Université Catholique de Louvain

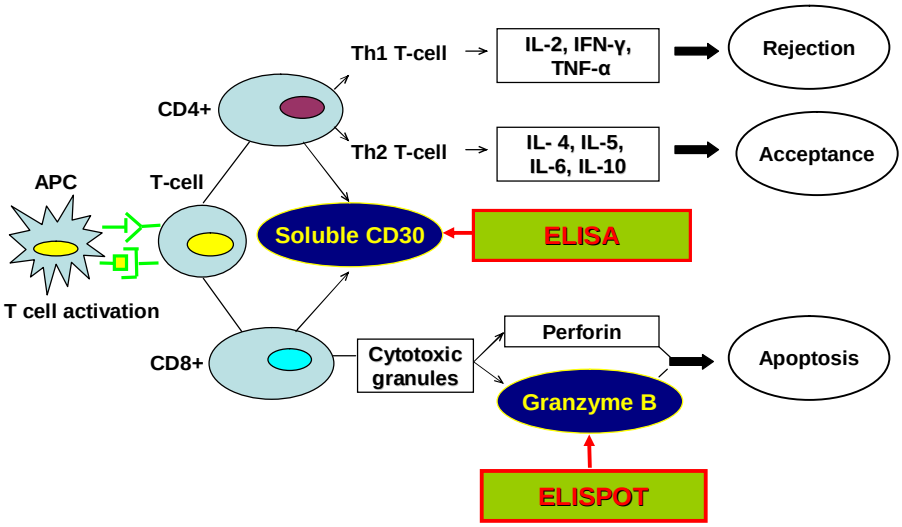
Faculty of Medicine, Brussels, Belgium

Pediatric Surgery and Transplant Unit & Laboratory of Transplant Immunology

THE IMMUNE MONITORING OF PEDIATRIC ORGAN TRANSPLANTS

POTENTIAL ROLE OF SOLUBLE CD30 PLASMA MEASUREMENT AND GRANZYME B ENZYME-LINKED IMMUNOSORBENT ASSAY

Dinh Quang TRUONG



Thesis for doctoral degree (PhD) in Pharmaceutical and Biomedical Sciences

Orientation: Pediatric Transplantation

Promoters: Professor Raymond Reding *&* Professor Dominique Latinne

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Université Catholique de Louvain
Faculté de Médecine

Unité de Chirurgie et Transplantation pédiatrique
Laboratoire de Biologie Hématologique

LE MONITORING IMMUNOLOGIQUE EN TRANSPLANTATION D'ORGANE PÉDIATRIQUE

LE RÔLE POTENTIEL DE DOSAGE DU CD30 SOLUBLE ET DU GRANZYME B ENZYME-LINKED IMMUNOSORBENT

Dinh Quang TRUONG

Promoteurs:
Professeur Raymond Reding *&* Professeur Dominique Latinne

Thèse présentée en vue de l'obtention du grade de docteur en sciences biomédicales et pharmaceutiques. Orientation: Transplantation pédiatrique
Octobre 2009

Viện Đại học Louvain
Trường Đại học Y khoa

Đơn vị Phẫu thuật và Ghép tạng nhi
Khoa Xét nghiệm Miễn dịch Ghép

THEO DÕI PHẢN ỨNG MIỄN DỊCH SAU GHÉP TẠNG Ở TRẺ EM

ĐÁNH GIÁ VAI TRÒ CỦA ĐỊNH LƯỢNG CD30 HÒA TAN VÀ GRANZYME B ENZYME-LINKED IMMUNOSORBENT

TRƯƠNG QUANG ĐỊNH

Người hướng dẫn:
Giáo sư Raymond Reding *&* Giáo sư Dominique Latinne

Luận văn Tiến sĩ Khoa học Y khoa
Chuyên ngành: Ghép tạng nhi
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Faculty of Medicine

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Composition of the jury:

President:	Professor Pierre Gianello
Promoters:	Professor Raymond Reding
	Professor Dominique Latinne
Members:	Professor Pierre Coulie
	Professor Michel Mourad
External member:	Professor Michel Toungouz

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Les médecins et les rois, deux espèces très respectables
avec lesquelles la vie humaine est quelquefois en danger
Voltaire (1694-1778)

Thầy thuốc và nhà vua, hai người được quý trọng nhất
nhưng cũng chính họ đôi khi làm nguy hại cho cuộc sống con người
Voltaire (1694-1778)

Medicine is an Art, based on Science
William Osler (1849-1919)

Y học là nghệ thuật, dựa trên nền tảng của khoa học
William Osler (1849-1919)

En hommage aux membres de ma famille disparus
Mon papa, ma sœur
Mes beaux-parents

Aux deux femmes les plus importantes dans ma vie
Ma mère, Nguyễn Thị Minh Vân
et mon épouse, Nguyễn Thúy Quỳnh Mai

Mille remerciements
pour mes frères et mes sœurs

Un merveilleux cadeau
pour Tony Gia Bảo, mon fils

Kính dâng cho những người đã khuất
Ba và chị tôi
Ba mẹ vợ tôi

Tri ân cho 2 người phụ nữ quan trọng nhất của đời tôi
Mẹ tôi, Nguyễn Thị Minh Vân
và vợ tôi, Nguyễn Thúy Quỳnh Mai

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Gửi đến các anh chị em trong gia đình

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Abbreviation:

Abs, antibodies; Ags, antigens; ALG, antilymphocyte globulin; APC, antigen-presenting cells; AR, acute rejection; ATG, antithymocyte globulin; ATP, adenosine 5-triphosphate; AZA, azathioprine; BA, biliary atresia; CD, cadaveric transplant; CDRs, complementary determining regions; CFSE, carboxyfluorescein succinimidyl ester; cpm, counts per minute; CNI, calcineurin inhibitors; CTL, cytotoxic T lymphocytes; CUD, Commission Universitaire pour le Développement; DC, dendritic cells; DTH, delayed-type hypersensitivity; ELISA, enzyme-linked immuno sorbent assay; ELISPOT, enzyme-linked immunosorbent spots; FBS, fetal bovine serum; GrB, granzyme B; HCM city, Ho Chi Minh City; HLA, human leukocyte antigen; IFN-γ, interferon-gamma; Ig, immunoglobulin; IL, interleukin; IS, immunosuppression; KT, kidney transplantation; LDA, limiting dilution assays; LT, liver transplantation; LRLT, living related liver transplant; MHC, major histocompatibility complex; mTOR, mammalian TOR; mRNA, messenger ribonucleic acid; MLR, mixed lymphocyte reaction; MMF, mycophenolate mofetil; MS-qPCR, quantitative methyl-specific PCR assay; NFAT, nuclear factor for activated T cells; NK, natural killer; PHA, phytohemagglutinin; PRA, panel reactive antibody; PBMC, peripheral blood mononuclear cell; PIC, Projet Interuniversitaire Ciblé; RT-PCR, reverse transcriptase-polymerase chain reaction; RT-QPCR, real-time quantitative PCR; PTLD, post-transplant lymphoproliferative disorder; sCD30, soluble CD30; TCR, T-cell receptor; TGF, transforming growth factor; Th, T helper; TNF, tumor necrosis factor; Tregs, T regulatory cells; TUNEL, terminal deoxynucleotide transferase-mediated dUTP Nick End Labeling.

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SUMMARY

Over the last half century, kidney and liver transplantation have been recognized as the treatment of choice for children with end-stage renal or liver failure. Greater understanding of the molecular mechanism of rejection and tolerance, as well as development of reliable assays that can measure accurately the status of the immune response, not only would help clinicians customize the prescription of immunosuppressive drugs in individual recipients, but also might contribute to optimization of outcome in pediatric organ transplantation. In addition, despite improved immunosuppression (IS) protocols, infants still face a variety of chronic complications such as drug toxicity, infection, and malignancies. Thus, a major goal in organ transplantation is to define the optimal immunosuppressive load, according to the individual immunological profile of the transplant patient. Ideally, from a clinical perspective, the *de novo* donor-recipient encounter should be monitored in pre-transplantation and the immediate post-transplantation period, allowing the early diagnosis of ongoing rejection processes.

In this thesis, we review the specificities of the immune system in infants; we then discuss the mechanisms of rejection and the terminology underlying the generic concept of "transplant tolerance". This review focuses on the several immunological assays studied or under development in pediatric organ transplantation. We subsequently attempted to find predictive tests in order to evaluate the patient's immunological risk of early rejection after organ transplantation. Forty pediatric patients (median age: 2.1 years, range: 4 months - 13 years) who received an orthotopic liver transplantation between 1994 and 2004 in the Pediatric Liver Transplant Program at Saint-Luc University Clinics in Brussels, Belgium, were included in this study. First, using antigen non-specific assays, we investigated the evolution of pre-transplant and post-transplant serum soluble CD30 (sCD30), interferon-gamma (IFN-γ) and interleukin-10 (IL-10) circulating levels in the recipients who developed acute rejection as compared to patients with early graft acceptance. The frozen serum of the patients was analyzed to measure sCD30, IL-10 and IFN-γ serum levels at pre-transplantation baseline (day 0) and post-transplantation at day 7. The delta value of serum sCD30 between day 0 and 7 was compared with the delta value of IFN-γ and of IL-10 circulating levels on the same day. This study showed that increased serum sCD30 could be correlated with increased IL-10 circulating levels, but not with IFN-γ levels in the post-transplantation period. Neither

pre-transplantation sCD30, nor sCD30 at day 7 post-transplantation could be correlated with acute rejection in liver graft recipient. The monitoring of sCD30 might constitute a tool to assess the risk of acute rejection in renal transplant but, at least in our limited series, did not appear to constitute a useful marker for early immunological monitoring in liver allograft recipients. Next, we used the enzyme-linked immunosorbent (ELISPOT) as an antigen-specific assay to analyze the detailed kinetics of granzyme-B (GrB) producing cells before and during the early post-transplantation period. We also investigated the pre- and post-transplantation secretion of GrB in pediatric liver recipients who developed acute rejection compared to patients with graft acceptance. Peripheral blood mononuclear cells (PBMC) from pediatric recipients were serially tested for GrB-producing donor-reactive cells at baseline and at days 7, 14, 28 post-transplantation. This study demonstrated that single GrB ELISPOT pre-transplantation could not predict the occurrence of early post-transplant acute rejection; similarly, GrB frequencies at days 7, 14 and 28 could not be correlated with acute rejection in pediatric liver recipients. However, a kinetic analysis of these data demonstrated that GrB increased significantly at day 7 from baseline in the rejection group. Thus, a kinetic monitoring of GrB ELISPOT variation was found helpful to predict or confirm early rejection in the liver allograft recipients.

In the future, further research should therefore be encouraged, to identify the role of sCD30 and GrB ELISPOT kinetic assays in combination with a number of techniques, e.g. regulatory T cell, proteomics transcriptional profiling, and DNA microarray assays. Accordingly, a multidisciplinary approach should be established through multicenter studies including assessment of dynamic profiles, so that ideal strategies can be identified for implemention in the immunological monitoring of kidney and liver transplant in pediatric recipients.

RÉSUMÉ

Au cours du dernier demi-siècle, la transplantation du rein et du foie a été identifiée comme traitement de choix pour les enfants atteints d'insuffisances rénales ou hépatiques terminales. L'étude du mécanisme moléculaire du rejet et de la tolérance, ainsi que le développement des tests fiables qui reflètent fidèlement les réponses immunitaires, aideraient non seulement les cliniciens à adapter les immunosuppresseurs pour chaque individu, mais pourraient également contribuer à optimiser de la survie des enfants transplantés. En outre, malgré l'amélioration des protocoles d'IS, les enfants doivent prendre des médicaments à vie avec un risque important de complications chroniques telles que la toxicité, l'infection et les cancers. Dès lors, en transplantation d'organe, il convient de définir la dose optimale des immunosuppresseurs, selon le profil immunologique de chaque patient. Idéalement, dans une perspective clinique, la réponse immunitaire du receveur vis-à-vis de son donneur devrait être suivie dans le décours de la transplantation afin de détecter immédiatement un rejet précoce post-transplantation ou un état de tolérance au décours de la greffe.

Dans cette thèse, nous avons réalisé une revue de la littérature sur les spécificités du système immunitaire chez l'enfant ; par la suite nous avons abordé les mécanismes du rejet et les terminologies du concept général de «tolérance au greffon». Cette revue se concentre sur plusieurs tests immunologiques étudiés ou développés en transplantation d'organe pédiatrique. Ensuite, nous avons essayé de trouver des tests prédictifs dans le but d'évaluer le risque du rejet précoce après transplantation d'organe. 40 enfants (âge médian : 2.1 ans, range : 4 mois - 13 ans) du programme de transplantation hépatique des Cliniques universitaires de Saint-Luc à Bruxelles, ayant été transplantés entre 1994 et 2004, ont été inclus dans cette étude. Tout d'abord, en utilisant un test « antigène non spécifique », nous avons étudié l'évolution des concentrations sériques de CD30 soluble, IFN-γ et IL-10 en pré- et post-transplantations dans deux groupes, l'un ayant présenté un épisode du rejet aigu, l'autre ayant une évolution sans épisode de rejet. Les sérums congelés des patients ont été utilisés pour mesurer les taux de sCD30, IL-10 et IFN-γ pré-transplant (jour 0) et post-transplant au jour 7. La valeur du delta de sCD30 sérique entre le jour 0 et 7 a été comparée à la valeur du delta d'IFN-γ et d'IL-10 circulant au même jour. Cette étude a démontré que l'augmentation du sCD30 pouvait être corrélée avec l'augmentation d'IL-10 circulant, mais pas avec l'IFN-γ dans la période post-transplantation. Ni le sCD30 pré-transplantion, ni le sCD30 post-transplantion au jour 7 n'ont

pu être corrélés avec le rejet aigu chez les receveurs de greffe de foie. Le monitoring du sCD30 pourrait bien constituer un marqueur utile pour évaluer le risque de rejet aigu chez les patients transplantés du rein mais, dans notre série limitée, ce marqueur n'a pas semblé valable pour la surveillance immunologique précoce des patients greffés du foie. Ensuite, nous avons utilisé « the enzyme-linked immunosorbent assay » (ELISPOT) comme test « antigène spécifique », pour analyser la cinétique du GrB, produit par les cellules du receveur stimulées avec les cellules du donneur avant et au cours de la période précoce post-transplantation. Nous avons également comparé les valeurs dans deux groupes ayant présenté ou non un épisode de rejet aigu. La production de GrB par les cellules mononucléées du sang périphérique des receveurs pédiatriques qui ont été stimulées avec les cellules du donneur, a été analysée au jour 0 avant transplantation ainsi qu'aux jours 7, 14 et 28 après transplantation. Cette étude a démontré que le GrB ELISPOT pré-transplantation ne permet pas de prévoir le rejet précoce, de même que la production du GrB aux jours 7, 14 et 28 ne peut être corrélée avec le développement d'un rejet aigu. Par contre, l'étude cinétique du GrB ELISPOT a démontré une augmentation significative du jour 0 au jour 7 dans le groupe de rejet. Le monitoring cinétique du GrB pourrait donc être un moyen utile afin de prévoir ou confirmer la survenue d'un rejet précoce chez les patients greffés du foie.

