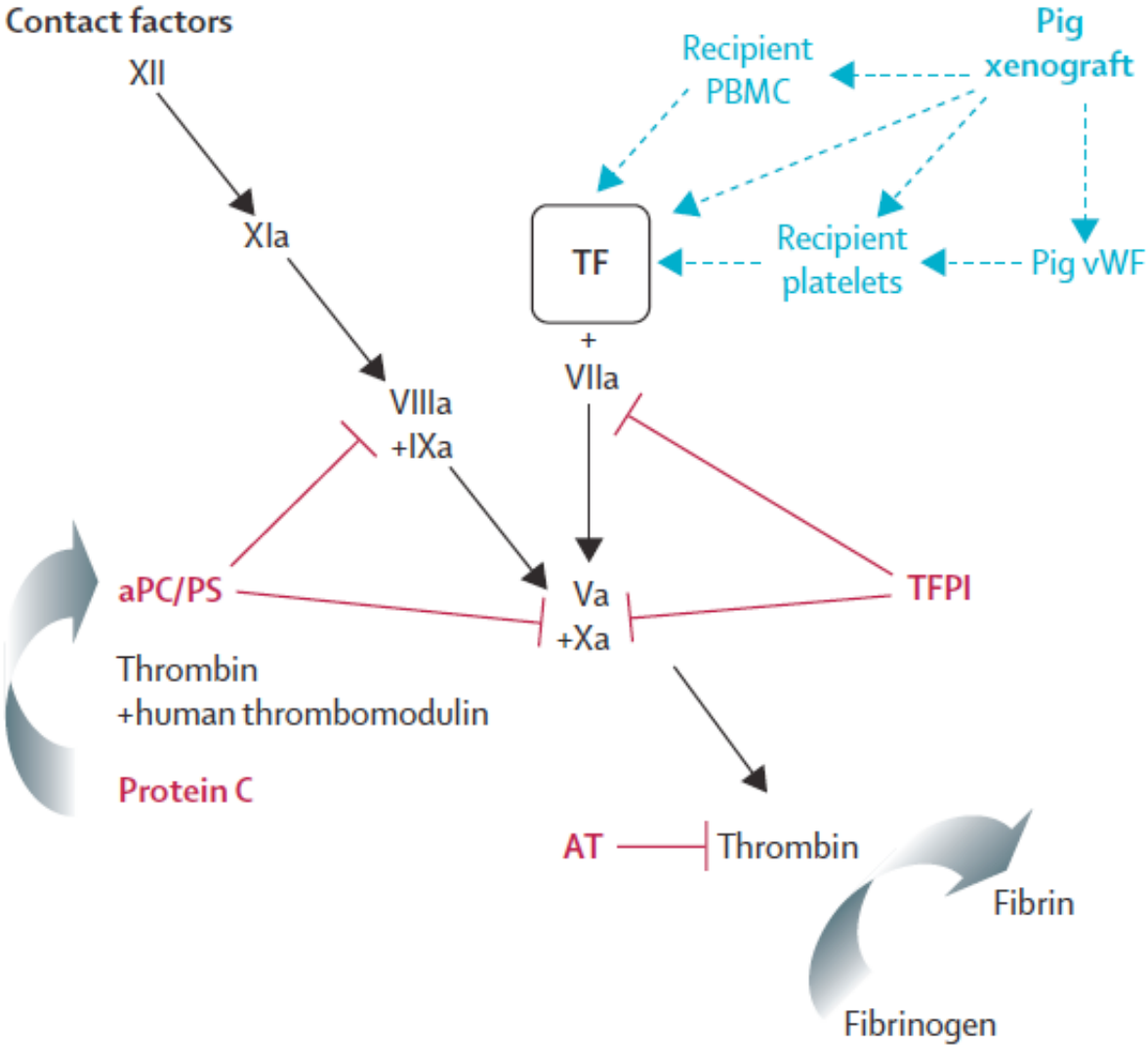


Legend – Figure 5: H&E stain, magnification x200. Fibrin deposition and platelet aggregation result in thrombosis within vessels of the graft (green arrow), leading to extensive myocyte death and fibrosis (white arrow). (Obtained from Ekser et al. Lancet 2012;379:672-683)

1.3.9 Molecular incompatibilities between pigs, primates and humans

Because of evolutionary divergence between primate and pig, there are some physiological barriers that remain to be overcome for successful xenotransplantation. Dysregulation of coagulation which is believed to contribute to consumptive coagulopathy after xenotransplantation is a major challenge. The coagulation cascade is tightly controlled by coagulation regulators, which are mainly TFPI, protein C system/ thrombomodulin and antithrombin. In the initiation stage of coagulation, activated FVII binds to TF and initiates a trickle of thrombin, which is regulated by the function of TFPI. However, *in vitro* porcine TFPI inefficiently inhibits human TF-dependent FX production and during AHXR, loss of porcine TF promotes the development of a procoagulant environment [**Kopp, 1997**]. This concept has been challenged by a recent study which showed porcine and human TFPI shared about 80% protein and over 80% in the functional Kunitz-1 and Kunitz-2 domains. The ability to inhibit human FVIIa/TF complex was also similar between both TFPIs [**Lee, 2008**]. In the propagation stage, the coagulation cascade is mainly regulated by thrombomodulin/protein C. Although porcine thrombomodulin shares 69% sequence identity with human thrombomodulin and binds to human thrombin, it is a poor cofactor to activate protein C and thrombin-activatable fibrinolysis inhibitor, contributing to insufficient cleavage of FV and FVIII [**Kopp, 1998a; Roussel, 2008**]. In terms of platelet activation, porcine vWF is known to spontaneously aggregate human platelet, in the absence of shear stress via GPIb because of difference in the O-linked glycosylation pattern between porcine and human vWF A1 domain [**Pareti, 1992; Schulte Am Esch, 2005**]. Major features of the human coagulation cascade in response to pig xenograft are explained in Figure 6 [**Ekser, 2012a**].

Figure 6: Major features of the human coagulation cascade in response to pig xenograft



Legend – Figure 6: Interaction between pig cells and recipient cells is shown in blue. When pig endothelium is activated, pig TF is expressed and released into the circulation. After interaction with pig endothelium, recipient platelets and PBMC express primate TF. Pig vWF also binds to platelet Gp1b. Coagulation cascade is shown in black. The extrinsic pathway is initiated by TF, which forms complexes with factor VIIa, activating factors IX and X, which activates thrombin downstream. Thrombin cleaves fibrinogen into fibrin monomers, and activates factor XIII, which cross-links fibrin monomers into an insoluble clot (not shown). The natural inhibitors of coagulation are shown in red. TF/VIIa complex is rapidly inhibited by TFPI, which rapidly deactivates factor Xa and the VIIa/TF complex. AT inhibits thrombin by forming a complex with its active site. After binding to human thrombomodulin, thrombin's ability to convert protein C into aPC is enhanced. aPC and its cofactor PS deactivate factors Va and VIIIa. Pig TFPI can inhibit human TF. Pig thrombomodulin can bind human thrombin and protein C, but has poor cofactor activity. Pig vWF can bind human platelet GP1b in vitro more strongly than can human vWF, resulting in greater platelet activation. TF=tissue factor. PBMC=peripheral blood mononuclear cell. vWF=von Willebrand factor. TFPI=tissue factor pathway inhibitor. AT=anti-thrombin. aPC=activated protein C. PS=protein S. (Obtained from Ekser et al. Lancet 2012;379:672-683)

Transgenic expression of CD39 on the surface of mouse ECs was developed to prolong graft survival without overt bleeding tendency in the rodent model [**Dwyer, 2004**]. Transgenic expression of CD39 and human thrombomodulin significantly decreased platelet loss and fibrinogen consumption, and therefore amelioration or delay in thrombotic microangiopathy is seen in genetically-engineered pig-to-baboon heart xenotransplantation [**Ekser, 2013a; Iwase, 2013a**]. Human cytokines produced by the infiltrating mononuclear cells in AHXR trigger the interaction between proinflammatory cells. One unpublished data described that human IFN-γ do not stimulate PAECs and such incompatibilities might attenuate immune response of xenografts [**Bach, 1996**]. Nevertheless, mouse renal allografts lacking IFN-γ receptors rapidly developed ischemic necrosis because of massive damage to the microcirculation of the graft by infiltrating cytotoxic T lymphocytes [**Sis, 2007**]. These observations support the hypothesis that IFN-γ induced major histocompatibility complex class I proteins on donor tissue engage inhibitory receptors on recipient cytotoxic T lymphocytes and prevent them from injuring the microcirculation by perforin–granzyme mechanisms. Therefore, incompatibility of cytokines may indirectly promote coagulopathy in xenotransplantation.

1.4 LIVER XENOTRANSPLANTATION

1.4.1 Pig liver anatomy

Pigs are commonly used in experimental surgery owing to the numerous similarities between porcine and human anatomy and physiology. Their comparable size to human anatomy allows for greater scope in surgical procedures than is the case for many small animal models. However, in case of liver xenotransplantation, size-mismatch needs particular attention [**Ekser, 2010b**]. Moreover, pigs are readily available, relatively

inexpensive and generally robust for use as a surgical model. To perform liver resection or transplantation in a porcine model, a detailed knowledge of the anatomy is required. The intraparenchymal vascular and biliary anatomy and the segmental anatomy of the liver are important.

The pig liver is classically described as having three main lobes: the right lateral, the median and the left lateral lobes. These lobes are divided by deep interlobular fissures. The median lobe is further subdivided by a deep umbilical fissure, which extends almost up to the hilum, giving the appearance of two separate median lobes, which can be assigned the terms left and right median lobes. Unlike the human liver, the left lateral lobe is consistently the largest of all the lobes – if the median lobe is to be considered as two separate halves (Figure 7). The caudate lobe adjoins the right lateral lobe on the visceral surface and is usually identified by the presence of a small fissure, which partially separates it from the right lateral lobe. The IVC is intraparenchymal and runs within the caudate lobe [**Court, 2003**].

The gallbladder lies partly within the substance of the right median lobe, with a small impression on the adjacent left median lobe. The cystic duct runs in a thin layer of connective tissue along the visceral surface of the liver to the hilum, where it joins a short common hepatic duct, which lies within the hilar plate. The cystic duct has a spiral valve at the entry to the gallbladder, making cannulation and injection difficult. Owing to the prominent fissures in the pig, it is much easier to divide the liver anatomically into right and left hemi-livers than in the human liver. Also, as in humans, no branches of the bile duct, hepatic artery or portal vein are seen to cross between the left and right hemi-livers in the majority of cases (Figure 8).

Figure 7: External macroscopic appearance of a pig liver

Obtained from Court et al, Brit J Surg 2003;90:440-444.

Figure 8: Cast after trimming, showing main vasculature and biliary anatomy of a pig liver

Obtained from Court et al, Brit J Surg 2003;90:440-444.

Segmental anatomy of the pig liver

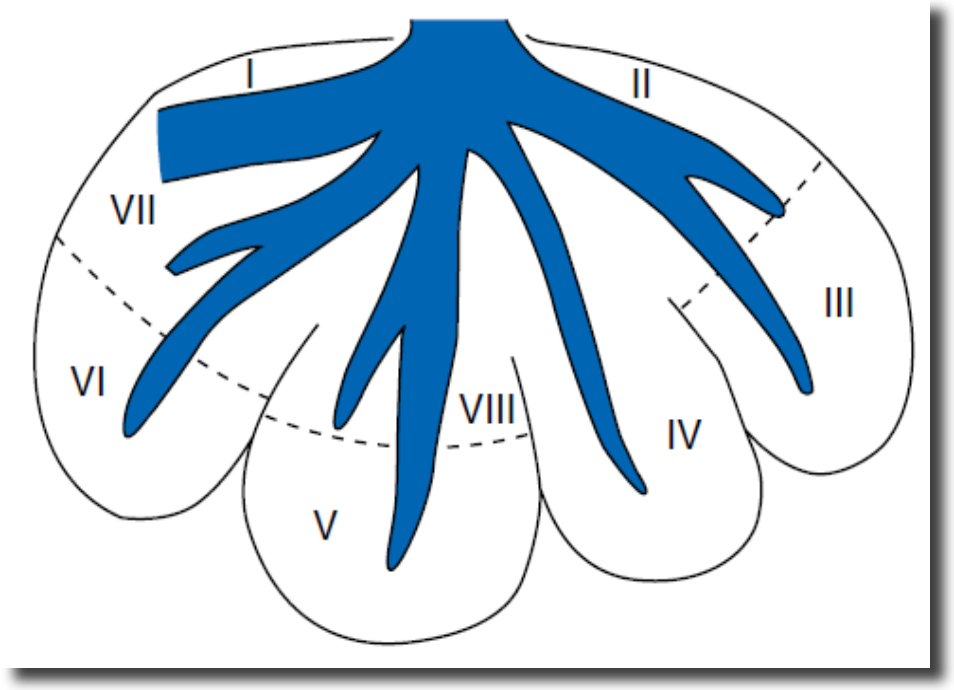
The pig liver can be divided into eight segments roughly similar to those described in the human liver by Couinaud [**Couinaud, 1954**]. Each segment has its own arterial supply, venous and biliary drainage. The left lateral lobe can be divided into segments II and III, and the right lateral lobe into segments VI, VII and I. The left medial lobe consists of segment IV, and the right medial lobe can be divided into segments V and VIII (Table 4).

Table 4: Segmental anatomy of the pig liver

Lobar Anatomy	Segmental Anatomy
Left lateral lobe	**Segments II and III**
Left medial lobe	**Segment IV**
Right medial lobe	**Segments V and VIII**
Right lateral lobe	**Segments VI and VII**
Caudate lobe	**Segment I**

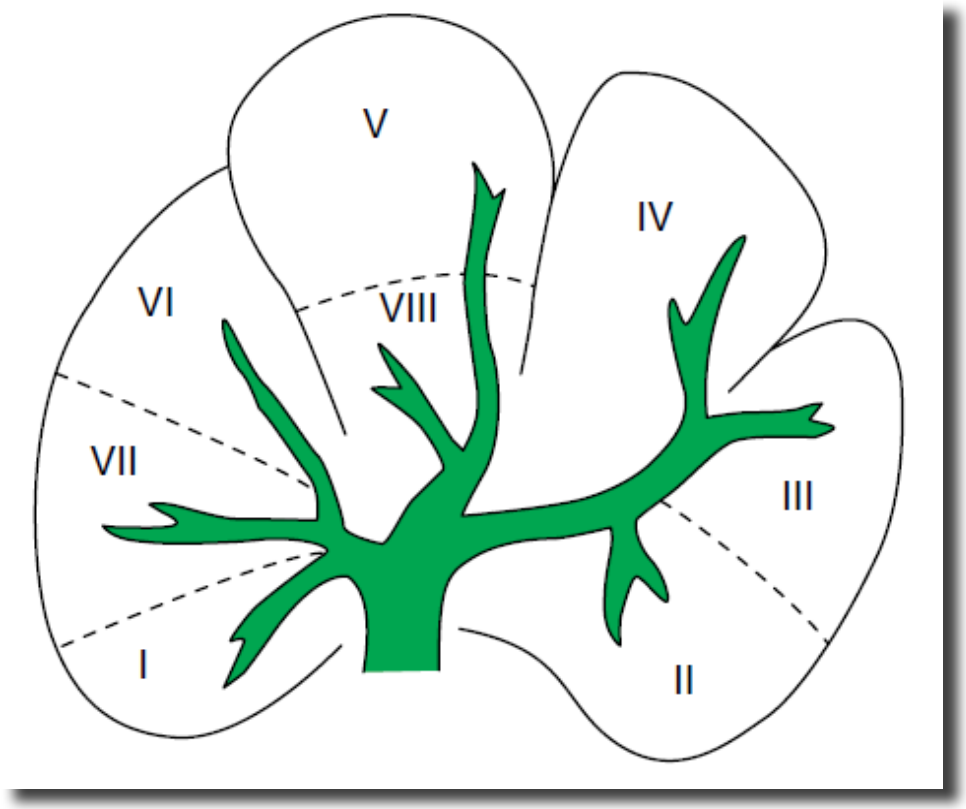
This segmentation is almost identical to that of the human, with the exception of segment I, or caudate lobe, which is adjacent to the visceral surface of the right lateral lobe and contains the cava within the parenchyma. There is also a marked difference in the relative volumes of the segments when compared with those in the human (Figure 9, and 10) [**Bismuth, 1993; Kahn, 1988**].

Figure 9: Segmental arrangement of hepatic veins of a pig liver



Obtained from Court et al, Brit J Surg 2003;90:440-444.

Figure 10: Segmental arrangement of portal veins of a pig liver



Obtained from Court et al, Brit J Surg 2003;90:440-444.

Hepatic vein of the pig liver

The hepatic vein has four main branches coming directly from the IVC and draining the left lateral, left medial, right medial and right lateral lobes respectively. Segment I drains directly into the IVC. The IVC and the hepatic vein confluence is intraparenchymal, thereby posing some problems during resection. The IVC and intraparenchymal hepatic veins have extremely thin walls and are easily damaged. The hepatic veins supplying the left lateral and left median lobes often have a common confluence at the cava, joining just proximal to the left fissure. The left lateral hepatic vein then has several short branches that drain segment II, and a longer branch that drains segment III. The left median hepatic vein supplies the entire left median lobe, which constitutes segment IV. The right median hepatic vein is formed from two tributaries draining segments V and VIII, which join just distal to the IVC confluence. The right hepatic vein is formed by several short branches that drain segment VII and a longer branch that drains segment VI (Figure 8, and 10).

Figure 11: Hepatic veins of pig liver (shown in blue)

Obtained from Court et al, Brit J Surg 2003;90:440-444.

Portal vein of the pig liver

The portal vein has two main branches, which divide at the hilum very close to the liver parenchyma. There are commonly one or even two communicating branches of the portal vein, which traverse the fissure between the right lateral and right median lobes of the liver. The most consistent of these is found at the origin of the right fissure and is a branch of portal vein supplying the right median lobe. There are no communicating branches between the right and left median lobes, thus allowing complete partition of right and left hemi-livers. The right portal trunk originates from the main portal trunk and divides into three branches supplying segments I, VII and VI respectively. The main portal trunk arches medially and gives off branches to the right median lobe, supplying segments VIII and V. The left portal trunk then continues intraparenchymally for 2–3 cm before dividing into two branches that supply the left median and left lateral lobes. The first of these two divisions supplies segment IV, but also gives off a branch that crosses the fissure and supplies segment III. The second division supplies segment II only (Figure 10, and 12).

Hepatic arteries of the pig liver

The main hepatic artery splits into a variable number of branches, but most commonly there are three branches visible anterior to the portal trunk. Two of these lie in the median edge of the porta hepatis and supply the left and median lobes respectively. However, they may be divided further before entering the liver parenchyma, thus resulting in three or even four branches in this area. The main branch supplying the right lateral lobe usually crosses the portal vein posteriorly, although there is often an accessory branch that lies anteriorly. The intraparenchymal hepatic arteries invariably follow the portal veins and supply the same segments (Figure 12).

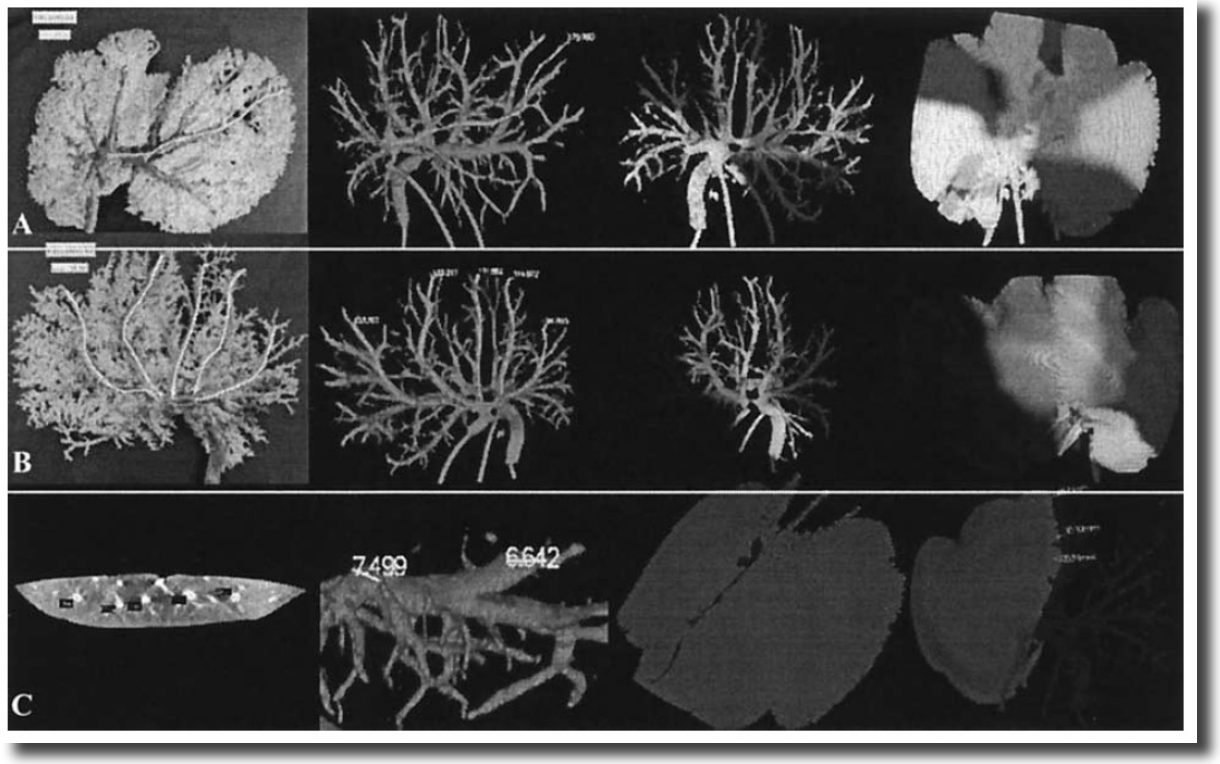
Biliary tree of the pig liver

The right and left hepatic ducts generally drain their respective halves of the liver before emptying into the common hepatic duct. Again, as in the human liver, the segmental distribution of the hepatic ducts follows that of the portal vein as they lie together within the Glissonian sheaths (Figure 8). The left hepatic duct is generally of much larger calibre than the right, and drains both the left median and left lateral lobes. The right hemi-liver is drained by two separate hepatic ducts, similar to the arrangement in the human liver. The smaller anterior duct drains the right median lobe before emptying into the common hepatic duct medial to the middle branch of the portal vein. The larger posterior duct drains the right lateral lobe and caudate, and usually unites with the anterior duct to form a single right hepatic duct. Occasionally, however, it is seen to drain directly into the common hepatic duct or even into the left hepatic duct. The right posterior duct traverses along the under surface of the left portal trunk from the right lateral lobe to the left side of the middle portal branch. This duct can therefore be damaged easily during dissection and ligation of the left and middle branches of the portal vein or hepatic ducts (Figure 12 and 13).

Figure 12: Portal veins (green), hepatic arteries (red) of pig liver

Obtained from Court et al, Brit J Surg 2003;90:440-444.

Figure 13: Images from the angiogram, acrylic photography and the software LIVER3D.



Legend – Figure 13: ***A*** *Pig liver segments in acrylic model and 3D images.* ***B*** *Pig liver sectors in acrylic model and 3D images.* ***C*** *Bidimensional angiogram, zoom of the vessels, VHM identification, and hepatectomy planning. Obtained from Zanchet et al, Transplant Proc 2005;37:198-200*

1.4.2 History of clinical liver xenotransplantation

There have been 7 reported cases in which auxiliary (heterotopic) liver xenografts have provided short-term support for patients in hepatic failure, although in no case did the patient survive longer than 3 days (Table 5) [**Taniguchi, 1997a, Taniguchi, 1997b**]. Heterotopic liver transplantation is accomplished by the insertion of the graft in parallel, or in continuity, with the recipient's own liver. This operation has usually been performed with the purpose of supporting the patient during the period of liver failure but with the retention of the native liver in the hope of its spontaneous recovery.

The first of such procedures was performed in 1966 by Starzl et al. who attached a chimpanzee liver to the femoral vessels of a young child in hepatic coma. The graft functioned for 24 hours, during which period the patient's coma was partially resolved, and clearance of bilirubin and alkaline phosphatase was documented [**Starzl, 1966**]. The loss of the graft was associated with the formation of thrombi in the hepatic artery and several smaller vessels. This result is typical of subsequent experience (Table 5).

Makowka and his colleagues [**Makowka, 1994, Makowka, 1995**] performed the only heterotopic liver xenotransplantation using a pig organ to support a patient with advanced liver failure (Table 5), with the aim of performing orthotopic allotransplantation when a human donor became available, at which time the auxiliary pig liver would have been removed. The patient was a 26-year-old woman with a 14-year history of autoimmune hepatitis that had been controlled by treatment with prednisone and azathioprine. Ten

days before the transplantation, she developed jaundice, diarrhea, abdominal pain, and lassitude. She did not respond to high-dose oral prednisone and was subsequently admitted to the hospital with grade III encephalopathy. Hepatitis C was detected serologically. She was listed with the United Network for Organ Sharing at the highest priority for a human donor of any blood type and organ size. The patient continued to deteriorate, with increasing encephalopathy and coagulopathy. She was sedated and intubated, and aggressive treatment for cerebral edema was instituted. A pig liver was transplanted heterotopically. Before transplantation, circulating natural anti-pig antibodies were removed by plasmapheresis and *ex vivo* perfusion of the donor pig kidneys. After transplantation, the liver xenograft clearly functioned, as documented by active bile production, stabilization of prothrombin levels, and reduction in the levels of lactic acid and the enzymes AST and ALT. Unfortunately, the liver graft did not result in any improvement in the neurologic status of the patient, and she died after 34 hours from irreversible brain damage. Despite the removal of >90% of the recipient's natural xenoantibodies prior to transplantation, the antibody rapidly returned and was associated with complement-mediated rejection of the graft. This experience demonstrated the ability of a pig liver to function, at least temporarily, in a human recipient and to provide some metabolic support during ALF. However, the case highlighted a problem that may be met in future cases of xenotransplantation, namely, the risks associated with the transfusion of human plasma to a recipient of a pig xenograft. Pooled plasma contains anti-pig antibodies, which may bind to the transplanted pig organ and initiate rejection. As the authors pointed out, the generous administration of fresh frozen plasma to Makowka's patient may well have contributed to graft failure. In the future in such cases, steps will need to be taken to adsorb anti-pig antibodies from all relevant blood products before transfusion [**Makowka, 1995]**.

Table 5: World experience in clinical liver xenotransplantation

Year	Surgeon	Donor	Patient Survival (days)
Heterotopic (Auxiliary) Liver Xenotransplantation			
1966	Starzl	Chimpanzee	**<1**
1969	Bertoye	Baboon	**<1**
1970	Leger	Baboon	**3**
1970	Marion	Baboon	**<1**
1971	Poyet	Baboon	**<1**
1971	Motin	Baboon	**3**
1993	Makowka	Pig	**<2**
Orthotopic Liver Xenotransplantation			
1969	Starzl	Chimpanzee	**9, <2**
1974	Starzl	Chimpanzee	**14**
1992	Starzl	Baboon	**70**
1993	Starzl	Baboon	**26**

Reproduced from Hara et al, Liver Transpl 2008;14:425-434

Orthotopic allotransplantation of the liver is the most effective treatment for hepatic failure. There have been 5 cases of clinical orthotopic liver xenotransplantation. The sources of the organs were the chimpanzee in 3 cases and the baboon in 2 (Table 5). The 2 most recent patients, both with hepatic failure caused by hepatitis B, received livers from baboons. The 2 grafts functioned for 70 and 26 days, respectively, without evidence of graft infection by the hepatitis virus. The first case, in which the patient was also HIV+, can be considered a relative success in that there was little pathologic evidence of rejection in the liver, but this was achieved probably at the expense of overimmunosuppression, the patient dying of overwhelming sepsis. The second case was less successful as the patient did not regain consciousness or renal function during the postoperative period, but again there was little histopathologic evidence of rejection in the transplanted liver [**Starzl, 1993**].

1.4.3 Concordant nonhuman primate liver xenotransplantation

There are several reports of concordant liver xenotransplantation in nonhuman primates (Table 6). Orthotopic liver transplantation has been carried out between cynomolgus and rhesus monkeys (with survival of <1 to 20 days), between vervet (African Green) monkeys and baboons (with survival of 2 to 420 days) and between baboons and rhesus monkeys (with survival of 91 and 1076 days) [**Zhong, 1996; Zhong, 2003**]. The 2 baboon-to-rhesus monkey liver grafts survived longer than kidney grafts under the same regimen. The 91-day survivor developed post-transplantation lymphoproliferative disease and required euthanasia. In the second case, all immunosuppression was discontinued at 1 year, and chronic rejection developed after 900 days. Vervet monkey livers have been transplanted heterotopically into ABO-compatible baboons (with survival of 20 to 123 days) and ABO-incompatible baboons (with survival of 28 to 45 days). HAR was not observed in the xenografts, and in the early stages, the appearances were similar to those of allografts.

Table 6: Experience in experimental concordant liver xenotransplantation in primates

Donor	Recipient	Type of Transplant	No	Survival (days)
Cynomolgus Monkey	Rhesus Monkey	OLT	3	<1-20
Vervet Monkey	Baboon	OLT	13	2-240
Baboon	Vervet Monkey	OLT	2	91 and 1076
Vervet Monkey (ABO-compatible)	Baboon	HLT	8	20-123
Vervet Monkey (ABO-incompatible)	Baboon	HLT	6	28-45

Legend – Table 6:: HLT= heterotopic liver transplantation, OLT= orthotopic liver transplantation. Reproduced from Hara et al, Liver Transpl 2008;14:425-434

1.4.4 Discordant (pig-to-primate) liver xenotransplantation

The pig-to-nonhuman primate model is increasingly being utilized as the final preclinical method of assessing strategies aimed at allowing clinical xenoxenotransplantation. In the earliest documented study, Calne et al. [**Calne, 1968; Calne, 1970**] reported survival of pig livers in (1) baboons (for 6 hours to 3.5 days), (2) rhesus monkeys (for <12 hours), and (3) a chimpanzee (for 8 hours; Table 7). Powelson et al. [**Powelson, 1994**] reported survival for 2 hours to 3 days of orthotopic pig livers in cynomolgus monkeys and baboons with an intensive regimen (aimed toward inducing tolerance) that included whole body and thymic irradiation, antithymocyte globulin, extracorporeal plasmapheresis, and pig bone marrow transplantation. Mieles et al. and Luo et al. [**Miles, 1995; Luo, 1998**] reported orthotopic liver transplantation in the pig-to-rhesus monkey model and heterotopic liver transplantation in the pig-to-baboon model. In untreated nonhuman primates, HAR occurred in all cases and was characterized histologically primarily by severe congestion and hemorrhage. No intravascular fibrin aggregation was seen. In a report by Ramirez et al.,[**Ramirez, 2000; Ramirez, 2001**] 3 baboons transplanted with livers from unmodified pigs survived for <12 hours and showed features of HAR. Two baboons transplanted with livers from pigs transgenic for hDAF survived for 4 and 8 days, respectively. One underwent cardiac arrest on day 4 after an episode of vomiting and aspiration, and the other was euthanized on day 8 after the development of sepsis and coagulopathy. Neither liver xenograft demonstrated histopathologic features of rejection. This study indicated that, when HAR is abrogated (by the presence of hDAF), the porcine liver can maintain reasonable levels of coagulation factors and protein in the baboon for up to 8 days. Interestingly, porcine fibrinogen produced by the graft was detected 2 hours after transplantation and gradually replaced primate fibrinogen in the blood. The authors drew attention to previous rodent studies that established that the recipient progressively acquires the protein profile of the donor species. In a subsequent brief report, livers from

pigs transgenic for CD55, CD59, and H-transferase survived only 13-24 hours in baboons [**Ramirez, 2005**]

Table 7: World experience of pig-to-nonhuman primate liver xenotransplantation

Study (year)	Pig type	Recipient primate	Type of Tx	Tx (n)	Immunosuppression	Survival (h)
Calne *et al.* (1968)	WT	Baboon	Orthotopic	7	Azathioprine + Cs (n = 2), Cs (n = 2), none (n = 3)	6, 6, 9, 19, 30, 36, 84
Calne *et al.* (1970)	WT	Rhesus	Orthotopic	3	ALG	12, 12, 12
Calne *et al.* (1970)	WT	Chimpanzee	Orthotopic	1	None	8
Powelson *et al.* (1994)	WT	Cynomolgous	Orthotopic	4	EIA (organ) + WBI + TI + ATG + pig BM	2, 4, 5, 13, 72, 75
Powelson *et al.* (1994)	WT	Baboon	Orthotopic	2	EIA (organ) + WBI + TI + ATG + pig BM	2, 4, 5, 13, 72, 75
Luo *et al.* (1998)	WT	Rhesus	Orthotopic	6	None (n = 3), CsA + Cs + Dashen (n = 3)	2 – 5.5
Luo *et al.* (1998)	WT	Baboon	Heterotopic	2	CyP + CsA + Cs	2, 2
Ramírez *et al.* (2000)	WT	Baboon	Orthotopic	3	CyP + CsA + Cs	2, 3, 8
Ramírez *et al.* (2000)	hCD55	Baboon	Orthotopic	2	CyP + CsA + Cs	96, 192
Ramírez *et al.* (2005)	hCD55.CD59.HT	Baboon	Orthotopic	5	CyP + Daclizumab + Rituximab + CsA + MMF + Cs	13, 18, 20, 21, 24
Ekser *et al.* (2010)	WT	Baboon	Orthotopic	1	None	5
Ekser *et al.* (2010)	GTKO	Baboon	Orthotopic	2	ATG + Tacrolimus + MMF + Cs	3, 144
Ekser *et al.* (2010)	GTKO.hCD46	Baboon	Orthotopic	8	ATG + Tacrolimus + MMF + Cs (n = 5), CyP + Tacrolimus + MMF + Cs (n = 3)	3, 20, 24, 96, 120, 144, 144, 168
Schuetz *et al.* (2011)	GTKO	Baboon	Orthotopic	2	ATG + CVF + anti-CD154mAb + AZA + LoCD2b (only in one case) + Tacrolimus + Cs	144, 216

Legend – Table 7: ALG: Antilymphocyte globulin; ATG: Anti-thymocyte globulin; AZA: Azathioprine; BM: Bone marrow; CD154mAb: Monoclonal antibody for a protien member of the TNF superfamily which is expressed on actived T cells; CD46: Membrane cofactor protein; CD55: Decay accelerating factor; CD59: Homologous restriction factor (protectin); Cs: Corticosteroids; CsA: Cyclosporine; CVF: Cobra venom factor; CyP: Cyclophosphamide; EIA: Extracorporeal immunoaffinity column of αGal oligosaccharide with swine kidney or liver; GTKO: α1,3-galactosyltransferase gene-knockout; h: Human; HT: H-transferase (α1,2-fucosyltransferase); LoCD2b: (Rat antiprimate CD2b monoclonal antibody; MMF: Mycophenolate mofetil; TI: Thymic irradiation; Tx: Transplatation; WBI: Whole body irradiation; WT: Wild-type. (Obtained from Ekser et al. Expert Rev Clin Immunol 2012;8:621-634)

Since there are few studies on liver xenotransplantation, and graft survival has been uniformly short, the understanding of liver xenograft immunology is limited. Recently, Hara et al [**Hara, 2008**] came to the following conclusions.