De futures recherches sont à encourager afin d'identifier le rôle du sCD30 et du GrB ELISPOT en association avec la détection des lymphocytes T régulateurs, du profil de transcription protéomique, des microarrays, etc. Par conséquent, une approche multidisciplinaire et multicentrique, y compris l'évaluation des profils dynamique, devrait permettre de dégager, en vue de leur mise en oeuvre, des stratégies idéales dans le suivi immunitaire en greffe rénale et hépatique pédiatriques.

TÓM TẮT (SUMMARY IN VIETNAMESE)

Hơn nửa thế kỷ qua, ghép thận và ghép gan đã được công nhận như là phương thức điều trị chọn lọc cho trẻ em bị suy thận hoặc suy gan vào giai đoạn cuối. Hiểu biết sâu sắc về cơ chế phân tử của quá trình thảy ghép và dung nạp ghép, cũng như việc phát triển các xét nghiệm đáng tin cậy nhằm đánh giá chính xác về tình trạng đáp ứng miễn dịch, không chỉ sẽ giúp các bác sĩ điều chỉnh liều lượng thuốc ức chế miễn dịch trên mỗi cá thể người nhận, mà còn góp phần cải thiện kết quả điều trị trong ghép tạng ở trẻ em. Tuy vậy, trẻ em sau ghép tạng vẫn còn phải đối mặt với nhiều biến chứng mãn tính như ngộ độc thuốc, nhiễm trùng, và ung thư hóa, mặc dù đã có nhiều tiến bộ về thuốc ức chế miễn dịch. Vì vậy, một trong những mục tiêu quan trọng trong ghép tạng là phải xác định được liều lượng tối ưu của thuốc ức chế miễn dịch, dựa trên khả năng đáp ứng miễn dịch của từng bệnh nhân ghép. Lý tưởng nhất, từ góc độ lâm sàng, các cặp người cho và nhận trong ghép tạng, phải được theo dõi về miễn dịch trước và ngay sau khi cấy ghép để có thể chẩn đoán thảy ghép sớm ngay từ giai đoạn vừa khởi phát.

Trong luận văn này, trước hết chúng tôi khái quát các đặc trưng của hệ thống miễn dịch ở trẻ em; kế đến chúng tôi bàn luận về các các cơ chế thảy ghép và các thuật ngữ xung quanh các khái niệm chung của dung nạp ghép. Phần phân tích y văn này tập trung vào các thử nghiệm miễn dịch đã được nghiên cứu hoặc đang được phát triển trong lĩnh vực ghép tạng nhi khoa. Tiếp theo đó, chúng tôi giới thiệu kết quả phân tích các xét nghiệm có thể tiên đoán nguy cơ thảy ghép sớm trên bệnh nhân sau ghép tạng. Nghiên cứu của chúng tôi đã thực hiện với 40 bệnh nhi (từ 4 tháng đến 13 năm tuổi với độ tuổi trung bình là 2,1 năm), được ghép gan ở giai đoạn từ 1994- 2004 tại Trường viện Saint-Luc, Brussels, Vương quốc Bỉ.

Trước tiên, chúng tôi đã dùng thử nghiệm không- đặc hiệu đối với các kháng nguyên (antigen-non-specific assays), để khảo sát sự tiến triển trước và sau ghép của CD30 hòa tan (sCD30), interferon- gamma (IFN-γ) và interleukin-10 (IL-10), và so sánh giữa 2 nhóm bệnh nhân bị thảy ghép cấp và không bị thảy ghép. Nồng độ của sCD30, IL-10 và IFN-γ trong huyết thanh đông lạnh của bệnh nhân được định lượng tại thời điểm trước ghép (ngày 0) và ngày 7 sau ghép. Độ chênh lệch của nồng độ sCD30 giữa ngày 0 và ngày 7 được so sánh với độ chênh lệch nồng độ của IFN-γ và IL-10 tại cùng một thời điểm. Nghiên cứu này cho thấy, mức độ gia tăng nồng độ sCD30 có thể tương quan với mức gia tăng nồng độ IL-10, nhưng không tương ứng với sự thay đổi nồng độ của IFN-γ trong giai đoạn sau ghép. Nồng độ

sCD30 vào ngày 0 trước ghép và vào ngày thứ 7 sau ghép không tương ứng với tình trạng thảy ghép cấp trên bệnh nhân ghép gan. Như vậy, định lượng sCD30 có thể được theo dõi để đánh giá nguy cơ thảy ghép cấp trong ghép thận nhưng, ít nhất trong giới hạn nghiên cứu của chúng tôi, sCD30 không có giá trị hữu ích cho việc theo dõi phản ứng miễn dịch ở bệnh nhân sau ghép gan.

Tiếp theo, chúng tôi đã dùng test ELISPOT là một thử nghiệm- đặc hiệu (antigen-specific assay) đối với các kháng nguyên để phân tích nồng độ dao động của granzyme-B (GrB) do các tế bào lympho T của người nhận sản xuất trước và trong giai đoạn sớm sau ghép. Chúng tôi cũng khảo sát sự sản xuất GrB trước và sau ghép nơi trẻ em nhận gan, cũng như so sánh sự khác biệt về mức độ sản xuất GrB ở 2 nhóm trẻ có thảy ghép cấp và không thảy ghép. Các tế bào đơn nhân của máu ngoại vi từ bệnh nhi được tiến hành thử nghiệm về mức độ sản xuất GrB sau khi được kích thích bởi tế bào người cho vào ngày 0 trước ghép và tại các ngày 7, 14, 28 sau ghép. Nghiên cứu này đã chứng minh rằng hàm lượng GrB ELISPOT trước cấy ghép không thể tiên lượng sự xuất hiện của thảy ghép cấp ở giai đoạn sớm sau ghép; tương tự, hàm lượng GrB vào ngày 7, 14 và 28 không tương quan với tình trạng thảy ghép cấp nơi bệnh nhi ghép gan. Tuy vậy, khi phân tích sự biến động theo thời gian của GrB từ nghiên cứu này, đã chứng minh rằng GrB tăng lên đáng kể vào ngày 7 so với ngày 0 trong nhóm bệnh nhân thảy ghép. Do đó, theo dõi sự biến thiên dao động của GrB ELISPOT có thể hỗ trợ cho việc dự đoán hoặc xác nhận tình trạng thảy ghép trong ghép gan ở trẻ em.

Các nghiên cứu chuyên sâu hơn cần được khuyến khích thực hiện trong tương lai, để xác định vai trò của sCD30 và sự biến thiên của GrB ELISPOT, phối hợp với một số thử nghiệm khác như định lượng lymphocyte T điều hòa (Treg), proteomic, và microarray DNA. Theo đó, nên tiến hành nhiều thử nghiệm, nghiên cứu trên phạm vi đa trung tâm bao gồm cả việc đánh giá các đặc điểm biến thiên miễn dịch của từng bệnh nhân ghép sẽ là một chiến lược lý tưởng để theo dõi miễn dịch trên các bệnh nhân sau ghép thận và ghép gan ở trẻ em.

1- FOREWORD

The population of Vietnam is 86,116,560 (versus 10,414,336 for Belgium, estimation in July 2008, source: https://www.cia.gov/library/publications/the-world-factbook/geos/vm.html) with a birth rate at 16.47 births/1,000 population (versus 10.22 births/1,000 in Belgium). The incidence of biliary atresia (BA) is approximately 1 in 15,000 live births. Consequently, according to the hypothesis that this incidence is similar throughout the world, 96 new BA cases are expected to occur every year in Vietnam. Moreover, the pediatric surgical literature shows that Kasai portoenterostomy (the operative procedure currently proposed when BA is detected in an infant) is successful in less than 50% of the cases; accordingly it can be estimated that 40-50 children with BA would finally require liver transplantation every year in Vietnam. To the best of our knowledge as of 2003, no case of pediatric liver transplantation (LT) has been performed in the country.

Ho Chi Minh (HCM) City, located in South Vietnam, has a population of 8 millions inhabitants, whereas the city has only two pediatric hospitals. A "Projet Interuniversitaire Ciblé" (PIC), managed and financed by the Belgian "Commission Universitaire pour le Développement" (CUD) was established in HCM City in 2004. This project has the seal of approval of the Health Service of HCM City and the University Training Center, with the participation of Children's Hospital 2. This 1000 bed hospital includes 40 clinical services and departments, covering almost all pediatric specialities.

The management of hepato-biliary diseases in children has been identified as a priority in the Health Service of HCM City as well as in Children's Hospital 2. Hepato-biliary diseases comprise an estimated 8% of hospitalizations in Children's Hospital 2 (*1*), including congenital malformations and other miscellaneous conditions (BA, metabolic diseases,…) as well as acquired diseases (viral hepatitis, liver malignancies, …). The 2004-2009 PIC program focused its activities on teaching, clinical research and patient care in the field of pediatric diseases of the gastro-intestinal tract. In view of the progressive launch of transplant programs in Vietnam (Table 1), the Vietnamese Health and Hospital Authorities requested the PIC program to help organization of a pediatric liver transplant team in HCM City. Having particular expertise in this field, the Belgian medical team of the PIC program responded positively to this request and closely cooperated with the Vietnamese doctors to set up a full,

multidisciplinary liver transplant program at Children's Hospital 2, which effectively started in 2005 (*2*).

To date, six pediatric liver transplantation cases with parental living donors were performed between December 2005 and November 2008 at Children's Hospital 2, thanks to this Belgian-Vietnamese cooperation. In the light of the success of this program, plans are underway on the Vietnamese side to set up a pediatric organ transplant center at Children's Hospital 2.

From a medical perspective, and as in other transplant centers, the importance of monitoring immune responses in order to optimize pediatric transplantation outcomes was soon recognized and we have thus focused the research described in this thesis on this area. Indeed, we face several major challenges as we try to evaluate immune responses through immunological assays. One of the greatest limitations is our only partial understanding of the mechanisms of clinical tolerance, and why it is absent in some individual recipients but develops in others. In this thesis, we review the most promising candidate assays for immunological monitoring in organ transplantation; we also personally investigated predictive tests that might contribute to determining the patient's risk of early rejection.

Overall, the objective of this thesis was not only to participate in scientific research in the field of immunological monitoring after pediatric transplantation, but also to increase our knowledge and expertise with the aim of further developing organ transplantation programs at our hospital in HCM City, and throughout the country.

Table 1- Landmarks of development of organ transplantation in Vietnam

Date	Events	Place
Jan 31st 2004	First pediatric liver transplantation	The Army Medical Institute 103 in Hanoi
May 26th 2004	First pediatric kidney transplantation	The National Pediatric Institute in Hanoi
June 14th 2004	First pediatric kidney transplantation in cooperation with a team from Paris	The Children's Hospital 2 in Ho Chi Minh City
Dec 5th 2005	First pediatric liver transplantation with a Belgian team from Saint-Luc University Clinics, Université Catholique de Louvain.	The Children's Hospital 2 in Ho Chi Minh City

2- THE IMMUNOLOGICAL MONITORING OF KIDNEY AND LIVER TRANSPLANT IN ADULT AND PEDIATRIC RECIPIENTS

2.1- Introduction

Liver transplantation has been accepted as the standard treatment for pediatric patients affected by a variety of acute and chronic liver diseases, allowing outcomes equivalent if not superior to adult patients (*3-5*). Similarly, kidney transplantation has also been recognized as the treatment of choice for adult and children with end-stage renal disease. Advances in carrying out transplantation in small children and progress in IS have resulted in improved short and long-term results for these types of therapy (*6, 7*). However, most pediatric recipients require long term IS after transplantation. Despite improved IS protocols, transplanted infants and young children face a variety of chronic complications such as drug toxicity, infection, and malignancies. Accordingly, introduction of immune monitoring may constitute an important step in predicting which recipients are candidates for marked reduction or even for removal of IS without increasing the risk of acute or chronic rejection.

The structure and function of the immune system in infants and children are different when compared to the adult, an observation which should be taken into account when analyzing the immune response to transplantation. First, in contrast to adults, during the first year of life, young infants have a sizeable thymus with the capacity to produce new T cells in large numbers and all normal infants present higher absolute lymphocyte counts with higher circulating T and B cell populations. However, while adults have large populations of memory T and B cells reflecting past exposure to antigens, vaccines and microorganisms, pediatric subjects have an immune system with repertoires of B and T cells that are predominantly ‘‘naïve” (*8*). Younger patients have had less cumulative exposure to infectious agents and vaccines, and their responses after transplantation tend to more accurately reflect the impact on immune responses of transplantation, IS and other manipulations.

Next, unlike the immune system of the laboratory mouse, normal human neonates are thought to have diverse T and B cell repertoires, which would explain their full capacity to develop antigen-specific T cell response. In contrast to human infants, the newborn laboratory mouse has a markedly deficient capacity to mount T and B cell responses and its T cell subdivision tends toward the Th2 (T helper) cytokines production (*9*). That makes it possible for tolerance to occur spontaneously (i.e., newborn tolerance) in mice which can not occur in

human subjects. However, the capacity to mount a T-cell-*independent* humoral immune response to antigens, such as ABO blood group antigens, is notably deficient in human neonates (*10*). Furthermore, the complement system is not fully competent in the young infant. Thus, the primary factors that would initiate hyperacute rejection are not fully effective during early infancy (*11*). Therefore, ABO-incompatible heart and liver transplantations have been performed safely during infancy before the onset of isohemagglutinin production (*12*). Overall, the anticipated allogenic rejection phenomenon is expected to be less pronounced in infants whose immune system is immature. It remains the hope of some research teams that this would enhance the chances of newborns to survive after cross species xenogenic transplantation (*13*).

Although infants present a relative naïve immune system, they are capable of mounting both cellular and humoral immune responses to the foreign antigens presented by the allograft. Immune monitoring may constitute a way of measuring functional and molecular correlates of immune reactivity which may provide clinically useful information for the titration of immunosuppressive drugs in individual recipients. Immune monitoring would also be essential for identifying patients at increased risk of acute rejection once IS minimization has been initiated, or to detect acute rejection prior to clinical/biochemical signs so as to be able to perform pre-emptive intervention to reduce/prevent damage to the graft. Monitoring requires the implementation of advanced assays ideally according to common technical standards, enabling large-scale, multi-centric application. In the transplant setting, monitoring would be useful for measuring both alloimmune (e.g. donor reactivity) and nonalloimmune reactivity to the graft (*14*). This work first focuses on the specifics of the immune system in infants and the current understanding of allograft rejection and acquired tolerance. It then describes the most promising candidate assays for monitoring in organ transplantation. Finally, this review discusses potential role of sCD30 and Gr ELISPOT in kidney and liver transplantation.

2.2 - Mechanisms of allograft rejection

The process of graft rejection can be divided into two phases: (1) sensitization, during which antigen-reactive lymphocytes of recipient proliferate in response to alloantigens on the graft, and (2) effector, during which immune destruction of the graft takes place (*15*).