1. After xenotransplantation, complement proteins will be produced by the pig liver [**Ramirez, 2000**]. The pig CRPs on the organ will probably provide some protection against pig complement, even in the presence of human anti-pig antibodies [**Platt, 2001**]. hDAF-transgenic pig livers will express both human and pig CRPs [**Luo, 2002**]. There is evidence that human CRPs can efficiently neutralize activated porcine complement [**Rees, 2004**] (though Tai et al [**Tai, 2007**] have demonstrated that the combination of *pig* anti-human antibodies and *pig* complement can injure human tissues).
2. The porcine hepatic sinusoidal endothelial cells may be rather more resistant to injury by human serum than porcine aortic endothelial cells [**Tector, 1998**].
3. Inflammatory immune complexes may form when material secreted by a transplanted organ becomes bound by antibodies [**Kanai, 2000**], and may injure the graft. Other protein-protein interactions, such as reaction of porcine von Willebrand factor (vWF) with human platelet 1b receptors, could potentially lead to thrombocytopenia. However, the liver's ability to clear soluble immune complexes may diminish the injury caused to the liver [**Tector, 1997**].

Kupffer cells within the liver xenograft may phagocytose the red blood cells of the primate recipient, necessitating frequent blood transfusions to maintain a normal hematocrit [**Luo, 2002; Rees, 2002**].

With the recent data obtained from our experiments and Dr. Tector's laboratory of the Indiana University, we were able draw a conclusion in immunobiology of liver xenotransplantation with colleagues from the University of Pittsburgh, University of Catania, Indiana University, Harvard University, University of Toledo, and Revivicor Inc., which were [**Ekser, 2012e**];

(i) After pig liver xenotransplantation, the key current problem is platelet activation, aggregation and/or phagocytosis that cause immediate and severe thrombocytopenia, resulting in hemorrhage.

(ii) There is an evidence of platelet phagocytosis by pig liver sinusoidal endothelial cells, hepatocytes and Kupffer cells.

(iii) Platelets aggregate to each other, bind to white blood cells in the blood, sequestrated in the liver xenograft and removed from circulation in the native lungs.

(iv) There is an evidence of initial tissue factor expression on platelets and peripheral blood mononuclear cells, which causes platelet aggregation without the need for liver sinusoidal endothelial cells activation.

(v) Genetically engineered α1,3-galactosyltransferase gene-knockout pigs have brought liver xenotransplantation a step closer to the clinic, with no HAR or early AHXR, and normal or near-normal liver function and coagulation in the recipient nonhuman primate after xenotransplantation.

(vi) Further genetic modifications may be well required to avoid platelet activation and phagocytosis, such as expression of thromboregulatory genes (CD39, thrombomodulin, endothelial protein C receptor) or knockdown of specific pro-aggregatory elements (e.g.,

asialoglycoprotein receptor-1, CD18) or expression of a specific protein (e.g., human signal regulatory protein-α).

(vii) If immediate platelet activation and severe thrombocytopenia can be prevented, a clinical trial of genetically engineered pig liver xenotransplantation as a 'bridge' to allotransplantation in patients with acute liver failure may be justified.

(viii) The expense of developing genetically engineered pigs is considerable, as is testing their organs in nonhuman primate models, and therefore this is a limiting factor in advancing the field.

1.5 ACUTE LIVER FAILURE

Since the clinical pig liver xenotransplantation [**Ekser, 2011; 2012a, 2012e**], is offered to bridge acute liver failure (ALF) patients to allotransplantation, details in this subject is timely.

1.5.1 Definitions

ALF is potentially devastating and is associated with a high mortality from cerebral edema (encephalopathy), hemodynamic instability, and coagulopathy. Under the term of ALF there 3 other definitions:

1) Acute on chronic liver failure

Acute-on-chronic liver failure (ACLF), defined as acute deterioration in liver function over a short period, occurs in patients with well-compensated liver disease following a precipitating event, and is characterized by jaundice, hepatorenal syndrome, and hepatic encephalopathy. Liver transplantation is the treatment of choice for type-1 or type-2 hepatorenal syndrome [**Arroyo, 2008; Munoz, 2008; McCormick, 2008**]. The

hemodynamic and neurohumoral abnormalities associated with hepatorenal syndrome disappear within the first month post-transplant and patients regain the ability to excrete sodium and free water. Survival of patients with hepatorenal syndrome who undergo liver transplantation is 62% at 5 years [**Ruiz, 2007**]. The problem with liver transplantation in the hepatorenal syndrome, particularly for type-1, is that most patients die before a liver becomes available. The overall mortality in ACLF is 50-90% [**Kjaergard, 2003; Jalan, 2002**], and the need to develop bridge-to-transplantation therapy for such patients is obvious.

Extracorporeal liver perfusion with pig livers, initially carried out in the late 1960s and early 1970s [**Abouna, 1968; Eiseman, 1965; Abouna, 1997; Chari, 1994]** for patients with ACLF prior to the development of liver transplantation, provides strong supporting evidence that the pig liver can be used to improve the patient's clinical condition in this population. Acute decompensation of chronic liver disease is frequently caused by variceal hemorrhage secondary to long-standing portal hypertension that can be relieved by removing the native liver.

2) Primary allograft failure

The incidence of primary non-function in liver transplantation is 6% [**Uemura, 2007; Ploeg, 1993; Pokorny, 2000**]. Hepatic artery thrombosis occurs in 3% of adult liver transplants and 8.3% in children [**Bekker, 2009**]. These complications may result in as many as 10-15% of recipients requiring urgent re-transplantation. UNOS data [**UNOS**] and a recent systematic review [**Bekker, 2009**] suggest that approximately 50% of these adult patients and 62% of children undergo re-transplantation within 14 days of being listed as status 1, while 10-33% die within 14 days without the benefit of re-transplantation, and 32% are removed from the waiting list as they become too sick to undergo the procedure. Long-

term outcomes for early re-transplantation for hepatic artery thrombosis or primary non-function can be quite good if performed within a month of the initial transplant, with graft and patient survival approaching 75% at 3 years.

3) Acute (fulminant) liver failure

The etiologies of ALF vary greatly by country and have evolved over time. Acetaminophen toxicity accounts for almost 50% of all cases [**Lee, 2008**], (Figure 16), and its incidence in the USA is increasing. Additional causes include other drugs, viral hepatitis (mainly hepatitis A and B), autoimmune hepatitis, Budd-Chiari syndrome, complications of pregnancy, Wilson's disease, neonatal liver failure [**Grabhorn, 2008**], (Figure 14, and 15) as well as miscellaneous other causes, such as mushroom poisoning and heat stroke; the cause in some cases remains unknown [**Lee, 2008; Grabhorn, 2008**].

Figure 14: Etiology of acute liver failure in infants ages 0 to 3 years in the U.S.

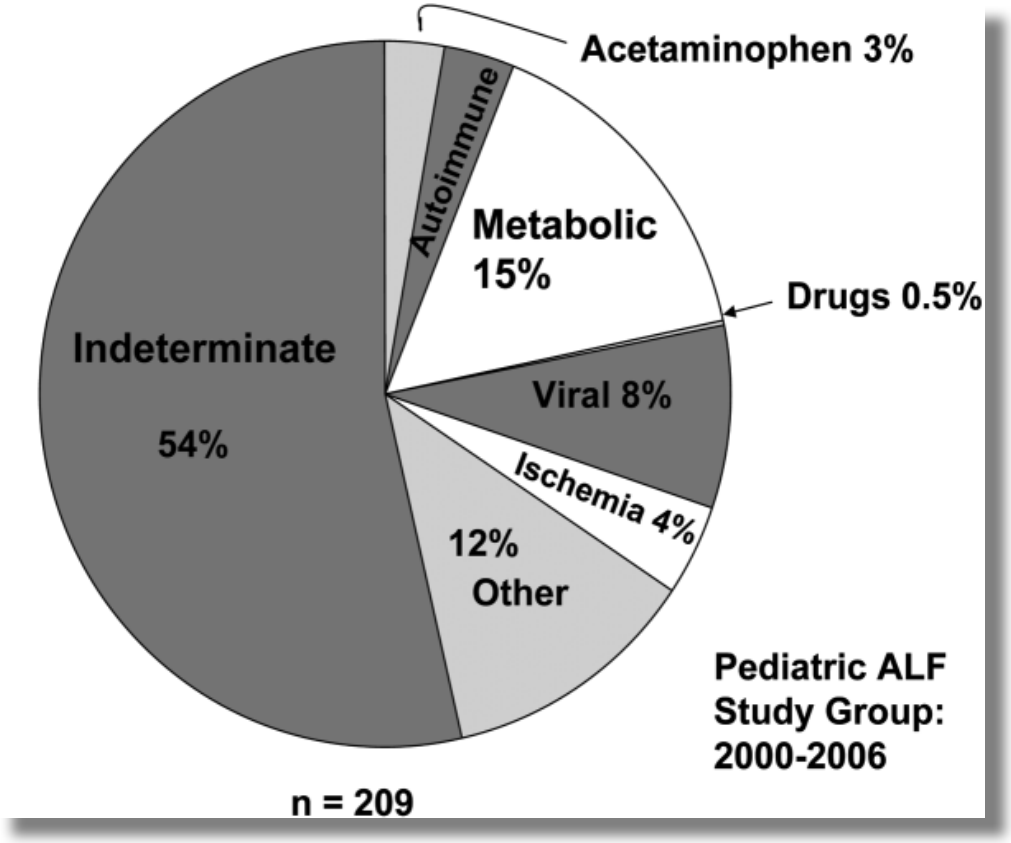


Figure 15: Etiology of acute liver failure in children ages 3 to 18 years in the U.S.

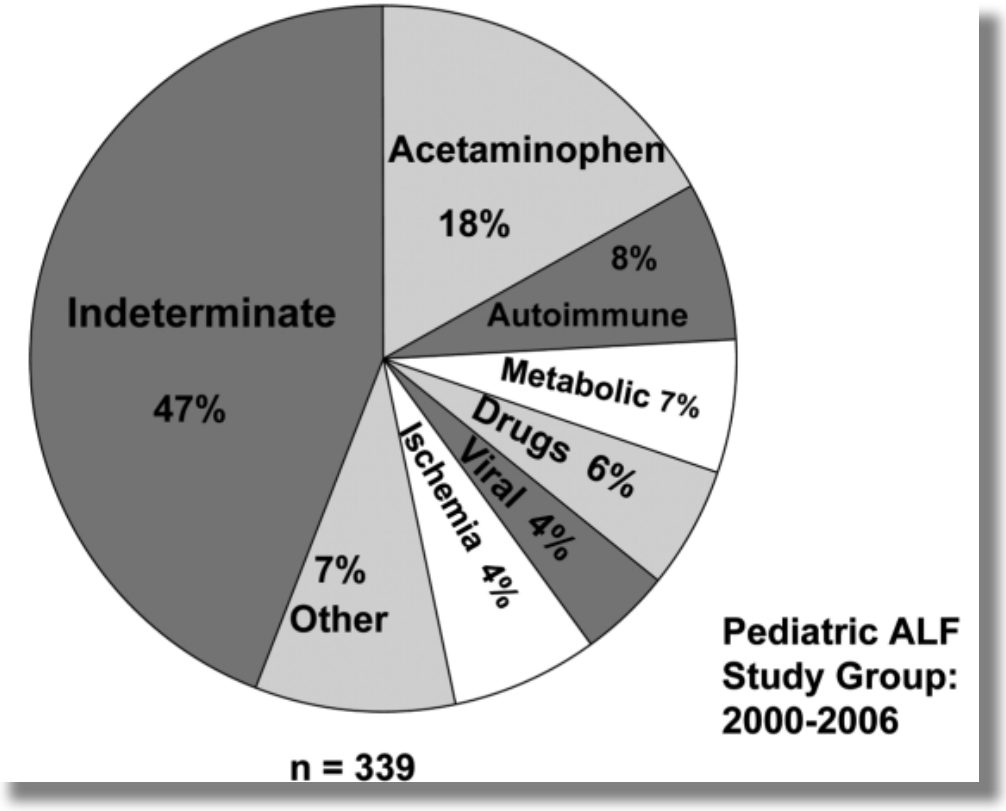
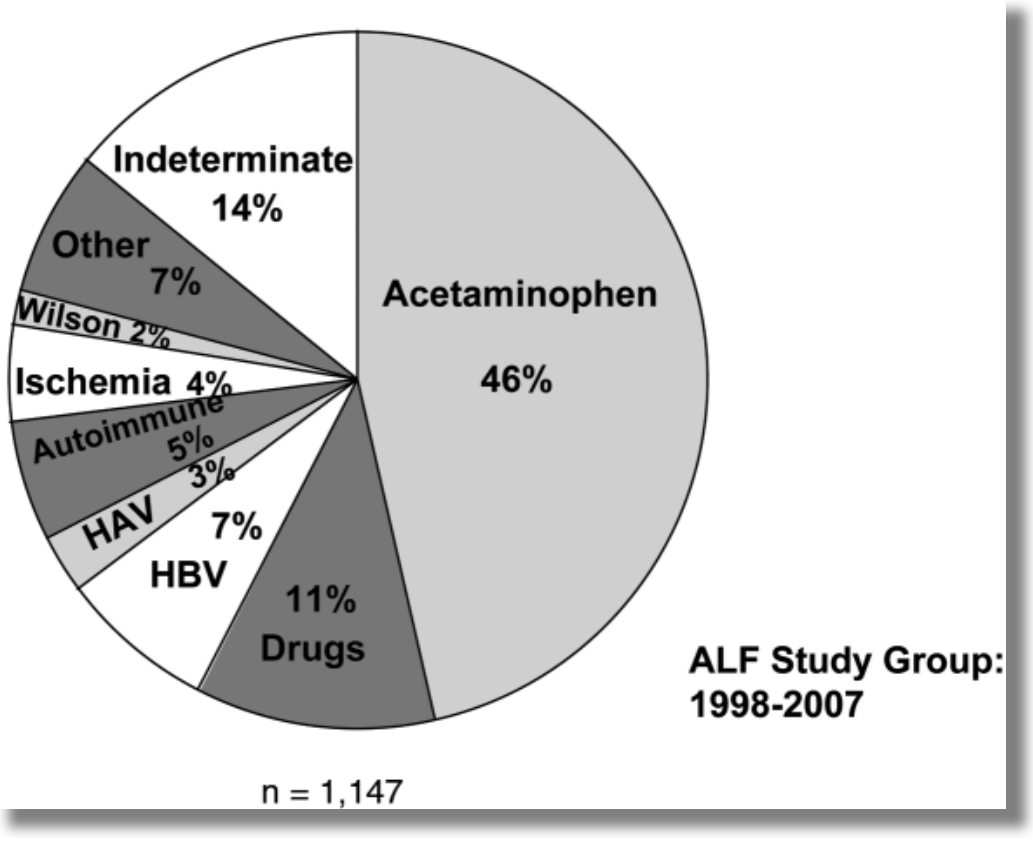


Figure 16: Etiology of acute liver failure in adults in the U.S.



1.5.2 The prompt need for liver transplantation for ALF

Prior to the successful introduction of liver transplantation for ALF, the mortality rate was 80-85% [**Bernuau, 1986**]. Currently in the US, there are approximately 1,600 patients with ALF each year [**Bower, 2007**]. In adults, recovery with medical therapy occurs in approximately 45%, liver transplantation is required in 25%, and death without transplantation occurs in 30%. Higher rates of recovery (56%) and transplantation (31%) are reported in children, with a concomitant reduced number of deaths (13%) [**Schiodt, 1999; Shakil, 2000**]. Thus, successful pig liver bridging may be life-saving in previously mentioned cases under the subchapter 1.5.1 Acute Liver Failure.There are specific criteria for urgent listing of these patients (e.g. UNOS category Status 1A (Table 8) and the United Kingdom Super Urgent Liver Scheme (Table 9). Despite the availability of several prognostic scoring systems for ALF, such as those of Clichy, King's College, APACHE II, and MELD, the American Association for the Study of Liver Diseases does not recommend reliance on any one [**Polson, 2005; Liou, 2008**]. However, in the setting of the donor organ shortage, it is important to identify patients who are too ill to benefit from liver transplantation and remove them from the waiting list.

Table 8: UNOS inclusion (and potential exclusion) criteria for liver transplantation in patients with ALF*

UNOS Inclusion Criteria for Adults (Status 1A)
1) Age ≥18 years
2) Life expectancy without liver Tx of <7 days
3) Onset of encephalopathy within 8 weeks of the first symptoms of liver disease
4) Absence of pre-existing liver disease (except for fulminant Wilson's disease)
5) Residence in the intensive care unit
6) At least one of the following: - ventilator-dependence, - renal replacement therapy - INR (international normalized ratio) >2.0
7) Complication of a transplanted liver within 7 days of implantation - primary non-function of a transplanted liver - hepatic artery thrombosis in a transplanted liver with evidence of severe liver injury
UNOS Inclusion Criteria for Children (Status 1A)
Same criteria as in adults except: - A PELD (Pediatric End-Stage Liver Disease) score of >25 in patients <12 years-old or a MELD (Model for End-Stage Liver Disease) score of >25 in patients 12-17 years-old. - Hepatic artery thrombosis in a transplanted liver within 14 days of implantation
Potential Exclusion Criteria
1) Age >70 years old (relative)
2) Certain malignancies outside of the liver
3) Severe cardiac, lung, or multiple organ failure
4) Uncontrolled septic shock / severe infection
5) Uncontrolled bleeding
6) Evidence of irreversible brain damage

Legend – Table 7: UNOS and Lee WM, 2008, Table adopted from Ekser, Transplantation 2009;88:1041-1049

Table 9: Selection criteria for urgent liver transplantation in the United Kingdom and Republic of Ireland. At least one of the following criteria must be met:-

Paracetamol poisoning
Category 1: pH <7.25 >24h after overdose and after fluid resuscitation
Category 2: co-existing PT >100 sec or INR>6.5, and serum creatinine >17.5 mg/dL (>300 µmol/l) or anuria, and Grade III-IV encephalopathy
Category 3: serum lactate >3.5 mmol/L on admission >24h after overdose or >3.0mmol/L after fluid resuscitation
Category 4: two of the 3 criteria from category 2 with clinical evidence of deterioration (e.g. increased intracranial pressure, FiO2 >50%, increasing inotropic requirements) in the absence of clinical sepsis
Seronegative hepatitis, Hepatitis A or B, or an idiosyncratic drug reaction
Category 5: PT >100 sec or INR >6.5, and any grade of encephalopathy
Category 6: any grade of encephalopathy and any 3 from the following;
a) unfavourable etiology (idiosyncratic drug reaction, seronegative hepatitis)
b) age >40 years
c) jaundice to encephalopathy time >7 days
d) serum bilirubin >17.5 mg/dL (>300 µmol/L)
e) PT >50 sec or INR >3.5
Acute presentation of Wilson's disease, or Budd-Chiari Syndrome
Category 7: a combination of coagulopathy, and any grade of encephalopathy
Post-liver transplantation complications
Category 8: hepatic artery thrombosis on days 0 to 14
Category 9: early graft dysfunction on days 0 to 7 with at least 2 of the following: AST >10,000 IU/L, INR >3.0, serum lactate >3.0 mmol/L, absence of bile production
Acute liver failure in children <2 years of age
Definition: multi-system disorder in which severe acute impairment of liver function with or without encephalopathy occurs in association with hepatocellular necrosis (in the absence of recognized underlying chronic liver disease)
INR >4.0 or Grade III/IV encephalopathy
paracetamol overdose - adult criteria apply
exclude leukemia/lymphoma, hemophagocytosis, disseminated intravascular coaguation
exclude ALF associated with conditions that will not be cured by liver Tx : a) mitochondrial disorder with neurological involvement, b) Niemann-Pick disease type C, c) Giant-cell hepatitis with Coombs' positive hemolytic anemia
AST = aspartate transaminase, INR = international normalized ratio

Table adopted from Ekser, Transplantation 2009;88:1041-1049

Unfortunately, clinical deterioration may develop rapidly, and many patients are removed from the urgent waiting list prior to a donor organ becoming available. This problem was demonstrated in patients with acetaminophen-induced ALF who fulfilled the King's College Hospital Criteria [**O'Grady, 1993**]. Thirty percent (30%) could not be listed for transplantation because of the rapid onset of complications, and 35% of those who were listed were eventually "delisted" because of rapid clinical deterioration. The majority (90%) who initially met the criteria for liver transplantation did not undergo the procedure and died [**Bernal, 1998**]. In the largest US study, 29% of patients with ALF underwent liver Tx, but 25% of those listed (10% of the entire group) died prior to receiving an organ [**Ostapowicz, 2002**].

1.5.3 Bridging ALF patients to liver allotransplantation

Alternatives to bridging to allotransplantation using a pig liver;

*a) Extracorporeal artificial liver devices*In recent years there has been renewed interest in techniques for providing temporary liver support to bridge the patient with rapidly deteriorating liver function to orthotopic liver transplantation or to allow time for liver regeneration. Such liver support can be non-biologic or biologic, and both methods of support are in clinical use [**reviewed in van de Kerkhove, 2004 and in Sgroi, 2009**].

The most promising non-biologic liver support therapies combine detoxification of water-soluble and protein-bound toxins in a dialysis system (e.g., the Molecular Adsorbents Recirculating System [MARS]) or an albumin dialysis system (the Artificial Liver Support System). Some beneficial effects on plasma toxin levels have been reported in uncontrolled trials of the albumin dialysis system in patients with ALF [**van de Kerkhove, 2002**]. However, in ALF patients, these systems have led to only limited or no improvement in patient survival [**van de Kerkhove, 2004; Sgroi, 2009**]; the nonspecific

removal of toxic compounds and the lack of capacity of the device to synthesize specific proteins and other hepatotrophic factors probably account for their limited effect.

Biologic approaches exploit hepatocytes of human or xenogeneic origin to support the patient with a BAL (bioartificial liver) device. Examples are the Extracorporeal Liver Assist Device that uses human hepatoblastoma cells [**Ellis, 1996**] and the HepatAssist device that uses cryopreserved porcine hepatocytes [**Demetriou, 2004**]. No clinical trial to date has shown convincing evidence of increased patient survival. Although several benefits have been reported during BAL support, no study has demonstrated significant improvement in plasma ammonia level and metabolic function. Several adverse events have been reported, such as tachypnea, tachycardia, hyperpyrexia, disseminated intravascular coagulation, thrombocytopenia, hypotension, hypoxia, sepsis, and, when porcine hepatocytes were used in the device, features of cell rejection [**van de Kerkhove, 2004; Ellis, 1996; Demetriou, 2004**].

Hepatocyte isolation and preservation is expensive, and long-term BAL support is likely to require a large number of cells. The cost-effectiveness of these procedures is therefore questionable. Furthermore, the number of hepatocytes in a BAL is far fewer than in a whole pig liver. The HepatAssist BAL contains an estimated 7 billion porcine hepatocytes (though the viability of these after cryopreservation is uncertain), whereas an average adult human liver may contain as many as 15-30 billion hepatocytes [**Li, 1992**]. In summary, the use of a BAL for bridging patients with ALF to liver transplantation is promising, but its clinical value awaits further improvement in the devices, and further assessment in controlled clinical trials.

*b) Extracorporeal liver perfusion (ECLP)*Initial experience using human livers was documented in the 1960s and 1970s [**Abouna, 1968; Eiseman, 1965**]. At least 141 ECLPs were performed to treat 87 patients with liver failure [**Abouna, 1997**]. The method

was then largely abandoned because of the successful development of orthotopic liver allotransplantation. However, with the increasing shortage of available donor organs, there has been renewed interest. Neurologic improvement to at least hepatic coma grade III or II has been documented in most patients. The results do not correlate with the underlying cause of the hepatic failure.

In 1994, Chari et al [**Chari, 1994**] reported their clinical experience with four patients treated by intermittent porcine liver perfusion, but only one survived to transplantation. In 2000, Horslen et al used human or unmodified (wild-type) miniature swine livers to bridge 14 patients to allotransplantation [**Horslen, 2000**]. Nine patients were supported successfully to orthotopic liver transplantation, the longest period of support being for >4 days, with seven surviving. ECLP maintained low ammonia levels for only 48h, and did not lower bilirubin levels.

In a small clinical trial by Levy et al [**Levy, 2000**], livers from pigs transgenic for the human complement-regulatory proteins, CD55 (hDAF) and CD59, were extracorporeally perfused in two patients with ALF for 6.5h and 10h, respectively, as bridging to successful allotransplantation.

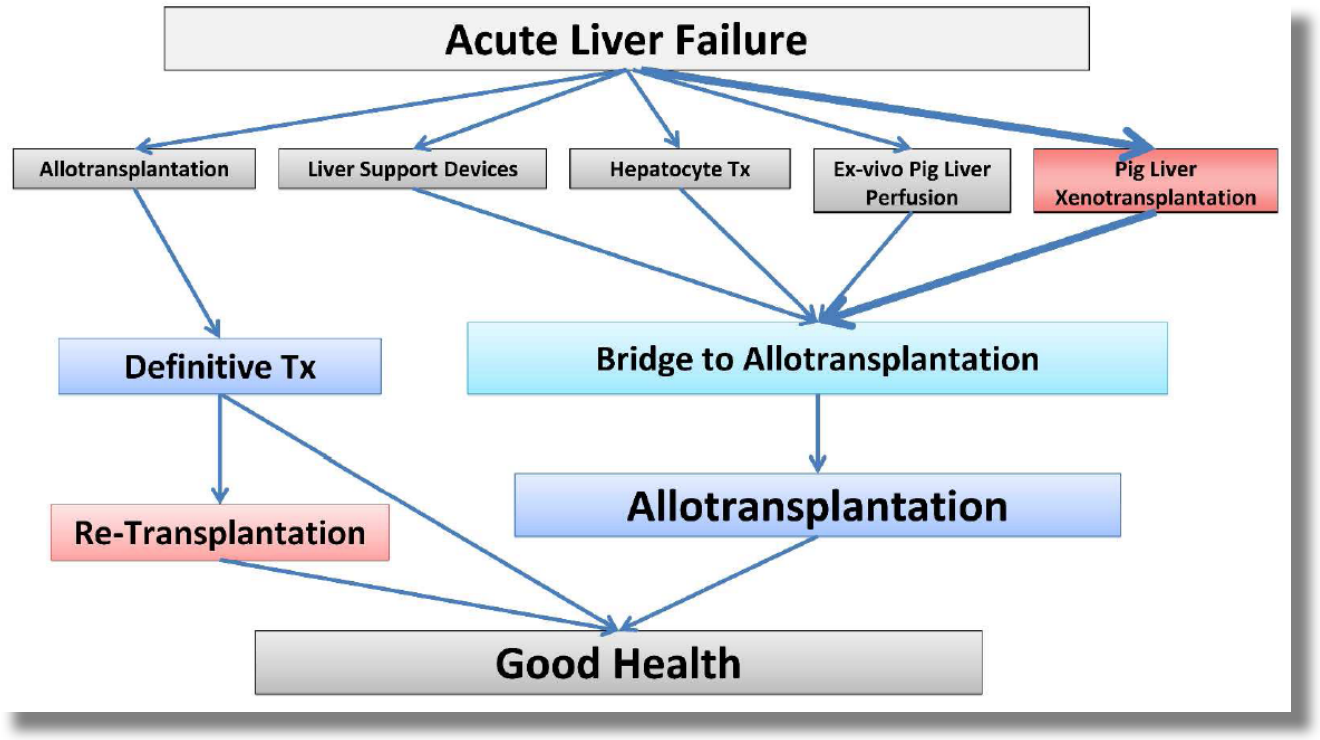
c) Hepatocyte transplantation

Hepatocyte transplantation could theoretically support the patient until the native liver recovers or could bridge to liver transplantation [**reviewed by Fisher, 2006, by Strom, 2006, and by Sgroi, 2009**]. It is doubtful, however, whether a sufficient number of donor hepatocytes could be infused to support a patient with severe liver failure, who may have increased metabolic needs through the presence of encephalopathy, coagulation, and toxic cytokine release from the necrotic native liver. Furthermore, there can be a 48-72h delay in function of the transplanted hepatocytes [**Bilir, 2000**], which could represent a major problem.

d) Liver xenotransplantation

The ready availability of a perfect pig liver would avoid (i) delay during which the patient's clinical condition may deteriorate further, (ii) prolonged cold ischemia, (iii) size-mismatch between liver and patient, (iv) transplantation of an inadequate number of hepatocytes, and (v) the harmful effects of leaving a necrotic native liver *in situ*. Patients would be able to receive a xenotransplant at the time of greatest clinical need, helping to avoid prolonged hospitalization and exposure to nosocomial pathogens.

Figure 17: Treatment decision algorithm for patients with rapidly deteriorating liver function when conventional medical treatment proves insufficient.



Adapted from Ekser, Transplantation 2009;88:1041-1049

Advantages of xenotransplantation over bridging by BAL, ECLP, or hepatocyte transplantation

For several reasons, xenotransplantation of a whole pig liver (Figure 17) as a bridge to allotransplantation may be preferable to BAL support:- (i) isolation of hepatocytes and preparation of the device may take time during which the clinical status of the patient may deteriorate, (ii) cryopreservation of porcine hepatocytes may impair their subsequent function, (iii) the number of hepatocytes in a BAL is far fewer than in a whole liver; metabolic function of a whole liver is likely to be superior to that of a relatively small number of hepatocytes, (iv) the BAL provides hepatocellular function, but without the structure of the liver or the other cell types, both of which are important in the function of the liver, and (v) use of a pig liver may be more cost-effective when compared with porcine

hepatocyte isolation and utilization in a BAL. Many of these concerns apply equally to hepatocyte transplantation.

In comparison to ECLP, the transplantation of a pig liver would (i) enable removal of the patient's necrotic liver, and thus avoid harmful cytokine release and prevent further clinical deterioration (although it is perhaps possible that removal of the diseased native liver would not be necessary in the presence of a well-functioning extracorporeal pig liver), (ii) reduce the risk of infectious complications associated with prolonged ECLP, and (iii) possibly maintain greater hemodynamic stability than can be achieved with ECLP.

CHAPTER 2 - MATERIALS AND METHODS - PROJECT 1 - HISTOPATHOLOGY OF LIVER XENOTRANSPLANTATION

2.1 ANIMALS

Baboons (Papio species) (Oklahoma University Health Sciences Center, Oklahoma City, OK) of either sex, weighing 7–12 kg, aged 2.70–3.47 years were recipients of one allograft and 9 xenografts, and donors of one liver (Table 14). Wild-type (genetically-unmodified, WT) Landrace/large white pigs (Country View Farm, Schellsburg, PA) were donors of liver allografts (n= 2) and a xenograft (n= 1), and recipients of 2 allografts. GTKO (n =1) or GTKO/CD46 (n= 7) pigs (Revivicor, Blacksburg, VA) were sources of xenograft livers (Table 14) [**Ekser, 2010b**]. From our original study [**Ekser, 2010b**], two liver xenotransplants have been excluded from the present report as donor-recipient size-mismatch precluded abdominal closure, necessitating early termination of the experiment. All animal care was in accordance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86–23, revised 1985). Protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC# 0706493). The animal experiments were conducted in strict compliance with animal welfare regulations, and the steps taken to ameliorate suffering were in accordance with the recommendations of the Weatherall Report on the use of non-human primates in research as well as with the ARRIVE guidelines on animal research. All recipient baboons placed in a tether-jacket system, as previously described, and so they did not require to be sedated for each blood draws throughout the experiment. Biopsy samples were taken under anesthesia or at the time of necropsy (see below) avoiding any

suffering to the animal. All animals were given Buprenorphine 0.01 mg/kg i.m. twice daily for 3 days after any surgical intervention for analgesia.

2.2 SURGICAL PRODECURES

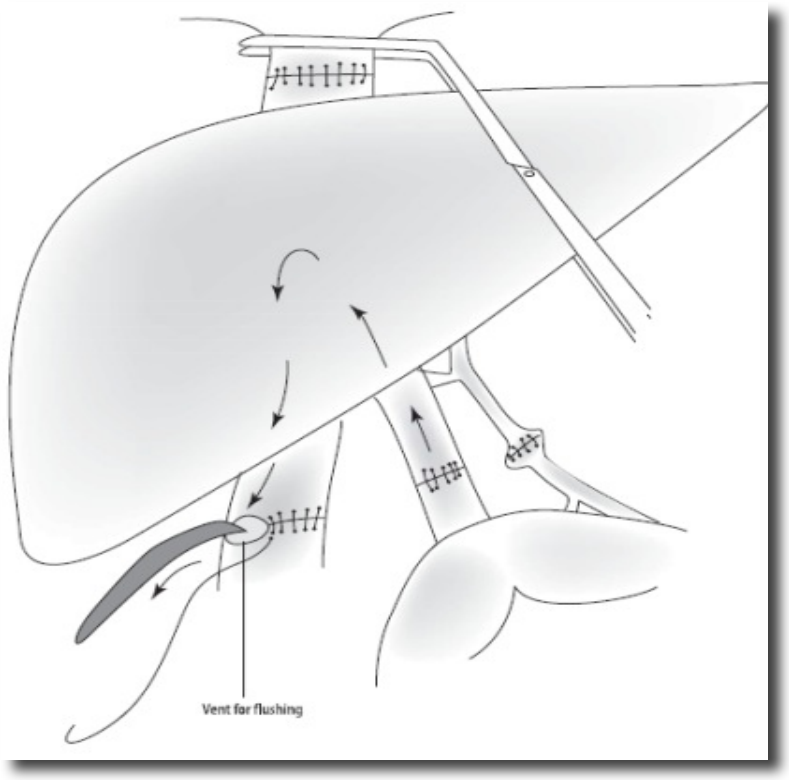
2.2.1 Donor liver procurement (pig and baboon)

Under full inhalatory anesthesia, through a midline incision and after flushing with cold University of Wisconsin Solution (ViaSpan Belzer UW, Fresenius Kabi, Graz, Austria, and Duramed Pharmaceuticals, Pomona, NY) the liver was procured using a standard clinical technique [**Abu-Elmagd, 2000**]. Cold ischemia time was <2h.

2.2.2 Recipient (pig and baboon) surgery

All recipients underwent intravascular catheter placement (internal and external jugular veins, carotid artery) prior to liver transplantation, as previously described [**Ekser, 2010b; Cooper, 1991**]. All transplants were carried out using the standard surgical technique for human orthotopic liver transplantation after recipient hepatectomy with sequential end-to-end anastomoses of the supra-hepatic and infra-hepatic inferior venae cavae and portal veins, followed by reperfusion of the liver, and end-to-end anastomoses of the hepatic arteries and common bile ducts; in two cases, a choledocho-jejunostomy was carried out to facilitate bile flow (B18508, B18908) (Figure 18). Due to the very small size of the bile ducts, the insertion of a 5F T-tube was not possible; in some cases, a 3.5F feeding tube was placed to facilitate bile flow. Veno-venous bypass was used in pig allotransplantation, but not when the baboon was the recipient. In some cases, splenectomy was performed after reperfusion of the liver. Intra-operative heparin (100units/kg) was administered to only two baboons just before liver reperfusion (B18508, B18908) (Table 14).

Figure 18: Liver transplant surgical procedure, after recipient hepatectomy



During the surgical procedures, the animal's status was monitored by a number of biochemical, blood gas, and hematologic parameters. Washed baboon red blood cells were available during the transplant, but not required. All recipients (pig or baboon) were extubated and recovered immediately after completion of the surgical procedure.

2.3 IMMUNOSUPPRESSIVE AND SUPPORTIVE THERAPY

2.3.1 Clinically applicable immunosuppressive therapy

No immunosuppression therapy was given to pig-to-pig liver allotransplantation. However, a clinically acceptable immunosuppressive regimen was used in baboon-to-baboon liver allotransplants and all xenotransplants except in the case of a WT-pig-to-baboon liver xenotransplant. The immunosuppressive therapy consisted of induction with thymoglobulin (Genzyme, Cambridge, MA) and maintenance with tacrolimus (Astellas Pharma US, Deerfield, IL), mycophenolate mofetil (MMF; Roche, Basel, Switzerland) and

methylprednisolone. In 3 baboons (B7908, B8108, and B18908) cyclophosphamide (Baxter, Deerfield, IL) replaced thymoglobulin. Details are given in Table 10.

Table 10: Immunosuppressive therapy

Therapy	**Dose**	**Duration**
INDUCTION THERAPY		
Antithymocyte globulin	5-10 mg/kg i.v.	Day -3 and -1 (if T cell count >500 cells/mm^3)
Cyclophosphamide	40 mg/kg i.v.	Day -2 (only in B7908, B8108, and B18908)
	20 mg/kg i.v.	Day -1 (only in B7908, B8108, and B18908)
Cobra venom factor	1-3 mg i.v.	Days -1 to 1 (only in B18508)
MAINTENANCE THERAPY		
Tacrolimus	0.05-0.1 mg/kg x2/day i.m.	From day -3 (to maintain 12h blood trough levels of 10-15 ng/mL)
Mycophenolate mofetil	110 mg/kg/day i.v. continuous infusion	From day -4 (to maintain blood levels of 3-5 µg/mL)
Methylprednisolone	10 mg/kg i.v.	Tapering from day 0

2.3.2 Immunosuppressive drug's levels

Tacrolimus levels were measured daily in baboons with the aim to maintain 12 hour blood trough levels of 10-15 ng/mL. Mycophenolate mofetil levels were measured at least 3 times in a week with the aim to maintain blood levels of 3-5 µg/mL.

2.3.3 Supportive therapy

In pig-to-pig liver allotransplantion only analgesia and cefazolin prophylaxis were administered. All baboons (allo or xenotransplantation recipients) received prophylactic

intravenous antibiotic and ganciclovir therapy throughout the post-Tx period of follow-up. Supportive therapy is listed in Table 11.

Table 11: Supportive therapy

Drug name	Dose	Duration
Famotidine	0.25 mg/kg x2/day i.v.	From day -7
Cefazolin	25 mg/kg x2 day i.v.	From day -7
Heparin	100 U/kg i.v.	Day 0 (only in B18508 and B18908)
Ganciclovir	5 mg/kg/day i.v.	From day -3
Dopamine	2-3 µg/kg/min i.v.	Days 0-7
Prostacycline	20 ng/kg/min i.v.	Days 0-7
Imipenem	500 mg x2/day i.v.	From day 0
Amikacin	150 mg /day i.v.	From day 0
Ketorolac	0.5 mg/kg/day i.v.	Days -1 to 1 (in B7708 and B7808) Days -1 to 6 (in B18508 and B18908)
Vitamin K	5 mg/day i.v.	From day 0 on alternate days (only in B18508 and B18908)

2.4 SPECIAL TREATMENTS

2.4.1 Clodronate liposomes in donor pigs

Clodronate liposomes (Cl_2MDP) (generously provided by Dr Robin Pierson of the University of Maryland) were used with the aim of depleting macrophages (including Kupffer cells) in two donor pigs on days -2 +/- day-1 (Table 12).

Table 12: Clodronate liposome treatment

Treatment of donor pig		
Drug name	**Dose**	**Duration**
Clodronate liposomes	90 mg/kg i.v.	Day -2 (only in donor pig of B7908) Day -2 and -1 (only in donor pig of B8108)

2.4.2 Complement depletion in baboons using Cobra Venom Factor

In one case (B18508), cobra venom factor was administered for 3 days in order to deplete complement activity (Table 13).

Table 13: Cobra venom factor treatment

Treatment of recipient baboon		
Drug name	**Dose**	**Duration**
Cobra venom factor	1-3 mg i.v.	Days -1 to 1 (only in B18508)

2.5 TIMING OF BIOPSIES OF LIVER GRAFTS AND MAJOR NATIVE ORGANS

Table 14 summarizes the timing of liver biopsies. All biopsies (wedge) were obtained under direct vision, except in the baboon allograft (B3408) on post-operative day 22, which was a transcutaneous Tru-cut needle biopsy. The WT pig liver underwent serial biopsies at 30 min, 1, 2, 3, 4 and 5 h. Biopsies of GTKO and GTKO/CD46 pig livers were obtained

pre-Tx, approximately 2 h after reperfusion (just before abdominal closure), and at necropsy. At necropsy, the macroscopic appearance of the liver graft was differentiated into (i) light areas, indicating relatively normal appearance, and (ii) dark areas that proved to be areas of hemorrhage. All major native organs were examined, and biopsies were taken from the areas that appeared most affected by pathogenic processes, e.g., hemorrhage.

Table 14: Recipient and donor information, immunosuppressive regimen and biopsy time points.

Recipient #	Donor #	Donor Species Type	Immunosuppressive Therapy	Survival (days)	Liver Biopsy Time-points
Allotransplantation					
P40307	P40107	WT	None	>3	Pre-Tx, 2 h, euthanasia
P40207	P40407	WT	None	>2	Pre-Tx, 2 h, euthanasia
B3408	B16407	Baboon	ATG+TAC+MMF+CS	>31	Pre-Tx, 2 h, day 22, euthanasia
Xenotransplantation					
B16907	P3008	WT	None	5 h	Pre-Tx, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h
B3108	P8208	GTKO	ATG+TAC+MMF+CS	6	Pre-Tx, 2 h, euthanasia#
B3208	P14508	GTKO/CD46	ATG+TAC+MMF+CS	4	Pre-Tx, 2 h, euthanasia#
B7708	P21708	GTKO/CD46	ATG+TAC+MMF+CS	7	Pre-Tx, 2 h, euthanasia#
B7808	P22108	GTKO/CD46	ATG+TAC+MMF+CS	6	Pre-Tx, 2 h, euthanasia#
B7908	P26708	GTKO/CD46	CyP+TAC+MMF+CS+(CL for donor)	<1	Pre-Tx, 2 h, euthanasia
B8108	P26608	GTKO/CD46	CyP+TAC+MMF+CS+(CL for donor)	1	Pre-Tx, 2 h, euthanasia
B18508	P3909	GTKO/CD46	ATG+TAC+MMF+CS+CVF	5	Pre-Tx, 2 h, euthanasia#
B18908	P4009	GTKO/CD46	CyP+TAC+MMF+CS	6	Pre-Tx, 2 h, euthanasia#

P = pig, B = baboon, ATG = antithymocyte globulin, TAC = tacrolimus, MMF=mycophenolate mofetil, CS = corticosteroids, CVF = cobra venom factor, CyP = cyclophosphamide, CL = clodronate liposomes, Tx = transplantation, #= native organs were biopsied at necropsy (euthanasia). (Obtained from Ekser et al, PLoS ONE 2012;7:29270.

2.6 LIGHT MICROSCOPY

Liver tissues were stored in 10% formalin for subsequent light microscopy. Paraffin blocks were prepared, 4 mm sections cut and stained with hematoxylin and eosin (H&E). On examination, particular attention was paid to (i) the presence of cellular infiltrates

(neutrophils, lymphocytes, eosinophils), (ii) blood vessels (congestion, hemorrhage, fibrin aggregation, venulitis or arteritis), (iii) hepatocytes (vacuolation, necrosis), (iv) bile ducts (inflammation, necrosis), and (v) interstitial tissue (fibrosis). All major recipient organs were examined. Staining for iron deposition was carried out in some cases.

2.7 IMMUNOHISTOPATHOLOGY

For immunohistochemistry studies, liver biopsies were stored at -80°C until processed. Cryosections (8 mm) were cut and mounted on to gelatin-coated slides. After being fixed in 2% paraformaldehyde in PBS for 15 min, sections were blocked with 20% nonimmune normal donkey serum for 1h at room temperature. Primary monoclonal antibodies were used to demonstrate (i) a cellular response (T cells [CD3], B cells [CD20], macrophages [CD68], neutrophils [CD97]), (ii) antibody and complement deposition (IgM, IgG, IgE, C3, C4d, C5b-9), and (iii) the presence of thrombotic microangiopathy (aggregation of platelets [CD41] and fibrin deposition) (Table 15), as previously described [**Luo, 1998; Shimizu, 2008; Ezzelarab, 2009**]. After washing in PBS, sections were incubated with different secondary antibodies. Table 16 summarizes the primary and secondary antibodies used, with their concentrations and sources. Images were viewed at x20 magnification and captured using a Nikon confocal microscope (Nikon D-ECLIPSE C1, Tokyo, Japan). Metaphor Image Analysis software was used for image analysis [**Balamurugan, 2005**]. At least 3 different areas from the same section were viewed and evaluated. An average of 12 different sections for each antibody stain was examined, and the positivity of staining was scored (Table 17). The percentages of positive staining area/total scanned area were calculated accordingly. Table 17 indicates the percentage of tissue stained by each antibody.

Table 15: Liver immunohistopathology at 2h and at necropsy

				Cellular Response					Antibody deposition			Complement deposition			Thrombotic microangiopathy	
Donor Species Type	**Pig#**	**Baboon#**	**Time of Bx**	**CD3**	**CD20**	**CD68 (pig)**	**CD68 (mon)**	**Neut**	**IgG**	**IgM**	**IgE**	**C3**	**C4d**	**C5b-9**	**CD41**	**Fibrin**
Biopsies at 2 h																
Allo Liver Tx	B16407	B3408	2 h	−	−	−	+	−	−	−	−	−	−	−	−	−
WT	P3008	B16907	2 h	++	+	++	−	++	+	+	−	++	−	+	+	+
GTKO/CD46	P21708	B7708	2 h	−	−	+	−	−	−	+	−	−	−	−	+	+
	P22108	B7808	2 h	−	−	++	−	+	+	+	−	−	−	++	−	++
	P4009	B18908	2 h	+	−	++	−	+++	−	++	−	−	−	−	+	++
Biopsies at necropsy																
Allo Liver Tx	B16407	B3408	31 d	−	+	−	+	++	−	−	−	−	−	−	−	−
GTKO/CD46	P21708	B7708	7 d	−	−	+	−	+++	−	+	−	−	−	−	+	++
	P22108	B7808	6 d	+	−	+	−	+++	+	+	−	−	−	−	+	+++
	P4009	B18908	6 d	−	−	++	−	+++	−	+	−	−	−	−	+	++
GTKO/CD46+CVF	P3909	B18508	5 d	−	−	++	−	+++	−	+	−	−	−	−	−	−
GTKO/CD46+CL	P26708	B7908	<1 d	−	−	+	−	++	−	−	−	−	−	−	++	+++
	P26608	B8108	1 d	+	−	+	−	++	−	+	−	−	−	+	−	+

P = pig, B = baboon, Bx = biopsy, Neut = neutrophil, CL = clodronate liposomes, CVF = cobra venom factor, mon = monkey.
(-) = none; (+) = mild; (++) = moderate; (+++) = severe.

Table 16: Primary and secondary antibodies used for immunofluorescence staining of liver xenografts and native organs

Name	Concentration	Source (Company)
Primary antibodies		
Rabbit anti-human CD3 (T-cell)	1:100	Dako, Denmark
Mouse anti-human CD20 (B-cell)	1:20	AbD Serotec, NC, USA
Mouse anti-pig CD68 (macrophage)	1:25	AbD Serotec, NC, USA
Mouse anti-monkey CD68 (macrophage)	1:100	Santa Cruz Biotech, CA, USA
Rabbit anti-human CD97 (neutrophil)	1:100	Thermo Scientific, PA, USA
Goat anti-human IgG	1:1000	K&P Lab, MD, USA
Goat anti-human IgM	1:1000	K&P Lab, MD, USA
Mouse anti-human IgE	1:100	Abcam, MA, USA
Mouse anti-human C3	1:100	Santa Cruz Biotech, CA, USA
Rabbit anti-human C4d	1:20	Abcam, MA, USA
Mouse anti-human C5b-9	1:100	Abcam, MA, USA
Mouse anti-human CD41 (platelet)	1:50	Abcam, MA, USA
Sheep anti-human fibrin	1:100	Affinity Biol, Canada
Secondary antibodies		
Donkey anti-rabbit Alexa 488	1:500	Molecular Probes, IL, USA
Donkey anti-mouse Cy3	1:250	Jackson IR Lab, PA, USA
Donkey anti-goat Alexa 488	1:500	Molecular Probes, IL, USA
Donkey anti-mouse Alexa 488	1:500	Molecular Probes, IL, USA
Donkey anti-sheep Cy2	1:100	Jackson IR Lab, PA, USA

Table 17: Extent of immunofluorescence of liver xenografts and native organs.

Type of antibody	−	+	++	+++
Rabbit anti-human CD3 (T-cell)	<0.19%	0.2–1.3%	1.4–5.08%	
Mouse anti-human CD20 (B-cell)	<2.8%	2.9–4.6%		
Mouse anti-pig CD68 (macrophage)	<0.19%	0.2–0.9%	1–2.2%	
Mouse anti-monkey CD68 (macrophage)	<0.13%	0.14–0.24%		
Rabbit anti-human CD97 (neutrophil)	<0.07%	0.08–0.9%	1–1.49%	1.5–2.3%
Goat anti-human IgG	<0.29%	0.3–0.84%		
Goat anti-human IgM	<0.09%	0.1–7.9%	8–10%	
Mouse anti-human IgE	<0.01%			
Mouse anti-human C3	<0.03%	0.04–1.99%	2.0–5.01%	
Rabbit anti-human C4d	<0.01%			
Mouse anti-human C5b-9	<0.06%	0.07–0.29%	0.3–0.46%	
Mouse anti-human CD41 (platelet)	<0.08%	0.09–0.7%	0.8–1.0%	
Sheep anti-human fibrin	<0.9%	1–4%	5–14%	15–20.8%

Calculation of the percentages of tissue or cells stained was specifically and independently made for each antibody using Metaphor Image Analysis software.

2.8 ELECTRON MICROSCOPY

Liver tissue was fixed with 2.5% glutaraldehyde in PBS. Transmission electron microscopy was performed, as previously described [**Wack, 2001**]. Particular attention was paid to the examination of (i) the sinusoidal endothelial cells, Kupffer cells, and hepatocytes, (ii) the interaction between platelets and these cells, and (iii) fibrin deposition.

CHAPTER 3 – RESULTS – PROJECT 1 - HISTOPATHOLOGY OF LIVER XENOTRANSPLANTATION

Donor livers were from pigs, except for one baboon alloTx. Thus, a brief description of normal pig liver histology is appropriate.

3.1 HISTOPATHOLOGICAL APPEARANCE OF NORMAL PIG LIVER

The hepatic lobules in the livers of pigs, unlike those of most other species, have a distinct, visually-recognizable envelope of connective tissue surrounding them, imparting a unique, individualized appearance to each structure (Figure 19A). In humans and nonhuman primates, this prominent fibrous margination (which creates these histomorphologically-recognizable boundaries in swine) is absent; therefore, in these species, the identification of individual lobules as distinct structures is seldom possible.

3.2 HISTOPATHOLOGY OF LIVER ALLOGRAFTS (PIG OR BABOON)

Recipients of two pig liver allografts (carried out to develop the surgical technique) did not receive any immunosuppression (Table 14), and were euthanized according to protocol on postoperative days 2 or 3. At necropsy, both livers showed minimal mononuclear portal and perivenular inflammation, and mild centrilobular congestion, suggesting early acute cellular rejection (not shown). One immunosuppressed baboon with a liver allograft (Table 14) survived 31 days and was euthanized according to protocol. Biopsies were obtained at 2 h post-perfusion, on day 22, and at euthanasia (Table 14). The 2 h post-transplant wedge biopsy showed mild patchy areas of acute sinusoidal congestion/ hemorrhage, possibly related to the biopsy procedure itself (not shown). Patchy, hepatocellular vacuolar change without evidence of an overt zonal patterning was also noted, possibly a

consequence of organ reperfusion. Tru-cut biopsy on post-operative day 22 showed unremarkable hepatocytes. One focal tiny nodular cluster of inflammatory cells was seen (Figure 19B). There was a mild increase in the number of sinusoidal granulocytes present, especially in the frozen section. These may have represented passenger leukocytes as opposed to a mild response to hepatocellular insult, the former being suggested by correlation with clinical, hematologic, and clinical chemistry findings, which were all normal [**Ekser, 2010c**]. At necropsy on post-operative day 31, the appearance of hepatocytes was unremarkable (Figure 19B). There were rare patchy mild-to-moderate periportal lymphocytic infiltrates. There was a mild $CD20^{+}$ infilitrate (Table 15). The correlation with normal clinical, hematologic, and chemistry findings suggested that early acute rejection was unlikely, and an ascending pericholangitis might be a more likely diagnosis. No evidence of bile stasis or thrombosis was seen.

3.3 HISTOPATHOLOGY OF LIVER XENOGRAFTS

3.3.1 WT pig-to-baboon liver xenotransplantation without immunosuppressive therapy

Serial wedge biopsies were performed after WT pig-to-baboon liver xenoTx (Table 14).

(i) At 30 min after reperfusion, significant hepatocellularvacuolar change consisting primarily of large, clear, generally solitary vacuoles often displacing nuclei eccentrically, was noted (Figure 19C). There was marked congestion in many of the larger interlobular vessels, and a lesser degree of mild patchy sinusoidal congestion. A mild degree of increased interlobular septal edema was noted, which prominently accentuated the

lobulated appearance of the organ. Infrequent small aggregates of polymorphonuclear cells were scattered throughout the sinusoidal spaces.

(ii) At 1 h, severe hepatocellular vacuolar change was present, similar to that seen in the 30 min biopsy, although more prominent and extensive, with a centrilobular distribution (Figure 19D). Infrequent focal hepatocyte necrosis was seen. Scattered increased numbers of neutrophils were present within the sinusoidal spaces without aggregation, as noted at 30 min. A very small number of thrombi were observed.

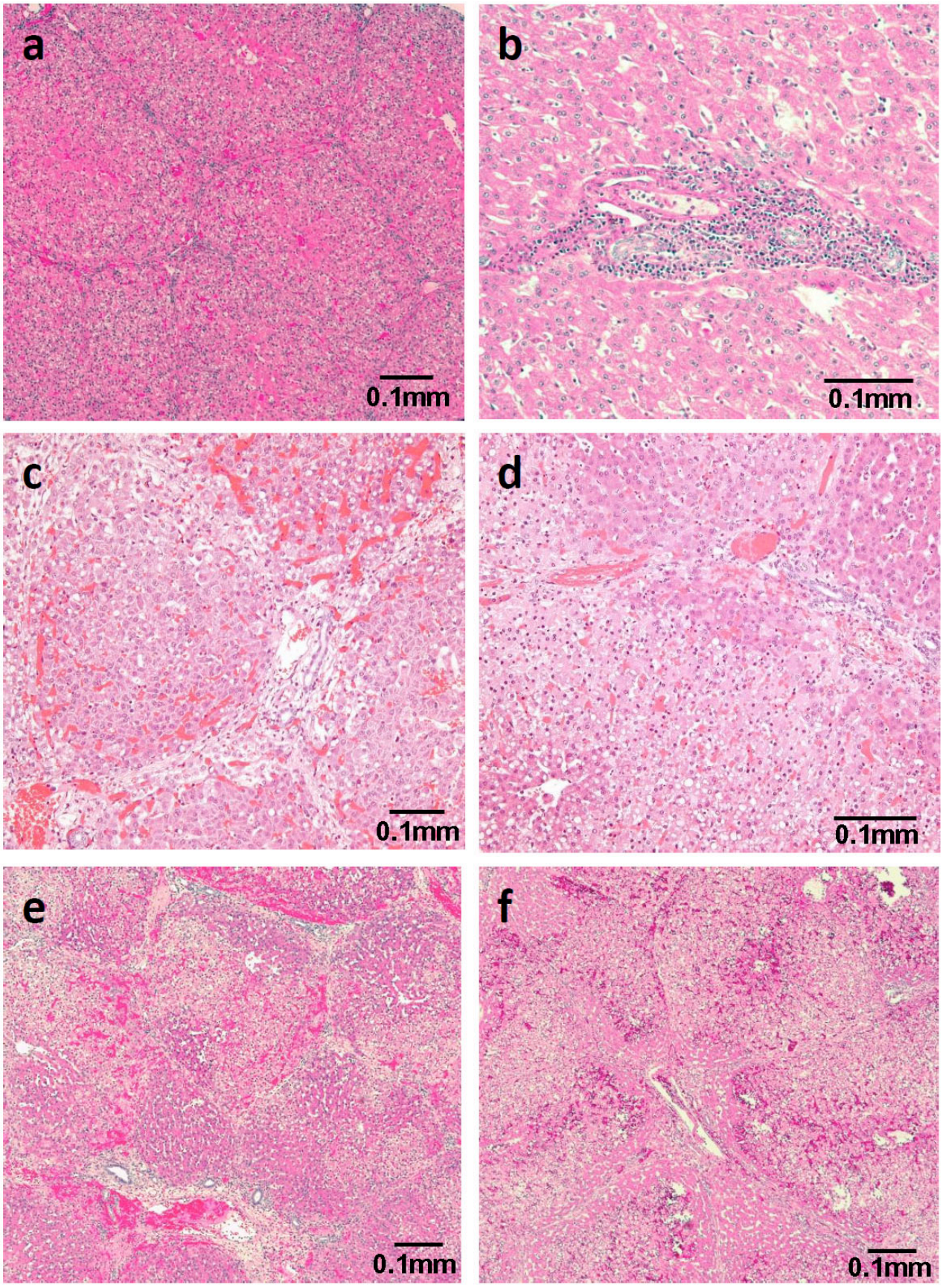
(iii) At 3 h, a distinct progression from vacuolation to early coagulative necrosis of the hepatocellular cytoplasm was observed (Figure 19E). This was characterized primarily by generally pale, more lightly eosinophilic staining. Scattered neutrophilic inflammatory cells remained present in these areas. Increased areas of sinusoidal congestion, extending in some regions to mild hemorrhage, were noted.

(iv) At 5 h, dark patchy areas were observed macroscopically. Therefore, normal (light) and abnormal (dark) areas were biopsied separately. In the light area biopsies, multiple regions located primarily in subcapsular areas were noted in which focal acute hemorrhagic coagulative necrosis was present, sometimes with a centrilobular distribution (not shown). In other more central lobules, there was more subtle evidence of hepatocellular vacuolar change with mild associated eosinophilic cytoplasmic pallor. One focus of subcapsular hemorrhagic necrosis had a distinct wedge shaped appearance, suggesting possible infarction. In the dark areas, although some relatively unaffected portions of hepatic parenchyma were noted, there was a focally extensive region of significant hepatocellular degeneration characterized by a combination of severe vacuolar

change and frank coagulative necrosis with extensive but patchy hemorrhage (Figure 19F). As with other sections, the same zonal patterning was noted, with periportal hepatic zones appearing spared of insult. A large early fibrin thrombus was noted in a portal vessel.

Figure 19: Histopathology (H&E) of (A) healthy (non-transplanted) pig liver, (B) baboon liver 31 days after allotransplantation, and (C–F) WT pig liver after pig-to-baboon xenotransplantation (Figure 19 is shown in next page).

Legend - Figure 19: A) Histomorphological appearance of a normal pig liver (x100). Distinct envelope of connective tissue surrounding hepatic lobules. B) Baboon-to-baboon allotransplantation (B3408) on post-operative day 31 (x200). Patchy, mild-to-moderate periportal lymphocytic infiltrates. C) WT pig-to-baboon liver xenoTx at 30 min (B16907) (x100). Significant hepatocellular vacuolar change, patchy sinusoidal congestion, and interlobular septal edema. D) WT pig-to-baboon liver xenoTx at 1 h (B16907) (x200). Severe hepatocellular vacuolar change, focal hepatocyte necrosis, and few thrombi. E) WT pig-to-baboon liver xenoTx at 3 h (B16907) (x100). Early coagulative necrosis, significant sinusoidal congestion, and large thrombus. F) WT pig-to-baboon liver xenoTx at 5 h (just before euthanasia) (B16907) (x100). Acute hemorrhagic coagulative necrosis, extensive hemorrhage, and large fibrin thrombi. (Obtained from Ekser et al. PLoS ONE 2012;7:e29720)


a
0.1mm
b
0.1mm
c
0.1mm
d
0.1mm
e
0.1mm
f
0.1mm

3.3.2 GTKO or GTKO/CD46 pig-to-baboon liver xenotransplantation with immunosuppressive therapy

Six longer-surviving (>1 day) recipient baboon liver biopsies (Tables 14 and 15) were examined at 2h and at necropsy (4–7 days).

At 2 h, all of the livers were well-perfused, although in one case (B18508) there was a macroscopic lobular darker area noted that could represent a possible embolic event during reperfusion (Figure 20A). Histomorphological changes at 2 h were minimal or mild. Differential sinusoidal dilatation was present in some cases, but generally did not differ from pre-Tx control biopsies. There was moderate variation in the number of neutrophils and neutrophilic clusters seen within the sinusoids, which was most prominent in B18908 (Figure 20B). In some cases this did not differ significantly from pre-Tx biopsies. In a similar fashion, some livers demonstrated what appeared to be patchy mild hepatocellular and canalicular bile stasis, but the pre-Tx 'controls' also showed similar foci, suggesting this was not an immediate experimental consequence. Of most apparent significance was the presence of intra-hepatocellular vacuolar change which was more extensive in the 2 h biopsies than in the pre-Tx samples (generally absent from the latter) (Figure 20C), and was thought likely to be due to ischemia/reperfusion injury. Based on the structural appearance of nuclei and the generally normal tinctorial nature of hepatocyte cytoplasm, there was no indication of frank hepatocellular necrosis.

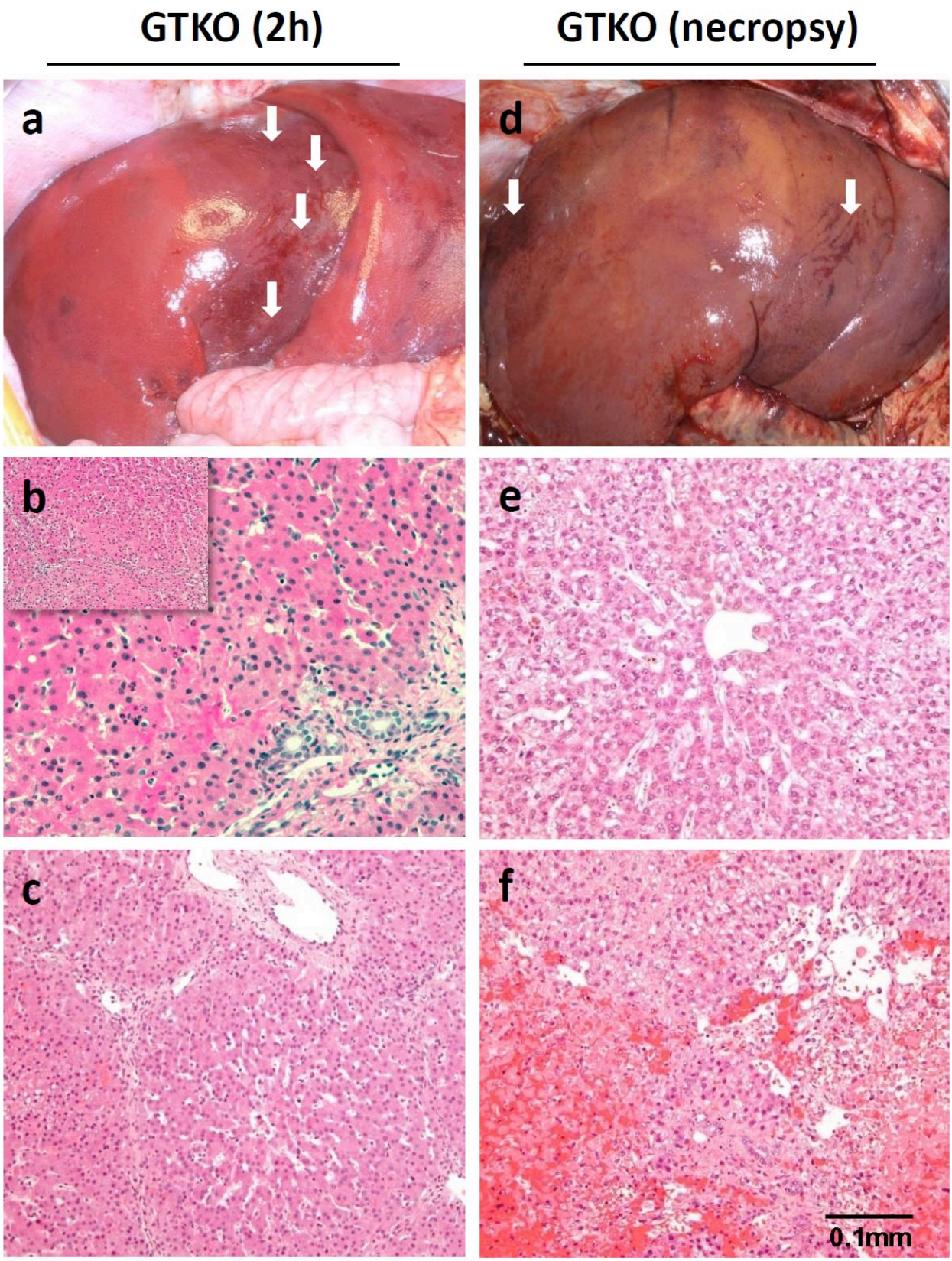
At necropsy, the macroscopic appearance of livers was generally normal or yellowish, indicating cholestasis (light areas), with patchy dark areas (Figure 20D). Therefore, individual side-by-side comparisons of light and dark areas were made. The synopsis below summarizes the changes and variations seen in each area, and also provides an

overall comparison between the two. Light areas: Although large regions of these sections were normal, occasional coalescent areas demonstrated distinct, multifocal areas of coagulative necrosis. Often admixed with this change were varying degrees of hemorrhage, though the extent and severity of such lesions varied significantly between livers. Frequently, a distinctive central lobular pattern was noted, although in some cases apparent foci of individual hepatocellular degeneration were also observed. Vacuolar hepatocellular cytoplasmic change (and in some cases distention) of unknown etiology was also present in several cases, being most prominent in B18908 (Figure 20E). Small fibrin thrombi were seen not infrequently in portal vessels, although typically not extensive in their distribution. Also present and variable in extent (although generally mild) were features of ductal and occasional canalicular bile stasis. Dark areas: The microscopic changes seen in the dark regions were generally similar to those in light areas, but with much more severe/extensive necrosis and evidence of vastly increased hemorrhage. Although variable in degree (as with light region biopsies), total lobular hemorrhagic necrosis was often seen or there was sparing only of focal groups of hepatocytes in the portal regions (Figure 20F). Again, thrombi were occasionally noted, but were not extensive. In areas in which there was not massive lobular hemorrhagic necrosis, predilection for central involvement of the lobule was again seen. In some cases in which viable parenchyma remained, hepatocellular cytoplasmic vacuolar change and distention (as seen in light areas) was seen, most prominently in B18908. In the pig liver in B18508, morphological change consistent with a distinct area of hepatic infarction was present (Figure 20A). This was characterized by large multifocal fibrin thrombi and severe hepatocyte disassociation/individualization. In regions of this section (often underlying the hepatic capsule), numerous punctate structures consistent with bacterial colonies were observed, presumed to be associated with pre-mortem retrograde migration of enteric

bacterial flora through thrombosed vessels. No inflammation was noted in association with the presence of these organisms.

Figure 20: Macroscopic and microscopic appearance of GTKO and GTKO/CD46 pig livers 2 hours after perfusion of the graft and at necropsy (Figure 20 is shown in next page)

Legend - Figure 20: A) B18508 (2-hour post-reperfusion). Macroscopic appearance of well-perfused pig liver. Lobular dark areas were noted in the middle of the liver (arrows) suggesting an arterio-embolic event. B) B18908 (2-hour post-reperfusion). Prominent increase in neutrophils (H&Ex200). Small panel at top left (H&Ex100). C) B18508 (2-hour postreperfusion). Intra-hepatocellular vacuolar change. The pig liver in B18508 demonstrated the most severe degree of involvement (although the change was still relatively mild and subtle in extent) (H&Ex200). D) B18908 (necropsy). Macroscopic appearance of livers was generally normal but with dark patchy areas (arrows). In some cases a yellowish color was noted, suggesting cholestasis (day 7). E) B18908 (light area at necropsy). Vacuolar hepatocellular cytoplasmic change (H&Ex200). F) B18508 (dark area at necropsy). Extensive hemorrhage and hemorrhagic necrosis, sparing only focal groups of hepatocytes in the portal regions (H&Ex200). Solid black bar indicates 0.1 mm for figures 2B, 2C, 2E, and 2F. (Obtained from Ekser et al. PLoS ONE 2012;7:e29720)


GTKO (2h)
GTKO (necropsy)
a
d
b
e
c
f
0.1mm

3.3.3 Immunohistopathology of liver xenografts

The liver grafts were examined at 2 h and at necropsy (Table 15).