2.2.1- Sensitization phase

The first step in the body's response to foreign antigen is T-cell recognition and activation. These activated T cells show a number of different outcomes. The first is differentiation into effector cells, which are responsible for orchestrating the immune response directed toward the target antigens. Some of the T cells will differentiate into memory cells. These are capable of providing rapid recall responses to antigen rechallenge. Other T cells may have their effector function silenced or terminated by anergy, apoptosis, or suppression, after interactions with other regulatory cells or soluble factors.

2.2.1.1- Allorecognition and the major histocompatibility complex

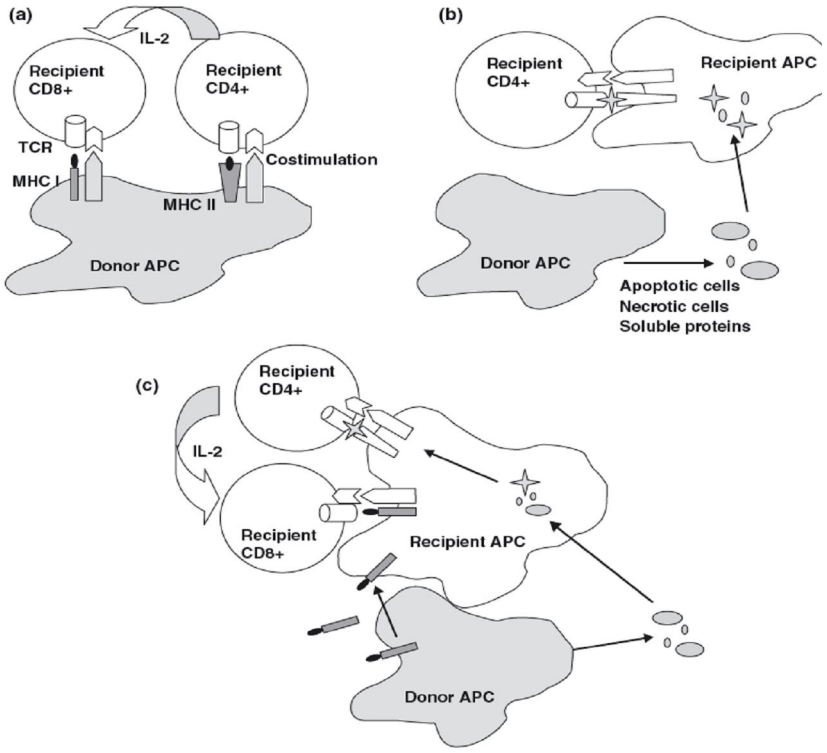
The time to rejection of a transplant as well as the duration of graft survival has been recognized as being dependent on the degree of genetic disparity between the donor and the recipient (*16, 17*). The genes responsible for this allogenic reaction have been defined as the major histocompatibility complex (MHC). In humans, this has been designated the human leukocyte antigen (HLA) system. The MHC in humans is found on chromosome 6, and comprises of many loci. The main relevant antigens in organ transplantation are coded by 3 of these loci: the class I HLA-A and HLA-B, as well as the class II HLA-DR antigens. Both the class I and class II HLA molecules consist of two different polypeptide chains. Class I molecules are constitutively expressed on the surface of almost all nucleated cells. They are important for recognition of target by CD8-positive cytotoxic T lymphocytes (CTL), and their expression can be upregulated by various cytokines such as tumor necrosis factor (TNF)-α and IFN-γ. However, class II molecules are expressed mainly on antigen-presenting cells (APC), such as macrophages, B lymphocytes, dendritic cells, and liver Kupffer cells. They are important for recognition of antigen by CD4-positive T cells. They can also be induced on other cell types by cytokines such as IFN-γ. These MHC molecules are responsible for presenting antigen to T cells via their peptide binding sites and hence are essential for allorecognition (recognition of antigens that are different among individuals of the same species).

T cells may recognize alloantigens via three distinct pathways: the direct, indirect and semi-direct. The direct pathway requires the recognition of intact donor MHC alloantigens on the surface of donor cells. This pathway could be an important driver of early acute transplant rejection. The second pathway of MHC allorecognition is generally referred to as the indirect

pathway and involves the internalization, processing, and presentation of alloantigens as peptides bound to recipient MHC molecules. There is evidence that indirect allorecognition is an important driver of transplant rejection and that the induction of tolerance in this pathway is a requirement for long-term transplant survival (*18*). A third pathway has been proposed based on the observation that if the trafficking recipient APCs acquire allogeneic MHC class I molecules from donor tissues, they can simultaneously stimulate indirect pathway $CD4^+$ and direct pathway $CD8^+$ T cells, thus allowing $CD4^+$ T cell help to be effective for the generation of cytotoxic T cells (*19*).

Over the past few years, the HLA class I molecule HLA-G, located in the HLA class I region of chromosome 6, has been described as having low polymorphism and having the main function in physiological conditions of abrogating maternal NK (natural killer) cell activity against foetal tissue and to establish immune tolerance at maternal-foetal interface (*20*). HLA-G can be viewed as a tolerance molecule from the following functional properties; (i) HLA-G can inhibit APC and $CD4^+$ which both express HLA-G receptors; (ii) HLA-G acts further upstream of the immune response by up-regulating inhibitory receptor expression which might subsequently render immune cell activation more difficult, and (iii) by inducing suppressor T cells, the inhibitory effect of HLA-G affects the reactivity of the immune system as a whole, by shaping its repertoire for the long term (*21*).

Figure 1*- Pathways of allorecognition. (a) Direct pathway. Recipient T cells recognize intact allogeneic $CD4^+$ MHC on APCs. Once primed by the donor APC, allospecific $CD4^+$ T cells can procure help for the effector function of $CD8^+$ T cells that have been activated by the same APC. (b) Indirect pathway. Allogeneic MHC molecules shed from the graft (soluble MHC molecules or dying/apoptotic cells) are taken up and processed by recipient APCs to be presented as peptides in the context of self-MHC molecules. (c) Semi-direct pathway. Recipient APCs acquire and present intact donor MHC I molecules to $CD8^+$ T cells through the direct pathway and simultaneously present internalized and processed donor MHC molecules to $CD4^+$ T cells through the indirect pathway. As T cells with direct and indirect allospecificity are primed by the same APC, linked-help can occur.



*Golshayan et al. *Transpl Int* 2007; **20**: 12-24.

2.2.1.2- T-cell receptor

There are two types of T-cell receptors (TCR): αβ and γδ. Each is formed by two distinct polypeptide chains. In organ transplantation, γδ-bearing T cells are thought to be the most important. The antigen specificity of any TCR is determined by the complementary determining regions (CDRs), which are coded for on the variable region of each receptor chain. The CDRs of the two chains of the TCR together form the antigen binding site.

2.2.1.3- T-cell activation and response

Complete activation of T cells requires two distinct, but synergistic, signals. The first signal is provided by a specific antigen and is delivered via the T-cell receptor. The second signal (co-stimulatory signal) is not antigen-specific. Instead, many T-cell molecules may serve as receptors for co-stimulation. The most well characterized co-stimulatory molecule is CD28, which has two ligands (B7-1 [CD80] and B7-2 [CD86]) that are expressed primarily on APCs. Another molecule, CTLA-4, is similar to CD28 and is also expressed on T cells. Although CTLA-4 binds CD80 and CD86, it transmits an inhibitory signal that serves to terminate the immune response. The interaction of IL-2 with its receptors constitutes signal 3 in T-cell activation. IL-2 acts both as an autocrine and paracrine growth factor. It results in a number of intracellular events that lead to DNA synthesis as well as T-cell differentiation.

T-cell activation results in the production of a variety of cytokines and clonal expansion. In this stage, CD30 is a membrane glycoprotein that belongs to the tumor necrosis factor TNF superfamily. It is expressed on activated cells, preferentially those secreting Th2-type cytokines, although the CD30 molecule is not considered as a physiologic marker of Th2 cells but rather as a co-stimulatory molecule regulating the balance between Th1/Th2 responses (*22*).

2.2.2- Effector stage

A variety of effector mechanisms participate in allograft rejection. The most common are cell-mediated reactions involving delayed-type hypersensitivity via macrophages and CTL-mediated cytotoxicity; less common mechanisms are antibody-plus-complement lysis and destruction by antibody-dependent cell-mediated cytotoxicity. In each of these effector mechanisms, the cytokine secretions play a central role after T-cell activation. IL-2 promotes T-cell proliferation and is generally necessary for the production of effector CTLs. IFN-γ is central to the development of a DTH response, promoting the influx of macrophages into the

graft and activation into more destructive cells. TNF-β has been shown to have a direct cytotoxic effect on the cells of a graft. A number of cytokines promote graft rejection by inducing expression of class I and II MHC molecules on graft cells. The interferon (α, β), TNF-α and TNF-β all increase class I MHC expression, and IFN-γ increases class II MHC expression as well. During a rejection episode, the levels of these cytokines increase, inducing a variety of cell types within the graft to express class I or class II MHC molecules. At this stage, GrB and perforin, proteins released by effector cells, can induce apoptosis in target cells by forming transmembrane pores and through cleavage of effector caspases such as caspase-3 (*23*).

2.3- Current immunosuppressive agents

Immunosuppressive drugs that have been introduced since 1985 have led to combination therapies that have significantly lowered the rates of acute rejection. All immunosuppressive drugs have specific side effects and additionally contribute to an overall state of IS, which lead to an increased risk of infections and cardiovascular disease and various specific malignant conditions. Over the past two decades, empirical trials have led to protocols of combination therapy that reduce side effect but yet maintain graft survival (*24*).The immunosuppressant agents act to inhibit the various pathways of this T-cell activation process. Their mechanism of action and side effects are summarized in Table 2.

Calcineurin inhibitors

Calcineurin inhibitors (CNI) continue to play a dominant role in maintenance IS in pediatric solid organ transplantation (*25*). The introduction of calcineurin inhibitors agents (cyclosporine A and tacrolimus) have radically improved the short-term outcomes in transplantation, their biggest impact being on the reduction in the incidence of acute rejection and enhancement of short-term graft survival (*26*). The current focus of clinical trials on this class of drug is therapeutic monitoring to minimize adverse events and specifically the nephrotoxicity associated with cyclosporine and tacrolimus.

Antimetabolites

Azathioprine (AZA) and mycophenolate mofetil (MMF) both inhibit purine synthesis, leading to failure of T-cell clonal expansion. However, azathioprine's antiproliferative effect is mediated via depletion of adenosine, and therefore is nonspecific in its inhibition of all

proliferating cells. MMF, on the other hand, blocks guanosine synthesis. Because lymphocytes cannot efficiently utilize the "salvage" pathway for guanosine synthesis, the antiproliferative action of MMF is relatively lymphocyte specific (*27*).

TOR inhibitors

Sirolimus (rapamycin, *Rapamune*) is a macrocyclic lactone produced by a strain of Streptomyces hygroscopicus. The TOR inhibitors, act by blocking the serine-threonine kinase mammalian TOR (mTOR). Sirolimus binds mTOR, making the enzyme unavailable to the activation pathway and effectively inhibits T and B cell proliferation. Blockade of mTOR diminishes lymphocyte responses to constimulatory signal 2 during G_0 and G_1 transition to cytokine signal 3 during the G_1 buildup. Sirolimus has also been shown to be an inhibitor of smooth muscle cell proliferation and intimal hyperplasia. With a 62-hour half-life, sirolimus is typically given once a day. One key difference in the pediatric population is the rapid metabolism of sirolimus which necessitates therapeutic drug monitoring via trough sirolimus levels in children (*28*).

Biological immunosuppressive agents

OKT3 is a murine monoclonal antibody against the human CD3 antigen. OKT3 is associated with significant clinical adverse events associated with its infusion. The cytokine release syndrome can be problematic despite adequate premedication (*29*). OKT3 has fallen out of favor as an induction agent in pediatric solid organ transplantation and has been surpassed by the antibody therapy agents: anti-IL-2 receptor antagonists and polyclonal antilymphocyte antibodies, antithymocyte globulin (ATG), and antilymphocyte globulin (ALG).

Recent research has focused on the role of the IL-2 receptor in acute allograft rejection. T-cell proliferation, a central event leading to graft rejection, is triggered by the interaction of IL-2 with its receptor on activated T cells. Anti-CD25 mAbs that selectively block IL-2 receptors (IL-2 receptor antagonists) on activated T helper cells are being used prophylactically as induction therapy. Compared with transplant-specific recipients of all ages, the use of anti-IL-2 receptor antagonists appears more common in pediatric kidney recipients, less common in pediatric heart recipients, and approximately equivalent in pediatric liver recipients (*25*).

Polyclonal antibodies are produced by immunizing animals with human lymphoid cells. The immunized animal produces numerous antibodies, each from an individual cell clone, in response to the various antigens contained on the immunogen. Cultured lymphoblasts (producing ALG) or human thymocytes (producing ATG) are the most commonly employed immunogens. ATG or ALG have been used to target multiple antigens on lymphocytes leading to modification of cell surface receptors and cell lysis. However, there has been no reported controlled trial in pediatric transplantation comparing polyclonal induction therapy to no induction or to monoclonal therapy (*30*).

Corticosteroids

Corticosteroids have been part of all immunosuppressive regimens since the early days of transplantation. Their development has been critical in both prevention and treatment of rejection episodes in all solid organ transplantation. To date, over 90% of all pediatric renal transplantation are on corticosteroids at the time of discharge and 30 days after transplantation. Corticosteroids are associated with significant morbidity that is both cumulative and dose-dependent. With reflect to children, chronic steroids lead to infection, arterial hypertension, blood cholesterol, and growth delay despite minimizing cumulative dosing (*31*). Corticosteroid minimization protocols have gained significant favor in the pediatric population, especially in the renal and liver transplant recipients. Overall, the various corticosteroid minimization protocols can be simplified into three strategies: alternating doses, tapering doses over extended period of time, or complete avoidance. Each of these protocols has inherent strengths and weaknesses. Alternate-day steroid regimens have promising initial results with equivalent graft function at 1 year post-transplantation (*32*). In pediatric liver transplantation, Reding et al compared liver transplantation under steroid–free IS in 20 children, who received combined tacrolimus and basiliximab, with 20 matched historical recipients as a historical control group receiving tacrolimus and steroids. This study showed steroid-free IS was clearly associated with growth catch-up starting in the first weeks after transplantation, whereas in children who received steroids, growth was delayed until the introduction of alternate-day steroid treatment (*33*).

IS minimization is a realistic goal for the pediatric patient, as it brings with it the benefit of reduced patient morbidity from the toxicities associated with many immunosuppressive drugs. IS avoidance for some of these drugs, especially steroids, is very

attractive and many single-center studies support the relative safety of this approach. Application of these IS minimization regimes to all pediatric patients, and the choice of the appropriate drug combinations to do this with a high safety index for rejection and infection, remains as current challenges for the transplant physician (*26*).

Experimental molecules: Targeting inhibitory costimulatory pathways

In addition to interruption of positive costimulatory signals, the transduction of negative signals to T cells through natural inhibitory molecules also shows promise in inhibiting graft rejection. Members of both the B7 family (including CD28 and CTLA4) and the TNF family, in which the CD40-CD154 pathway is preeminent, play key roles in the T cell response following alloantigen presentation (*34*).

CTLA4:B7 Pathway: The best studied of the inhibitory receptors is CTLA-4, which acts in part through competing with CD28 for its ligands B7.1 and B7.2, and in part through the transduction of signals through an immunoreceptor tyrosine-based inhibitory motif present in its cytoplasmic domain. The engagement of CTLA-4 transmits a negative signal to T cells, inhibiting activation. It has also been shown that CTLA-4 crosslinking can induce the production of transforming growth factor (TGF)-β and indoleamine 2, 3-dioxygenase, which act to downmodulate immune responses. Studies targeting this pathway for the prevention of transplant rejection are hindered by the fact that no soluble agents that are capable of inducing CTLA-4 signaling are currently available. In fact, it has been suggested that the use of soluble CTLA-4 fused to an immunoglobulin domain (CTLA-4Ig) may exacerbate immune responses in some models because of its blockade of inhibition through CTLA-4. However, the expression of crosslinking anti-CTLA-4 antibodies on cell membranes can prevent the rejection of allogeneic cells (*35*), suggesting that this may be a fertile area for future research.

CD40:CD154 pathway: CD40 is constitutively expressed on APCs such as B cells, macrophages, dendritic cells, and thymic epithelium, but can also be induced on endothelial cells and fibroblasts. CD154 is expressed on activated CD4 T cells, as well as on a subset of CD8 T cells, NK cells, and eosinophils. The CD40:CD154 interaction is remarkable in that this serves primarily to provide a costimulatory signal to the APC rather than to the T cell (reverse costimulation). In doing so, it significantly augments the ability of APCs to present antigen and to deliver positive costimulatory signals, which in turn indirectly promote T cell

activation. Importantly, antigen presentation by APCs lacking CD154 may be tolerogenic for T cells.

CD40 blockade using anti-CD154 promotes long-term allograft survival in a number of murine transplant models and synergizes with CD28 blockade. The effect requires prolonged administration, as abbreviated courses alone do not result in permanent engraftment of either islet cell or cardiac allografts. The addition of donor antigen at the time of transplantation augments the percentage of recipients obtaining long-term engraftment, promotes donor specific tolerance, and can prevent chronic arteriopathy. Donor specific tolerance in this setting is dependent on the presence of CD4 cells, IFN-γ, and CTLA4 signaling. In concordance with these findings, $CD154^{-/-}$ mice do not reject cardiac allografts but do develop chronic allograft vasculopathy suggestive of ongoing CD154-independent immune mediated allograft injury (*36*).

Table 2 - Immunosuppressive agents: Mechanism and side effects

Agent	Mechanism of action	Side effects
Anti- metabolite		
Mycophenolate mofetil	Inhibits purine synthesis	Gastrointestinal upset Bone Marrow Suppression
Azathioprine	Inhibits purine synthesis	Drug-Induced Hepatitis Bone Marrow Suppression
Cyclosporin	Inhibits calcineurin, thereby Inhibiting IL-2 production	Hirsutism Hypertension Nephrotoxicity Neurotoxicity Gingival Hyperplasia
Tacrolimus	Inhibits calcineurin, thereby Inhibiting IL-2 production	Nephrotoxicity Hypertension Glucose Intolerance Neurotoxicity

Sirolimus (Rapamycine)	TOR inhibitors Inhibits T and B cell proliferation	Hyperlipidemia Leukopenia, Thrombocytopenia Inhibits wound healing
Biological agents		
ATG/ALG	Binds multiple antigens on lymphoid cells, resulting in modification of cell surface receptors and cell lysis	Cytokine release syndrome Serum Sickness Thrombocytopenia Leucopenia Increased viral infections Increased PTLD
OKT3	Bind to T-lymphocyte surface antigen CD3	Same as ATG/ALG
IL-2 Receptors antibody	Block the stimulation of T cell IL-2 receptor sites by IL-2 and subsequent T cell proliferation	No major side effect
Corticosteroids	Block cytokine gene transcription via inhibition of NFAT	Glucose-intolerance Hypertension Growth retardation Osteopenia Dermatological complications Hyperlipidaemia

2.4- Transplantation tolerance

2.4.1- Terminology

A clear, widely accepted definition of the immune statuses underlying the term "tolerance" constitutes a minimal requirement for the study of immunological monitoring in solid-organ transplant recipients. Several concepts should be carefully distinguished, within a proper terminology. ***Transplantation tolerance*** is characterized by an absence of donor specific alloreactivity *in vivo* as well as *in vitro*, and it is believed to occur through central mechanisms within the thymus (*37*). ***Operational/clinical tolerance*** is defined as the absence of acute and chronic rejection, and indefinite graft survival with normal function in an IS-free, fully immunocompetent host, usually as the end result of a successful attempt at IS withdrawal (*38*). In contrast with transplantation tolerance, operational tolerance does not necessarily mean complete unresponsiveness of the recipient immune system toward the donor cell (split tolerance), but rather refers to the lack of a destructive immune response toward the graft despite the presence of generalized immune competence (*39*). ***Prope tolerance***, as proposed by Calne, describes a state of "almost tolerance" in patients who maintain normal allograft function and histology under minimal IS, usually a monotherapy calcineurin inhibition with infra-therapeutic blood levels (*40, 41*). ***Graft acceptance*** defines the common situation in which a transplant recipient has a normal immunosuppressive load, with absence of immune injury toward the graft.

2.4.2- Mechanisms of tolerance

Transplantation tolerance is typically divided into two categories: central and peripheral tolerance (Figure 2). In a clinical transplantation setting, tolerance is the result of at least three processes including deletion, anergy, and peripheral regulation (*42, 43*).

(1) *Deletion is a powerful mechanism,* as evidenced by the robust nature of neonatal tolerance and can occur centrally through the thymus (central deletion) or peripherally via passive death or activation-induced cell death (peripheral tolerance). Apoptosis of T cells leading to deletion, can be mediated through two distinct mechanisms. Firstly, engagement of death receptors such as Fas, most of which belong to the TNF receptor superfamily and contain an intracellular death domain, signal *via* the caspase pathway, resulting in cell death. Secondly, withdrawal of cytokines responsible for T cell survival such as IL-2, IL-4, IL-7, and IL-15 (all sharing the common γ chain) promotes T cell death by altering the activity of Bcl-2

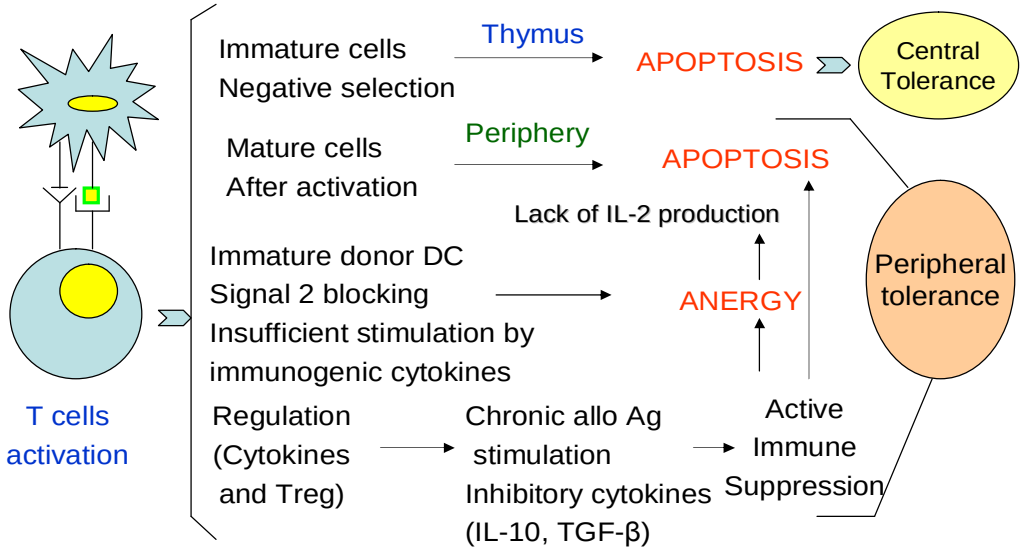
family proteins. Several strategies aiming to induce the death of donor-reactive recipient T cells have been devised and examined in both experimental and clinical models. Deletion may contribute to the long-term allograft survival that occurs after co-stimulation blockade (*44*). Combined bone marrow and organ transplantation in conditioned recipients who were treated with a short course of various immunosuppressants (*e.g.*, co-stimulation blockers, cyclosporine) has been shown to promote the development of robust tolerance in a number of rodent and preclinical transplant models as well as in humans (*45, 46*).

(2) *Anergy of allogenic T cells* can be defined as unresponsiveness to antigenic stimulus through lack of co-stimulation, characterized by functional inertia with inability to respond to subsequent antigenic stimuli including an impaired capacity to produce certain cytokines (*e.g.*, IL-2) or the presence of other cytokines (*e.g.*, IL-10) (*47*). Activation of naïve T cells requires a combination of TCR ligation plus a positive co-stimulatory signal. Early examples of co-stimulatory molecules that are expressed by T cells include CD28 and its ligands CD80/CD86 and CD154 and its ligand CD40. Subsequently, a number of additional co-stimulatory molecules (4-1BB and OX40), many of which belong to the TNF receptor superfamily, have been described (*48*). A number of inhibitory co-stimulatory molecules, such as CTLA4, which binds to CD80 and CD86, and PD-1 and its ligand PD-L, act to inhibit T cell activation and may contribute to development of anergy (*36*). Regardless of how lymphocytes become anergic, they persist in a functionally impaired state. Importantly, this state is reversible under certain circumstances, suggesting that transplantation tolerance that is mediated by anergy alone may not persist indefinitely. It should be noted that some but not all anergic T cells eventually will be deleted by the process of apoptosis (*49*). Recently, LEA29Y/Belatacept, a CTLA4-Ig fusion protein identified as an agent blocking T cell co-stimulatory signals, has been found to be beneficial when introduced into clinical transplantation procedures (*50*).

(3) *Peripheral regulation* of Tregs is emerging as a key mechanism for actively inducing and maintaining unresponsiveness to donor alloantigens (*51*). Several populations of cells have been identified in tolerance models as well as in clinical settings including $CD4^+CD25^+$, $CD3^+CD4^-CD8^-$, $CD8^+CD28^-$, and $NK1.1^+$ T cells. Recently, the transcription factor Foxp3 has been shown to be highly expressed in $CD4^+$ Tregs. In fact, mutation in the X chromosome–encoded Foxp3 gene is the cause of the fatal autoimmune disorder observed in

patients with IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) (*42*). The role of Tregs in general immune homeostasis and protection from autoimmune syndromes is now well established. Similarly, there has been increasing evidence for Tregs involvement in allograft rejection, current immunotherapies, and transplantation tolerance. However, despite significant advances in understanding the development, function, and therapeutic efficacy of Tregs in certain well-defined rodent models, the relevance of Tregs to clinical transplantation remains unclear (*52*).

Figure 2- Mechanisms of tolerance



2.5- Potential assays in immunological monitoring in adult and pediatric transplantation

Assays being developed for the immunological monitoring of the alloimmune response can be broadly divided into antigen-specific and antigen non-specific (Table 3). This review focuses on the several immunological assays already evaluated (Table 4) or in progress to be developed in pediatric organ transplantation.

2.5.1- Antigen-specific assays for immune monitoring

The development of immunologic memory and antigen specificity are hallmarks of the adaptive immune system. Assays that evaluate donor-specific responses of recipient lymphocytes are likely to be informative in transplantation. However, this method is also limited by the availability of stored lymphocytes of donor origin (in the context of cadaveric transplantation) to perform donor antigen-specific assays.

2.5.1.1- Cell proliferation assays

Assessment of proliferative responses of human lymphocytes is a fundamental technique for the assessment of their biological responses to various stimuli. Most simply, measurement of proliferation involves the measurement of the number of cells present in culture before and after the addition of a stimulating agent; however, this can be both labor intensive and difficult. The most common assessment of proliferation is performed by measuring new DNA synthesis, an essential process in cell division. The amount of new DNA synthesized can be assessed by measuring the incorporation of tritiated thymidine into DNA, a process which is closely related to underlying changes in cell number (15). *In vitro* induced proliferation assays have been used in the clinical setting for the assessment of an individual response to mitogens and specific antigens, including foreign alloantigens (15).

Table 3- Potential assays in immunological monitoring in adult and pediatric transplantation

Antigen-specific assays for immune monitoring
Cell proliferation assays
*Mixed lymphocyte reaction**
Limiting dilution assays
*Cell-mediated lymphotoxicity**
Tetramer technology
Measurement of cell proliferation by CFSE labeling
Enzyme-linked immunosorbent spots (ELISPOT)*
Delayed-type hypersensitivity (Trans- vivo DTH assay)
Detection of donor-specific-antibodies*
Detection of hematopoietic chimerism*
Non–antigen-specific assays for immune monitoring
Pre and post-transplant measurement of soluble immune mediators
*Circulating cytokines levels**
*Soluble CD30 levels**
Regulatory T cells*
Non–antigen-specific stimulation (Immuknow measurement)*
Analyses of T cell receptor repertoire
Proteomic biomarker
Detection of alloreactive T cell apoptosis
Detection of tolerogenic dendritic cells*
Gene analyses
*Gene polymorphisms**
*Detection of mRNA precursors**
*Microarray analysis of gene expression**
* : Evaluated in pediatric organ transplantation, references (*53*), (*54*), (*55*), (*56*), (*46*), (*57*), (*58*), (*59*), (*60*), (*61*), (*62*), (*63*), (*64*), (*65*), (*66*), (*67*)

2.5.1.1.1- Mixed lymphocyte reaction (MLR)

The primary *in vitro* response to the direct recognition of allogeneic molecules emerges in MLR. In this assay, PBMC of recipients are incubated with irradiated or mitomycin-C treated donor cells acting as stimulators for a period of 5–7 days. In the last 18 h of incubation, the cells are pulsed with tritiated thymidine. Following incubation, the cells are then harvested and lysed. The lysate including DNA is subsequently transferred to a scintillation counter for counting. The mean counts per minute (cpm), (a direct correlate of newly synthesized DNA and an indirect measure of the amount of cellular proliferation), are determined for background cultures and for each experimental condition. To correct for differences in background proliferation, the data may be presented as Δ cpm (i.e., experimental cpm– background cpm) or as the stimulation index, (i.e., experimental cpm/background cpm). One of the limits of MLR using tritiated thymidine incorporation is that it provides information on the entire cell population, not on individual cells or subsets of cells. It is not possible to establish specific functional characterization of proliferating cells (*68*).

Using MLR in pediatric living-donor liver transplantation, Koshiba et al showed that in operational tolerant recipients, increased proliferation of recipient T cells following stimulation with both irradiated donor APC and third party APC was observed in the presence of recipient $CD4^{+}CD25^{-}$ cells. However, suppression of proliferation following donor stimulation compared to third party stimulation was obtained in the presence of recipient $CD4^{+}CD25^{+}$ cells (*53*). This observation suggests the presence in the recipient of reactive T cells potentially to donor antigens which are suppressed by regulatory T cells.

This assay is a classical *in vitro* analysis of T-cell proliferation but has very little predictive value in the context of transplantation (*69*). For this reason, several approaches have been proposed to assess more accurately the reactivity of recipient cells toward donor antigens in pediatric organ transplantation.