Cellular infiltrates. When HAR occurred in the WT pig liver xenograft, there was a significant cellular response, as well as antibody and complement deposition (Table 15 and Figure 21). In the GTKO/CD46 pig liver xenografts at 2 h, there was no T or B cell infiltrate except a very mild $CD3^+$ infiltrate in one case (B18908) (which correlated with light microscopy findings in pre-Tx and 2 h biopsies with similar cellular infiltration and neutrophil clusters [see above]). This liver showed the most prominent neutrophil-staining among the 2 h biopsies (Table 15). At necropsy, there was a mild increase in $CD3^+$ staining in one case (B7808). Between the 2h and necropsy biopsies, we observed a significant increase in neutrophil-staining (even in the liver allograft recipient which was negative pre-transplantation [pre-Tx]) (Table 15). To decrease liver macrophages (Kupffer cells), in 2 cases (B7908 and B8108) clodronate liposomes were administered to the organ source pig 24 h before excision and Tx of the liver, as described above. The combination of clodronate liposomes followed by ischemia/reperfusion appeared to be toxic to the liver since primary nonfunction occurred in both cases [**Ekser, 2010b**]. Immunohistological staining confirmed that macrophage depletion was not completely successful (Table 15), though there were fewer macrophages in the 2 h biopsies than in other pig livers.

Antibody and complement deposition. The WT pig liver showed deposition of IgG, IgM, C3, and C5b-9 (Table 15 and Figure 21). There was no IgG deposition in GTKO/CD46 pig livers at 2h and at necropsy, except in one case (B7808), which showed minimal

deposition (+ =<1%, Tables X and X). IgM deposition was observed in almost all liver xenografts at 2 h and at necropsy, although deposition was minimal in many cases (+=1–8%, Table 17); in one case (B18908), it reached up to 10% (++) on the 2 h biopsy (Figure 21, Tables 15 and 17). There was no correlation between euthanasia time-points and IgM deposition. In an effort to understand whether the immediate thrombocytopenia seen after reperfusion was related to mast or eosinophil cell activation, liver tissues were stained with an IgE antibody; all 2 h and necropsy biopsies stained negative (Table 15). Both 2 h and necropsy samples from GTKO/CD46 pig livers were negative for complement deposition (C3, C4d, and C5b-9), except in B7808 at 2 h and B8108 at 1 day (Table 15 and Figure 21), where deposition was minimal (<1%) (Table 15).

Thrombotic microangiopathy. To assess the extent of thrombotic microangiopathy (platelet aggregation and fibrin deposition) in the grafts, the grafts were stained for CD41 (platelets) and fibrin. All, including the WT graft, stained positive for platelets and fibrin (Table 15) even in the 2h biopsies. (The 2h and necropsy biopsies of the baboon allograft (B3408) showed no platelet or fibrin staining, suggesting that there was neither platelet aggregation nor fibrin deposition after allotransplantation) (Table 15). In some pig liver xenografts (B7808 and B7908), fibrin deposition reached up to 20% (+++) at necropsy (Tables 15 and 17 and Figure 21). However, no platelet or fibrin staining was seen in the pig liver in B18508, which had been treated with cobra venom factor (Table 15), although this baboon showed the same degree of thrombocytopenia as the other recipients [**Ekser, 2010b, 2012c**].

3.3.4 Ultrastructure of liver xenografts

Electron microscopy was not carried out in the WT liver that underwent HAR. With GTKO/CD46 pig livers, comparisons were made between pre-perfusion (pre-Tx), 2 h post-perfusion, and necropsy (light and dark area) biopsies (Figure 22). At 2 h, hepatocytes and sinusoidal endothelial cells were relatively normal (Figure 22B). However, deposition of platelets was observed along the sinusoid walls. At necropsy, a significant presence of red blood cells and fibrin was observed in dark areas, indicating large fibrin clots (Figure 22C), whereas light areas showed deposition of platelets along the sinusoidal endothelial cells together with mononuclear cells and other unidentified necrotic cells (Figure 22D). In the light areas, patchy endothelial cell death was documented. We did not observe any platelet phagocytosis by hepatocytes or liver endothelial cells, as previously shown by Burlak et al. [**Burlak, 2010**] and Peng et al. [**Peng, 2012**] in their in vitro models. However, our biopsies were limited and randomized, which could explain the lack of this finding.

Figure 21: Immunological assessment of liver xenografts. Figure 21 is shown in next page

Legend - Figure 21: Cellular infiltrates (CD3, CD20), antibody (IgG and IgM) and complement (C3, C4d, C5b-9) deposition were obvious in the WT pig liver at 2 hours, but were minimal or absent in GTKO and GTKO/CD46 pig livers. Fibrin and platelets were present in WT or GTKO and GTKO/CD46 pig livers regardless of post-Tx time, immunosuppression, and genetic modification of the organ-source pig. For details, see Table 2. (g) = green, (r) = red. Solid white bar indicates 0.1 mm for all figures. (Obtained from Ekser et al. PLoS ONE 2012;7:e29720)

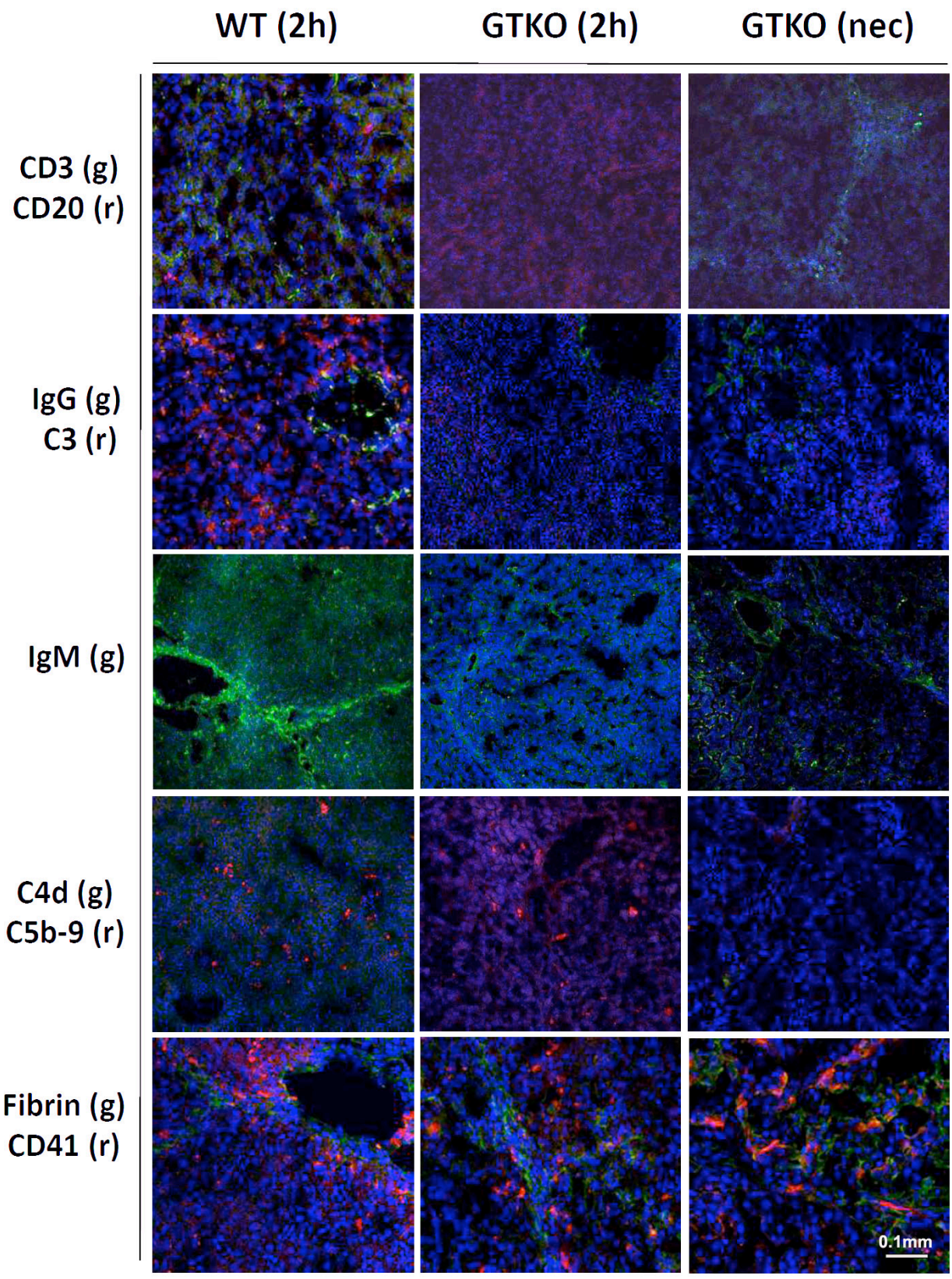
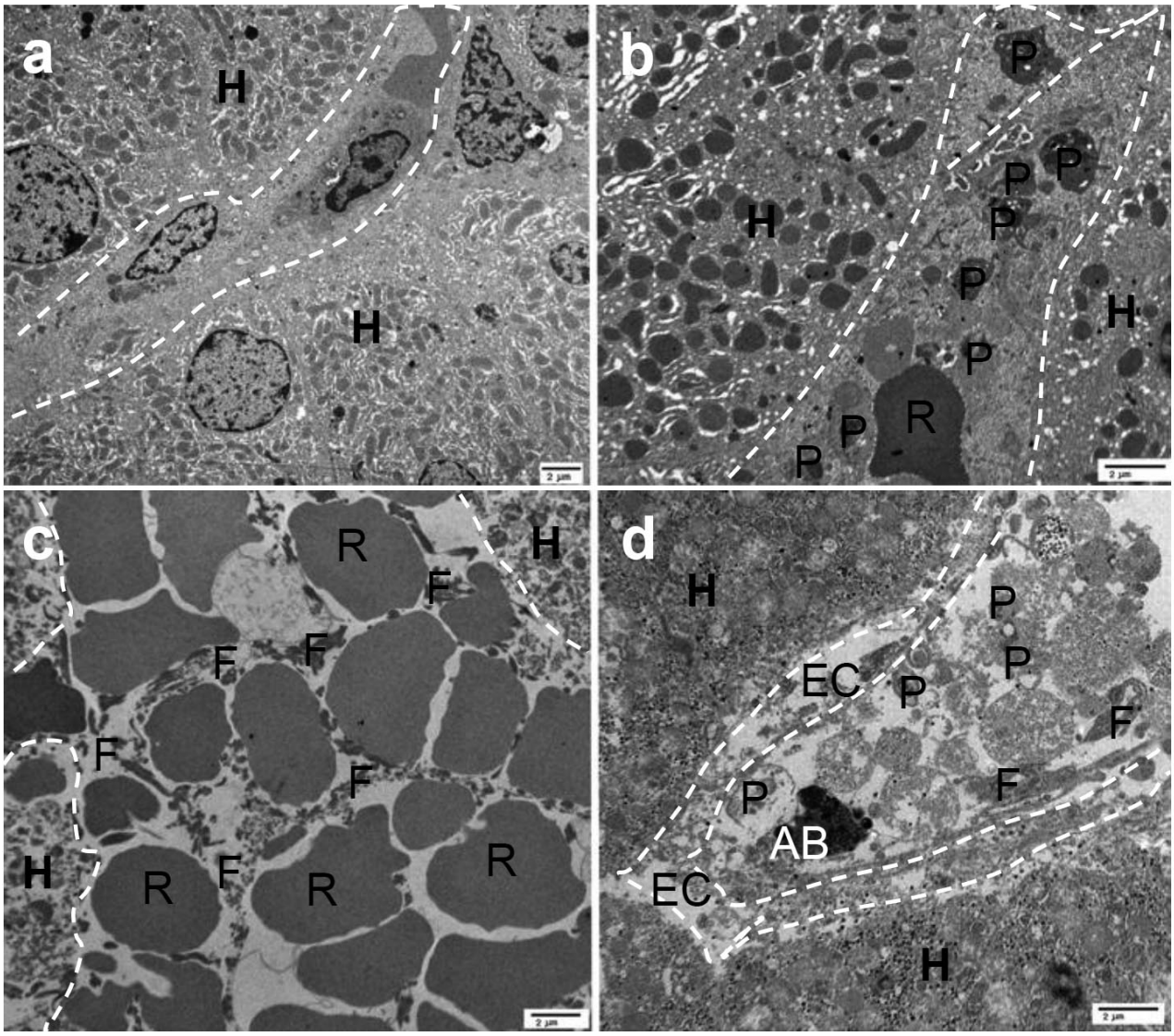

WT (2h)
GTKO (2h)
GTKO (nec)
CD3 (g)
CD20 (r)
IgG (g)
C3 (r)
IgM (g)
C4d (g)
C5b-9 (r)
Fibrin (g)
CD41 (r)
0.1mm

Figure 22: Electron microscopic appearance of liver xenografts



Legend - Figure 22: A) Ultrastructure of GTKO/CD46 liver before excision from the pig. B) (2 h post-reperfusion). Normal appearance of hepatocytes (H) with widespread platelet (P) aggregation and deposition along liver sinusoidal endothelial cells. C) (necropsy, dark area). Red blood cells (R) with fibrin deposition (F) indicating fibrin clots in the sinusoids. D) (necropsy, light area). Platelet, platelet/monocyte and platelet/necrotic cell aggregates along liver sinusoidal endothelial cells, and patchy endothelial cell death. Dashed lines indicate endothelial cells lining the sinusoids. AB = apoptotic body, EC = endothelial cells, F = fibrin, H= hepatocytes, P = platelets, R = red blood cells. The line at the bottom right indicates 2 µm. (Obtained from Ekser et al. PLoS ONE 2012;7:e29720)

3.4 HISTOPATHOLOGY OF THE NATIVE ORGANS IN LIVER XENOGRAFT RECIPIENTS

Hemorrhage was sometimes clinically manifest by petechiae in the skin and buccal mucosa, and by melena stools [**Ekser, 2010b, 2012c**]. Native organs of 6 longer-surviving (>1 day) recipients were macroscopically assessed and biopsied (wedge) under direct vision at necropsy (Tables 14 and 18), and were examined by light microscopy for morphological changes. In particular, macroscopic evaluation of hemorrhage in body cavities and native organs was carried out (Table 19). In 3 of the 6 recipients, the native organs were stained for platelet aggregation and fibrin deposition (Table 18). Clear signs of hemorrhage were observed in several sites in every baboon, presumably a direct result of severe thrombocytopenia. Blood-stained fluid was present in the thoracic cavity (from very mild-to-moderate) and in the abdominal cavity (moderate-to-severe) (Table 19). In order to identify whether there was a major bleeding site intraperitoneally, we performed relaparotomy in one case on post-operative day 4 (B18508). We observed that there was no major bleeding site, and all anastomoses were intact, but blood was oozing from the peritoneum and other soft tissues. All anastomoses remained intact in all baboons at necropsy.

3.4.1 Heart

Macroscopically, there was no hemorrhage observed in the myocardium, except in B18908 (Table 19 and Figure 23A). Microscopically, sections of heart examined were essentially normal with the exception of two (see below). There were no features suggesting inflammation, myofiber degeneration, or fibrin thrombi. Microscopically, B3108 showed several patchy areas of minimal-to-mild congestion and hemorrhage. Consistent

with the macroscopic necropsy appearance of the heart, B18908 had extensive hemorrhage throughout the organ (Figure 23A). This was most prominent in one large subendocardial area, although patchy foci of hemorrhage were also noted within the myocardium as well as on the epicardial surface. In numerous areas the hemorrhage and related edema caused a mild separation of individual myofibers by expanding the interstitial space between them. No platelet aggregation or fibrin deposition was seen, except in B18908, where there had been extensive hemorrhage (Table 18). The hemorrhage seen in these two cases (mild or severe) was likely spontaneous as a result of severe thrombocytopenia in the baboon, and therefore a secondary manifestation of a systemic problem (rather than representing primary cardiac disease).

3.4.2 Lungs

Macroscopically, hemorrhage in the lungs was patchy, but moderate-to-severe (Table 19 and Figure 23B). Microscopy showed patchy mild acute congestion, occasional focally-extensive atelactasis (B3108), multiple (numerous) thrombi in small interstitial vessels (most dense frequency in B7808), with no features of inflammation. No morphological evidence of platelets was noted under light microscopy, but immunohistological staining was always significantly positive (Table 18 and Figure 23C). In one case (B3208), there was focally-extensive hemorrhage (the most severely hemorrhagic lung of all baboons) (Figure 23B); scattered alveolar macrophages engulfing granular material were also observed, which could indicate platelets.

3.4.3 Small Intestine

Macroscopically, hemorrhage was extensive in the walls of the small intestine (Table 19), and 3 of 6 recipients had melena stools. Microscopically, there was moderately extensive

submucosal (B7708, B18908) and mild patchy serosal hemorrhage and congestion with no evidence of any mucosal involvement, even in the presence of melena stools. By causing dissection, submucosal hemorrhage distended the submucosal space (especially in B18908) (Figure 23D). There were no thrombi, and the mucosa looked normal. Immunofluorescence staining was negative, except for platelets in B18908, which had the most prominent hemorrhage (Table 18).

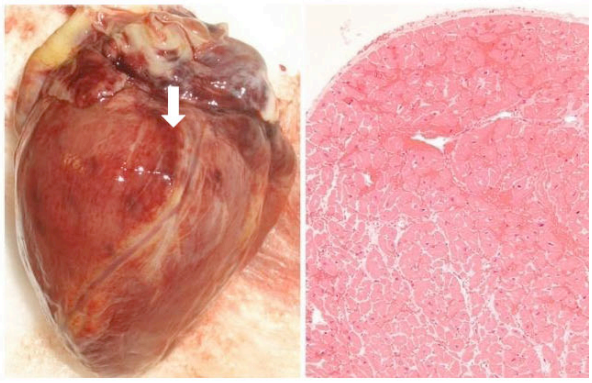
Table 18: Immunohistological features of thrombotic microangiopathy in recipient native organs at necropsy.

Baboon#	Time of Bx	CD41 (Platelets)	Fibrin
Heart			
B7708	day 7	–	–
B7808	day 6	–	–
B18908	day 6	++	–
Lungs			
B7708	day 7	++	–
B7808	day 6	++	–
B18908	day 6	++	–
Small intestine			
B7708	day 7	–	–
B7808	day 6	–	–
B18908	day 6	+	–
Kidneys			
B7708	day 7	–	–
B7808	day 6	–	–
B18908	day 6	++	+
Lymph nodes (mesenteric)			
B7708	day 7	–	–
B7808	day 6	–	–
B18908	day 6	+	–

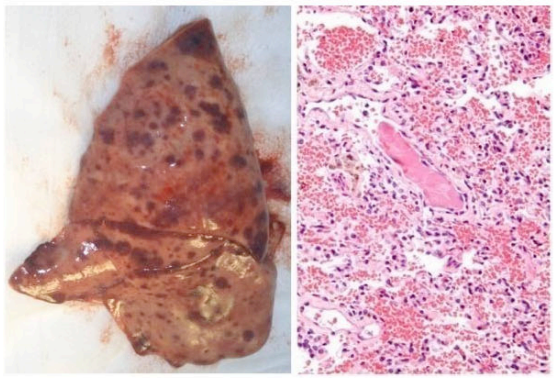
B = baboon, Bx = biopsy, n.a = not available. (-) = none; (+) = mild; (++) = moderate; (+++) = severe.

Figure 23: Macroscopic and microscopic appearances of native organs

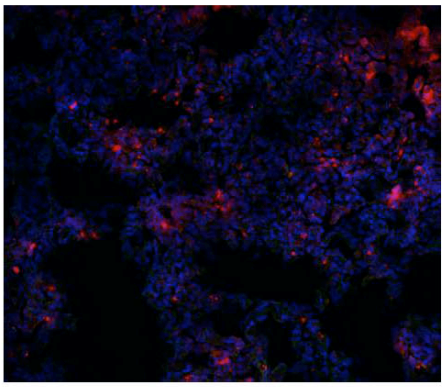
a) heart



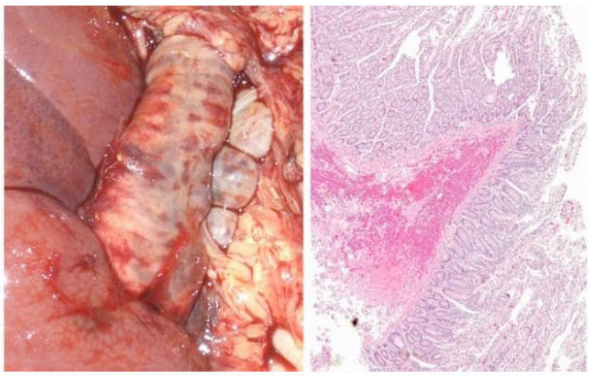
b) lung



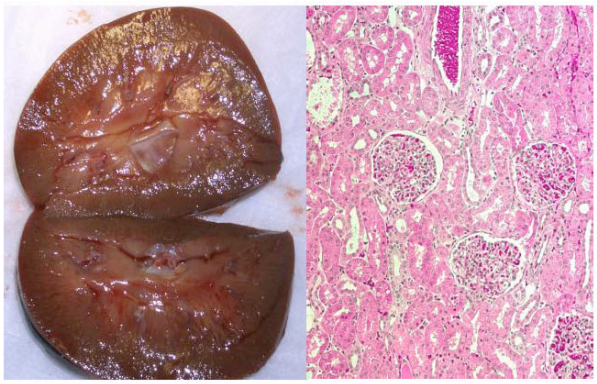
c) lung – platelet/fibrin staining



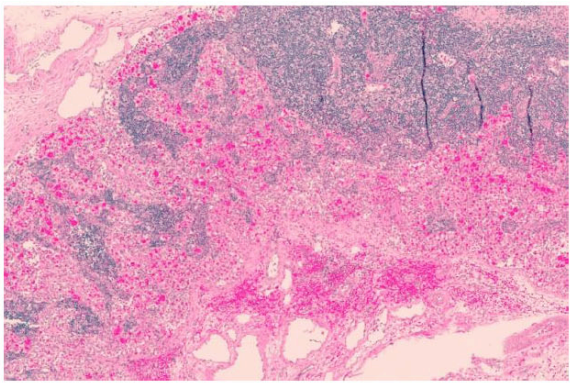
d) small intestine



e) kidney



f) lymph node



Legend - Figure 23: A) Heart: Left panel, macroscopic appearance of heart (B18908). Arrow indicates macroscopic bleeding in the myocardium (B18908). Right panel, subendocardial hemorrhage within the myocardium as well as on the epicardial surface (H&E 6100). B) Lungs: Left panel, macroscopic appearance of lung in which there was patchy bleeding (B3208) (Table 19). Right panel, acute congestion, focal atelactasis, multiple thrombi with no features of inflammation (H&E x200). C) Staining for platelets (CD41+) was positive in lungs, suggesting platelet aggregation and migration. This phenomenon was observed in lungs regardless of bleeding, but was present in other native organs only when bleeding had occurred (Table 19) (x100). D) Small intestine: Left panel, macroscopic hemorrhage in the wall of small intestine. Right panel, although the mucosa appeared normal, prominent submucosal hemorrhage was noted (B18908) (H&E x100). E) Kidneys: Left panel, kidneys were macroscopically normal, except in one case (B18908) which showed small, patchy petechiae. Right panel, acute glomerular congestion associated with tubular and interstitial hemorrhage (H&E x100). F) Lymph nodes: Although the macroscopic appearance was normal, lymph nodes in one case (B18508) showed hemorrhage in the hilar and medullary regions (H&E x200).

3.4.4 Kidneys

Macroscopically, the kidneys showed no signs of hemorrhage, except in one case (B18908) (Table 19). On light microscopy, there was mild capillary congestion, mild tubular proteinosis, and, in one case, infrequent small fibrin-microthrombi (B3108) (both interstitial and glomerular vessels). The patchy mild-to-moderate tubular proteinosis could have been an effect of the infusion of human albumin or of protein loss (B7808 and B18908). There were no fibrin thrombi in any case. In the only kidney that showed hemorrhage (B18908), acute severe glomerular capillary congestion associated with tubular and interstitial hemorrhage was present (Table 19 and Figure 23E). Granular and hyaline-appearing glomerular proteinaceous casts possibly suggested moderate hemoglobinuric nephrosis. This kidney was positive for platelet and fibrin staining (Table 18).

Table 19: Macroscopic observations at necropsy of hemorrhage in native organs and body cavities after genetically-engineered pig liver xenotransplantation in longest-surviving baboon recipients

Baboon #	Survival (days)	Blood-stained fluid in the chest	Heart	Lung	Blood-stained fluid in the abdomen	Melena stools	Small intestine	Kidney	Lymph nodes (mesenteric)
B3108	6	+/-	-	-	+++	+	++	-	-
B3208	4	++	-	+++	++	-	+	-	-
B7708	7	++	-	+++	++	-	-	-	-
B7808	6	+/-	-	-	++	+	++	-	-
B18508	5	+/-	-	++	+++	-	-	+/-	-
B18908	6	+	+	-	+++	+	+++	+	-

Legend: (-) = none; (+/-) = very mild; (+) = mild; (++) = moderate; (+++) = severe. All necropsies were performed by the same surgeon (B.E) and pathologist (E.K). The spleen was not studied since splenectomy was performed in all recipients immediately after reperfusion of the liver graft, except in B3108.

3.4.5 Lymph nodes

Mesenteric lymph nodes were collected during necropsy. Although macroscopic evaluation was not easy, no obvious hemorrhage was noted (Table 19). Microscopically, patchy mild cortical hemorrhage and congestion was present in B3108, and significant congestion and bleeding in the hilum and deeper medullary region was present in B18508. In other cases, no features of hemorrhage were seen. No thrombi were observed in any cases (Figure 23F).

CHAPTER 4 – DISCUSSION – PROJECT 1 - HISTOPATHOLOGY OF LIVER XENOTRANSPLANTATION

The main aim in the pig allotransplants was to study pig liver anatomy and investigate the surgical technique, and therefore no immunosuppressive therapy was administered. As expected, early features of acute cellular rejection were observed on days 2 and 3.
The baboon allotransplant was carried out (i) to study baboon liver anatomy and surgical technique, and (ii) to test our clinically-applicable immunosuppressive regimen (Tables 10 and 14). No significant features of humoral or cellular rejection were seen. The one WT pig-to-baboon liver xenotransplantation was carried out to study the development of HAR. Cellular infiltration and antibody and complement deposition were confirmed (Table 15 and Figure 21), as previously reported by Luo et al. [**Luo, 1998**]. In their experience, more rapid vascular rejection was seen in the WT pig-to-baboon model than in the WT pig-to-rhesus monkey model. However, heterotopic transplantation was performed in the former model, but orthotopic transplantation in the latter. The removal of the native liver almost certainly reduced the amount of native complement (90% of which is produced by the liver) that could participate in the rejection process; Hara et al have demonstrated that pig complement has a significantly reduced effect on a pig xenograft than human complement [**Hara, 2010**]. There have been no previous studies in which sequential liver biopsies have been taken during the development of HAR.

Therefore, the present study describes, for the first time, the evolution of HAR. Ramirez et al. [**Ramirez 2000, 2005**] observed severe edema and sinusoidal congestion in 3 WT pig liver transplants in baboons, with destruction of vascular and sinusoidal endothelium and parenchymal hemorrhage. Their immunohistological findings were similar to those in the present study [**Ekser, 2012c**]. Biopsies obtained from GTKO or GTKO/CD46 pig livers did

not show signs of HAR in the 2 h biopsies. At this time-point, light microscopic findings were generally normal and there was no cellular infiltrate or antibody and complement deposition, except minimal IgM deposition (Tables 15 and 17). Variable extents of sinusoidal dilatation and hepatocellular vacuolation were seen, but were not severe. Electron microscopy confirmed almost normal hepatocytes and endothelial cells.

There is scarce experience of genetically-engineered pig liver xenotransplantation in the literature [**Ekser, 2012e**; **2012a**]. Ramirez et al, using human CD55 or CD55/CD59/H-transferase-expressing pigs [**Ramirez 2000, 2005**], demonstrated the absence of HAR in 2 h liver biopsies. However, although they reported no deposition of C3 and C5b-9, there was positivity for immunoglobulin and C4 deposition without any information regarding the isotype of the immunoglobulin or extent of deposition [**Ramirez, 2000**].

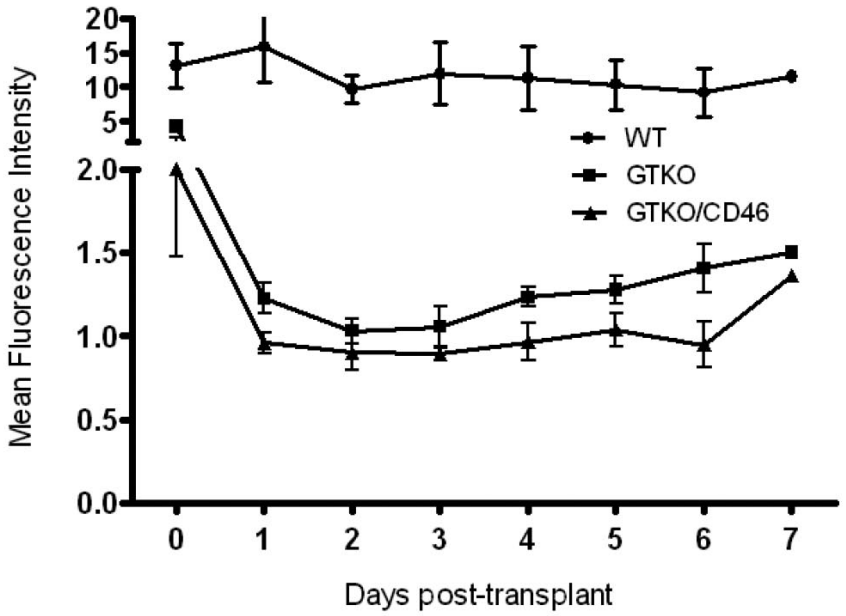
In the present study, there were significant changes in the livers at necropsy, but these were largely related to hemorrhage within the liver, rather than a result of an immune response, i.e., antibody-mediated or cellular rejection. Some lesions may have been associated with hepatocellular ischemia due to anemia and organ hypoperfusion. The hemorrhage noted was most likely directly associated with the profound thrombocytopenia that developed rapidly in all baboons, though a role for immune-mediated injury cannot be totally excluded. Except for minimal IgM deposition, however, there was no or minimal cellular infiltration or antibody and/or complement deposition (Tables 15 and 17 and Figure 21). Nevertheless, there is a possibility that patches of hemorrhagic necrosis may have masked features of immune injury.

The observation that anti-nonGal antibody levels did not increase (although this might not be expected within 4–7 days) lends support to this conclusion (Figure 24). In vitro antibody

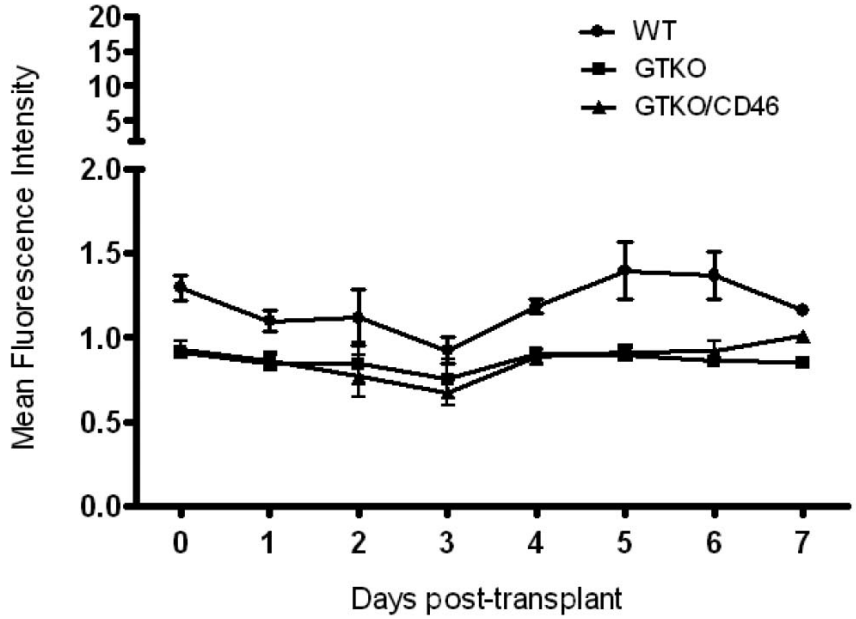
binding to porcine aortic endothelial cells from GTKO and GTKO/CD46 pigs was also measured, confirming minimal or absent antibody binding. This further finding suggests (though does not prove) that antibody-mediated injury did not cause the changes seen in the liver grafts.

Figure 24: Average anti-nonGal antibody levels (IgM and IgG) in 4 baboons.

a) IgM binding to WT, GTKO, and GTKO/CD46 pig PBMCs



b) IgG binding to WT, GTKO, and GTKO/CD46 pig PBMCs



*Legend – Figure 24: Serum from the longest-surviving recipient baboons (n = 4; B7708, B7808, B18508, and B18908) was drawn daily (pre-Tx to postoperative day 7), and stored at -80^{o}C until processed. Binding of (i) anti-nonGal IgM and IgG antibodies to GTKO and GTKO/CD46, and of (ii) anti-pig antibodies (Gal and nonGal), to WT pig peripheral blood mononuclear cells (PBMCs) was measured by flow cytometry [**Hara, 2008**]. A) IgM binding against WT, GTKO, and GTKO/CD46 pig PBMCs: Significantly increased binding to WT pig PBMCs. However, GTKO and GTKO/CD46 pig PBMCs showed decreased binding throughout the study. B)*
IgG binding against WT, GTKO, and GTKO/CD46 pig PBMCs: Although IgG binding against WT pig PBMCs was slightly increased; there was no significant difference between bindings against GTKO and GTKO/CD46 pig PBMCs. (Obtained from Ekser et al. PLoS ONE 2012;7:e29720)

Similar observations were made by Ramirez et al. [**Ramirez, 2000**]. In their 4-day survivor, there were no features of rejection, and liver lobular architecture was preserved, although hemorrhage was noted. Similar to our findings, in Ramirez et al's 8-day survivor, hemorrhage and hemorrhagic/ischemic necrosis in the liver was seen, but they emphasized the absence of features of rejection [**Ramirez, 2000**]. Observations in the present study with light and electron microscopy and with immunohistological studies with confocal microscopy indicated that there was deposition of fibrin and platelets in the liver grafts, which increased between the 2h biopsies and necropsy (Tables 15 and 17 and Figure 21). Significant platelet aggregation was seen in the liver sinusoids. Electron microscopy confirmed fibrin deposition in the liver especially at necropsy in the dark areas. In the light areas, fibrin deposition was noted as well, but was significantly less than in the dark areas. Fibrin deposition was not seen particularly in the native organs, except in the kidneys of B18908 [Table 18]). Flow cytometry studies by Ezzelarab et al have demonstrated extensive platelet-WBC aggregation in the peripheral blood [**Ezzelarab, 2012**] Peng et al. [**Peng, 2012**] recently showed in vitro that pig hepatocytes, liver

sinusoidal and aortic endothelial cells from GTKO or WT pigs induced moderate aggregation of baboon platelets, which correlates with the findings of Burlak et al. [**Burlak, 2010**]. Although recent in vitro studies have indicated (by electron and confocal microscopy) platelet phagocytosis by hepatocytes and liver sinusoidal endothelial cells [**Burlak, 2010; Peng, 2012**], we were unable to confirm this phenomenon in our ultrastructural studies. We suggest this may be a result of differences between in vitro and in vivo models. In vitro studies may provide better and more repeated access to biopsies for electron microscopy [**Burlak, 2010**], and platelets can be more easily tagged to follow their destiny [**Burlak, 2010; Peng, 2012**]. Nevertheless, it is possibly surprising that platelets are phagocytosed by liver sinusoidal endothelial cells. Soluble components and colloidal particles are cleared from the blood by hepatic endothelial cells by receptor-mediated endocytosis [**Shiratori, 1993; Elvevold, 2008**], but these particles are <0.23 μm in diameter (almost 10-fold less than the normal platelet diameter). Furthermore, in our in vivo studies, platelet-platelet and/or platelet-WBC aggregation was documented in the blood and in the graft, possibly making it more unlikely that platelets would be successfully phagocytosed [**Ezzelarab, 2012**]. However, platelets may migrate through the fenestrations or between the sinusoidal endothelial cells, which could possibly enlarge under certain circumstances, and these cells may change their behavior, possibly as a result of activation, ischemia/reperfusion injury, inflammation, etc. How platelets become activated (possibly through a specific receptor, such as the Ashwell receptor [**Rumjantseva, 2009**] or through CD47-SIRPalpha interaction [**Ekser, 2012e**]) or aggregate on the sinusoidal endothelial cells remains uncertain and require investigation. We also studied the native organs of the recipient baboons. Macroscopic and microscopic assessments strongly suggested bleeding had occurred as a result of the severe thrombocytopenia. The most affected organs were the lungs and small intestine (Table 19, Figure 23), but petechiae of the skin, and melena stools were common.