2.5.1.1.2- Limiting dilution assays (LDA)

These assays provide more precise quantification of immunity to a given stimulus and allow the estimation of frequencies of antigen specific cells participating in an immune response. The technique consists of setting up multiple replicates of graded

dilutions of responder cells (recipient's PBMC) in wells containing a non-limiting stimulus (donor stimulator cells). The readout from a particular well is only considered positive if the measured result of the selected well exceeds the mean results of controls (cultures lacking responder cells) by a factor of three or more. The number of "negative" wells at each dilution of responder cells is determined. As the concentration of the responder cells increases, the proportion of "negative" wells will tend to decrease; the relation between the number of precursors can be plotted and a frequency obtained (*70*).

The ability of a LDA assay to predict the frequency of precursors depends on the number of replicates and the number of responder cells added per dilution (*71*). Different effector functions can be measured at different time points, including proliferation, cytotoxicity (determination of cytotoxic T-lymphocytes precursors) and cytokine secretion (determination of helper T-lymphocytes precursors). In each case, each well is classified as positive or negative, and the frequency of precursor cells able to mount such response can be, thus, calculated.

Cytokine production from recipient PBMC before and after donor-specific antigen stimulation may constitute a useful test for monitoring of acute allograft rejection. The production of different cytokines can be measured by MLR or LDA in the presence of supernatant cultures such as IFN-γ, IL-5, IL-4, IL-10, IL-13, or TNF-α present in the well. As a result, the validity of the Th1/Th2 paradigm in transplantation has been questioned. With this assay, Chung et al showed a highly significant correlation between the donor-specific- and third-party-stimulated IL-4 and IL-10 production from recipient PBMC with stable liver graft function as assessed by histopathology and/or biochemistry (*72*). $CD4^+$ Th cells can be divided into Th1, Th2 or Th7 on the basis of their cytokine production pattern. Nowadays, Th1/Th2 immune deviation tends to be seen purely in terms of the relative balance of cytokine accumulation, but it may well be that the relative chronology and intensity of the individual cytokine response is in fact more important than the overall relative balance of cytokine (*73*). Th1-type cytokines, including IL-2, IL-3, TNF-α, and INF-γ, mediate cellular immune responses and are pro-inflammatory, while Th2-type cytokines such as IL-4 and IL-10 have been shown to inhibit the development and function of Th1-cells, to suppress inflammation, and to enhance the humoral pathways of the immune response. Th17- type cytokines have been

shown to be important in the pathogenesis of autoimmunity. Their role in transplantation immunity is still unclear. Along with Th1 cells, Th17 cells may mediate allograft rejection, and their role may be more important when Th1 responses are suppressed (*74*).Th1-cytokines are mainly involved in allograft rejection, by up-regulation of MHC class I and class II expression, stimulating macrophage function (TNF-α, and INF-γ) by endothelial cell activation, up-regulation of cell adhesion molecules, and facilitating the recruitment and activation of leukocytes (TNF-α). Conversely, evidence from animal transplant models has suggested a role for Th2-cytokines such as IL-4, IL-5, IL-6 and IL-10 in promoting graft survival. Their mechanism of action would be in down-regulating pro-inflammatory cytokine production (*75*). IL-10 may also down-regulate T-cell activation by decreasing MHC class II and CD80/CD86 expression on APC, even if all of these hypotheses have been challenged by data showing that mice deficient in INF-γ rapidly reject fully mismatched cardiac allograft (*76*). Mice lacking STAT4, a transcription factor associated with the development of Th1 responses, also rejected a graft (*77*). On the other hand, Th2 cytokines have also been noted in grafts undergoing acute vascular rejection (*78*). As a result, the validity of the Th1/Th2 paradigm in transplantation has been questioned.

2.5.1.1.3- Cell-mediated lymphotoxicity

CTL assay is widely used to quantify the cytolytic activity of recipient T cells primed *in vivo* after transplantation (*54*). Specifically, recipient T cells are cultured with Cr^{51}-labeled target cells that express donor or irrelevant alloantigens for a short period, and lysis is determined by measurement of the amount of Cr^{51} released. In an early report that described a patient who displayed functional tolerance after kidney transplantation, Burlingham et al reported hyporesponsiveness to donor alloantigens in a CTL assay (*79*). More recently, Weimer et al reported using hyporesponsiveness in the CTL assays to guide drug withdrawal after renal transplantation (*80*). These data suggest that measures of the donor-specific cytotoxicity of recipient T cells after transplantation may be useful for guiding decisions about immunosuppressive drug management and for the identification of tolerant transplant recipients. Nevertheless, the assay typically assesses $CD8^+$ T cell–mediated activity only and does not give a comprehensive picture of alloreactivity (*49*).

2.5.1.1.4- Tetramer technology

Tetramers consist of four MHC-peptide complexes that are linked covalently to a fluorochrome. The binding of the MHC-peptide complex to the TCR of T cells that are specific for the given peptide-MHC molecule complex or allo-MHC allows the identification of antigen-specific T-cells *ex vivo* by flow cytometry, regardless of their ability to produce cytokines. The construction of class II MHC peptide tetramers has been technically more demanding than construction of class I MHC peptide tetramers. Additionally, the use of class II MHC peptide tetramers to detect and monitor antigen-specific $CD4^+$ T cells is less straightforward than that of class I tetramers for $CD8^+$ $CD4^+$. T cells specific for particular antigens typically circulate at very low frequencies, which are below the detection limit of flow cytometry. In order to detect these low frequency $CD4^+$ T-cells, an *in vitro* amplification step involving stimulation of the $CD4^+$ T-cells of interest using the studied antigen is necessary (*81*). Although peptide MHC tetramers are powerful tools, they have certain limitations. They can only be used to detect immune responses to known antigens, because the peptide of interest must be loaded into the peptide MHC tetramer and thus must already be known and synthesized. Within the field of solid organ transplantation, the difficulty in using this technique for immune monitoring is that unique tetramers will be needed for each donor/recipient combination and for direct and indirect immune responses (*68*).

2.5.1.1.5- Measurement of cell division by CFSE labeling

This assay measures the proliferative response of recipient lymphocytes that are cultured or stimulated with inactivated donor cells for a period of several days. In this assay, carboxyfluorescein succinimidyl ester (CFSE), an intracellular fluorescent label that divides equally between daughter cells, has been used to study cell division. Accurate counting of dividing cells can be achieved by the use of internal standards such as microspheres that allow enumeration of absolute cells as opposed to percentages (*82*). This approach offers several advantages, including the avoidance of radioisotopes, the ability to determine whether all cells undergo a few divisions or some cells undergo many divisions, and the ability to characterize the phenotype of the dividing cells using multicolor flow cytometric techniques. The limit of detection of this method is established by background proliferation and the number of cells acquired; a higher

sensitivity can be achieved if the dividing cells can be identified by a surface marker. CFSE labeling to assess alloantigen responses has been used widely in murine systems. In order to monitor antidonor alloreactivity for accurate diagnosis of acute rejection after living-donor liver transplantation (LT), Tanaka et al used a MLR-CFSE assay to distinguish rejection on suspicious biopsies (*83*). Despite the theoretical appeal of this assay, to date, no human studies have demonstrated a correlation between donor antigen–induced proliferation and the ability to wean off IS or the development of tolerance (*49*).

2.5.1.2- Enzyme-Linked Immunosorbent Spots (ELISPOT)

ELISPOT quantifies the frequency of previously activated or memory T cells that respond to donor antigens by producing a selected cytokine *in vitro*. In this assay, responder/recipient T cells are cultured with inactivated stimulator/donor or third-party cells on tissue culture plates that are coated with an antibody that is specific for the cytokine of interest including IFN-γ, GrB, IL-2, IL-4, IL-5, and IL-10. After a short incubation, the cells are washed away and the bound cytokine is detected, using labeled secondary antibodies and an automated plate reader. Because of the short culture period, each spot is detected represents a cell that had been primed to the stimulating antigen(s) *in vivo* (effector or memory T cells)

This assay is capable of detecting cytokine secretion by individual, antigen-reactive T cells within a population of PBMC. A second advantage is that ELISPOT can detect an immune response to donor antigens presented either through the direct pathway (recipient T cells cultured with donor stimulators) or the indirect pathway (recipient T cells cultured with recipient APC plus donor cell lysates or pulsed with peptides derived from donor cells). However, this assay detects and quantifies antidonor response but does not determine the mechanism responsible for negative response (*84*). Obtaining and storing sufficient number of recipient and donor cells to perform this assay repeatedly is another limitation especially in pediatric patients who usually have T lymphopenia during the early post-transplant period.

According to literature, monitoring of IFN-γ ELISPOT has been widely used in renal transplant. Van Besouw et al found that the IFN-γ ELISPOT assay is superior to the GrB ELISPOT assay as surrogate marker for CTL activity after third-party stimulation (*85*). Some studies demonstrated that IFN-γ could be a predictive marker for the risk of

rejection when tested before transplantation (*86-89*), and several authors confirmed the good correlation between IFN-γ-producing lymphocytes both with acute rejection (*89*) and with chronic rejection (*90*) in post-transplant episodes. In liver transplant recipients, the IFN-γ ELISPOT technique has been used to assess hepatitis C virus eradication and Epstein-Barr virus cytotoxic T cells (*91*). To the best of our knowledge, no report of GrB ELISPOT producing cells is available in the field of pediatric organ transplantation.

2.5.1.3- Delayed-type hypersensitivity (Trans- vivo DTH assay)

The *trans-vivo* DTH assay, as defined by Burlingham, has the ability to identify donor-specific unresponsiveness with linked recognition. In this assay, recipient PBMC plus donor antigen are transferred to the pinnae or footpads of "naïve" mice. If previously sensitized, recipient PBMC responds with a measurable DTH-like swelling response. As control, saline, third-party cells, and recall antigens such as tetanus or Epstein-Barr virus are injected into different footpads (*92*). The absence of a DTH response when donor and recipient cells are injected at a single site could represent the failure of recipient T cells to be primed *in vivo* in response to donor antigens or could be the result of an active regulatory mechanism that inhibits the antidonor response. To distinguish between these two possibilities, recipient T cells can be injected together with both donor antigen and recall antigens. The loss of a DTH response to recall antigens in this setting could be attributed to bystander suppression that is mediated by donor-reactive regulatory T cells. That this is indeed the case has been demonstrated by the recovery of DTH responses to donor antigen or donor antigen/recall antigen combinations after the injection of neutralizing antibodies to TGF-β and/or IL-10. The *trans-vivo* DTH assay detects the presence of donor reactive T cells primed through the direct or indirect pathways. Moreover, this assay has the potential to distinguish between deletional tolerance and tolerance maintained through regulation. As in the ELISPOT assay, the *trans-vivo* DTH assay requires donor antigen in order to quantify donor-specific responses. In addition, because of the dependence of this assay on mice, it is difficult to envision its application for routine clinical monitoring (*84*). In a small cohort of transplant recipients, clinical tolerance may be associated with active immune regulation characterized by donor antigen–linked DTH unresponsiveness (bystander suppression) mediated in part by TGF-β or IL-10 (*93*). However, in their study including 420 recipients of a primary kidney or

simultaneous kidney/pancreas organ(s) transplant, Pelletier et al found that the presence of an anti-inflammatory DTH response to donor antigens did not correlate with an improved clinical outcome at a median of nearly 5 years after transplantation. So these authors suggested that detection of an anti-inflammatory T cell response to donor antigens may not allow the identification of patients that have developed graft protective cellular regulatory responses (*94*).

2.5.1.4- Detection of donor-specific-antibodies

Crossmatching is routinely performed to detect recipient antibodies specific for donor antigens using the complement-dependent cytotoxicity assay in which recipient sera are mixed with donor cells and complement with or without antihuman globulin. The crossmatch technique can also be performed using flow cytometry or ELISA which have been shown to be more sensitive (*95*). Antibodies specific for HLA have a greater impact on allograft survival than do antibodies against non-HLA molecules. Recently, the Luminex bead-based screening assays have been used to identify both complement-binding and non-complement-binding HLA class I and II antibodies in recipient sera. The validity of this technique has been shown for class I as well as for class II alloantigens with high sensitivity and specificity. The clinical impact of class II DQ and DP antibodies is still unknown (*96*).The ability of donor-specific MHC complex alloantibodies to destroy a transplanted organ within minutes, the so-called hyperacute rejection phenomenon, has been known for a long time. Increasing evidence now suggests that humoral responses to alloantigens could play an important role in both acute and chronic alloimmunity, particularly following activation of the indirect pathway; consequently, the detection of alloantibodies should be mentioned in the list of candidate assays for the immunological monitoring of transplant recipients (*97*). A positive correlation between anti-HLA antibodies and poor graft outcome was established in kidney, lung and liver recipients, especially in pediatric heart transplantation whether those antibodies were present before grafting or appear after transplantation (*56, 98*). In the future, rather than detecting donor-specific antibodies that indirectly reflect the number and function of plasma cells, it might be preferable to develop assays that directly measure the frequency and function of donor-antigen-specific B cells by identifying the precursor frequency of

alloantibody-secreting B cells and simultaneously determining the specificity of the anti-HLA antibodies produced (*84*).

2.5.1.5- Detection of hematopoietic chimerism

Chimerism is defined as the existence of replicating cells from different genetic backgrounds in a single organism (*99*). In *microchimerism*, donor-specific cells, usually dendritic cells (DC), are present early post-transplant at low frequencies ($\leq 1/10^4$ to 10^5 cells) in recipients, especially after LT, and usually disappear within the first 3 weeks post-transplant. In contrast, *macrochimerism* or *mixed chimerism* is defined by the persistence of more than 5% of circulating donor-derived cells, as observed following hematopoietic stem cell grafting, a condition classically associated with intrathymic deletion of donor-reactive T cells and central tolerance (*44*). Complete hematopoietic chimerism classically occurs in bone marrow transplantation, during which all bone marrow–derived cells in the recipient are eliminated and replaced by donor cells (*45*). In LT patients, high levels of early microchimerism did not abrogate the persistence of an alloreactive response at 1 year posttransplant, such microchimerism being now considered as a consequence of graft acceptance under maintenance IS rather than as a marker of allogenic unresponsiveness (*100, 101*). Recently, Alexender et al reported mixed hematopoietic chimerism followed by full tolerance of a liver allograft from a deceased young male donor which developed in a 9-year-old girl, with no evidence of graft-versus-host disease 17 months after transplantation (*46*). Some studies presented recently that combined kidney/ bone marrow transplantation in HLA-mismatched patients with nonmyeloablative conditioning to achieve persistent mixed chimerism can reach renal allograft tolerance as well as excellent myeloma ablation responses (*102-104*).

2.5.2- Non–antigen-specific assays for immune monitoring

Besides the assays mentioned above which studied the response of T cells to donor antigens, a number of non-antigen-specific assays for immunological monitoring in pediatric transplantation have also been described. More sophisticated approaches have been suggested in the light of technological advances.

2.5.2.1- Pre and post-transplant measurement of circulating cytokines levels

This assay is an ELISA measuring production of soluble immune mediators such as circulating cytokines which are secreted by the Th cells which can be activated *in vivo* by donor antigens but the *ex vivo* assay is not donor specific.

The validity of monitoring peripheral cytokines to identify recipients accepting or rejecting their grafts has already been tested in pediatric transplantation. However, the analysis of the literature correlating circulating cytokines levels to the post-transplant immunological status (rejection versus graft acceptance versus operational tolerance) has remained confusing, providing contradictory results (*37*). The immunological and clinical relevance of circulating cytokine levels in organ transplantation is subject to several drawbacks, including the presence of confounding factors (surgical stress, blood transfusions, ischemic-reperfusion injury, hepatic regeneration, infectious complications), and the lack of serial cytokine profiles in most published literatures (*105*). Moreover, circulating cytokines levels should only be regarded as an indirect evaluation which may not necessarily reflect the exposure to cytokine locally within the allograft or the lymphoid organs of the recipient. Indeed, these works, most of them published in the mid 1990s, have not resulted in standard guidelines for the immunological monitoring in clinical organ transplantation.

Considering some of the limitations of blood cytokines measurements listed above, it has been hypothesized that the kinetic analysis (rather than individual values) of blood cytokines in the early post-liver transplantation period may provide interesting clues as to the immunological evolution of the allograft. The study of 40 pediatric LT recipients at Saint-Luc University Clinics, Brussels, showed that patients with early graft acceptance have a statistically significant IFN-γ and TNF-α decrement observed as soon as one hour following portal reperfusion. This work also confirmed that the IL-10 peak occurs within the first hours after LT. These results suggested that combination of seric IL-10, IFN-γ and TNF-α monitoring could be an important tool in the diagnosis of graft acceptance in children (*57*). Another promising avenue of cytokine research focuses on Th17, a recently identified T lymphocyte subset producing high levels of IL-17 which has been associated with acute rejection in kidney transplantation (*106-108*). More over, it

has been showed that CD4 Th17 cells are a major mediator of vascular inflammation (*109*).

2.5.2.1- Regulatory T cells

Regulatory T cells (Tregs) constitute an important mechanism of immune regulation and are essential for the induction and maintenance of tolerance against self-antigens in the periphery. Depending upon the experimental systems studied, a variety of Tregs, differing in phenotype and mode of suppression, have been identified.

2.5.2.1.1- Naturally occurring Tregs

Naturally occurring $CD4^{+}CD25^{high+}$ Tregs develop in the thymus and are predominantly found in the lymphoid organs. $CD4^{+}CD25^{high+}$ Tregs are known to suppress effector T cell proliferation and differentiation *in vitro* through a contact–dependent cell mechanism that is largely cytokine independent (*52*, *110*). Other cell-surface markers, such as CD45RB, CTLA-4, glucocorticoid-induced TNF receptor family–related receptor (GITR or TNFRSF18), CD122, CD103 ($\alpha_E\beta_7$ integrin), CD134 (OX40), and CD62L (L-selectin), whose relative expression levels can be used to define and isolate $CD4^{+}CD25^{high+}$ Tregs have also been identified. Transcription factor Foxp3 is critical for the development and function of Treg cells. Gene Foxp3 is expressed constitutively in human $CD4^{+}CD25^{high+}$ T cells with suppressor function, but it is also expressed in other human T cells after activation. Therefore, expression of Foxp3 mRNA and protein do not represent reliable markers for the identification of Treg cells in humans. Recently a human Treg marker of unprecedented specificity was identified through the analysis of epigenetic modifications of gene Foxp3 (*111*). Constitutive expression of Foxp3 in murine and human Treg cells was shown to correlate with the unmethylated status of CpG dinucleotides located in a conserved region of Foxp3 intron 1. In contrast, these CpG dinucleotides were methylated in other $CD4^{+}$ T cells, and remained methylated after activation even though gene Foxp3 was transiently expressed. Quantitative methyl-specific PCR assay (MS-qPCR) have been developed to analyse the frequency of cells with demethylated Foxp3 intron 1 in human PBMC samples (*112*). Such a MS-qPCR might be used to monitor Treg frequency in transplanted patients.

2.5.2.1.2- Inducible $CD4^{+}$ Tregs

There are 2 populations: Th3 cells and Tr1 cells. Th3 cells, through the secretion of TGF-β, promote immune tolerance following the ingestion of antigens. Tr1 cells are similar to Th3 cells, but they secrete large amounts of IL-10 and tend to migrate toward sites of inflammation (*113*). Induced Tregs depend on peripheral factors such as the maturity or type of the stimulating APC and the availability of cytokines such as TGF-β. Both Th3 and Tr1 cells appear to function independently of cell-to-cell contact and suppress immune responses through the secretion of immunosuppressive cytokines, such as IL-10 and TGF-β (*114*). Tr1 cells maintain peripheral tolerance, control autoimmunity, and prevent allograft rejection and graft versus host disease. Cellular therapy with *ex vivo* generated Tr1 cells has been proven to be effective in several preclinical models of T cell-mediated pathologies and therefore represents a promising approach for clinical application (*115*).

2.5.2.1.3- Other Treg types

Treg subsets have also been described outside the $CD4^+$ compartment including $CD3^+CD4^-CD8^-$ (double negative) cells and $CD8^+$CTLA4+ $Foxp3^+$ cells (*116-118*). Natural killer (NK) T cells are a distinct population of T cells. NK T cells were originally thought to mediate the innate immune responses that lyse tumor cells and pathogens, but are also involved in autoimmune diseases. When stimulated by contact with antigen, NK T cells develop heightened killer-cell activity and produce large amounts of IL-4, INF-γ, TNF-β, and IL-10, all known to be involved in the activation of cells that mediate inflammation, innate immunity, and Th2-type immunity (*42*). These cells have been detected in autoimmune patients (i.e. diabetes) and are thought to form part of the patients' disease defense mechanism.

2.5.2.1.4- Regulatory T cells in transplantation

Both experimental and clinical studies indicate that manipulating the balance between regulatory and responder T cells is an effective strategy to control immune responsiveness after transplantation. The kinetics and function of Tregs have also been investigated by using *in vitro* functional assays and *in vivo* Treg adoptive transfer assays in a mouse model of orthotopic corneal transplantation. This study demonstrated that it is the level of Foxp3 expression in the cells which is far more relevant than Treg frequency. Foxp3 expression is directly associated with the potential of Tregs to prevent allograft

rejection by producing regulatory cytokines and suppressing effector T cell activation (*119*). In another experiment, Baecher-Allan et al reported the identification of a $CD4^+$ population of Tregs in the circulation of humans expressing high levels of $CD25^+$ exhibiting *in vitro* characteristics identical with those of the $CD4^+CD25^{high+}$ regulatory cells isolated in mice. With TCR cross-linking, $CD4^+CD25^{high+}$ cells did not proliferate but instead totally inhibited proliferation and cytokine secretion by activated $CD4^+CD25^-$ responder T cells in a contact-dependent manner (*120*). Based on some of most recent work from the Pittsburgh group, these authors proposed several new strategies to achieve transplant tolerance in rodent animals via manipulating Treg function, including the use of histone deacetylase inhibitor to regulate Foxp3 transcription and to enhance Treg suppression, induction of Treg-sparing apoptosis via Nur77, and identification of the co-inhibitory molecule herpes virus entry mediator as an effector molecule for Treg function (*121*). In adult allograft patients, several studies have shown that recipient-derived Treg cells are involved in transplantation tolerance by directly inhibiting effector T cells, and keeping their proliferation under control (*119, 122, 123*). In the setting of pediatric living-donor LT, where 87 patients successfully achieved complete withdrawal of IS, Koshiba et al showed that operationally tolerant patients exhibited a significantly higher proportion of $CD4^+CD25^{high+}$ cells within peripheral blood lymphocytes, compared with patients under immunosuppressants and age-matched healthy volunteers (*53*). Similarly, in pediatric liver recipients with acute rejection, Sternard et al demonstrated that the levels of circulating $CD4^+CD25^{high+}$ $Foxp3^+$ Tregs significantly decrease in these patients. In addition, this study also showed that $Foxp3^+$ Tregs were increased in the portal region of livers with histopathologic evidence of rejection and were localized primarily within the inflammatory infiltrate (*59*). This finding correlated with another report of immunohistochemical identification of $Foxp3^+$ cells in human grafts, with Veronese et al who detected elevations in $CD4^+$ $Foxp3^+$ cells in renal allografts during cellular graft acute rejection as compared to humoral rejection, suggesting that the Tregs presence in the graft may be regulating the immune process (*124*). In contrast, the Kyoto LT program found high expression of $Foxp3^+$ in liver grafts derived from tolerant patients, implying the presence of Tregs within tolerant grafts (*125*). To address the question as to where and how Tregs exert their suppressive activity, further research will be required, to

determine the balance between Foxp3 and innate/adaptive immunity, and the linkage between Foxp3 and cytokines/co-stimulatory factors in tolerant graft (*53*).

2.5.2.3- Non–antigen-specific stimulation (Immuknow measurement)

Immuknow assay directly measures cellular immune function by quantification of intracellular adenosine 5-triphosphate levels in $CD4^+$ lymphocytes after phytohemagglutinin stimulation. This assay has the ability to discern between immune profiles of over-IS and under-IS by looking at the actual responsiveness of the patient's $CD4^+$ lymphocytes, as a surrogate marker of the patient's immune function. The immuknow assay showed a better correlation than drug-level measurements in patients undergoing a stable post-transplant clinical course. Furthermore, immunosuppresion drug levels measurements did not reliably reflect the immunological quiescence of these patients but rather misleadingly depicted these patients as either over- or under-immunosuppressed. Comprehensive reports have described its utilization in the immune monitoring of adult transplant recipients (*126*), and smaller scale studies have demonstrated its relevance in pediatric renal transplant recipients (*60*). The pilot study in pediatric liver transplantation showed that the Immuknow assay could serve as a reliable and unique parameter of the cellular immune function (*61*).

2.5.2.4- Analyses of T cell receptor repertoire

It has been hypothesized that immune responses to autoantigens, tumor antigens, and alloantigens perturb the T cell repertoire as indicated by the relative increase or decrease in the number of T cells expressing a given TCR Vβ. This assay uses PCR to analyze the T cell repertoire with respect to Vβ chain use and CDR3 length. During T-cell ontogeny, the β chain undergoes somatic rearrangement of four noncontiguous gene clusters, V, D, J, and C, resulting in a large repertoire of TCR molecules. The TCR antigen-binding site is formed by three CDR, CDR 1 and 2 being encoded by sequences of C genes alone; CDR 3 consists of rearranged sequences of V, D, and J genes, plus the random insertion of nucleotides either side of D region, and represents the most variable TCR region in contact with the central residues of the bound peptide. Therefore, the analysis of TCR-Vβ usage by determining CDR 3 may provide valuable information on the composition of the T-cell repertoire selected during an immune response. Comparing TCR Vβ usage in long-term kidney transplant recipients with variable levels of graft

acceptance from chronic rejection to operational tolerance, as well as in healthy individuals, Alvarez et al evidenced strongly altered Vβ usage, including an increased frequency of oligoclonality and a decreased frequency of polyclonality (*127*). Similarly, Brouard et al in Nantes showed in a limited number of patients a unique blood TCR pattern characterized by a restricted CDR 3 length distribution (*128*). Longitudinal studies will be necessary to determine the stability of the repertoire changes observed. Also, functional studies focusing on subsets of T cells (i.e., naïve vs memory, $CD4^+$ vs $CD8^+$, and Tregs) will be critical for increasing the sensitivity of the assay and gaining deeper insight into the mechanisms (*84*).

2.5.2.5- Proteomic biomarker

In recent years, proteomics have been applied in the search for biological markers of acute allograft rejection. As proteins are involved in different cellular processes, an understanding of proteins inside the cell provides an insight into the cellular events. The proteomics approach might provide an unbiased high-throughput approach to identify differentially expressed proteins in the healthy and diseased states. Therefore proteomic analysis for biomarker discovery has been extensively applied to many fields of biomedical research, including oncology, diabetes, renal and urine-related diseases, and solid organ transplantation. Different proteomic methods are available for biomarker discovery efforts. Proteomic methods are broadly classified into gel-based and gel-free methods (*129*). In the context of organ transplantation, acute tubular necrosis, glomerulopathies, urinary tract infections, and cytomegalovirus viremia were not confounding variables in proteomic assay. However, protein arrays are subject to a number of challenges that are not encountered in designing DNA arrays. Firstly, proteins may undergo post-translational modification or require multimerization to function normally. Furthermore, proteins can not be amplified as can RNA, and they tend to be unstable (*49*). A recent report has demonstrated that proteomic technology using mass spectrometry can define those proteins present in the urine of renal transplant recipients that are associated with acute and chronic cellular rejection (*130, 131*).

2.5.2.6- Detection of alloreactive T cell apoptosis

Clonal deletion may occur in the thymus or peripherally by apoptosis, that is the process of cell death during allograft rejection, not only in the animal models, but also in

clinical transplantation. During this apoptosis process, the cellular DNA is fragmented into pieces of approximately 180 bases pair length; these fragments can be nick-end labelled by biotinylated dUTP after the addition of terminal deoxynucleotidyl transferase. The biotinylated dUTP can be detected by enzyme- or fluorochrome-labelled avidin or streptavidin. This method for detection of apoptosis, known as the TUNEL assays (Terminal deoxynucleotide transferase-mediated dUTP Nick End Labeling), can be used for flow cytometry, or performed directly in the allograft or lymphoid tissues of the recipient using immunohistology. Whereas the TUNEL assay detects effectively the advanced stage of cell death, as evidenced by DNA fragmentation, the annexin-V assay can be performed alternatively to detect ongoing apoptosis in its early stage (*132*). Such approaches provided substantial evidence that apoptosis of graft-infiltrating T cells contributes to the spontaneous acceptance of mouse and rat liver allografts. The TUNEL assay is also used to investigate the effect of IS on hepatocyte proliferation and apoptosis in a young animal model of liver regeneration (*133*). Apoptosis can also be detected by molecular imaging techniques considered non-invasive, to quantify and monitor immune events potentially affecting the graft. Given the vast number of ligands and enzymatic precursors that can be radiolabeled, nuclear methods are well suited for imaging of molecular events. Some studies in an experimental rodent model showed that radiolabeled annexin V can be used to image apoptosis in acute transplant rejection (*134*, *135*). So far, to the best of our knowledge, no report of lymphocyte apoptosis detection is available in the context of clinical pediatric transplantation.