Immunohistological studies confirmed platelets in the lungs regardless of the presence of bleeding, and in other native organs in which bleeding had occurred (Figure 23B and Table 18).

In conclusion, after GTKO and GTKO/CD46 pig liver transplantation in baboons, the rapid development of a profound thrombocytopenia was by far the major problem seen, and was suggested as the major causative factor in the hemorrhage that occurred, not only in the pig liver grafts, but also in several native organs and body cavities. The histopathologic features described can largely be explained on this basis. However, the factors influencing the activation and consumption of platelets, and therefore the knowledge of the development of coagulopathy and the factors influencing the coagulopathy are extremely important [**Ekser, 2012a, 2012d**], need to be studied. Since the features of rejection were minimal or absent, we believe that, if platelet activation and aggregation/phagocytosis can be prevented, possibly by further genetic modification of the pigs or by novel therapeutic agents, bridging by a pig liver to allotransplantation may become a feasible clinical option.

CHAPTER 5 – INTRODUCTION – PROJECT 2 – STRATEGIES TO PREVENT ADAPTIVE IMMUNE RESPONSE IN XENOTRANSPLANTATION

The introduction of genetically-engineered pigs (e.g., GTKO [**Phelps, 2003**] , or transgenic for a human complement-regulatory protein [CD55, CD46 or CD59] [**Cozzi, 1995; Loveland, 2004; Cooper, 1993, 2007; Ekser, 2012a**]), has contributed to a significant increase in the survival of pig organs transplanted into nonhuman primates. Progress has also been made in understanding the mechanisms of the innate and adaptive immune responses [**Ezzelarab 2009; Satyananda, 2013**], and by the introduction of novel immunosuppressive agents, e.g., those that inhibit costimulation.

Subsequently, however, Chen et al [**Chen, 2005**] reported both HAR and AHXR in renal grafts from GTKO pigs in baboons that received an immunosuppressive regimen that did not prevent an elicited antibody response. We have also seen early graft failure from AHXR when immunosuppressive therapy has been inadequate, particularly when an adaptive immune response developed [**Ezzelarab, 2009**]. From this experience, we would conclude that an immunosuppressive regimen that successfully prevents the adaptive immune response is essential for prolonged GTKO graft survival.

In the studies of Kuwaki [**Kuwaki, 2005**] and Tseng [**Tseng, 2005**], the immunosuppressive regimen consisted of induction with anti-thymocyte globulin (ATG), and maintenance with an anti-CD154mAb, mycophenolate mofetil (MMF), and methylprednisolone. It was uncertain whether all of the agents in the immunosuppressive regimen were essential to prevent an adaptive immune response. Yamada et al [**Yamada, 2005**], using a GTKO pig-to-baboon kidney transplantation model and the same costimulation blockade-based regimen with additional therapy aimed at inducing tolerance,

has demonstrated that maintenance methylprednisolone may not be essential [**Yamada, 2005**].

Our interest has been directed to two topics – (i) costimulation blockade as a form of immunosuppression [**Ezzelarab, 2009, 2012**], and (ii) genetic manipulation of the pig to provide protection from the primate adaptive immune response [**Phelps, 2009; Hara, 2013**].

Effective immunosuppressive regimens based on blockade of the CD40/CD154 (CD40 ligand) pathway by targeting with an anti-CD154 monoclonal antibody (mAb) have been shown to be successful in preventing the adaptive response to allografts and xenografts contributing to prolonged graft survival [**Kirk, 1997; Buhler, 2000; Kuwaki, 2004, 2005; Tseng, 2005; Cardona, 2006, 2007; Hering, 2006; Ezzelarab, 2009, 2012; van der Windt, 2009; Ford, 2009; Mohiuddin, 2012**]. However, clinical use of anti-CD154mAbs has currently been abandoned due to thrombotic complications [**Kirk, 1997, 2001; Kawai, 2000; Kuwaki, 2004; Knosalla, 2003**]. Anti-CD154mAbs are unlikely to be available for clinical use. However, the CD40/CD154 interaction remains a promising target, and CD40-specific therapies have been suggested as an alternative approach. Various CD40-specific antibodies have been shown to extend kidney and islet allograft survival in nonhuman primates [**Pearson, 2002; Haanstra, 2003, 2005; Aoyagi, 2009; Adams, 2005; Watanabe, 2013**]. Thompson et al. have demonstrated that human CD40-specific antibodies effectively promoted pig islet engraftment and survival in monkeys without thromboembolic phenomena [**Thompson, 2011**].

CD28/B7 costimulatory pathway blockade has also been tested in clinical trials of organ allotransplantation with encouraging results. For example, belatacept-based regimens

have improved patient and allograft survival by avoiding the chronic toxicities of the current pharmacologic immunosuppressive therapies [**Larsen, 2005; Vincenti, 2010**].

We developed a simple artery patch transplantation procedure in the pig-to-baboon model, which successfully exposes the baboon to sufficient pig antigens to induce an adaptive immune response [**Ezzelarab, 2012**]. In this model, we were able to assess the humoral and cellular immune responses to xenografts in the absence of the coagulation dysfunction that is seen after organ transplantation and which can complicate assessment of the extent of the strength of the adaptive immune response [**Ezzelarab, 2012**]. When no or inadequate immunosuppressive therapy is administered, a GTKO pig xenograft induces significant T cell proliferative and elicited IgM and IgG responses [**Ezzelarab, 2009**]. An anti-CD154mAb-based, but not a CTLA4-Ig (abatacept)-based, regimen prevented sensitization to a GTKO pig xenograft [**Ezzelarab, 2012**]. (*See details in 5.2 Preliminary Studies*).

An alternative or adjunctive approach is to transplant cells, tissues, or organs from pigs that have been genetically engineered to protect from the primate T cell-dependent adaptive immune response. This might at least allow a reduction in the exogenous immunosuppressive therapy required to prevent T cell-mediated rejection. To date, this approach has included the production of pigs (i) expressing a costimulation-blockade agent, e.g., CTLA4-Ig, either constitutively [**Phelps, 2009; Koshika, 2011**] or in specific cells **[Martin, 2005; Leveque, 2011, Ayares, 2011**] or (ii) transgenic for a human mutant MHC class II transactivator (CIITA-DN pigs), which reduces swine leukocyte antigen class II expression both when the cell is quiescent and when activated [**Hara 2013**].

We have now explored the efficacy of a graft from a CIITA-DN pig in reducing the adaptive response in this model, and have further investigated the ability of CD28/B7 pathway blockade or blockade of both (CD28/B7 and CD40/CD154) pathways to prevent the adaptive immune response to a pig artery patch graft as an alternative to CD154-specific mAb therapy.

5.1 COSTIMULATORY PATHWAYS

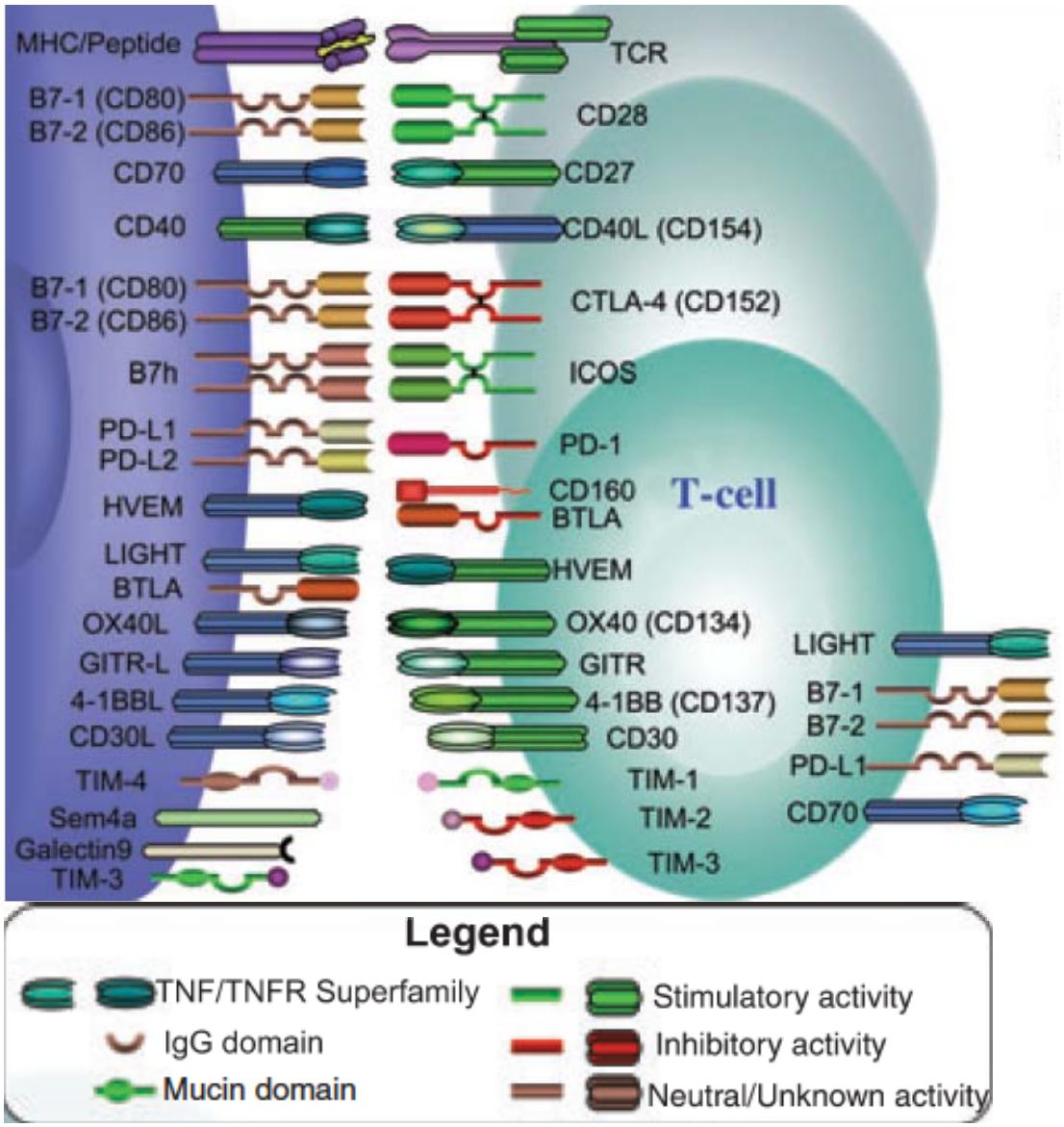
As both rejection and regulation directed against allo- or xenoantigens require T cell activation, costimulatory molecules play an important role in regulating both processes and ultimately fate of the graft. Antigen-mediated stimulation of T cells through their clonotypic T cell receptor (TCR) delivers signal 1, which initiates the process of T cell activation and defines the specificity of the immune response. A second signal, delivered by a constellation of cell surface structures collectively called costimulation molecules, is critical to drive clonal expansion, survival, and differentiation of activated T cells [**Janeway, 1994**]. Figure 25 and Table 20 describe the costimulatory molecules in details.

5.1.1 CD28/B7 pathway

CD28 is constitutively expressed on all naïve $CD4^+$ and $CD8^+$ T cells. CD28 has two known ligands, B7-1 (CD80) and B7-2 (CD86), both of which are type I transmembrane glycoproteins of the Ig superfamily [**Li, 2009**]. B7-1 expression is inducible and can be expressed by APCs and many other cell types including activated T cells. While B7-2 is constitutively expressed by antigen-presenting cells (APCs), its levels of expression are upregulated by cell activation. One agent that has been widely used to block CD28 costimulation is CTLA-4-Ig, a recombinant fusion protein consisting of the extracellular domain of CTLA-4 and the Fc portion of IgG [**Lenschow, 1992**]. CTLA4 (CD152), whi h

although structurally homologous to CD28, has a ~20-fold higher affinity for CD80/CD86, and thus can out-compete CD28/B7 binding. There are two different CTLA4-Ig molecules known as Abatacept and Belatacept.

Figure 25: Costimulatory molecules



Legend – Figure 25: Naïve T cells on top, followed by early activated T cells in the middle, and effector or memory T cells at the bottom. Obtained from Li et al. Immunological Reviews 2009;229:271-293

Table 20: Expression and function of costimulatory pairs in the immune response

Receptor	Ligand	Receptor expression	Ligand expression	Function of interaction on the immune response
CD28	CD80	Naïve T cells	APC (induced upon activation)	Promotes increased stability of IL-2 mRNA and increases levels of anti-apoptotic molecules e.g. Bcl-xl , increases proliferation
	CD86	APC (constitutively)		
CTLA-4 (CD152)	CD80/CD86	Naive T cells, Treg		Decreased survival, cytokine production, increases inhibitory function of Treg
ICOS (CD278)	ICOSL	Activated T cells	B cells, monocytes	T cell activation and differentiation , splenic germinal centre formation and immunoglobulin class switching
PD-1 (CD279)	PD-L1	Activated T cells, B cells and myeloid cells	Resting T cells, B cells, DC, macrophage, endothelial cells	Inhibits TCR signals resulting in decreased survival, proliferation and cytokine production
	PD-L2	DC, macrophages		
OX40 (CD134)	OX40L	Activated T cells	B cells, macrophages, dendritic cells, vascular endothelial cell, mast cells, activated NK cells	Late expansion and survival of the immune response, involved in memory responses
41BB (CD137)	41BBL	$CD4^+$ and $CD8^+$ T cells and NK cells	Mature DC, activated B cells and macrophages	Promotes proliferation, cytokine production , B cell proliferation
CD40	CD154	B cells, DC, macrophages	T cells, B cells, activated platelets, DC, eosinophils	DC maturation, upregulation of CD80, CD86 and ICAM-1 and B cell activation
CD27	CD70	Activated T and B cells, NK cells	Activated T and B cells, NK cells, DC	Promotes survival of effector T cells and increases memory generation

Obtained from Kinnear et al. Transplantation 2013;95:527-535

Although Abatacept and Belatacept are both CTLA4-Ig, they differ from each other for 2 amino acids [**Wekerle, 2011**]. Abatacept has a higher affinity to CD80 than CD86. Belatacept is a second generation of CTLA4-Ig with increased affinity and prolonged binding time to B7 molecules [**Wekerle, 2011**].

5.1.2 CD40/CD154 pathway

CD40 and CD154 belong to tumor necrosis factor receptpr (TNFR) and TNF familes, respectively. Although CD40 is constitutively expressed on APCs at low levels, its expression is upregulated upon cellular activation. CD154 is a ligand for CD40 and expressed on activated T cells, NK cells, eosinophils and platelets [**Li, 2009**]. As discussed

earlier, although anti-CD154mAb was found to be effective on blocking adaptive immune response, its clinical use of anti-CD154mAbs has currently been abandoned due to thrombotic complications. More recently reagents which target CD40 rather than CD154, have been developed. Anti-CD40 was found to synergize with CTLA4-Ig to promote long-term graft survival in mouse models of skin and bone marrow transplantation. Anti-CD40 (4D11) showed significant prolongation of renal allograft survival in nonhuman primates and prevented the development of alloantibodies [**Imai, 2007**]. Anti-CD40mAb is also used in xenotransplantation with good results [**Mohuiddin, 2013**].

5.2 PRELIMINARY STUDIES FOR ARTERY PATCH XENOTRANSPLANTATION

We have carried out a series of experiments to determine whether the costimulation-based regimen used by Kuwaki and Tseng can be simplified and yet still prevent the adaptive immune response since anti-CD154mAb will likely not be available for clinical use due to its side effects, such as thromboembolic events. We, therefore, used CTLA4-Ig to determine if this agent could replace anti-CD154mAb in the above-mentioned regimen.

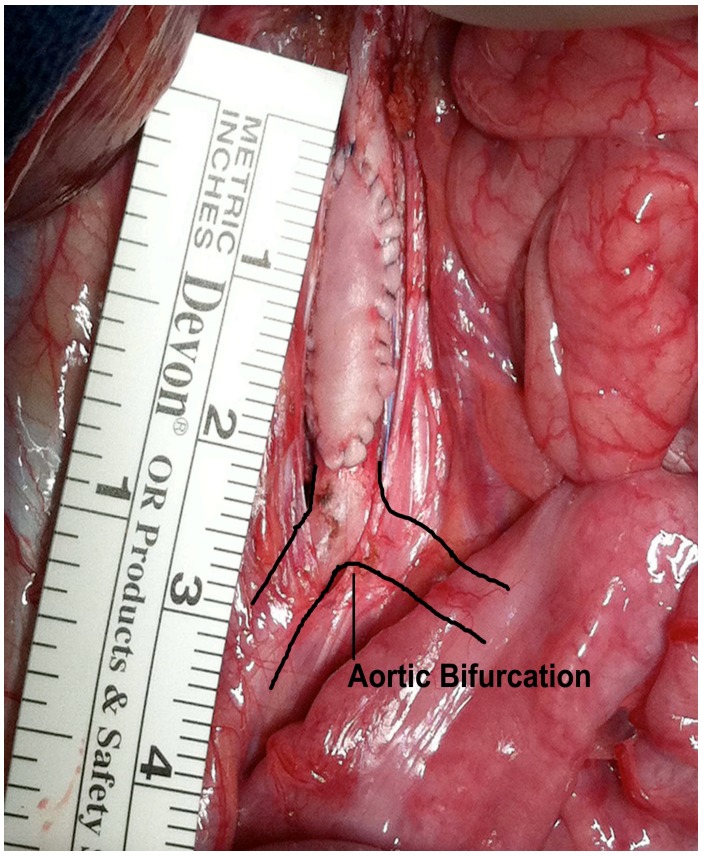
5.2.1 Animals and Surgical Procedures

Fourteen baboons, weighing 6-10kg and of known ABO blood type, were recipients of GTKO pig artery grafts. GTKO pigs (n=7) of blood group O (nonA) weighing 7-20kg**,** generated by nuclear transfer/embryo transfer from modified fibroblasts from Large White/Landrace/Duroc cross-breed pigs [**Phelps 2003**], served as sources of carotid artery (Table 21).

Anesthesia in pigs and baboons, and intravascular catheter placements in baboons have been described previously [**Cooper, 1991; Kozlowski, 1999; Buhler, 2001**]. In the source

GTKO pig, after heparinization (300U/kg i.v.), a length of carotid artery was excised, and stored in University of Wisconsin solution in ice (at approximately 4°C) until transplanted into the baboons. The ischemic period was approximately 6h in 10 experiments and 20h in 4 experiments. The artery was divided longitudinally to form a patch, which was cut into two for insertion into two baboons. Each patch was approximately 2x1cm. In the recipient baboon, the abdomen was opened through a midline incision, and the abdominal aorta was exposed. After partial heparinization (100U/kg i.v.) the aorta was clamped immediately distal to the renal vessels and at the bifurcation, and opened longitudinally. The artery patch was sutured in place as an onlay graft using 5.0 polypropylene sutures (Figure 26). The clamps were then released, and, after determining there was good flow to both legs, the abdomen was closed.

Figure 26: GTKO pig artery patch xenotransplantation



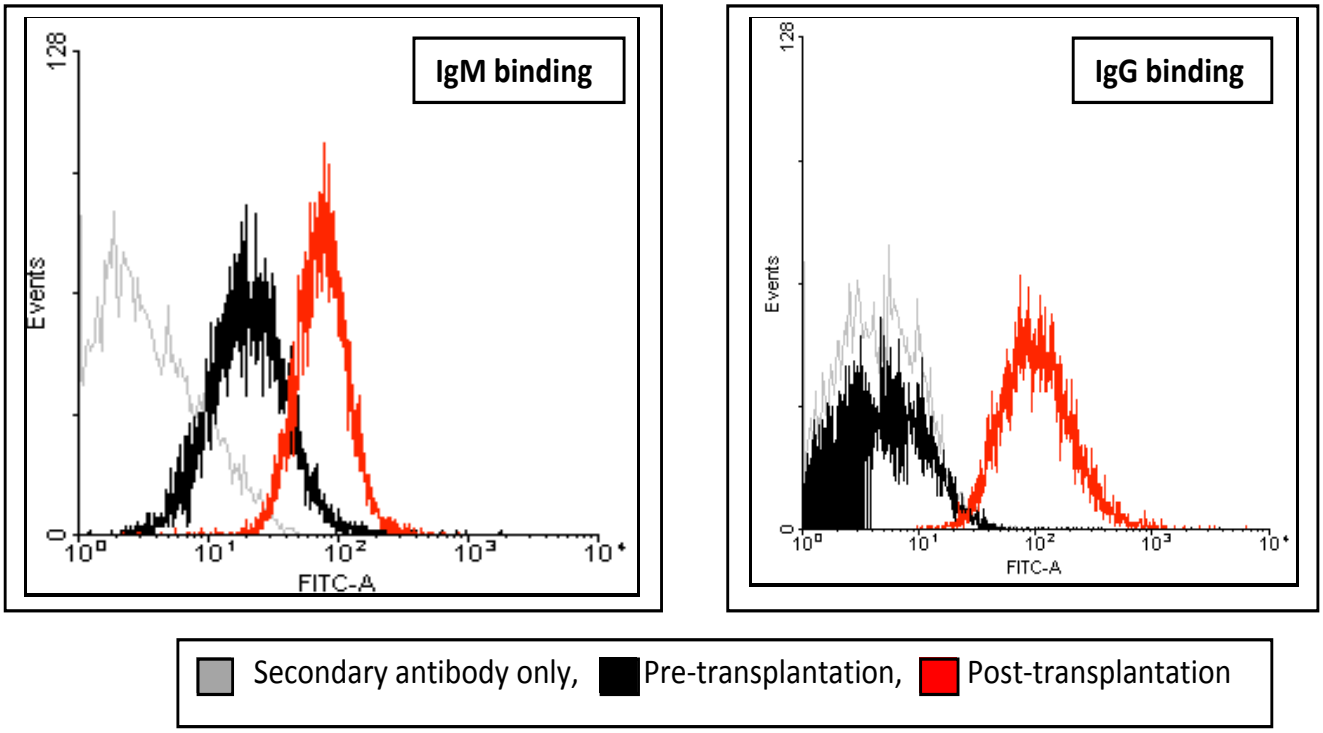
Legend – Figure 26: After exposure of the recipient abdominal aorta (below the renal arteries and above the bifurcation), the pig artery patch (2 x 1 cm) was sutured in place as an onlay graft. Arrows indicate the extent of the graft. (B16011).

Table 21: Immunosuppressive regimen, graft survival and complications after GTKO pig artery transplantation

Group	N.	Immunosuppressive therapy	Survival (days)	Complications
1	3	None	14 (n=1)	Ruptured graft
			>28 (n=2)	
2	6	Anti-CD154mAb (n= 2)	<1 (n=1)	Aortic thrombosis
			>28 (n=1)	
		ATG+anti-CD154mAb (n=3)	>28	
		ATG+anti-CD154mAb+MMF (n=1)	>28	
3	5	CTLA4-Ig (n=1)	>28	
		ATG+CTLA4-Ig+MMF (n=3)	14 (n=1)	Death from disconnected vascular lines
			>28 (n=2)	
		ATG+anti-CD154mAb+CTLA4-Ig +MMF (n=1)	>28	

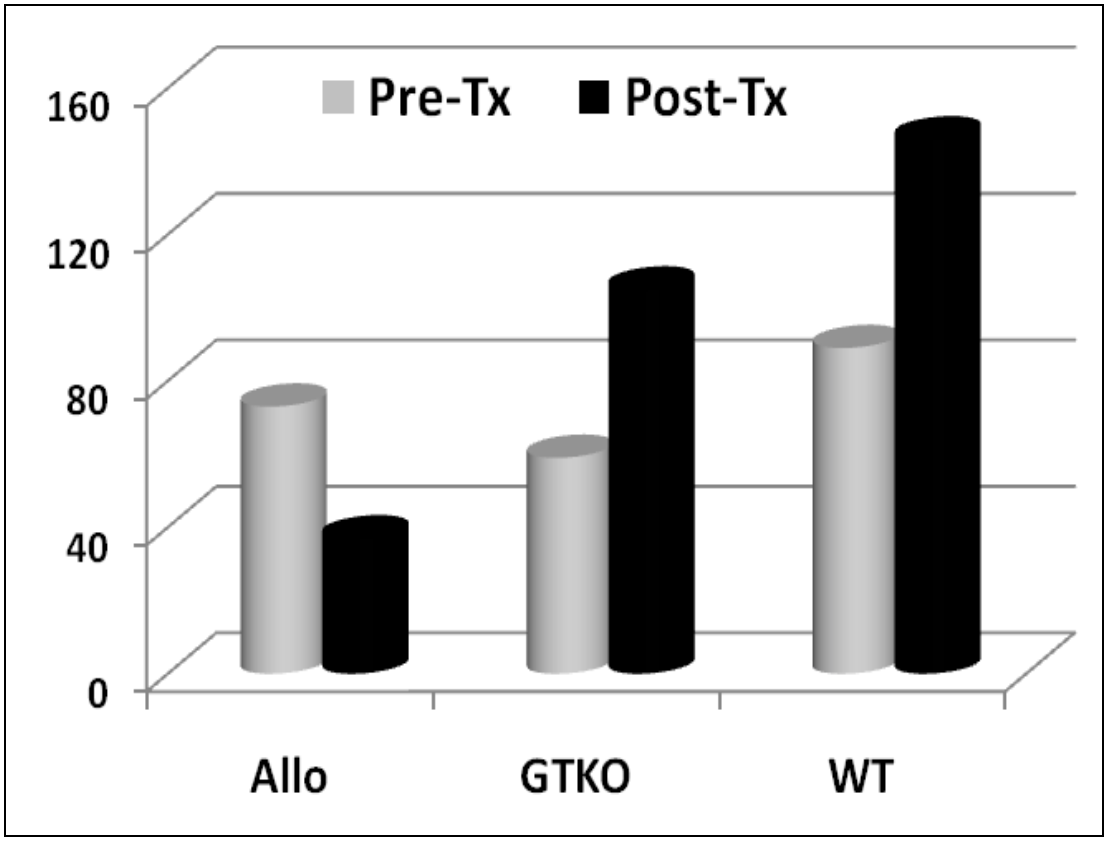
In Group 1**,** when we did not administer immunosuppression, we observed an increased humoral response (IgG and IgM binding to nonGal antigens on the peripheral blood mononuclear cells [PBMCs]) which was measured by flow cytometry (Figure 27) and an increased cellular response measured by mixed leukocyte reaction (MLR) (Figure 28) [**Ezzelarab, 2009**].

Figure 27: Humoral response without immunosuppression in GTKO pig arterial transplantation in baboons



In Group 2, the grafts showed significant cellular infiltration even though sensitization did not develop when anti-CD154mAb alone was used (data not shown). If the full regimen was employed (ATG+anti-CD154mAb +/- MMF) we did not see any infiltrates.

Figure 28: Cellular immune responses without immunosuppression in GTKO pig arterial transplantation in baboons



Legend – Figure 28: Baboon PBMCs before and after the GTKO pig artery exposure (sensitization) were tested against allo (3rd party), GTKO and WT pig PBMCs. Since was no immunosuppression used, both GTKO and WT responses increased (WT more than GTKO due to anti-Gal antibodies). However, we did not observe sensitization against allo-baboon PBMCs after the transplantation.

In Group 3, we found that CTLA4-Ig alone was insufficient to prevent sensitization or an increased MLR response, and there was a significant cellular infiltrate in the graft (not shown). If a full CTLA4-Ig-based immunosuppressive regimen (ATG+CTLA4-Ig+MMF) was used, detectable anti-nonGal antibodies were seen but there was no increase in MLR response and a reduced cellular infiltration (Figure 29 and 30).

Figure 29: Humoral immune response with full anti-CD154mAb-based (Group 2) vs full CTLA4-Ig-based (Group 3) immunosuppressive regimens in GTKO pig arterial transplantation in baboons

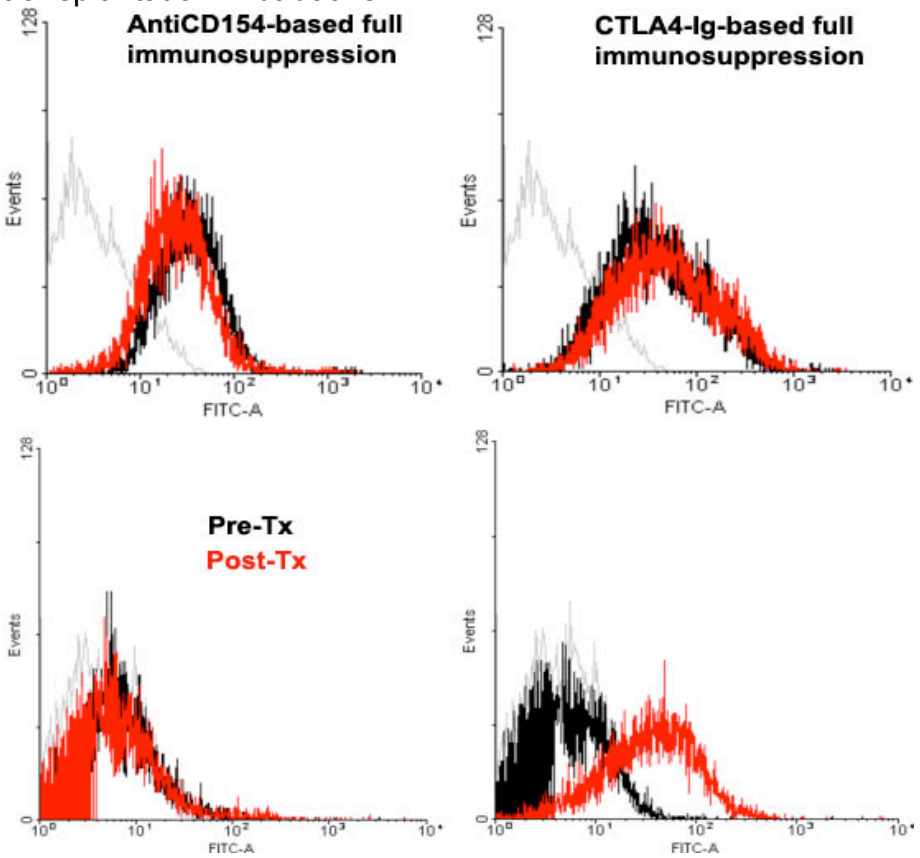
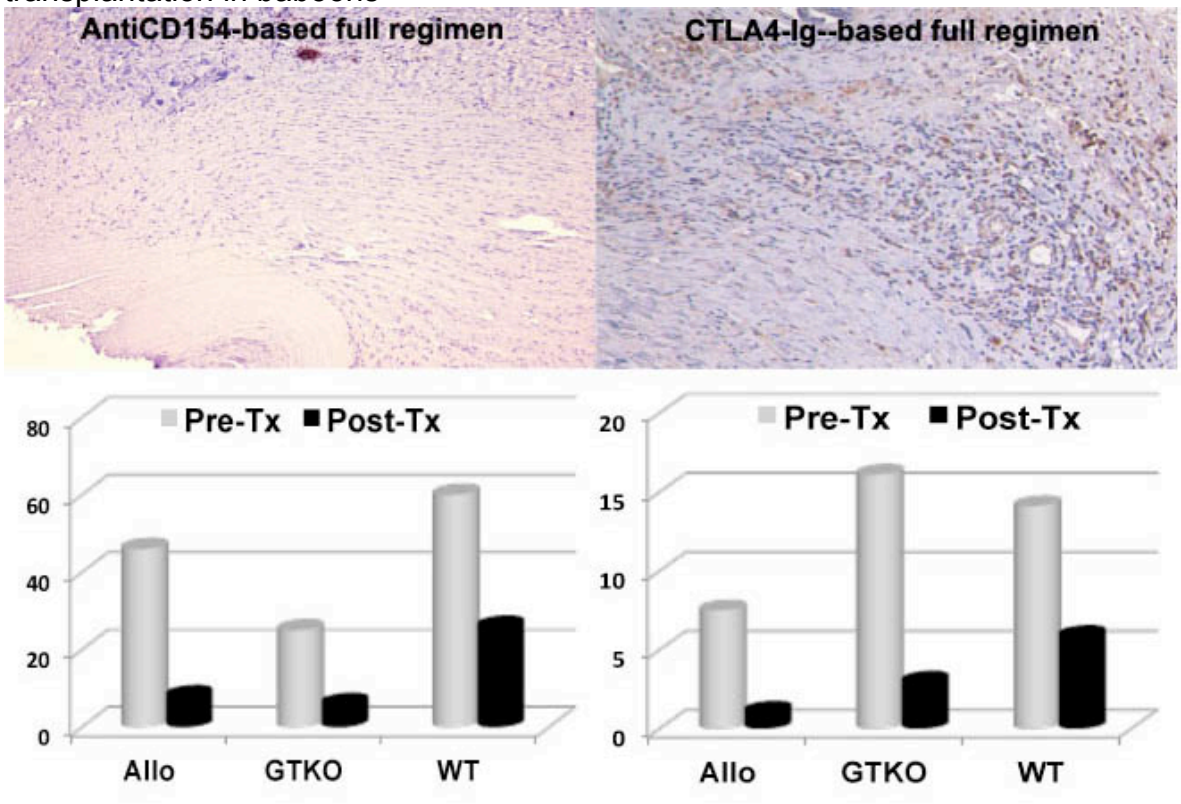


Figure 30: Cellular immune response with full anti-CD154mAb-based (Group 2) vs full CTLA4-Ig-based (Group 3) immunosuppressive regimens in GTKO pig arterial transplantation in baboons



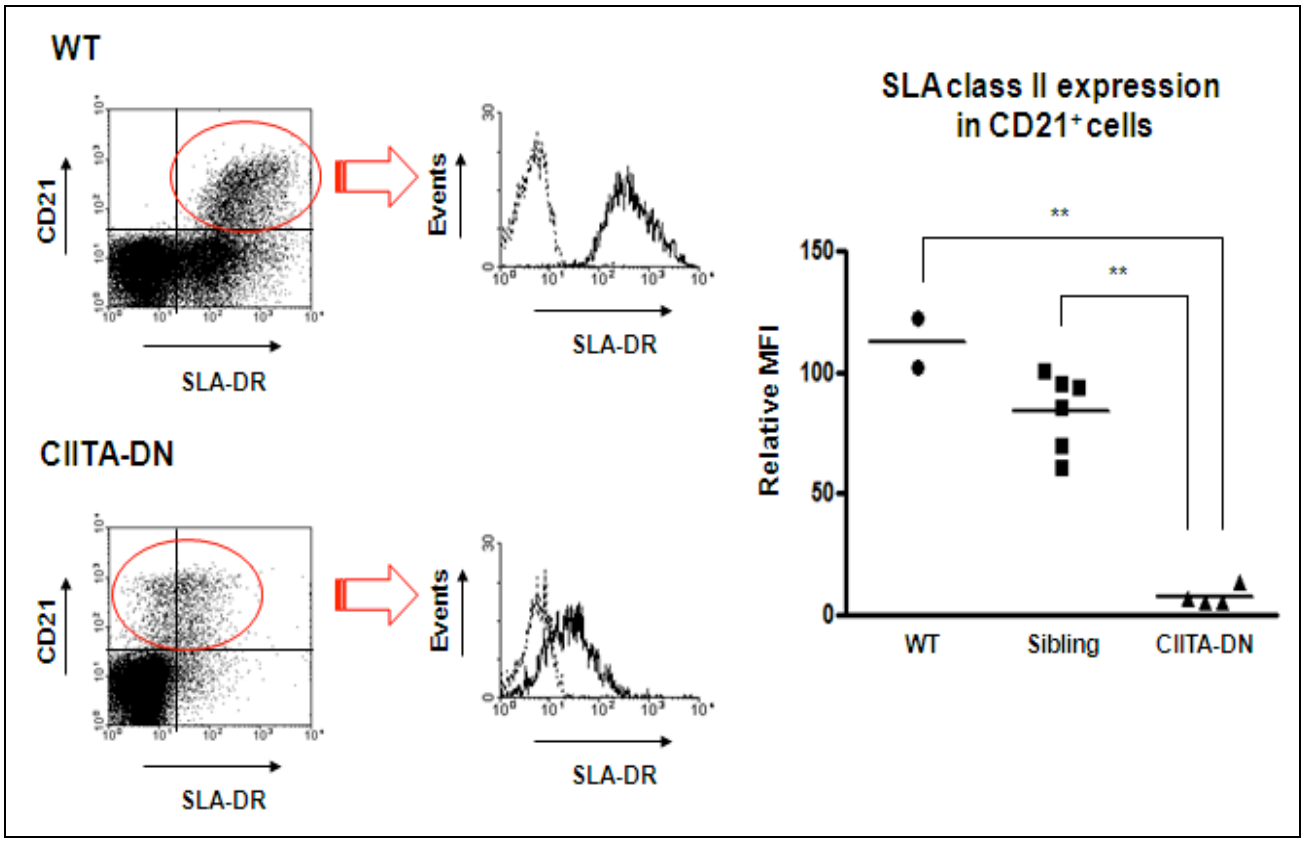
After this experience, we concluded that anti-CD154mAb is essential to prevent the primate humoral immune reponse in xenografts. If CTLA4-Ig is to replace anti-CD154mAb, a more effective form (e.g. LEA29) or additional treatment, such as B-cell depletion, is needed [**Ezzelarab 2009**].

5.3 THE DEVELOPMENT OF MHC CLASS II KNOCKDOWN PIGS

Swine leucocyte antigen (SLA) class II molecules on porcine (p) cells play a crucial role in xenotransplantation as activators of recipient human $CD4^+$ T cells. A human dominant-negative mutant class II transactivator (CIITA-DN) transgene under a CAG promoter with an endotheliumspecific Tie2 enhancer was constructed [**Hara 2013**]. CIITA-DN transgenic pigs were produced by nuclear transfer/embryo transfer. CIITA-DN pig cells were evaluated for expression of SLA class II with/without activation, and the human $CD4^+$ T-cell response to cells from CIITA-DN and wild-type (WT) pigs was compared.

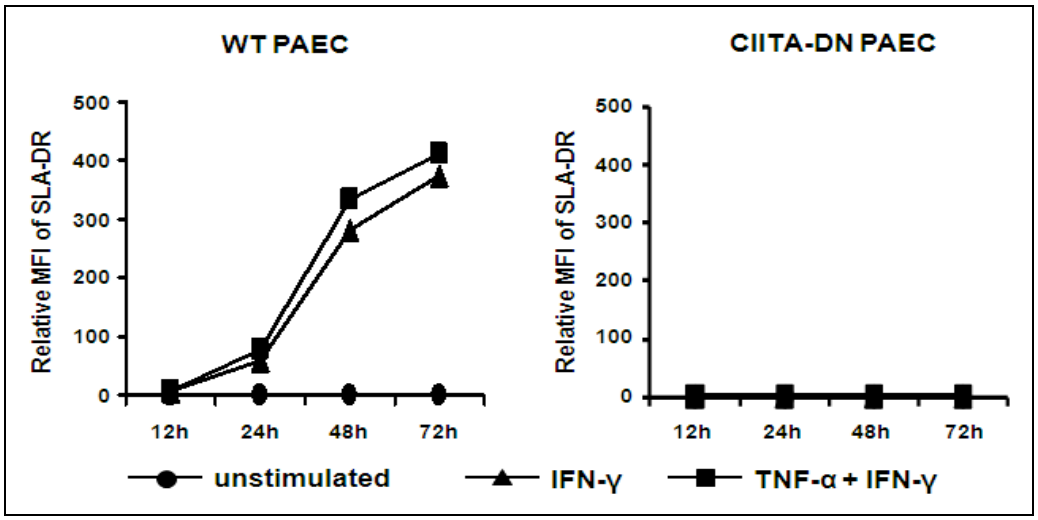
We saw that swine leukocyte antigen (SLA) class II expression on B cells and PAECs was significantly down-regulated (Figure 31 and 32).

Figure 31: Down-regulation of SLA class II expression on APCs (B cells) from CIITA-DN pig



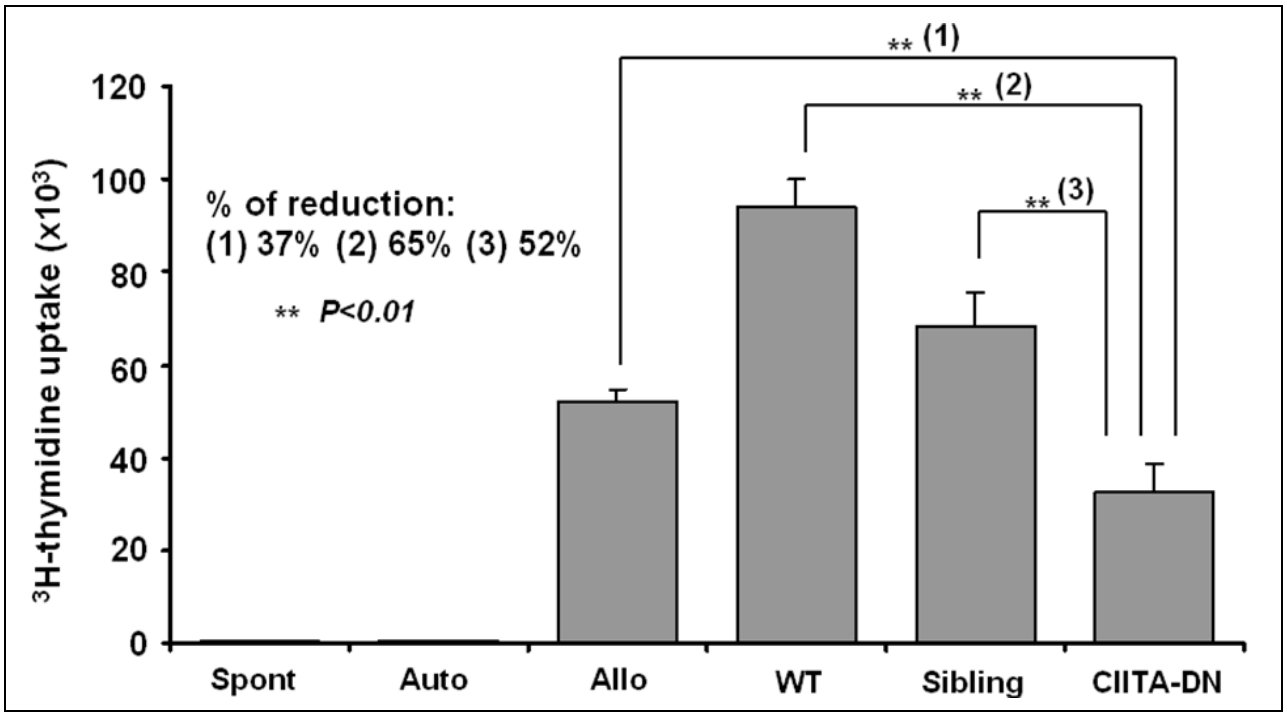
Legend – Figure 31: WT= wild-type pig, CD21= B cell marker, sibling= non-CIITA-DN sibling of CIITA-DN pig

Figure 32: Down-regulation of SLA class II expression on CIITA-DN PAECs after activation with IFN-gamma and TNF-alpha + IFN-gamma



When we tested the human CD4+ T cell response to CIITA-DN pig PBMC, we obtained a significantly weaker response in comparison to that to allo-human, wild-type and sibling (non-CIITA) pig PBMC (Figure 33).

Figure 33: The response of human $CD4^+$T cells to CIITA-DN pig PBMC



Legend – Figure 33: Spont= spontaneous, WT= wild-type pig, CD21= B cell marker, sibling= non-CIITA-DN sibling of CIITA-DN pig

In vitro testing of the human $CD4^+$ T cell response to cells from CIITA-DN pigs indicated a much diminished human T cell response which is weaker than human PBMC, suggesting that organs and cells from these pigs should be significantly protected against the human/nonhuman primates cellular immune response [**Hara 2013**].

CHAPTER 6 – MATERIALS AND METHODS – PROJECT 2 – STRATEGIES TO PREVENT ADAPTIVE IMMUNE RESPONSE IN XENOTRANSPLANTATION

6.1. ANIMALS AND SURGICAL PROCEDURES

Baboons (*Papio* species, n=12; Division of Animal Resources, Oklahoma University Health Sciences Center, Oklahoma City, OK), 3-4 years-old, weighing 6-9kg and of known AB blood type, were recipients of pig artery grafts. GTKO/CD46 (n=4) or GTKO/CD46/CIITA (n=5) pigs of blood group O (nonA), weighing 30-80kg (Revivicor, Blacksburg, VA), generated by nuclear transfer/embryo transfer from modified fibroblasts from Large White/Landrace/Duroc cross-breed pigs [**Phelps, 2003; Dai, 2002; Hara, 2013**], served as sources of carotid artery patches.

Surgical procedures are done as previously explained in Chapter 5 **[Ezzelarab, 2012]**.

6.2. EXPERIMENTAL GROUPS

The studies were divided into two groups, based on the nature of the graft-source pig. In Group 1 (n=8), baboons received grafts from GTKO/CD46/CIITA-DN pigs. In Group 2 (n=4), baboons received grafts from GTKO/CD46 pigs. Table 22 shows experimental groups in details.

6.2.1 Immunosuppressive and supportive therapy

Anti-CD40mAb (2C10R4) was obtained from the NIH NHP Resource Center, Boston, MA), CTLA4-Ig (Abatacept; from Bristol-Myers Squipp, Princeton, NJ, USA), and CTLA4-Ig (Belatacept; from Bristol-Myers Squipp, Princeton, NJ, USA). The doses of the

costimulation-blockade agents were based on previous studies by us [**Ezzelarab, 2012**] and others [**Larsen, 2011**].

One baboon in Group 1 (that received grafts from GTKO/CD46/CIITA-DN pigs) received no immunosuppressive therapy (as a control). Two baboons (Group 1A) received CD28/B7 pathway blockade alone, that was abatacept-based (n=2) and three baboons (Group 1B) received belatacept-based (n=3), and two received combined CD28/B7 and CD40/CD154 pathway blockade with belatacept and an anti-CD40mAb (2C10R4) (Group 1C) (n=2).

In order to determine the role of the CIITA-DN mutation, baboons in Group 2 (that received grafts from GTKO/CD46 pigs) received the immunosuppressive regimen as Group 1C which was based on blockade of both the CD28/B7 and CD40/CD154 pathways, i.e., belatacept+anti-CD40mAb (2C10R4).

In order to ensure that HAR did not occur, in the early part of this study 5 baboons received cobra venom factor, but this was later considered unnecessary. The decision not to include corticosteroids for maintenance in the regimen (except in one case) was based on the observations of Yamada et al [**Yamada, 2005; Griesemer, 2009**]. Rapamycin replaced MMF in some experiments simply because we have found it necessary to administer MMF by a continuous i.v. infusion, whereas rapamycin can be administered by i.m. injection.

Table 22: Details of donor pigs and recipient baboons, immunosuppressive regimen and survival after pig artery patch xenotransplantation

Recipient Baboon	Donor Pig	Induction therapy	Immunosuppressive Regimen	Survival (days)
B19310	GTKO/CD46/CIITA	no IS	**No immunosuppression**	30
Group IA				
B15911	GTKO/CD46/CIITA	ATG	Abatacept (50mg/ml), MMF	30
B16011	GTKO/CD46/CIITA	ATG	Abatacept (25mg/ml), MMF	21
Group IB				
B12612	GTKO/CD46/CIITA	ATG	Belatacept (20mg/ml), Rapamycin, Actemra	48
B12712	GTKO/CD46/CIITA	ATG	Belatacept (20mg/ml), Rapamycin	48
B5812	GTKO/CD46/CIITA	ATG	Belatacept (20mg/ml), Rapamycin, CS	49
Group IC				
B16111	GTKO/CD46/CIITA	ATG	Belatacept (20mg/ml), Anti-CD40 (25mg/kg), MMF	28
B16211	GTKO/CD46/CIITA	ATG	Belatacept (20mg/ml), Anti-CD40 (50mg/kg), MMF	28
Group II				
B5412	GTKO/CD46	ATG	Belatacept (20mg/ml), Anti-CD40 (25mg/kg), MMF	28
B5912	GTKO/CD46	ATG	Belatacept (20mg/ml), Anti-CD40 (25mg/kg), Rapamycin	29
B128-12	GTKO/CD46	ATG	Belatacept (20mg/ml), Anti-CD40 (25mg/kg)	49
B129-12	GTKO/CD46	ATG	Belatacept (20mg/ml), Anti-CD40 (25mg/kg), Rapamycin	49

IS: Immunosuppression, ATG: Antithymocyte globulin, MMF: mycophenolate mofetil

6.2.2 Measurement of anti-pig (nonGal) IgM and IgG antibodies

Anti-pig (nonGal) antibody levels were measured by flow cytometry using GTKO porcine aortic endothelial cells (pAEC) as target cells [**Ezzelarab, 2012**]. Briefly, baboon sera (20µL) were heat-inactivated for 30min at 56ºC, and then incubated with 0.1-0.2x10^6 pAEC for 30min at 4ºC. pAEC were washed and incubated for 30min at 4ºC with secondary antibodies (1:20) - anti-human FITC-IgM (µ-chain-specific) and FITC-IgG (γ-chain-specific) (Invitrogen, Carlsbad, CA). Negative controls were obtained by incubating the target cells with secondary anti-human antibodies only (and no serum). Binding of IgM and IgG was assessed using relative mean fluorescence intensity (MFI), which was calculated by dividing the MFI value for each sample by the negative control. Data acquisition was performed with a LSR II flow cytometer (BD), and data were analyzed using Flowjo or WinMDI software.

6.2.3 CFSE-Mixed lymphocyte reaction (MLR)

MLR were carried out before transplantation and between days 21-28 or 41-48 after transplantation. Baboon and pig peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by centrifugation at 700g for 30min over Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ). The total mononuclear cell fraction was washed twice with phosphate-buffered saline (PBS, Invitrogen) and contaminating red cells were lysed with ammonium chloride/potassium solution, when necessary. PBMC were then resuspended in AIM V medium (Invitrogen).

CFSE Labeling

PBMC (10×10^6 cells/mL) were resuspended in PBS. CFSE (Molecular Probes, Eugene, OR) was added to make a final concentration of 5µM, and the cells were gently mixed and incubated for 30min at 37°C in a CO_2 incubator protected from light. Labeling of cells was

stopped by adding cold PBS with 2% fetal bovine serum (Sanko, Tokyo, Japan), and the cells were then washed and resuspended in AIMV medium (Invitrogen).

MLR Assay

The PBMC prepared as stimulator cells ($0.4x10^6$ cells/well; i.e., autologous baboon, donor pig, and third-party pig with the same genetic manipulation) were irradiated with 2,800cGy, and those as responder cells ($0.4x10^6$ cells/well) from baboon recipients were labeled with CFSE, as described above. The responder:stimulator ratio was 1:1. Both the stimulator and responder cells in the AIMV medium were adjusted to 2×10^6 cells/mL of medium and cocultured in a total volume of 1mL of medium (BD Labware, Franklin Lakes, NJ) at 37°C in a 5%CO_2 incubator in the dark for 6 days. After culture, nonadherent cells were harvested and stained with either phycoerythrin (PE)-Cy7-conjugated CD4 or PE-conjugated CD8 mAbs (BD Pharmingen, San Diego, CA) together with a Pacific Blue-conjugated CD3e mAb (BD Pharmingen). Flow cytometry was performed on a FACS Calibur dual-laser cytometer (Becton Dickinson, Mountain View, CA) using LSR II flow and analyzed by FlowJo software. Dead cells were excluded from the analysis by light-scatter or propidium iodide.

Samples were tested in quadruplicate. Stimulation index (SI) was calculated by dividing the value of proliferation in response to GTKO PBMC by the value of proliferation in response to autologous PBMC.

6.2.4 Monitoring of baboon $CD4^+$ and $CD8^+$ T cells

Blood samples were collected before any therapy, and on days -2, 0 (day of the transplant), 7, 14, 21, 28 and 42 for flow cytometric analysis. FITC-conjugated mouse anti-human CD3ε (Cat# 556611), PEcy7-conjugated anti-CD4 (Cat# 557852), and PE-

conjugated anti-CD8 (Cat# 555367) antibodies were obtained from BD Pharmingen. Percentages of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells were determined by flow cytometry. Data acquisition was performed with a LSR II flowcytometer, and data were analyzed using Flowjo or WinMDI software.

6.2.5 Histopathology and immunohistopathology of pig artery grafts

The pig artery patch xenografts (with surrounding baboon aortic tissue) were excised at euthanasia. For conventional histology, tissues were fixed in 10% formalin, embedded in paraffin, and sections were stained with hematoxylin and eosin (H&E). For immunohistochemistry studies, cryosections (4mm) were stained by using primary monoclonal antibodies to demonstrate (i) a cellular response (T cells [$CD3^+$, $CD4^+$, $CD8^+$], B cells [$CD20^+$], macrophages [$CD68^+$], neutrophils [myeloperoxidase]), (ii) antibody and complement deposition (IgM, IgG, C3d), and (iii) the aggregation of platelets ($CD42^+$) and fibrin deposition, as previously described [**Chen G, 2005; Ezzelarab, 2009; Shimizu, 2008**].

CHAPTER 7 – RESULTS – PROJECT 2 – STRATEGIES TO PREVENT ADAPTIVE IMMUNE RESPONSE IN XENOTRANSPLANTATION

7.1 RECIPIENT BABOON SURVIVAL AND CLINICAL COMPLICATIONS

All 12 baboons remained healthy and active throughout the follow-up. There were no complications from the surgical procedure or immunosuppressive regimen. Follow-up was electively for either 28 or 48 days, except in one case when the baboon was euthanized on day 21 (Table 21). Follow-up was extended from 28 to 48 days to ensure we were not missing a gradual or late elicited anti-pig antibody response.

7.2 IMMUNOLOGICAL MONITORING

7.2.1 T and B cell counts

In all baboons (with all regimens), after the administration of ATG on day -3, a profound depletion of T cells ($CD3^+$, $CD4^+$, $CD8^+$) was obtained, and was maintained for approximately one week, after which there was some recovery, irrespective of differences in regimen or maintenance therapy (Figures 34, 35, and 36). However, $CD3^+$T cell counts were generally maintained <500/mm^3, with $CD4^+$ and $CD8^+$ cell numbers frequently less than half of this throughout the period of follow-up. B cell counts ($CD20^+$, $CD21^+$, $CD22^+$) remained lower than pre-transplantation values (Figures 37, 38, and 39) for a month after transplantation. They recovered only on those with longer follow-up up to 50 days (Groups 1B and 2). Despite the recovery of B cells in Groups 1B and 2, there was no significant antibody binding (Figure 41). In recipients of GTKO.CD46 pig grafts (Group 2) (without CIITA-DN) where more T cells were expressed, depletion of $CD3^+$ and $CD4^+$ was more prominent with same immunosuppressive regimen as in Group 1C.

Figure 34: CD3^{+}T cell counts in non-immunosuppressed, Group 1 and Group 2 baboons.

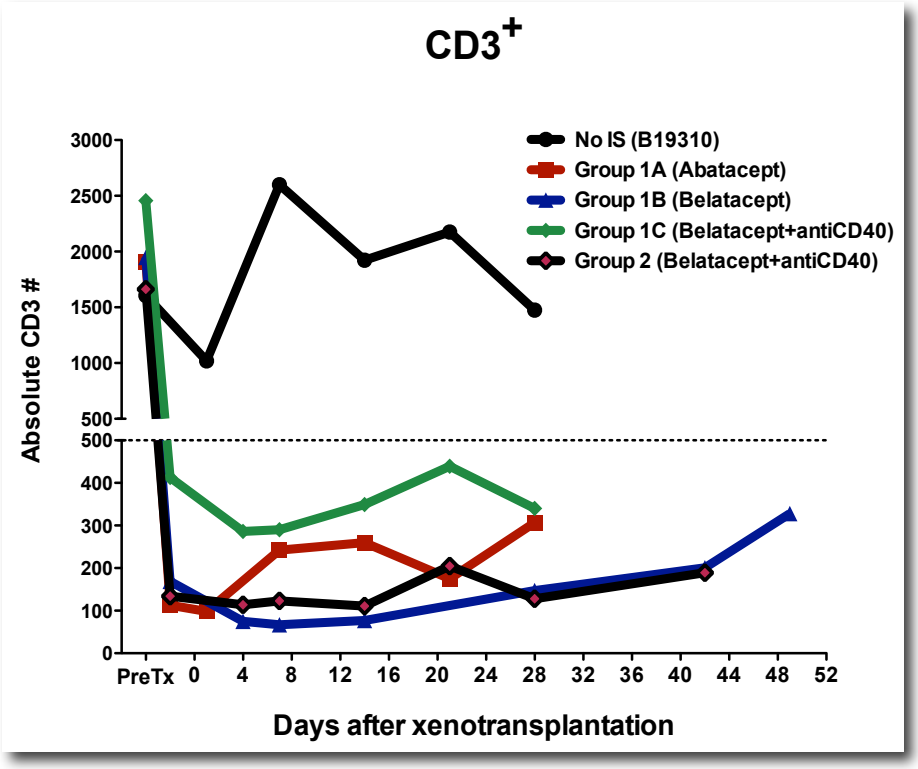


Figure 35: CD4^{+}T cell counts in non-immunosuppressed, Group 1 and Group 2 baboons.

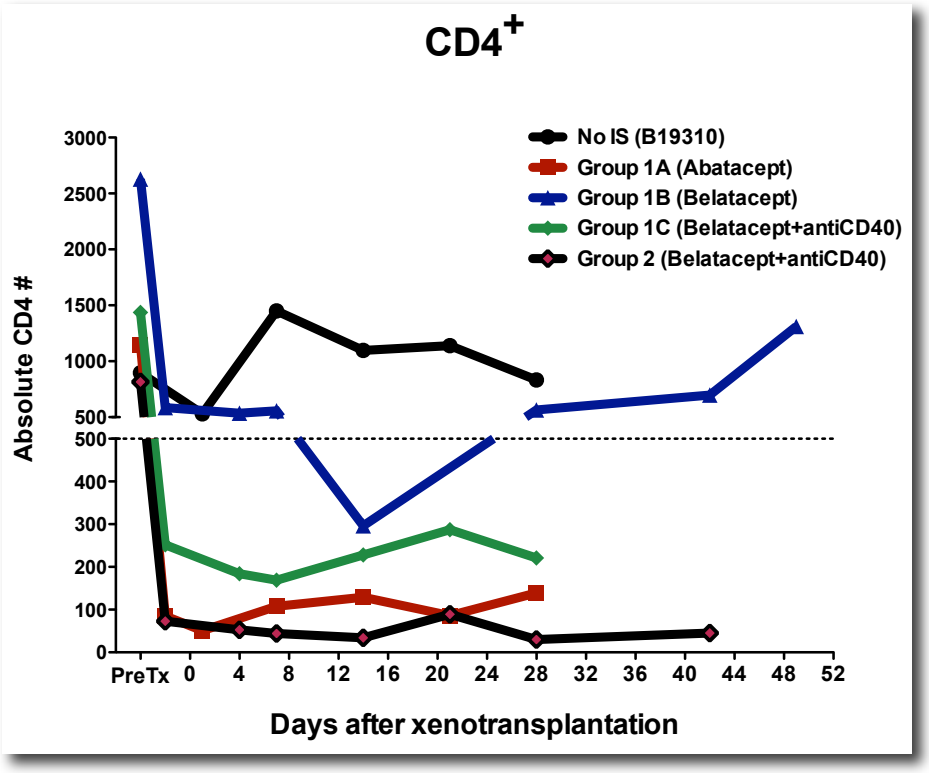


Figure 36: $CD8^+$T cell counts in non-immunosuppressed, Group 1 and Group 2 baboons.

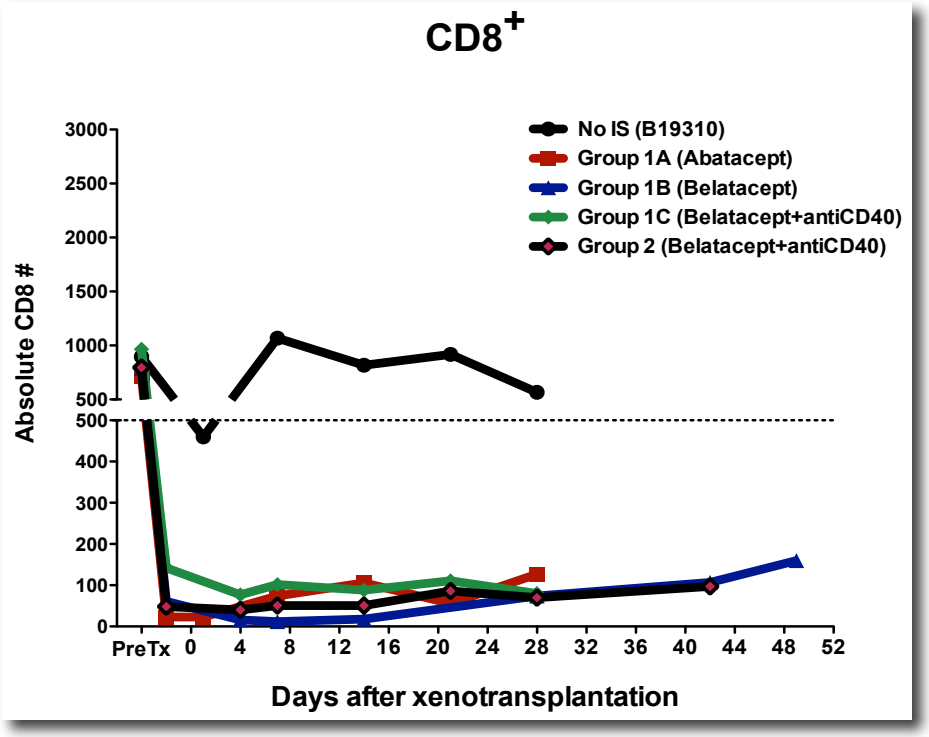


Figure 37: $CD20^+$ B cell counts in non-immunosuppressed, Group 1 and Group 2 baboons.

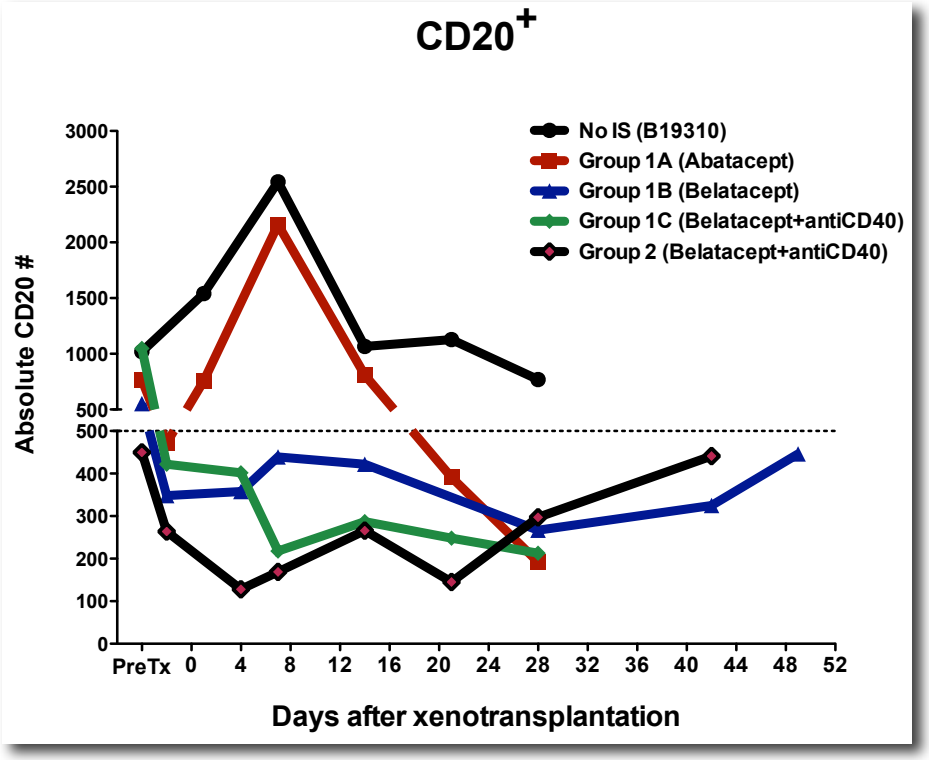


Figure 38: CD21^{+} B cell counts in non-immunosuppressed, Group 1 and Group 2 baboons.

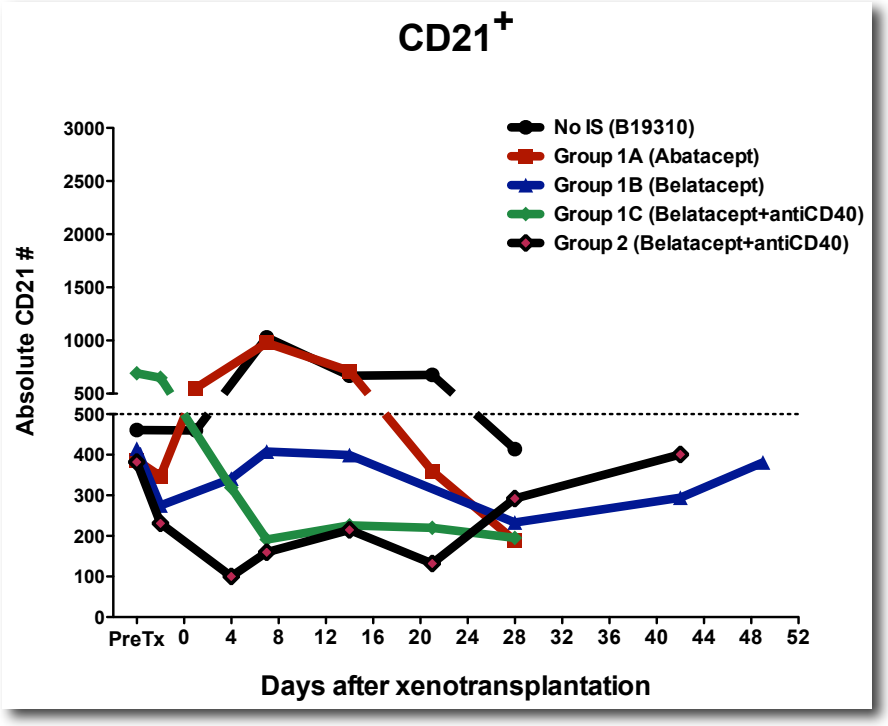
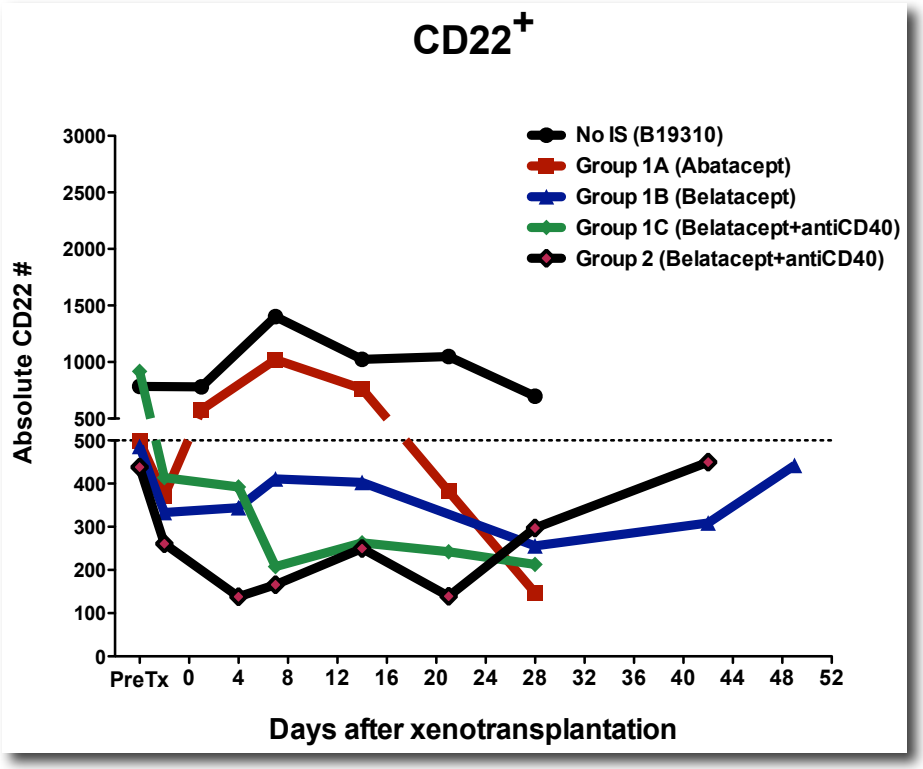


Figure 39: CD22^{+} B cell counts in non-immunosuppressed, Group 1 and Group 2 baboons.