2.5.2.7- Detection of tolerogenic dendritic cells

Dendritic cells (DCs) are phagocytic and migratory leucocytes that process and convey antigens from the periphery for presentation to naïve T cells in secondary lymphoid organs. Evidence of animal experience suggests that immature DC may play a role in the regulation of T cell responses and can promote organ transplant tolerance. In humans, two major subpopulations of precursors (p) of DC have been described: monocytoid pDC and plasmacytoid pDC. Monocytoid DC ($CD11c^+$) can be derived from circulating monocytes in response to granulocyte-macrophage colony-stimulating factor and IL-4, whereas plasmacytoid DC ($CD123^+$) develop after stimulation with IL-3 and CD40L. Monocytoid DC, which induce Th1 cell differentiation *in vitro*, and

plasmacytoid DC, which promote Th2 cell responses, have been designated DC1 ($CD11c^{+}$ $CD123^{-/low}$) and DC2 ($CD11c^{-}CD123^{high}$) respectively, which may be specialized for the induction of immunity and tolerance, respectively (*136*). Mazariegos et al showed that precursors of plasmacytoid DC are increased in operationally tolerant LT recipients and in those being weaned from IS when compared to those receiving chronic IS (*62*). Accordingly these authors found that IS drugs themselves were not shown to affect the number of pDC2, but a pDC2/pDC1 subset ratio could serve to identify that a patient might be considered for IS weaning (*63*). Similarly, in pediatric LT, the Pittsburgh group also hypothesized that patients with operational tolerance and/or undergoing successful immunosuppressive drug weaning might exhibit an increased incidence of DC2 precursors relative to DC1 precursors (*137*). Longitudinal studies will be necessary to determine the stability of changes in the number of pDC2 and the pDC2/pDC1 ratio. It has still to be demonstrated that the higher ratio pDC2/pDC1 is a good marker of tolerance. If the number of pDC2 is increased in tolerant recipients before transplantation or there is an absence of donor specificity, this would imply that alterations in the balance of pDC2 and pDC1 reflect an inherent immunodeficiency rather than acquired tolerance (*84*).

2.5.2.8- Gene analyses

2.5.2.8.1- Gene polymorphisms

Gene polymorphisms, including both single-nucleotide polymorphisms and microsatellite regions, that involve regulators of immune responses such as cytokines, chemokines, adhesion molecules, co-stimulatory molecules, or their receptors have been reported to affect autoimmunity, and tumor immunity. Although gene polymorphisms provide an attractive explanation for the marked variability in the outcome of organ transplantation, the field is still in its early stages (*84*). Gene polymorphisms affecting TNF-α, IL-10, TGF-β, and INF-γ have been reported to alter the immune response to transplanted organs (*138, 139*). Recently, polymorphisms in CTLA4 and CCR5 were correlated with respectively rejection and overall survival after renal transplantation (*140*). It has been reported that circulating cytokine levels are associated with polymorphism in cytokine genes. Furthermore, the expression of single or multiple cytokine gene polymorphisms may also aid in identifying potential candidates for IS

weaning (*137*). In pediatric LT, children successfully maintained off IS are more likely to have a genetic predisposition toward low TNF-α and high/intermediate IL-10 production (*64*). In pediatric kidney transplant, Mondoza-Carrera et al found that patients with a high, compared with low-production TNF-α allele, experienced earlier acute graft rejection, and those with high-production alleles of both TNF-α and IFN-γ showed a two-fold higher risk for acute graft rejection than TNF-α alone. These findings support the notion that a single genotype cannot by itself explain an event as complex as acute graft rejection. The sum or combination of different specific alleles of these genes could better account for the immune response to an allograft (*65*). However, it was difficult to evaluate whether *in vitro* cytokine production profiles can be reproducibly deduced from a particular cytokine. Analyzing the relationship between cytokine gene polymorphisms and *in vitro* TNF-α, IFN-γ, and IL-10 and IL-13 production in healthy volunteers, Warlé et al observed a significant relationship between polymorphisms of TNF-α and IL-10 with *in vitro* production of TNF-α and IL-10, respectively, whereas no significant associations were found for the other tested cytokine gene polymorphisms. For LT recipients, no significant relationship could be established between any of the cytokine gene polymorphisms and *in vitro* production of corresponding cytokines (*141*).

2.5.2.8.2- Quantification of mRNA precursors

The analysis of cytokine messenger ribonucleic acid (mRNA) precursors using the reverse transcriptase-polymerase chain reaction (RT-PCR) technology was proposed to mitigate the limitations of analyzing the circulating cytokine levels as surrogate marker of tissue exposure to cytokines within the transplant and/or the recipient lymphoid organs. In the clinic, such analysis has been essentially limited to the allograft, with the requirement to obtain control biopsy samples before graft reperfusion. Unfortunately, the published studies providing data were again rather conflicting, particularly concerning IL-2, IL-4, IL-15, and IFN-γ mRNA in graft acceptance as well as in acute and chronic rejection states (*142, 143*). As suggested for circulating cytokines, these contradictory results make very hazardous the use of intragraft cytokines precursors determination to predict the level of allogenic responsiveness of a given patient in order to propose IS withdrawal. In pediatric LT, the analysis of circulating cytokine levels and their mRNA precursors in liver graft biopsies showed the intraoperative peak of IL-10 plasmatic levels

could be significantly correlated with increased levels of corresponding mRNA precursors within the liver graft at 2 hours following portal unclamping and on day seven post-LT. This study also showed that significantly higher amounts of IL-10 precursors on day seven in non-rejectors as compared with rejectors (*57*). Recently, in adult liver graft recipients, it has been shown that certain chemokine polymorphisms (CCR5Δ32) may correspond to ischemic type biliary lesions leading to chronic graft dysfunction. However, in the study of CCR5Δ32 polymorphism in a cohort of pediatric LT recipients, Fischer-Mass et al found no significant correlation between acute graft rejection or chronic graft dysfunction and the CCR5Δ32 allele (*67*).

2.5.2.8.3- Microarray analysis of gene expression

Gene expression may be quantified by gene array or real-time quantitative PCR (RT-QPCR). The term *DNA microarray* refers to a high-density array of oligonucleotides or polymerase chain reaction-products immobilized onto a solid support such as glass slides; the immobilized DNA selectively retrieves genes or sequences of interest when the array is hybridized to a mixture of complementary sequences obtained from tissue or blood samples of clinical relevance. In the context of transplant recipients, gene chips hold great promise for discovering noninvasive biomarkers for monitoring of intragraft events, and for stratifying patients toward more individualized treatment regimes, particularly using comparative analyses of the peripheral blood, of the graft and its local environment (bile in LT or urine in renal transplant). There are only limited data available describing the patterns of gene expression displayed in organ transplant recipients. In a small study investigating pediatric renal transplant recipients, microarray analysis found no differences between subclinical allograft rejection and normal patients' gene expression as assayed with RT-QPCR in 17 genes (*66*). However, to design a clinically applicable molecular test for operational tolerance in liver transplantation, Martínez-Llordella et al studied transcriptional patterns in the peripheral blood of 80 liver transplant recipients and 16 nontransplanted healthy individuals by employing oligonucleotide microarrays and RT-QPCR. This resulted in the discovery and validation of several gene signatures comprising a modest number of genes capable of identifying tolerant and nontolerant recipients with high accuracy. Multiple peripheral blood lymphocyte subsets contributed to the tolerance-associated transcriptional patterns,

although NK and γδTCR$^+$ T cells exerted the predominant influence. These data suggest that transcriptional profiling of peripheral blood can be employed to identify liver transplant recipients who can discontinue immunosuppressive therapy and that innate immune cells are likely to play a major role in the maintenance of operational tolerance in LT (*144*). Comparing microarray versus RT-QPCR assay of renal allograft biopsies, Allanach et al demonstrated that both microarrays and RT-QPCR assessments agree strongly with one another and histopathology in assessing transplant inflammation. However, the costs of the two systems depend on the number of genes analysed: RT-QPCR is much less expensive if the assessment can be made with one gene, but microarrays are cost-effective, if many genes can be assessed (or the whole genome), and have the advantage of high standardization (*145*).

Table 4- Assessment of immunological assays previously evaluated in pediatric organ transplantation

Assays	Authors, (pulication year), reference	Types of transplants	Number of infants included in clinical trial	Aim of study	Outcome
MLR Detection of Tregs	Koshiba et al 2007) (*53*)	Liver transplant	87	Investigating role of Tregs in alloreactivity responses	- $CD4^+$ $CD25^+$ cells suppress proliferation following donor stimulation
Cell- mediated lymphotoxicity	Oei et al (2000) (*54*)	Heart transplant	6 children and 5 adults	measuring CTLp frequencies and their avidity for donor antigens	The shift towards more destructive high-avidity CTLp in the peripheral blood indicates their potential damaging effect on the heart valve allograft
ELISPOT	Truong et al (2008) (*55*)	Liver transplantat	28	Analysis of the detailed kinetics of GrB before and during the early post-transplantation	- Single GrB ELISPOT could not be predictive and correlated with acute rejection - A kinetic study could be helpful to predict or confirm early rejection

Detection of donor-specific-antibodies	Wright et al (2007) (*56*)	Heart transplant	148	Assessment of outcomes of recipients with either positive PRA before transplant or positive retrospective crossmatch	A positive correlation between anti-HLA antibodies and poor graft outcome
Detection of hematopoietic chimerism	Alexender et al (2008) (*46*)	Liver transplant	1 case report	Analysis of complete hematopoietic chimerism	- Mixed hematopoietic chimerism followed by development of full tolerance in a 9-year-old girl
Circulating cytokines levels mRNA precursors	Gras et al (2007) (*57*)	Liver transplant	40	Analysis of circulating cytokine levels and their mRNA precursors in liver biopsy samples	The occurrence of cytokine immune deviation may be related to early graft acceptance
Determination of sCD30	Truong et al (2007) (*58*)	Kidney and liver transplant	12 children and 12 adults	Investigation of potential role of sCD30 plasma levels	sCD30 could not be correlated with acute rejection in pediatric liver transplantation

Detection of Tregs	Stenard et al (2009) (*59*)	Liver transplant	11	- Examination of Treg following transplantation - Determination of the relationship between Treg cell levels in the blood and in the graft	- $CD4^{+}CD25^{high+}Foxp3+$ Tregs decrease in recipients with acute rejection - Foxp3+ Tregs were increased in the portal region of livers with histopathologic evidence of rejection
Immuknow measurement	Hooper et al (2005) (*60*) Israeli et al (2008) (*61*)	Kidney transplant Liver transplant	50 healthy and 37 stable recipients 23	Assessment of the ImmuKnow assay's relevance and reliability in the immune monitoring of pediatric transplant recipients	ImmuKnow assay allows the immune monitoring of pediatric recipients and provide to prevent over- or under-IS
Detection of tolerogenic dendritic cells	Mazariegos et al (2005) (63)	Liver transplant	58 children and 17 adults	Analysis of circulating pDC1 and pDC2 of DC subsets	- Plasmacytoid DC increased in operational tolerance - A pDC2/pDC1 subset ratio could serve to identify patient for IS weaning

Gene polymorphism	Mazariegos et al (2002) (*64*) Mendoza-Carrera et al (2008) (*65*)	Liver transplant Kidney transplant	56 51	Analysis of the correlation between gene polymorphism and tolerant recipients.	- Low TNF-α and high/intermediate IL-10 production in patients maintained off immunosuppresion - The sum or combination of different specific alleles of these genes could better account for the immune response to an allograft
Microarray	Alakulppi et al (2008) (*66*)	Kidney transplant	8	Using a whole genome microarray analysis to identify all potentially useful genes	- No robust whole blood gene expression biomarker for subclinical allograft rejection was found

2.6- Potential role of soluble CD30 and granzyme B in kidney and liver transplantation

2.6.1- Soluble CD30

CD30 is a 105- to 120-kDa transmembrane glycoprotein, a member of the TNF /nerve growth factor receptor family, and was originally identified on the surface of Hodgkin's and Reed Sternberg cells (*146*). Normal PBMCs do not express CD30, but a subset of CD45RO+ T lymphocytes can express CD30 after mitogenic stimulation (*147*). The function of CD30 in mature, peripheral T lymphocytes is unclear, but there is evidence that CD30 can act as a signal transduction molecule. Ligation of CD30 by anti-CD30 agonistic monoclonal antibodies leads to the rapid activation of NF-$_k$B in T cells mediated through TNF receptor-associated factor (TRAF)-2 and TRAF-5 (*148*), whereas cross-linking of CD30 with anti-CD30 monoclonal antibodies increases the levels of intracellular Ca^{++} in Jurkat cells (*149*). Furthermore, signaling through CD30 promotes the development of Th2 cell (*150*).

Analysis of a panel of human $CD4^+$ Th clones revealed that CD30 is preferentially expressed on cells that produce Th2 type cytokines; whereas Th1 clones express little if any CD30 and Th0 clones have intermediate CD30 expression (*151*). A similar association between CD30 expression and the production of Th2 cytokines by $CD8^+$ cells has also been reported. In diseases in which Th2-type immune responses predominate, such as erythematous or atopic dermatitis, elevated serum sCD30 was found to be associated with increased disease activity. Elevated serum sCD30 in early stages of HIV-1 infection predicted a more rapid progression to AIDS. In multiple sclerosis, a disease in which Th1-type immune responses predominate increased sCD30 serum levels were correlated with disease remission. These findings prompted the suggestion that CD30 may serve as a marker for human T lymphocytes that produce Th2 cytokines (*152*). However, other studies have failed to demonstrate a strict association between CD30 expression and Th2 cytokines. Alzona et al reported that CD30+ T calls were the predominant source of IFN-γ and IL-5 in response to anti-CD mAbs, ionomycin, or PHA and IL-2, whereas CD30- T cells produced high levels of IL-2 (*147*). In addition, CD30+ T cells contributed to the production of IFN-γ during Th1-like responses and CD30 expression was observed on antigen-specific Th0, Th1, and Th2 clones (*153*). Recently,

Pellegrini *et al* found that CD30 may be an important co-stimulatory molecule and marker for the physiological balance between Th1/Th2 immune response (22). A soluble form of CD30 (sCD30) is released into the bloodstream after activation of $CD30^+$ T cells (154) (Figure 3).

2.6.2- Pre-and post-transplant sCD30 plasma level: a new immunological marker for organ transplant monitoring?

The identification of pre- and post-transplant biological parameters of recipients bearing an increased risk of allograft rejection is an important prerequisite for the successful implementation of individually tailored IS of transplant patients. In current clinical practice, a panel reactive antibody (PRA) screening is the only established immunological parameter that provides clinically useful information concerning the responder status of a cadaver kidney recipient. For recipients without PRA, a categorization into high or low immunological risk is not possible. PRA screening, which at present is exclusively used as indicator of an increased risk of graft rejection, is not sufficient to characterise the immunological profile of transplant recipient. For this reason, novel markers are urgently needed for proper monitoring of pre- and post-transplant risks (155). Recently, there has been some evidence that high pre-transplant serum levels of sCD30 could be a risk marker of an impaired graft outcome in kidney recipients (156, 157). Thus, up-regulated sCD30 levels were shown to be indicative for an increased risk of transplant loss emphasizing their clinical relevance and the implementation of sCD30 as a predictive biomarker for allograft rejection upon transplantation of different solid organs. Furthermore, to compare the accuracy of pretransplant PRA and serum level of sCD30 in predicting early (<6 months) acute rejection in living-donor and deceased-donor kidney transplantation (KT) patients, Cinti et al showed that pretransplant sCD30 could be a more accurate predictor of acute rejection (AR) when compared with PRA in a retrospective study (158). In a large series of nearly 3900 kidney transplants performed at 29 centers, Susan et al were able to demonstrate that pretransplant determination of sCD30 is a powerful indicator for estimating the risk of graft rejection not only in presensitized but also in nonsensitized recipients. Based on these published results, pre-transplant sCD30 serum levels higher than 100 U/ml have been classified as a risk factor and patients possessing both PRA and

high sCD30 in their pre-transplant serum had a particularly poor graft outcome (159). However, by analysis of heterogeneous sCD30 concentrations from individual kidney transplant recipients when measured quarterly over 1 year was shown by Altermann et al not to allow the stratification of patients into high and low immunological risk groups based on a single sCD30 value > 100 U/ml because of the high degree of variation (155).