7.2.2 Antibody responses to GTKO/CD46 and GTKO/CD46/CIITA-DN pig artery grafts

The single baboon in Group 1 that received no immunosuppression developed all of the features of an adaptive immune response;

(i) T cell proliferative response on MLR (Figure 40),

(ii) elicited anti-pig antibody response (Figure 41), and

(iii) intense cellular infiltration of the graft (Figure 42).

When CD28/B7 pathway blockade was administered (Group 1A), no proliferative response was seen in T cells on MLR (Figure 40), and the elicited antibody response was greatly attenuated (Figure 41). However, there remains a modest response, and also some graft infiltration (Figure 43). It was only when both pathways were blocked that no elicited antibody response was documented (Groups 1C and 2) (Figure 41). In these cases, cell infiltration of the graft was minimal or absent (Figures 44 and 45). In recipients of CIITA-DN grafts with both CD28/B7 and CD40/CD154 costimulatory pathway blockades (Group 1C), there was no proliferative response on T cells post-transplantation (Figure 40), elicited antibody response was the lowest (almost none) (Figure 41), and there was no cellular infiltration in the graft (Figure 44).

Figure 40: MLR reaction to PBMCs in non-immunosuppressed, Group 1 and Group 2 baboons

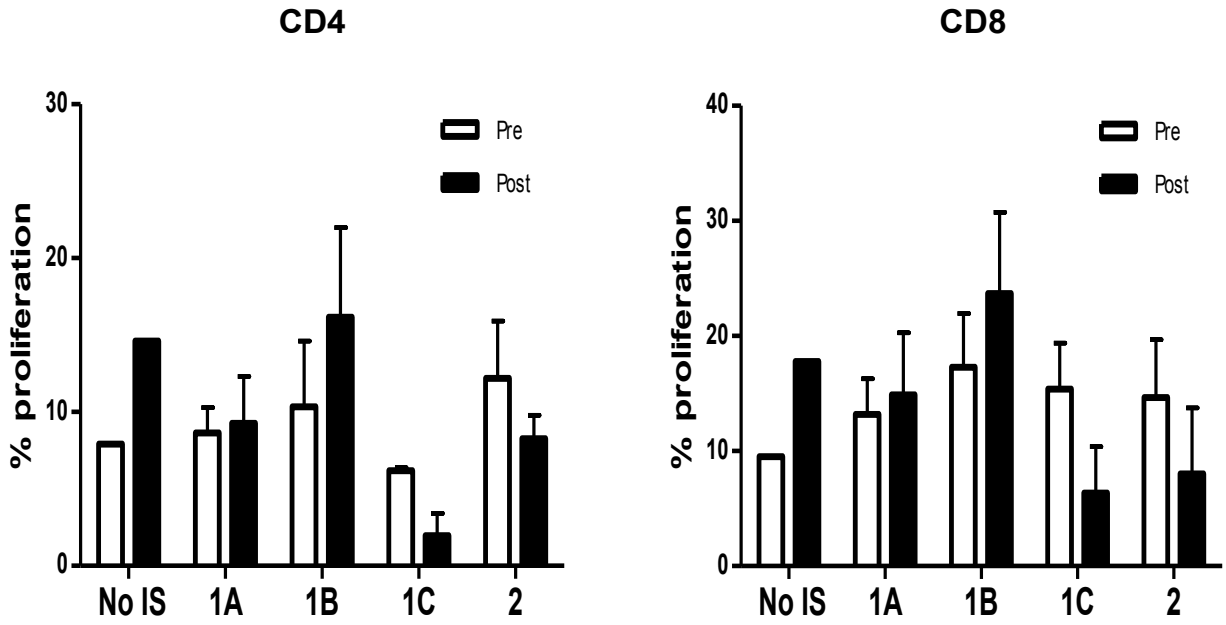


Figure 41: IgG binding to PAEC in non-immunosuppressed, Group 1 and Group 2 baboons

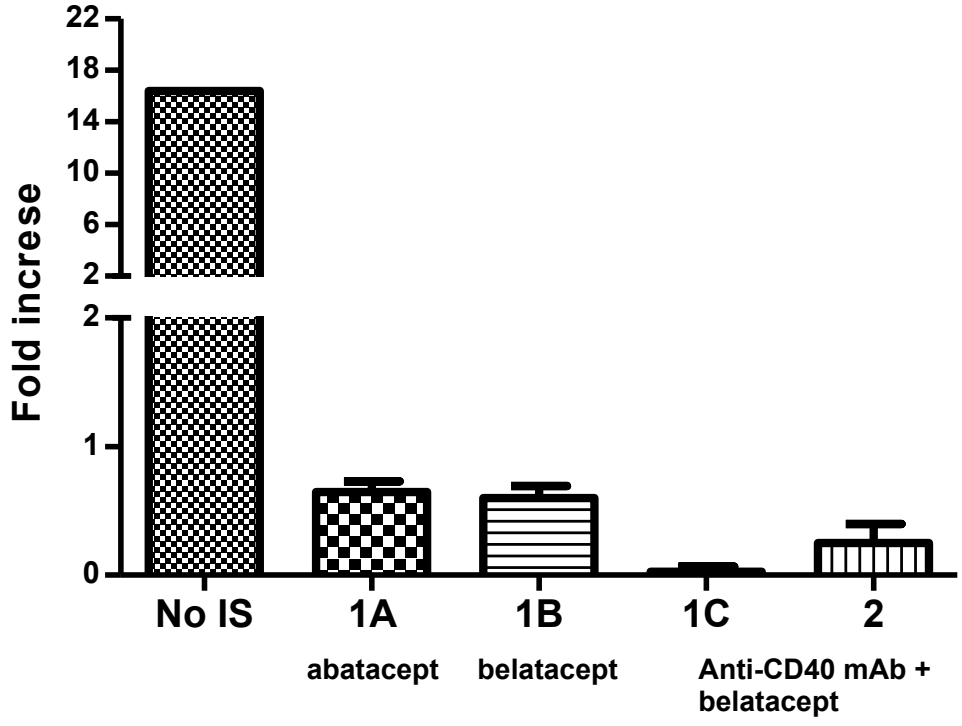
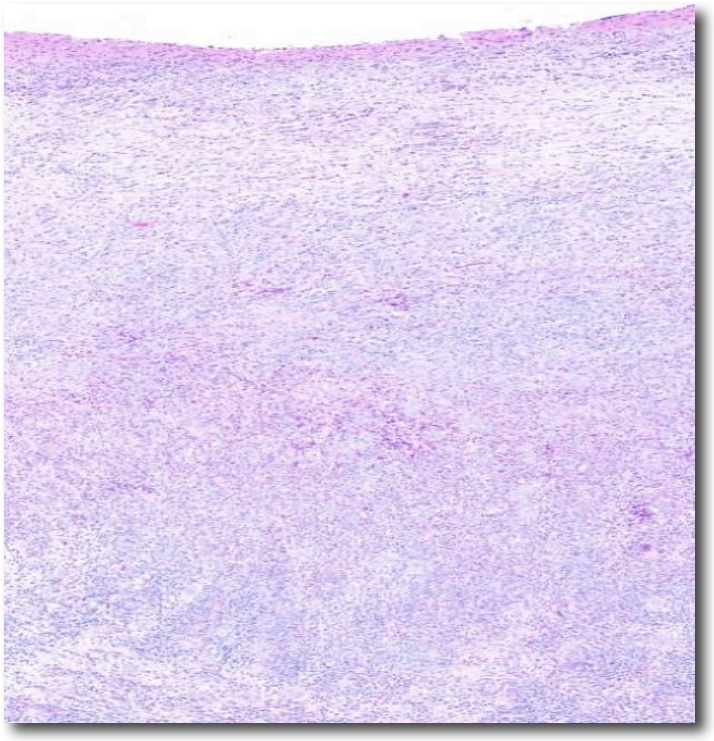
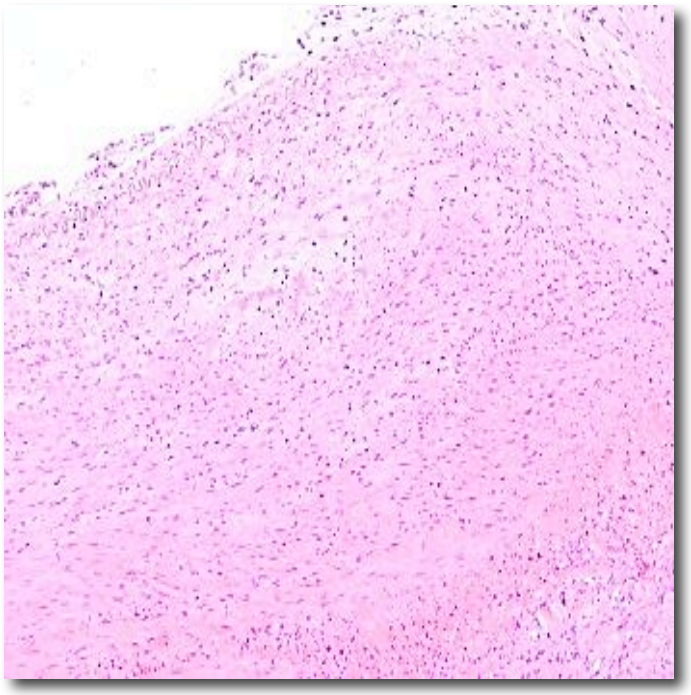


Figure 42: H&E of pig artery patch in baboon with no immunosuppression



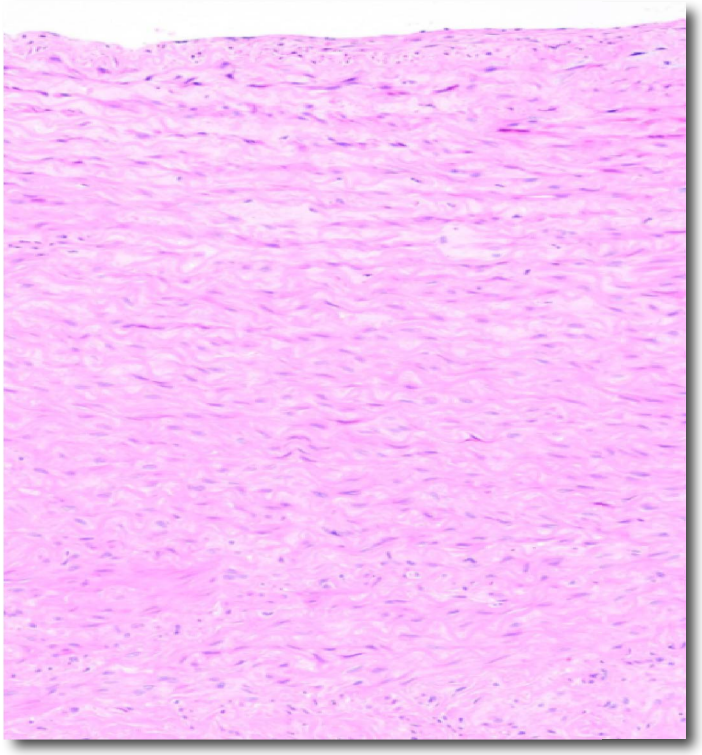
Legend – Figure 42: x4 magnification. Intense cellular infiltration in the graft

Figure 43: H&E of pig artery patch in baboons in Group 1A (abatacept-based) and Group 1B (belatacept-based)



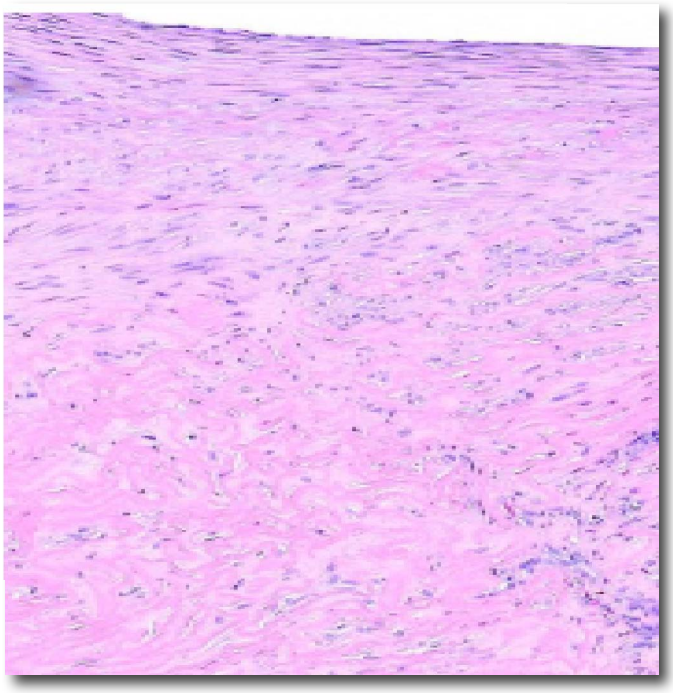
Legend – Figure 43: x4 magnification. Modest cellular infiltration in the graft

Figure 44: H&E of pig artery patch in baboons in Group 1C (belatacept and anti-CD40)



Legend – Figure 44: x10 magnification. No cellular infiltration in the graft

Figure 45: H&E of pig artery patch in baboons in Group 2 (GTKO/CD46 pigs) with (belatacept and anti-CD40) immunosuppression



Legend – Figure 45: x10 magnification. Minimal cellular infiltration in the graft

In order to determine the role of the CIITA-DN mutation, baboons in Group 2 (that received grafts from GTKO/CD46 pigs) received the identical or similar Group 1C regimen. In both groups, although there were some differences with CIITA-DN mutation grafts, similar results were obtained. There were no features of an adaptive immune response, indicating that the immunosuppressive regimen might be enough to prevent the response even against grafts from pigs that did not express the mutation.

However, when the results in Group 1A were compared with those of a historic group in which CD28/B7 pathway blockade was administered [**Ezzelarab 2012**], the elicited antibody response was significantly reduced when the graft expressed the CIITA mutation.

CHAPTER 8 – DISCUSSION – PROJECT 2 – STRATEGIES TO PREVENT ADAPTIVE IMMUNE RESPONSE IN XENOTRANSPLANTATION

Our initial studies using the GTKO pig artery patch transplantation model indicated that;

(i) a patch graft in a non-immunosuppressed baboon was sufficient to induce an adaptive immune response (manifest by a proliferative response on MLR, an induced anti-pig IgM and IgG antibody response, and intense T cell infiltration of the graft with some B cells, neutrophils and macrophages);

(ii) anti-CD154mAb-based regimen prevented virtually all of the features of an adaptive response (with no proliferation on MLR, no elicited IgM or IgG response, and no infiltrate in the graft except for minimal macrophages) and

(iii) a CTLA4-Ig (abatacept)-based regimen was not successful in preventing an adaptive response (with no proliferative response on MLR, but a slight increase in IgM, a clear increase in IgG in some baboons, and a cellular infiltrate in the graft consisting of a few T cells, no B cells, but extensive neutrophils and macrophages).

(iv) treatment with anti-CD154mAb alone (without additional MMF) was associated with a minimal elicited antibody response but a moderate cell infiltrate, and treatment with abatacept alone was unsuccessful.

In the present study, using GTKO/CD46 pigs (rather than GTKO pigs), we investigated whether a belatacept-based regimen could replace an anti-CD154mAb-based regimen, and found this not to be quite so effective. Even when the graft was taken from a GTKO/CD46/CIITA-DN pig, this regimen was not totally successful (Group 1B), although

there was evidence that the CIITA-DN mutation resulted in a reduced response (reduced antibody response, reduced cell infiltration).

However, when belatacept was combined with an anti-CD40mAb, the regimen was fully successful when the graft was from either a GTKO/CD46/CIITA-DN or a GTKO/CD46 pig, there being no cell proliferation on MLR, no elicited antibody response, and no significant cell infiltrate in the graft.

We did not test an anti-CD40mAb-based regimen in the absence of belatacept. Our in vitro studies [**Hara and Satyananda, manuscript in preparation**] indicate that belatacept has a stronger suppressive effect on MLR than the anti-CD40mAb, and so we did not put a priority on testing this latter agent in vivo. However, Lee et al [**Lee, 2000**] reported an in vitro study that demonstrated that belatacept was a more powerful suppressive agent than anti-CD154mAb, which is clearly not the case in vivo, and therefore in vivo testing of the anti-CD40mAb may well be worthwhile.

Like many studies in nonhuman primate models, the present study is limited by the relatively small number of experiments in each group. However, each regimen was tested at least twice (in the presence of grafts from each type of genetically-engineered pig), and the results obtained were consistent, and so we believe our conclusions are justified.

Furthermore, we have tested the combined anti-CD40mAb-belatacept-based regimen in the pig-to-baboon heart transplantation model [**Iwase 2013, Ekser 2013**] and have demonstrated it to prevent an adaptive response as successfully as in the artery patch model. This outcome was achieved in the absence of a continuous heparin infusion, which we have always felt to be necessary when administering an anti-CD154mAb-based

regimen [**Knosalla 2002; Ezzelarab 2009; 2012**]**.** We have also demonstrated an abatacept-based regimen to be less successful [**Iwase 2013, Ekser 2013**], although we have not yet tested a belatacept-based regimen.

In conclusion,

(i) The presence of a CIITA-DN graft reduced the baboon T cell-dependent anti-nonGal IgG response.

(ii) This was particularly obvious and beneficial when the immunosuppressive therapy administered was inadequate to control the T cell response, e.g., when abatacept was administered.

(iii) To successfully replace anti-CD154mAb as immunosuppressive therapy, blockade of both CD28/B7 and CD40/CD154 pathways was necessary in preventing a primate T cell response to transplanted pig tissue.

(iv) When immunosuppressive regimen was more potent, e.g., anti-CD40mAb+belatacept, the beneficial effect of CIITA-DN was less obvious, but still appeared to be present

(v) Although we currently have no data on long-term for chronic rejection, we suggest that, through the reducing the effects of graft endothelial activation by inhibiting the upregulation of SLA Class II, CIITA-DN may prove beneficial inhibiting the development of graft vasculopathy (chronic rejection).

CHAPTER 9 - REFERENCES

A

Abouna GM, Garry R, Hull C, et al. Pig-liver perfusion in hepatic coma. Lancet, 2, 590-610, (1968).

Abouna GM. Extracorporeal xenogeneic liver perfusion for the treatment of liver failure. In: Cooper DKC, Kemp E, Platt JL, White DJG, eds. Xenotransplantation. 2nd ed. Germany: Springer, 785-792 (1997).

Abu-Elmagd K, Fung J, Bueno J, Martin D, Madariaga JR, Mazariegos G, et al. Logistics and technique for procurement of intestinal, pancreatic, and hepatic grafts from the same donor. Ann Surg, 232, 680-687 (2000).

Adams AB, Shirasugi N, Jones TR et al. Development of a chimeric anti-CD40 monoclonal antibody that synergizes with LEA29Y to prolong islet allograft survival. J Immunol .174, 542–550 (2005).

Adham M, Sab JM, Ducerf C, et al. Correction of acute liver failure disorders through liver xenoperfusion: experimental study. Transplant Proc, 29, 3013-3014 (1997).

Aoyagi T, Yamashita K, Suzuki T et al. A human anti-CD40 monoclonal antibody, 4D11, for kidney transplantation in cynomolgus monkeys: Induction and maintenance therapy. Am J Transplant. 9, 1732–1741 (2009)

Al-Mohanna F, Saleh S, Parhar RS, et al. Human neutrophil gene expression profiling following xenogeneic encounter with porcine aortic endothelial cells: the occult role of neutrophils in xenograft rejection revealed. *J Leukoc Biol*. 78, 51-61 (2005).

Alexandre GPJ, Gianello P, Latinne D, et al. Plasmapheresis and splenectomy in experimental renal xenotransplantation. In: Hardy MA (ed) *Xenograft. 25*, 259-266 (1989).

Arroyo V, Fernandez J, Gines P. Pathogenesis and treatment of hepatorenal syndrome. Semin Liver Dis, 28, 81-95 (2008).

Auchincloss H, Jr., Sachs DH. Xenogeneic transplantation. Annu Rev Immunol 16, 433-470 (1998).

Ayares D, Vaught T, Ball S, et al. Islet-specific expression of TFPI, CD39, and CTLA4Ig in transgenic pigs designed for xenoislet transplantation [abstract #120]. Xenotransplantation. 18, 269 (2011).

Azimzadeh A, Kelishadi SS, Ezzelarab M, et al. Early graft of GTKO pig organs in baboons reduced by hCPRP expression. Xenotransplantation, 16, 356 (abstract) (2009).

B

Bach FH, Robson SC, Ferran C, et al. Endothelial cell activation and thromboregulation during xenograft rejection. Immunol Rev 141, 5-30. (1994).

Bach FH, Robson SC, Winkler H, et al. Barriers to xenotransplantation. Nat Med 1, 869-873 (1995)

Bach FH, Winkler H, Ferran C, et al. Delayed xenograft rejection. Immunol Today *17*, 379-384, (1996).

Bachli EB, Schuepbach RA, Maggiorini M, et al. Artificial liver support with the molecular adsorbent recirculating system: activation of coagulation and bleeding complications. Liver Int, 27, 475-484 (2007).

Bailey LL, Nehlsen-Cannarella SL, Concepcion W, Jolley WB. Baboon-to-human cardiac xenotransplantation in a neonate. JAMA, 254, 3321-3329, (1985).

Balamurugan AN, He J, Guo F, et al. Harmful delayed effects of exogenous isolation enzymes on isolated human islets: relevance to clinical transplantation. Am J Transplant 5: 2671–2681 (2005)

Baumann BC, Forte P, Hawley RJ, et al. Lack of galactose-α1,3-galactose expression on porcine endothelial cells prevents complement-induced lysis but not direct xenogeneic NK cytotoxicity. *J Immunol.* 172, 6460-6467 (2004).

Baumann BC, Stussi G, Huggel K, et al. Reactivity of human natural antibodies to endothelial cells from Galalpha(1,3)Gal-deficient pigs. *Transplantation*. 83, 193-201 (2007).

Bekker J, Ploem S, de Jong KP. Early hepatic artery thrombosis after liver transplantation: a systematic review of the incidence, outcome and risk factors. Am J Transplant, 9, 1-13 (2 009).

Bernal W, Wendon J, Rela M, et al. Use and outcome of liver transplantation in acetaminophen induced acute liver failure. Hepatology, 27, 1050-1055 (1998).

Bernuau J, Rueff B, Benhamou J. Fulminant and subfulminant liver failure: definition and causes. Semin Liver Dis, 6, 97-106 (1986)

Bilir BM, Guinette D, Karrer F, et al. Hepatocyte transplantation in acute liver failure. Liver Transplant, 6, 32-40, (2000).

Bismuth H, Dennison AR. Segmental liver resection. *Adv Surg,* 26, 189–208, (1993).

Blakely ML, Van der Werf WJ, Berndt MC, Dalmasso AP, Bach FH, Hancock WW. Activation of intragraft endothelial and mononuclear cells during discordant xenograft rejection. *Transplantation.* 58, 1059-1066 (1994).

Bower WA, Johns M, Margolis HS, et al. Population-based surveillance for acute liver failure. Am J Gastroenterol, 102, 2459–2463 (2007).

Buhler L, Awwad M, Basker M, et al. High-dose porcine hematopoietic cell transplantation combined with CD40 ligand blockade in baboons prevents an induced anti-pig humoral response. *Transplantation*. 69, 2296-2304 (2000).

Buhler L, Yamada K, Kitamura H, et al. Pig kidney transplantation in baboons: anti-Galα1-3Gal IgM alone is associated with acute humoral xenograft rejection and disseminated intravascular coagulation. *Transplantation.* 72, 1743–1752 (2001).

Buhler LH, Cooper DKC. How strong is the T cell response in the pig-to-primate model? *Xenotransplantation.* 12, 85-87 (2005).

Burlak C, Paris LL, Chihara RK, et al. The fate of human platelets perfused through the pig liver: implications for xenotransplantation. Xenotransplantation 17: 350–361 (2010)

Byrne GW, McCurry KR, Martin MJ, et al. Transgenic pigs expressing human CD59 and decay-accelerating factor produce an intrinsic barrier to complement-mediated damage. *Transplantation*. 63, 149-155 (1997).

Byrne GW, Davies WR, Oi K, et al. Increased immunosuppression, not anticoagulation, extends cardiac xenograft survival. *Transplantation*. 82, 1787-1791 (2006).

Byrne GW, Azimzadeh AM, Ezzelarab M, et al. Histopathological insights into the mechanism of anti-non-Gal antibody-mediated pig cardiac xenograft rejection. *Xenoransplantation*. 20, 292-307 (2013).

C

Calne RY, White HJ, Herbertson BM, et al. Pig-to-baboon liver xenografts. Lancet, 1, 1176-1178 (1968).

Calne RY. Organ transplantation between widely disparate species. Transplant Proc. 4, 550-556, (1970).

Cardozo LAM, Rouw DB, Ambrose LR, et al. The neutrophil: unnoticed threat in xenotransplantation. *Transplantation*. 78. 1721-1728 (2004).

Castalino DJ, Salem HH. Natural anticoagulants and the liver. J Gastorenterol Hepatol, 12, 77-83 (1997).

Chari RS, Collins BH, Magee JC, et al. Brief report: treatment of hepatic failure with ex vivo pig-liver perfusion followed by liver transplantation. N Eng J Med, 331, 234-237 (1994).

Chen D, Weber M, McVey JH, et al. Complete inhibition of acute humoral rejection using regulated expression of membrane-tethered anticoagulants on xenograft endothelium. *Am J Transplant*. 4, 1958-1963. (2004).

Chen G, Qian H, Starzl T, et al. Induced anti-non-Gal antibodies lead to acute humoral xenograft rejection in baboons using α1,3-galactosyltransferase gene-knockout pigs as kidney donors. *Nat Med.* 11, 1295-1298 (2005).

Chen Y, Qiao J, Tan W, et al. Characterization of porcine factor VII, X and comparison with human factor VII, X. Blood Cells Mol Dis, 43, 111-118 (2009).

Cooper DKC, Human PA, Lexer G, et al. Effects of cyclosporine and antibody adsorption on pig cardiac xenograft survival in the baboon. *J Heart Transplant.* 7, 238–246 (1988).

Cooper DKC, Ye Y, Rolf LL, Zuhdi N. The pig as potential organ donor for man. In Cooper DKC, Kemp E, Reemtsma K, White DJG, eds. Xenotransplantation. Springer, Heidelberg, pp. 481-500, (1991).

Cooper DKC, Good AH, Koren E, et al. Identification of alpha-galactosyl and other carbohydrate epitopes that are bound by human anti-pig antibodies: relevance to discordant xenografting in man. Transpl Immunol 1, 198-205, (1993).

Cooper DKC, Tseng, Y-L, Saidman, SL. Allo- and xeno-antibody cross-reactivity in transplantation. Transplantation, 77, 1-5 (2004).

Cooper DKC, Dorling A, Pierson RN III, et al. α1,3-galactosyltransferase gene knockout pigs for xenotransplantation: where do we go from here? Transplantation, 84, 1-7 (2007).

Cardona K, Korbutt GS, Milas Z, et al. Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. *Nat Med.* 12, 304-306 (2006).

Cardona K, Milas Z, Strobert E et al. Engraftment of adult porcine islet xenografts in diabetic nonhuman primates through targeting of costimulation pathways. Am J Transplant. 7, 2260–2268 (2007)

Couinaud C. Lobes de segments hepatiques: notes sur l'architecture anatomique et chirurgicale du foie. *Presse Med,* 62: 709–712, (1954).

Court FG, Wemyss-Holden SA, Morrison CP, et al. Segmental nature of the porcine liver and its potential as a model for experimental partial hepatectomy. Brit J Surg, 90, 440-444, (2003).

Cowan PJ, d'Apice AJ. The coagulation in xenotransplantation: incompabilities and strategies to overcome them. Curr Opin Organ Transplant, 13, 178-183 (2008).

Cozzi E, White DJ. The generation of transgenic pigs as potential organ donors for humans. Nat Med. 1, 964–966 (1995).

Cozzi E, Bhatti F, Schmoeckel M, et al. Long-term survival of non-human primates receiving life-supporting transgenic porcine kidney xenografts. *Transplantation.* 70, 15-21 (2000).

Cozzi E, Vial C, Ostlie D, et al. Maintenance triple immunosuppression with cyclosporin A, mycophenolate sodium and steroids allows prolonged survival of primate recipients of hDAF porcine renal xenografts. *Xenotransplantation.* 10, 300-310 (2003).

Cozzi E, Bosio E, Seveso M, et al. Xenotransplantation as a model of integrated, multidisciplinary research. *Organogenesis*. 5, 288-296 (2009)

D

Daar AS. Ethics of xenotransplantation: animal issues, consent, and likely transformation of transplant ethics. World J Surg. 21, 975-82 (1997).

Dai Y, Vaught TD, Boone J, et al. Targeted disruption of the alpha1,3-galactosyltransferase gene in cloned pigs. Nat Biotechnol *20*, 251-255 (2002).

Dalmasso AP, Vercellotti GM, Platt JL, Bach FH. Inhibition of complement-mediated endothelial cell cytotoxicity be decay accelerating factor. Potential for prevention of xenograft hyperacute rejection. *Transplantation.* 52, 530–533 (1991).

Demetriou AA, Brown RS, Busuttil RW, et al. Perspective, randomized, multicenter, controlled trial of a bioartificial liver in treating acute liver failure. Ann Surg, 239, 660-670 (2004).

Demetris AJ, Jaffe R, Tzakis A, et al. Antibody-mediated rejection of human orthotopic liver allografts. A study of liver transplantation across ABO blood group barriers. Am J Pathol, 132, 489-502 (1988).

Dieckhoff B, Petersen B, Kues WA, et al. Knockdown of porcine endogenous retrovirus (PERV) expression by PERV-specific siRNA in transgenic pigs. *Xenotransplantation.* 15, 36-45 (2008).

Dorling A, Lechler RI. T cell-mediated xenograft rejection: specific tolerance is probably required for long term xenograft survival. *Xenotransplantation.* 5, 234-245 (1998).

Dwyer KM, Robson SC, Nandurkar HH, et al. Thromboregulatory manifestations in human CD39 transgenic mice and the implications for thrombotic disease and transplantation. *J Clin Invest.* 113, 1440-1446 (2004).

E

Eiseman B, Liem DS, Raffucci F. Heterologous liver perfusion in treatment of hepatic failure. Ann Surg, 162, 329-345 (1965).

Ekser B, Gridelli B, Tector AJ, Cooper DK. Pig liver xenotransplantation as a bridge to allotransplantation: which patients might benefit? Transplantation. 88, 1041-1049 (2009)

Ekser B, Cooper DKC. Overcoming the barriers of xenotransplantation: prospects for the future. Expert Rev Clin Immunol, 6, 219-230 (2010a)

Ekser B, Long C, Echeverri GJ, et al. Impact of thrombocytopenia on survival of baboons with genetically-modified pig liver transplants: clinical relevance. Am J Transplant. 10, 273-285 (2010b)

Ekser B, Echeverri GJ, Cortese Hassett A, et al. Hepatic function after genetically-engineered pig liver transplantation in baboons. Transplantation. 90, 483-493 (2010c)

Ekser B, Gridelli B, Veroux M, Cooper DK. Clinical pig liver xenotransplantation: how far do we hae to go? Xenotransplantation. 18, 158-167 (2011)

Ekser B, Ezzelarab M, Hara H, et al. Clinical xenotransplantation: the next medical revolution? Lancet, 379, 672-683 (2012a)

Ekser B, Bianchi J, Ball S, et al. Comparison of hematologic, biochemical, and coagulation parameters in α1,3-galactosyltransferase gene-knockout pigs, wild-type pigs and 4 primate species. Xenotransplantation. 19: 342-354 (2012b)

Ekser B, Klein E, He J, Stolz DB, et al. Genetically-engineered pig-to-baboon liver xenotransplantation: histopathology of xenografts and native organs. PLoS ONE. *7, e29720 (2012c)*

Ekser B, Lin CC*, Long C, et al. Potential factors influencing thrombocytopenia and consumptive coagulopathy after genetically-modified pig liver xenotransplantation (*shared first authors). Transplant Int. 25, 882-896 (2012d)

Ekser B, Burlak C, Waldman JP, et al. Immunobiology of liver xenotransplantation. Expert Reviews in Clinical Immunology. 8, 621-634 (2012e)

Ekser B, Walters E, Nottle MB, et al. Initial experience of xenotransplantation with hearts from pigs expressing coagulation-regulatory genes. In preparation (2013a)

Elliott RB, Escobar L, Garkavenko O, et al. No evidence of infection with porcine endogenous retrovirus in recipients of encapsulated porcine islet xenografts. Cell Transplant *9*, 895-901 (2000).

Ellis AJ, Hughes RD, Wendon JA, et al. Pilot-controlled trial of the extracorporeal liver assist device in acute liver failure. Hepatology, 24, 1446-1451 (1996).

Elvevold K, Smedsrod B, Martinez I. The liver sinusoidal endothelial cell:
a cell type of controversial and confusing identity: Am J Physiol Gastrointest
Liver Physiol 294: G391–400 (2008)

Ezzelarab M, Hara H, Busch J, et al. Antibodies directed to pig nonGal antigens in naïve and sensitized baboons. *Xenotransplantation,* 13, 400-407 (2006a).

Ezzelarab M, Cortese-Hassett A, Cooper DK, Yazer MH. Extended coagulation profiles of healthy baboons and of baboons rejecting GT-KO pig heart grafts. Xenotransplantation, 13, 522-528 (2006b)

Ezzelarab M, Garcia B, Azimzadeh A, et al. The innate immune response and activation of coagulation in α1,3-galactosyltransferase gene-knockout xenograft recipients. *Transplantation*. 87, 805-812 (2009).

Ezzelarab MB, Ekser B, Echeverri G, et al. Costimulation blockade in pig artery patch xenotransplantation - a simple model to monitor the adaptive immune response in nonhuman primates. Xenotransplantation. 19, 221-232 (2012).

F

Fischel RJ, Bolman RM, 3rd, Platt JL, et al. Removal of IgM anti-endothelial antibodies results in prolonged cardiac xenograft survival. Transplant Proc *22*, 1077-1078 (1990).

Fisher RA, Strom SC. Human hepatocyte transplantation: worldwide results. Transplantation, 82, 441-449 (2006).

Fishman JA, Patience C. Xenotransplantation: infectious risk revisited. Am J Transplant, 4, 1383-1390, (2004).

Fishman JA. Xenosis and xenotransplantation: current concepts and challenges (Abstract PL5:2). *Xenotransplantation.* 12, 370 (2005).

Ford ML, Larsen CP. Translating costimulation blockade to the clinic: Lessons learned from three pathways. Immunol Rev. 229, 294–306 (2009)

Fox A, Mountford J, Braakhuis A, et al. Innate and adaptive immune responses to nonvascular xenografts: evidence that macrophages are direct effectors of xenograft rejection. *J Immunol.* 166, 2133-2140 (2001).

G

Galili U, Rachmilewitz EA, Peleg A, Flechner I. A unique natural human IgG antibody with anti-alpha-galactosyl specificity. J Exp Med *160*, 1519-1531 (1984).

Galili U, Basbaum C., Shohet SB, et al. Identification of erythrocyte Gal alpha 1-3Gal glycosphingolipids with a mouse monoclonal antibody, Gal-13. J Biol Chem *262*, 4683-4688. (1987a).

Galili U, Buehler J, Shohet SB, Macher BA. The human natural anti-Gal IgG. III. The subtlety of immune tolerance in man as demonstrated by crossreactivity between natural anti-Gal and anti-B antibodies. J Exp Med *165*, 693-704 (1987b).

Galili U, Shohet SB, Kobrin E, Stults CL, Macher BA. Man, apes, and Old World monkeys differ from other mammals in the expression of alpha-galactosyl epitopes on nucleated cells. *J Biol Chem.* 263, 17755–17762 (1988).

Gibson T, Medewar PB. The fate of skin homografts in man. J Anat, 77, 299-310, (1943).

Gilli UO, Schneider MKJ, Loetscher P, et al. Human polymorphonuclear neutrophils are recruited by porcine chemokines acting on CXC chemokine receptor 2, and platelet-activating factor. *Transplantation*. 79, 1344-1331 (2005).

Gollackner B, Mueller NJ, Houser S, et al. Porcine cytomegalovirus and coagulopathy in pig-to-primate xenotransplantation. *Transplantation*. 75, 1841-1847 (2003).

Gollackner B, Goh S-K, Qawi I, et al. Acute vascular rejection of xenografts: roles of natural and elicited xenoreactive antibodies in activation of vascular endothelial cells and induction of procoagulant activity. *Transplantation.* 77, 1735-1741 (2004).

Grabhorn E, Richter A, Fischer L, et al. Emergency liver transplantation in neonates with acute liver failure: long-term follow up. Transplantation, 86, 932-936 (2008).

Gridelli B, Gatti S, Piazzini A, et al. Xenogeneic orthotopic liver transplantation from sheep to pig. Transplant Proc, 24, 614-616 (1992).

Gridelli B, Gatti S, Piazzini A, et al. Xenogeneic orthotopic liver transplantation in nonhuman primates. Transplant Proc, 25, 457-461 (1993).

Gridelli B, Gatti S, Andreani P, et al. Xenogeneic orthotopic liver transplantation from sheep to pig: delay of rejection by FUT 175 infusion. Transplant Proc, 28, 784-787 (1996).

Griesemer AD, Hirakata A, Shimizu A, et al. Results of gal-knockout porcine thymokidney xenografts. Am J Transplant. 9, 2669-2678 (2009).

Gustavsson ML, Steen LM. Transmission of carbohydrate-compatible viruses from xenografts. Transplantation, 66, 939 (1998).

H

Haanstra KG, Ringers J, Sick EA et al. Prevention of kidney allograft rejection using anti-CD40 and anti-CD86 in primates. Transplantation. 75, 637–643 (2003)

Haanstra KG, Sick EA, Ringers J et al. Costimulation blockade followed by a 12-week period of cyclosporine a facilitates prolonged drug-free survival of rhesus monkey kidney allografts. Transplantation. 79, 1623–1626 (2005)

Hara H, Ezzelarab M, Rood PP, et al. Allosensitized humans are at no greater risk of humoral rejection of GT-KO pig organs than other humans. Xenotransplantation, 13, 357-365 (2006).

Hara H, Long C, Lin YJ, et al. *In vitro* investigation of pig cells for resistance to human antibody-mediated rejection. *Transpl Int.* 21, 1163-1174 (2008).

Hara H, Gridelli B, Lin YJ, Marcos A, Cooper DKC. Liver xenografts for the treatment of acute liver failure: clinical and experimental experience and remaining immunologic barriers. Liver Transplant, 14, 425-434 (2008b).

Hara H, Campanile N, Tai HC, Long C, Ekser B, et al. An in vitro model of pig liver transplantation in humans – pig complement is associated with reduced lysis of wild-type and genetically-modified pig cells. Xenotransplantation. 17, 370-378 (2010)

Hara H, Witt W, Crossley T, et al. Human dominant-negative class II transactivator transgenic pigs - effect on the human anti-pig T-cell immune response and immune status. Immunology. 140, 39-46 (2013)

Hasan RI, van den Bogaerde J, Forty J, et al. Prolonged survival of hamster to rat heart xenografts with cyclophosphamide therapy. Transplant Proc. 24, 517-518, (1992).

Heneine W, Tibell A, Switzer WM, et al. No evidence of infection with porcine endogenous retrovirus in recipients of porcine islet-cell xenografts. Lancet *352*, 695-699 (1998).

Hering BJ, Wijkstrom M, Graham ML, et al. Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. *Nat Med.* 12, 301-303 (2006).

Horslen SP, Hammel JM, Fristoe LW, et al. Extracorporeal liver perfusion using human and pig livers for acute liver failure. Transplantation, 70, 1472-1478 (2000).

I

Ibrahim Z, Busch J, Awwad M, et al. Selected physiologic compatibilities and incompatibilities between human and porcine organ systems. Xenotransplantation, 13, 488-499, (2006).

Imai A, Suzuki T, Sugitani A, et al. A novel fully human anti-CD40 monoclonal antibody, 4D11, for kidney transplantation in cynomolgus monkeys. Transplantation 84, 1020 (2007).

Inverardi L, Clissi B, Stolzer AL, et al. Human natural killer lymphocytes directly recognize evolutionarily conserved oligosaccharide ligands expressed by xenogeneic tissues. *Transplantation.* 63, 1318-1330 (1997).

Iwamura S, Enzan H, Saibara T, Onishi S, Yamamoto Y. Hepatic sinusoidal endothelial cells can store and metabolize serum immuglobulin. Hepatology, 22, 1456-1461 (1995).

Iwase H*, Ekser B*, Satyananda V, et al. Pig-to-baboon heart transplantation – first experience with pigs transgenic for human thrombomodulin andc omparison of three costimulation blockade-based regimens (*contributed equally). In preparation (2013a)

J

Jalan R, Williams R. Acute-on-chronic liver failure: pathophysiological basis of therapeutic options. Blood Purif, 20, 252-61 (2002).

Janeway CA, Bottomly K. Signals and signs for lymphocyte responses. Cell. 76, 275–285 (1994).

K

Kahn D, Hickman R, Terblanche J, von Sommoggy S. Partial hepatectomy and liver regeneration in pigs – the response to different resection sizes. *J Surg Res,* 45, 176–180, (1988).

Kanai N, Platt JL. Xenotransplantation of the liver. Clin Liver Dis, 4, 731-746 (2000).

Kawai T, Andrews D, Colvin RB, SachsDH, Cosimi AB. Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. Nat Med. 6: 114 (2000)

Katopodis AG, Warner RG, Duthaler R, et al. Removal of anti-Galalpha1,3Gal xenoantibodies with an injectable polymer. J Clin Invest *110*, 1869-1877 (2002).

Kasahara M, Kaihara S, Oike F, et al. Living-donor liver transplantation with monosegments. Transplantation, 76, 694-696 (2003).

Kinnear G, Jones ND, Wood KJ. Costimulation blockade: current prespectives and implications for therapy. Transplantation. 95, 527-535 (2013).

Kirk AD, Harlan DM, Armstrong NN et al. CTLA 4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. Proc Natl Acad Sci USA. 94: 8789–8794 (1997)

Kirk AD, Knechtle SJ, Sollinger HW. Preliminary results of the use of humanized anti-CD154 in human renal allotransplantation (abstract). Am J Transplant. 1(Suppl 1): 1. (2001)

Kjaergard LL, Liu J, Als-Nielsen B, et al. Artificial and bioartificial support systems for acute and acute-on-chronic liver failure: a systematic review. JAMA, 289, 217-222 (2003).

Knosalla C, Gollackner B, Buhler L et al. Correlation of biochemical and hematological changes with graft failure following pig heart and kidney transplantation in baboons. Am J Transplant. 3, 1510–1519 (2003)

Kobayashi T, Cooper DKC. Anti-Gal, α-Gal epitopes, and xenotransplantation. In: αGal and Anti-Gal: α-1,3-Galactosyltransferase, α-Gal Epitopes, and the Natural Anti-Gal Antibody. Galili U, Avila JL (eds). *Subcellular Biochemistry Series (Volume 32)*, Kluwer Academic/Plenum, New York, London, 229-257 (1999).

Kolln J, Bredehorst R, Spillner E. Engineering of human complement component C3 for catalytic inhibition of complement. Immunol Lett, 98, 49-56 (2005).

Kopp CW, Siegel JB, Hancock WW, et al. Effect of porcine endothelial tissue factor pathway inhibitor on human coagulation factors. Transplantation *63*, 749-758, (1997).

Kopp CW, Grey ST, Siegel JB, et al. Expression of human thrombomodulin cofactor activity in porcine endothelial cells. Transplantation *66*, 244-251, (1998a).

Koshika T, Phelps C, Fang J, et al. Relative efficiency of porcine and human cytotoxic T-lymphocyte antigen 4 immunoglobulin in inhibiting human CD4+ T-cell responses co-stimulated by porcine and human B7 molecules. Immunology. 134, 386-397 (2011).

Kozlowski T, Ierino FL, Lambrigts D, et al. Depletion of anti-Gal(alpha)1-3Gal antibody in baboons by specific alpha-Gal immunoaffinity columns. Xenotransplantation *5*, 122-131 (1998).

Kroshus TJ, Dalmasso AP, Leventhal JR, et al. Antibody removal by column immunoabsorption prevents tissue injury in an ex vivo model of pig-to-human xenograft hyperacute rejection. J Surg Res *59*, 43-50 (1995).

Kuwaki K, Knosalla C, Dor FJ et al. Suppression of natural and elicited antibodies in pig-to-baboon heart transplantation using a human anti-human CD154 mAb based regimen. Am J Transplant. 4, 363–372 (2004)

Kuwaki K, Tseng YL, Dor FJMF, et al. Heart transplantation in baboons using α1,3-galactosyltransferase gene-knockout pigs as donors: initial experience. *Nat Med.* 11, 29-31 (2005).

L

Lai L, Kolber-Simonds D, Park KW, Cheong HT, et al. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science *295*, 1089-1092 (2002).

Larsen CP, Elwood ET, Alexander DZ et al. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. Nature. 381, 434–438 (1996).

Larsen CP, Pearson TC, Adams AB et al. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. Am J Transplant. 5, 443–453 (2005).

Larsen CP, Elwood ET, Alexander DZ, et al. Pillars article: long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. Nature. 1996. 381: 434-438. 1996. J Immunol. 186, 2693-2697 (2011).

Lee RS, Yamada K, Womer KL, et al. Blockade of CD28-B7, but not CD40-CD154, prevents costimulation of allogeneic porcine and xenogeneic human anti-porcine T cell responses. J Immunol. 164, 3434-3444 (2000).

Lee KF, Salvaris EJ, Roussel JC, et al. Recombinant pig TFPI efficiently regulates human tissue factor pathways. Xenotransplantation *15*, 191-197, (2008).

Lee WM. Etiologies of acute liver failure. Semin Liver Dis, 28, 142-152 (2008).

Lenschow DJ, et al. Long term survival of xenogeneic pancreatic islet grafts induced by CTLA-4-Ig. Science. 257, 789–792 (1992).

Leventhal JR, John R, Fryer JP, et al. Removal of baboon and human antiporcine IgG and IgM natural antibodies by immunoadsorption. Results of in vitro and in vivo studies. Transplantation *59*, 294-300 (1995).

Lévêque X, Cozzi E, Naveilhan P, Neveu I. Intracerebral xenotransplantation: recent findings and perspectives for local immunosuppression. *Curr Opin Organ Transplant*. 16, 190-194 (2011).

Levy MF, Crippin J, Sutton S, et al. Liver allotransplantation after extracorporeal hepatic support with transgenic (hCD55/hCD59) porcine livers: clinical results and lack of pig-to-human transmission of the porcine endogenous retrovirus. Transplantation, 69, 272-280, (2000).

Lewis JH. Comparative hemostasis in vertebrates. Kluwer Academic Publisher, Springer , p285-298 (1996).

Lexer G, Cooper DKC, Rose AG, et al. Hyperacute rejection in a discordant (pig to baboon) cardiac xenograft model. J Heart Transplant. 5, 411–418 (1986).

Li AP, Roque MA, Beck DJ, et al. Isolation and culturing of hepatocytes from human livers. J Tiss Cult Meth, 14, 136-146 (1992).

Li XC, Rothstein DM, Sayegh MH. Costimulatory pathways in transplantation: challenges and new developments. Immunol Rev. 229, 271-293 (2009).

Lin YJ, Hara H, Tai H-C, et al. Suppressive efficacy and proliferative capacity of human regulatory T cells in allogeneic and xenogeneic responses. *Transplantation.* 86, 1452-1462 (2008).

Lin CC, Chen D, McVey JH, Cooper DKC, Dorling A. Expression of tissue factor and initiation of clotting by human platelets and monocytes after incubation with porcine endothelial cells. *Transplantation.* 86, 702-709 (2008).

Lin CC, Cooper DK, Dorling A. Coagulation dysregulation as a barrier to xenotransplantation in the primate. *Transpl Immunol.* 21, 75-80 (2009a).

Lin CC, Ezzelarab M, Shapiro R, et al. Tissue factor expression on recipient platelets initiates consumptive coagulopathy in pig-to-primate kidney xenotransplantation. Submitted for publication (2009b).

Lin CC, Ekser B, Long C, et al. Consumptive coagulopathy is not associated with a humoral immune response after genetically-modified pig liver transplantation in baboons Submitted for publication (2009c).

Liou IW, Larson AM. Role of liver transplantation in acute liver failure. Semin Liver Dis, 28, 201-209 (2008).

Long C, Hara H, Pawlikowski Z, et al. Genetically-engineered pig red blood cells for clinical transfusion: initial in vitro studies. *Transfusion, 49, 2418-2429,* (2009).

Loveland BE, Milland J, Kyriakou P, et al. Characterization of a CD46 transgenic pig and protection of transgenic kidneys against hyperacute rejection in non-immunosuppressed baboons. *Xenotransplantation*. 11, 171-183 (2004).

Luo Y, Kosanke S, Mieles L, et al. Comparative histopathology of hepatic allografts and xenografts in the nonhuman primate. Xenotransplantation, 5, 197-206 (1998).

Luo Y, Levy G, Ding J, et al. HDAF transgenic pig livers are protected from hyperacute rejection during ex vivo perfusion with human blood. Xenotransplantation, 9, 36-44, (2002).

M

Makowka L, Wu GD, Hoffman A, et al. Immunohistopathologic lesions associated with the rejection of a pig-to-human liver xenograft. Transplant Proc, 26, 1074-1075 (1994).

Makowka L, Cramer DV, Hoffman A, et al. The use of a pig liver xenograft for temporary support of a patient with fulminant hepatic failure. Transplantation, 59, 1654-1659 (1995).

Martin C, Plat M, Nerriére-Daguin V, et al. Transgenic expression of CTLA4-Ig by fetal pig neurons for xenotransplantation. *Transgenic Res*. 14, 373-384 (2005).

Mazariegos G, Chen Y, Squires R. Biological and artificial liver support system in children: a new perspective. Pediatr Crit Care Med, 6, 616-617 (2005).

McCormick PA, Donnelly C. Management of hepatorenal syndrome. Pharmacol Ther, 119, 1-6 (2008).

McCurry KR, Parker W, Cotterell AH, et al. Humoral responses to pig-to-baboon cardiac transplantation: implications for the pathogenesis and treatment of acute vascular rejection and for accommodation. *Hum Immunol.* 58, 91-105 (1997).

McGregor CG, Davies WR, Oi K, et al. Cardiac xenotransplantation: recent preclinical progress with 3-month median survival. J Thorac Cardiovasc Surg, 130, 844-851 (2005).

Medevar PB. Tests by tissue culture methods on the nature of immunity to transplanted skin. Q J Microsc Sci. 89, 239-252, (1948).

Merrill JP, Murray JE, Harrison JH, Guild WR. Successful homotransplantation of the human kidney between identical twins. J Am Med Assoc 160, 277-282 (1956).

Mieles L, Ye Y, Luo Y, et al. Auxiliary liver allografting and xenografting in the nonhuman primate. Transplantation, 59, 1670-1676 (1995).

Milland J, Christiansen D, Lazarus BD, et al. The molecular basis for galalpha(1,3)gal expression in animals with a deletion of the alpha1,3galactosyltransferase gene. J Immunol *176*, 2448-2454 (2006).

Miyata R, Shimazu M, Tanabe M, et al. Clinical characteristics of thrombotic microangiopathy following ABO incompatible living donor liver transplantation. Liver Transpl, 13, 1455-1462 (2007).

Mohiuddin MM, Corcoran PC, Singh AK, et al. B-cell depletion extends the survival of GTKO.hCD46Tg pig heart xenografts in baboons for up to 8 months. Am J Trans. 12, 763-771 (2012).

Mohiuddin MM, Singh AK, Corcoran PC, et al. Role of anti-CD40 antibody-mediated costimulation blockade on non-Gal antibody production and heterotopic cardiac xenograft survival in a GTKO.hCD46Tg pig-to-baboon model. Xenotransplantation. doi: 10.1111/xen.12066. [Epub ahead of print] (2013).

Mueller NJ, Barth RN, Yamamoto S, et al. Activation of cytomegalovirus in pig-to-primate organ xenotransplantation. J Virol, 76, 4737-4740, (2002).

Munoz SJ. Hepatorenal syndrome. Med Clin North Am, 92, 813-37 (2008).

O

O'Grady JG, Portmann B, Wiliamms R. Fulminant hepatic failure. In: Schiff L, Schiff R, eds. Diseases of the liver. JB Lippincott, Philadelphia: (1993).

Oglesby TJ, White D, Tedja I, et al. Protection of mammalian cells from complement-mediated lysis by transfection of human membrane cofactor protein and decay-accelerating factor. *Trans Assoc Am Phys*.104, 164–172 (1991).

Olthoff KM, Merion RM, Ghobrial RM, et al. Outcomes of 385 adult-to-adult living donor liver transplant recipients: a report from the A2ALL Consortium. Ann Surg. 242, 314-

323, (2005).

Onions D, Cooper DKC, Alexander TJL, et al. An assessment of the risk of xenozoonotic disease in pig-to-human xenotransplantation. *Xenotransplantation.* 7, 143-155 (2000).

Oriol R, Ye Y, Koren E, Cooper DKC. Carbohydrate antigens of pig tissues reacting with human natural antibodies as potential targets for hyperacute vascular rejection in pig-to-man organ xenotransplantation. *Transplantation*. 56, 1433-1442 (1993).

Ostapowicz G, Fontana R, Schiodt F, et al. Results of a prospective study of acute liver failure at 17 tertiary care center in the United States. Ann Intern Med, 137, 947-954 (2002)

P

Paradis K, Langford G, Long Z, et al. Search for cross-species transmission of porcine endogenous retrovirus in patients with living pig tissue. The XEN 111 Study Group. *Science.* 285, 1236-1241 (1999).

Pareti FI, Mazzucato M, Bottini E, Mannucci PM. Interaction of porcine von Willebrand factor with the platelet glycoproteins Ib and IIb/IIIa complex. Br J Haematol *82*, 81-86, (1992).

Patience C, Takeuchi Y, Weiss RA. Infection of human cells by an endogenous retrovirus of pigs. Nat Med 3, 282-286. (1997).

Patience C, Patton GS, Takeuchi Y, et al. No evidence of pig DNA or retroviral infection in patients with short-term extracorporeal connection to pig kidneys. *Lancet.* 352, 699-701 (1998).

Pearson TC, Trambley J, Odom K et al. Anti-CD40 therapy extends renal allograft survival in rhesus macaques. Transplantation. 74, 933–940 (2002)

Peng Q, Yeh H, Wei L, et al. Mechanisms of xenogeneic baboon platelet aggregation and phagocytosis by porcine liver sinusoidal endothelial cells. PLoS ONE. 7, e47273 (2012)

Pfeiffer S, Zorn GL 3rd, Zhang JP, et al. Hyperacute lung rejection in the pig-to-human model. III. Platelet receptor inhibitors synergistically modulate complement activation and lung injury. Transplantation, 75, 953-959 (2003).

Phelps CJ, Koike C, Vaught TD, et al. Production of α1,3-galactosyltransferase-deficient pigs. *Science* 299, 411-414 (2003).

Phelps C, Ball S, Vaught T, et al. Production and characterization of transgenic pigs expressing porcine CTLA4-Ig. *Xenotransplantation.* 16, 477-485 (2009)

Platt JL, Vercellotti GM, Lindman BJ, et al. Release of heparan sulfate from endothelial cells. Implications for pathogenesis of hyperacute rejection. J Exp Med 171, 1363-1368 (1990).

Platt JL. Xenotransplantation of the liver: is more complement control needed? Liver Transpl, 7, 933-934 (2001).

Ploeg RJ, D'Alessandro AM, Knechtle SJ, et al. Risk factors for primary dysfunction after liver transplantation – a multivariate analysis. Transplantation, 55, 807-813 (1993).

Pokorny H, Gruenberger T, Soliman T, et al. Organ survival after primary dysfunction of liver grafts in clinical orthotopic liver transplantation. Transplant Int, 13(suppl 1): S154-S157 (2000).

Polson J, Lee W. AASLD position paper: the management of acute liver failure. Hepatology, 41, 1179-1197 (2005).

Powelson J, Cosimi AB, Austen W Jr, et al. Porcine-to-primate orthotopic liver transplantation. Transplant Proc, 26 ,1353-1354 (1994).

R

Ramirez P, Chavez R, Majado M, et al. Life-supporting human complement regulator decay accelerating factor transgenic pig liver xenograft maintains the metabolic function and coagulation in the nonhuman primate for up to 8 days. Transplantation,70, 989-998, (2000).

Ramirez P, Yelamos J, Parrilla P, Chavez R. Hepatic xenotransplantation will benefit from strategies aimed to reduce complement activation. Liver Transpl, 7, 562-563 (2001).

Ramirez P, Montoya MJ, Rios A, et al. Prevention of hyperacute rejection in a model of orthotopic liver xenotransplantation from pig to baboon using polytransgenic pig livers (CD55, CD59, and H-transferase). Transplant Proc, 37, 4103-4106 (2005).

Ramsoondar J, Vaught T, Ball S, et al. Production of transgenic pigs that express porcine endogenous retrovirus small interfering RNAs. *Xenotransplantation.* 16, 164-180 (2009).

Rees MA, Butler AJ, Chavez-Cartaya G, et al. Prolonged function of extracorporeal hDAF transgenic pig livers perfused with human blood. Transplantation, 73, 1194-1202 (2002).

Rees MA, Butler AJ, Negus MC et al. Classical pathway complement destruction is not responsible for the loss of human erythrocytes during porcine liver perfusion. Transplantation, 77, 1416-1423 (2004).

Rieben R, Seebach JD. Xenograft rejection: IgG1, complement and NK cells team up to activate and destroy the endothelium. *Trends Immunol.* 26, 2-5 (2005).

Robson SC, Cooper DKC, d'Apice AJF. Disordered regulation of coagulation and platelet activation in xenotransplantation. (Invited paper). Xenotransplantation, 7, 166-176 (2000).

Rose AG, Cooper DKC, Human P, et al. Histopathology of hyperacute rejection of the heart - experimental and clinical observations in allografts and xenografts. *J Heart Lung Transplant.* 10 223-234 (1991).

Rose AG, Cooper DKC. A histopathologic grading system of hyperacute (humoral, antibody-mediated) cardiac xenograft and allograft rejection. *J Heart Lung Transplant.* 15, 804-817 (1996).

Rose AG, Cooper DKC. Venular thrombosis is the key event in the pathogenesis of antibody-mediated cardiac rejection. *Xenotransplantation.* 7, 31-41 (2000).

Roussel JC, Moran CJ, Salvaris EJ, et al. Pig thrombomodulin binds human thrombin but is a poor cofactor for activation of human protein C and TAFI. Am J Transplant *8*, 1101-1112, (2008).

Ruiz R, Barri YM, Jennings LW, et al. Hepatorenal syndrome: a proposal for kidney after liver transplantation. Liver Transplant, 13, 838-843 (2007).

Rumjantseva V, Grewal PK, Wandall HH, et al. Dual roles for hepatic lectin receptors in the clearance of chilled platelets. Nat Med. 15, 1273–1280 (2009)

S

Saadi S, Platt JL. Role of complement in xenotransplantation. *Clin Exp Pharmacol Physiol.* 26, 1016-1019 (1999).

Satyananda V, Ekser B, van der Windt DJ, Hara H, Ezzelarab M, Schuurman HJ, Cooper DKC. Progress in pig-to-nonhuman primate transplantation models (1998-2012). In preparation (2013)

Schiodt FV, Atillasoy E, Shakil AO, et al. Etiology and outcome for 295 patients with acute liver in the United States. Liver Transpl Surg, 5, 29-34 (1999).

Schirmer JM, Fass DN, Byrne GW, et al. Effective antiplatelet therapy does not prolong transgenic pig to baboon cardiac xenograft survival. Xenotransplantation *11*, 436-443 (2004).

Schulte Am Esch J, 2nd, Robson SC, Knoefel WT, et al. O-linked glycosylation and functional incompatibility of porcine von Willebrand factor for human platelet GPIb receptors. Xenotransplantation *12*, 30-37, (2005).

Schuurman HJ. Regulatory aspects of pig-to-human islet transplantation. *Xenotransplantation.* 15, 116-120 (2008).

Sgroi A, Serre-Beinier V, Morel P, et al. What clinical alternatives to whole liver transplantation? Current status of artificial devices and hepatocyte transplantation. Transplantation, 87, 457-466 (2009).

Shakil AO, Kramer D, Mazariegos GV, et al. Acute liver failure: clinical features, outcome analysis, and applicability of prognostic criteria. Liver Transpl, 6, 163-169 (2000).

Sharma A, Naziruddin B, Cui C, *et al.* Pig cells that lack the gene for alpha1-3 galactosyltransferase express low levels of the gal antigen. Transplantation *75*, 430-436 (2003).

Shiratori Y, Tananka M, Kawase T, et al. Quantification of sinusoidal cell function in vivo. Semin Liver Dis 13: 39–49 (1993)

Sis B, Famulski KS, Allanach KL, et al. IFN-gamma prevents early perforin-granzyme-mediated destruction of kidney allografts by inducing donor class I products in the kidney. Am J Transplant *7*, 2301-2310, (2007).

Sosef MN, Van De Kerkhove MP, Abrahamse SL, et al. Blood coagulation in anhepatic pigs: effects of treatment with the AMC-bioartificial liver. J Thromb Haemost, 1, 511-515 (2003).

Starzl TE, Marchioro TL, Faris TD, et al. Avenues of future research in homotransplantation of the liver with particular reference to hepatic supportive procedures, antilymphocyte serum, and tissue typing. Am J Surg, 112, 391-400 (1966).

Starzl TE, Marchioro TL, Porter KA, Brettschneider L. Homotransplantation of the liver. Transplantation. 5 (Suppl), 790-803 (1967).

Starzl TE, Fung J, Tzakis A, et al. Baboon-to-human liver transplantation. Lancet, 341, 65-71, (1993).

Strom SC, Bruzzone P, Cai H, et al. Hepatocyte transplantation: clinical experience and potential for future use. Cell Transplant, 15(suppl 1), s105-s110, (2006).

Sykes M, d'Apice A, Sandrin M; IXA ethics committee. Position paper of the ethics committee of the International Xenotransplantation Association. Transplantation, 78, 1101-1107 (2004).

T

Tai HC, Zhu X, Hara H, et al. The pig-to-primate immune response: relevance for xenotransplantation. Xenotransplantation, 14, 227-235 (2007).

Taniguchi S, Cooper DKC. Clinical xenotransplantation—a brief review of the world experience. In: Cooper DKC, Kemp E, Platt JL, White DJG, eds. Xenotransplantation. 2nd ed. Heidelberg, Germany: Springer, 776-784 (1997).

Taniguchi S, Cooper DK. Clinical xenotransplantation: past, present and future. Ann R Coll Surg Engl, 79, 13-19 (1997).

Taylor SG, McKenzie IF, Sandrin MS. Characterization of the rat alpha(1,3)-galactosyltransferase: evidence for two independent genes encoding glycosyltransferases that synthesize Galalpha(1,3)Gal by two separate glycosylation pathways. Glycobiology *13*, 327-337 (2003).

Tector AJ, Elias N, Rosenberg L, et al. Mechanisms of resistance to injury in pig livers perfused with blood from patients in liver failure. Transplant Proc, 29, 966-999 (1997).

Tector AJ, Chen X, Soderland C, et al. Complement activation in discordant hepatic xenotransplantation. Xenotransplantation, 5, 257-261 (1998).

Tector AJ, Fridell JA, Watanabe T, Forbes RD, Salazar J, Grienke D, et al. Pulmonary injury in recipients of discordant hepatic and renal xenografts in the dog-to-pig model. Xenotransplantation, 5, 44-49 (1998b).

Tector AJ, Fridell JA, Elias N, et al. Aberrations in hemostasis and coagulation in untreated discordant hepatic xenotransplantation: studies in the dog-to-pig model. Liver Transpl, 8, 153-159, (2002).

Teranishi K, Gollackner B, Buhler L, et al. Depletion of anti-gal antibodies in 217 baboons by intravenous therapy with bovine serum albumin conjugated to gal oligosaccharides. Transplantation *73*, 129-139. (2002).

Thompson P, Cardona K, Russell M, et al. CD40-Specific costimulation blockade enhances neonatal porcine islet survival in nonhuman primates. Am J Transplant. 11, 947-957 (2011).

Trotter JF, Adam R, Lo CM, Kenison J. Documented deaths of hepatic lobe donors for living donor liver transplantation. Liver Transpl. 12, 1485-1488, (2006).

U

Uemura T, Randall HB, Sanchez EQ, et al. Liver retransplantation for primary nonfunction: analysis of a 20-year single-center experience. Liver Transpl, 13, 227-233 (2007).

UNOS - United Network for Organ Sharing (UNOS). http://www.unos.org (last accessed on November 26, 2013)

Ustransplant – www.ustransplant.com (last accessed on November 26, 2013)

V

van den Bogaerde JB, Aspinall R, White DJ. Hamster to rat xenografts are not rejected primarily by rat T cells. Transplant Proc. 23, 2277-2279, (1991).

van de Kerkhove MP, Di Lorio E, Scuderi V, et al. Phase I clinical trial with AMC-bioartificial liver. Academic Medical Center. Int J Artif Organs, 25, 950-959 (2002).

van de Kerkhove MP, Hoekstra R, Chamuleau RA, et al. Clinical application of bioartificial liver support systems. Ann Surg, 240, 216-230 (2004).

van der Windt DJ, Bottino R, Casu A, et al. Long-term controlled normoglycemia in diabetic non-human primates after transplantation with hCD46 transgenic porcine islets. *Am J Transplant*. E-pub October 21, (2009).

Vincenti F, Charpentier B, Vanrenterghem Y et al. A phase III study of belatacept-based immunosuppression regimens versus cyclosporine in renal transplant recipients (BENEFIT study). Am J Transplant. 10, 535–546 (2010).

W

Wack KE, Ross MA, Zegarra V, et al. Sinusoidal ultrastructure evaluated during the revascularization of regenerating rat liver. Hepatology. 33, 363-378 (2001)

Watanabe M, Yamashita K, Suzuki T, et al. ASKP1240, a fully human anti-CD40 monoclonal antibody prolongs pancreatic islet allograft survival in nonhuman primates. Am J Transplant.13, 1976-1988 (2013)

Watts A, Foley A, Awwad M, et al. Plasma perfusion by apheresis through a Gal immunoaffinity column successfully depletes anti-Gal antibody: experience with 320 aphereses in baboons. Xenotransplantation *7*, 181-185 (2000).

Weiss RA. Xenografts and retroviruses. Science, 285, 1221-1222, (1999).

Wekerle T, Grinyo JM. Belatacept: from rational design to clinical application. Transpl Int. 25, 139-150 (2012)

White DJ. Transplantation of organs between species. Int Arch Allergy Immunol, 98, 1-5, (1992).

Y

Yamada K, Sachs DH, DerSimonian H. Human anti-porcine xenogeneic T cell response. Evidence for allelic specificity of mixed leukocyte reaction and for both direct and indirect pathways of recognition. *J Immunol.* 155, 5249-5256 (1995).

Yamada K, Yazawa K, Shimizu A, et al. Marked prolongation of porcine renal xenograft survival in baboons through the use of α1,3-galactosyltransferase gene-knockout donors and the cotransplantation of vascularized thymic tissue. *Nat Med.* 11, 32-34 (2005).

Z

Zanchet DJ, Montero EFS, Marques AM, et al. Personal computer software evaluation in interactive generation of pig liver three-dimensional anatomical images. Transplant Proc, 37, 198-200, (2005).

Zhang L, Li Y, Jiang H, et al. Comparison of hepatic coagulant, fibrinolytic, and anticoagulant functions between banna minipig inbred line and humans. Transplantation, 79, 1128-1131 (2005).

Zhong R, Tucker J, Grant D, et al. Long-term survival and functional tolerance of baboon to monkey kidney and liver transplantation: a preliminary report. Transplant Proc, 28, 762, (1996).

Zhong R, Tucker J, Zhang Z, et al. The long-term survival of baboon-to-monkey kidney and liver xenografts. Xenotransplantation, 10, 398-409 (2003).

Zhou NN, Senne DA, Landgraf JS, et al. Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. J Virol, 73, 8851-8856, (1999).

Zhu X, Dor FJMF, Cooper DKC. Pig-to-non-human primate heart transplantation: immunologic progress over 20 years. *J Heart Lung Transplant.* 26, 210-218 (2007).