In pediatric liver transplantation, to the best of our knowledge as of 2005, when we performed this investigation, no data had been reported. Significantly, more recently in a study of liver transplant in adults in 2007 from Santander, Spain, it was observed that the concentrations of sCD30 were similar in the rejection and non-rejection groups on postoperative day 1. During the entire postoperative period, sCD30 seric levels were also significantly higher in all the patients, with or without rejection, however, a significant increase in postoperative sCD30 levels was found in the rejection group at day 7 (160).

2.6.3- Granzyme B

CTL and NK cell granules contain a number of proteins, including perforin and granzymes, with GrB being the most abundant granzyme present (161). Upon recognition and conjunction of the effector cell with the target, preformed granules containing GrB polarize in cytolytic lymphocytes at the point of contact and are secreted into the intercellular space formed between the effector and target cells (162). Polymers of perforin are inserted into the cytoplasm of the target cells where they form pores through which GrB can penetrate the cell. Upon entry, GrB molecules pass into the cytoplasm of the target cell, where they act on specific substrates that active the apoptosis pathway, and/or GrB is transported into the nucleus, where it directly cleaves and activates substrates involved in cell suicide (163). The secretion of GrB occurs quite rapidly, is Ca^{2+}-dependent, and mediates the lethal hit that kills virus-infected and tumor cells (164).

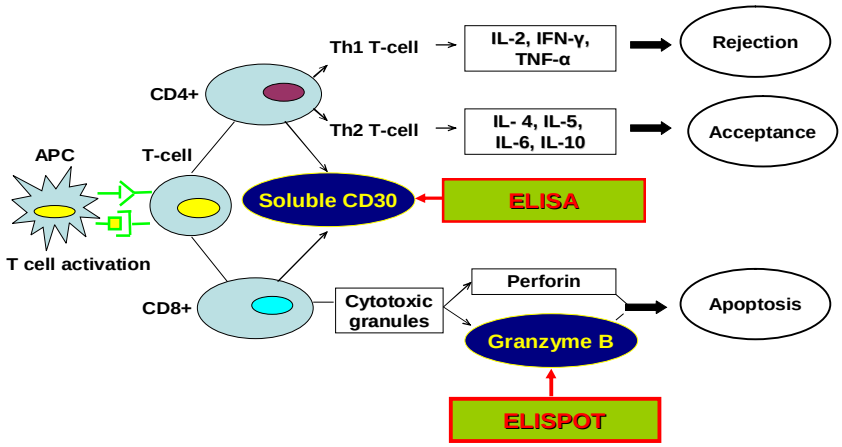
The ELISPOT method has been successfully applied to measure GrB secretion. It has been shown to be highly sensitive in detecting molecules secreted by individual cells present in low frequencies. The higher sensitivity of ELISPOT in comparison to that of ELISA or intracellular staining is due to the plate-bound antibodies directly capturing the product secreted around the cell before it is diluted in the supernatant, absorbed by high affinity receptors, or degraded by proteases. Since the spot in the ELISPOT assays

represent individual cells that have actually secreted the product measured, the number of spots per well reflects their frequency in the test population (163).

2.6.4- Pre-and post-transplant frequency of granzyme B enzyme-linked immunosorbent spot assay as immune parameters for recipients

Regarding GrB, only the gene expression in rejection following organ transplantation has been investigated. In human allograft biopsy, GrB expression seems to be related to acute rejection in renal (165, 166), hepatic (167, 168) and intestinal transplant (169). In peripheral blood, the genetic marker (mRNA) of GrB has been detected, predicting kidney (170, 171) and pancreas transplant rejection (172). Furthermore, Clement et al have reported identifying GrB in bronchoalveolar lavage specimens when evaluating lung rejection (173) and Li et al have claimed that measuring mRNA encoding cytotoxic proteins in urinary cells offers a non invasive mean of diagnosing acute rejection of a renal allograft (174). For the GrB ELISPOT technique, some authors have demonstrated that GrB ELISPOT assays could be an alternative to the standard 51Cr-release assay for measuring granule-mediated cytotoxicity (163), monitoring in cancer vaccine trials (175) or in kidney transplantation (85, 176). They have also shown that GrB is a more specific candidate marker than IFN-γ for measuring the cytotoxic capacity of immune effector cells such as NK cells (85, 176, 177), but not yet, as far as we know, for exploring allogenic responsiveness in LT.

Figure 3- Role of sCD30 and Granzyme B in mechanisms of rejection



3- CLINICAL RESEARCH HYPOTHESIS

The first aim of this study was to investigate the evolution of pre-transplant and post-transplant serum sCD30, IFN-γ and IL-10 cytokines circulating levels in liver and kidney recipients who developed acute rejection, when compared to the patients with early graft acceptance. It was hypothesized that peri-transplant sCD30 serum levels may be predictive of the immune reactivity status within the first week post-transplant.

The second aim of this study was to analyze the detailed kinetics of GrB-producing cells before and during the early post-transplantation period. We also investigated the pre- and post-transplantation secretion of GrB in pediatric liver recipients who developed acute rejection compared to patients with graft acceptance. We hypothesized that GrB ELISPOT could be a marker for recipient alloreactivity for immunological monitoring in organ transplantation.

4- PERSONAL WORKS

4.1- Immunological monitoring after organ transplantation: potential role of soluble CD30 blood level measurement

4.1.1- Introduction

4.1.1.1- Soluble CD30 in organ transplant

Despite the improvement in IS protocols, most transplant recipients face a variety of drug-related complications. Early post-transplant acute rejection and infection are major causes of morbidity and mortality, whereas lack of full rehabilitation, drug toxicity, chronic rejection and malignancies constitute debilitating long-term complications. All problems described above may be related (directly or indirectly) to the lack of immunological tests for reproducibly predicting the risk of rejection and for recognizing ongoing rejections after transplantation as early as possible. Until now, panel reactive antibodies are the only established indicators of increased immunologic responsiveness before transplantation, related to increased risk of early acute rejection (158, 178). The CD30 molecule is a member of the tumor necrosis factor/nerve growth factor receptor superfamily and was originally identified as a cell surface antigen on Hodgkin and Reed Sternberg cells (*146*). Both CD4 and CD8 T cells expressed CD30 after primary alloantigenic stimulation (*152*). Some studies suggested that CD30 may serve as a marker for human T lymphocytes that produce Th2 cytokines (*150*)**,** while others demonstrated a strict association between CD30 expression and Th1 cytokine production (*152*). However, Pellegrini *et al* found that CD30 may be an important co-stimulatory molecule and marker for the physiological balance between Th1/Th2 immune response (22). A soluble form of CD30 is released into the bloodstream after activation of $CD30^+$ T cells (154). In recent publications, some authors showed that the pre-transplantation assessment of serum sCD30 may provide highly clinically relevant information concerning the risk of rejection of subsequent kidney transplants (156, 179).

4.1.1.2- ELISA technology

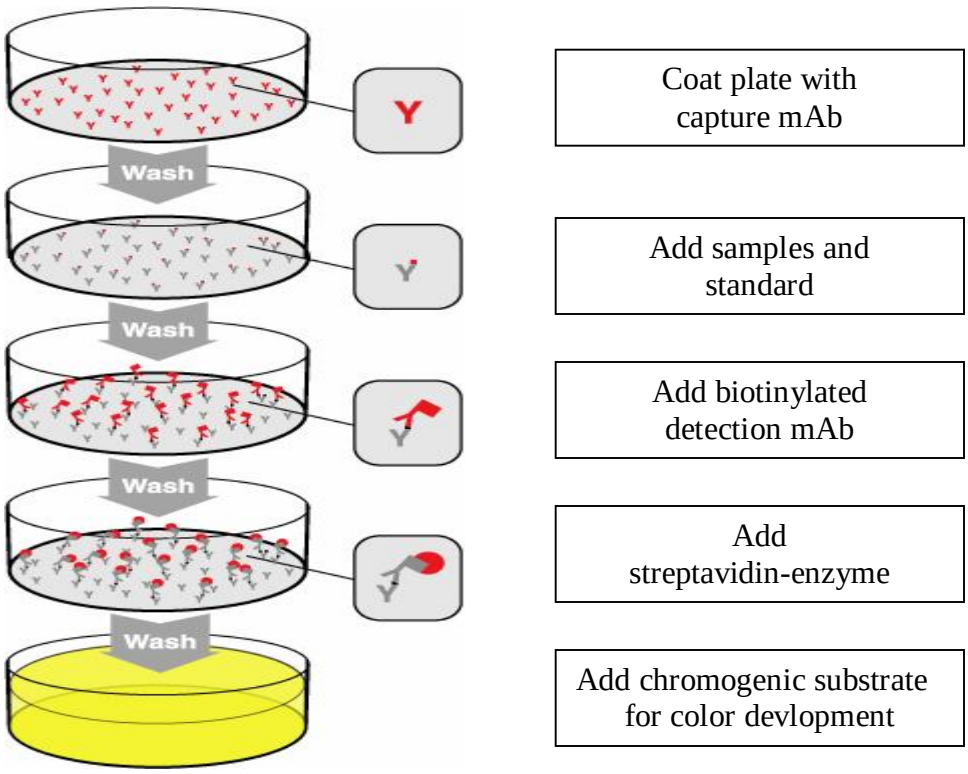
ELISA is a specific and highly sensitive method for detecting and quantifying antibodies (Abs) or antigens (Ags) against viruses, bacteria or other analytes in solutions (Figure 4).

In ELISA technology, the solid phase consists of a 96-well polystyrene plate, although other materials can be used. Plates are coated with Abs or Ags, as they bind to the solid phase. After incubation with serum samples containing Ags or Abs to detect, the plates are washed to remove any unbound material. Conjugate is then added to the plate and allowed to incubate.

The conjugate consists of either an Ag or Ab that has been labeled with an enzyme. Depending upon the assay format, the immunologically reactive portion of the conjugate binds to the Ags oe Abs fixed on the coated plates. The enzyme portion of the conjugate enables detection.

The plates are washed again and an enzyme substrate (hydrogen peroxide and a chromogen) is added and allowed to incubate. Color develops in the presence of bound enzyme and the optical density is read with an ELISA plate reader.

Figure 4- The ELISA technique illustrated



4.1.2- Objectives of study

The aim of this study was to investigate the evolution of pre-transplant and post-transplant serum sCD30, IFN-γ and IL-10 cytokines circulating levels in liver and kidney recipients who developed acute rejection, when compared to the patients with early graft acceptance. It was hypothesized that peri-transplant sCD30 serum levels may be predictive of the immune reactivity status within the first week post-transplant.

4.1.3- Patients and methods

Organ recipients transplanted between 1994 and 2004 at Saint-Luc university clinics, Brussels, Belgium, were selected with respect to the availability of plasma samples on day 0 before transplantation and on day 7 post-transplantation. 27 patients (15 pediatric liver graft recipients and 12 adult kidney graft recipients) could be considered in the present study (Table 5). IS protocols administered to the patients are detailed in Table 6. The diagnosis of acute liver and kidney rejection was confirmed histologically in all cases. The frozen serum of the patients was analysed to measure sCD30 content using a commercially available ELISA kit (Biotest, Dreieich, Germany) on pre-transplantation baseline (day 0) and post-transplantation at day 7. IL-10 and IFN-γ serum levels were measured by flow cytometry with Th1/Th2 cytometric bead array kit (BD Biosciences, CA, USA). Delta value of serum sCD30 between day 0 and 7 was compared with delta value of IFN-γ and of IL-10 cytokines circulating levels on the same day. Blood samples at day 7 could not be collected in the six kidney recipients without rejection for logistical reasons. IL-10, IFN-γ, and sCD30 levels were reported as geometric mean and SD because of lognormal distributions. Data were log-transformed before statistical analyses. Paired Student t tests were used to compare levels at days 0 and 7. Unpaired Student t tests were used to compare patients with rejection with those without rejection. Changes in IL10 or IFN-γ were compared with changes in sCD30 using linear regression. All tests were two tailed and a p-value <0.05 was considered as a statistical significance.

Table 5- Demographic data of 15 pediatric liver and 12 kidney transplant recipients submitted to pre-and post-transplant sCD30 monitoring

	Pediatric liver transplant (n=15)	Adult kidney transplant (n=12)
Median age (range)	3.4 years (0.4-13)	49 years (28-66)
Sex (M/F)	7/8	7/5
Indication for transplantation	- Biliary atresia: 6 - Familial cholestasis: 4 - Hepatic malignancies: 3 - Metabolic diseases: 1 - Other diseases: 1	- Chronic glomerulonephritis 2 - Malignant arterial hypertension 2 - Interstitial nephritis 2 - Obstructive uropathy 1 - Polycystic kidney disease 1 - Systemic immunologic disease 1 - Unknown 3
Immuno-suppressive protocol	- Steroid, tacrolimus: 13 - Steroid- free, tacrolimus, basiliximab 2	- Steroid, tacrolimus, mycophenolate mofetil 9 - Steroid, tacrolimus, mycophenolate mofetil, rapamycine 3

4.1.4- Results

When comparing day 0 and day 7 sCD30 plasma levels, a decrement was observed in all patients whatever the type of organ transplanted (Table 6). In the liver graft group, the patients who developed acute rejection during the first month (n=9) had a slightly (although not significantly) higher sCD30 value on pre-transplantation baseline (day 0) and post-transplantation (day 7), when compared to patients with normal early graft function (n=6) (day 0: 118(1.5) U/ml versus 102(1.6) U/ml, mean (SD), respectively: p=0.52) and (day 7: 83(1.6) U/ml versus 69(1.5) U/ml, respectively: p=0.47). Regarding the serum levels of IFN-γ and IL-10, there was a statistical difference between pre-transplantation baseline (day 0) and post-transplantation (day 7) levels in liver graft recipients. At day 7, IL-10 serum levels increased up to 336 % (11(6.2) pg/ml versus 48(2.1) pg/ml, respectively: p=0.03) and IFN-γ serum levels decreased up to 62% from baseline value (102(15.0) pg/ml versus 39(8.8) pg/ml, respectively: p=0.03) (Table 7). Increased serum sCD30 was shown to correlate with increased IL-10 circulating levels between day 0 and day 7 (r= 0.53; p=0.04), whereas, no correlation could be evidenced between sCD30 and IFN-γ (r= 0.02; p=0.47) (Figure 5). In the kidney transplantation group, no significant difference was found in pre-transplant sCD30 level in patients with or without early graft rejection (77(1.6) U/ml versus 85(1.3) U/ml, respectively: p=0.66). However, at day 7 there was a decrease up to 55% of sCD30 levels from baseline value in the group of recipients with rejection (77(1.6) U/ml versus 35(1.4) U/ml; respectively: p=0.02).

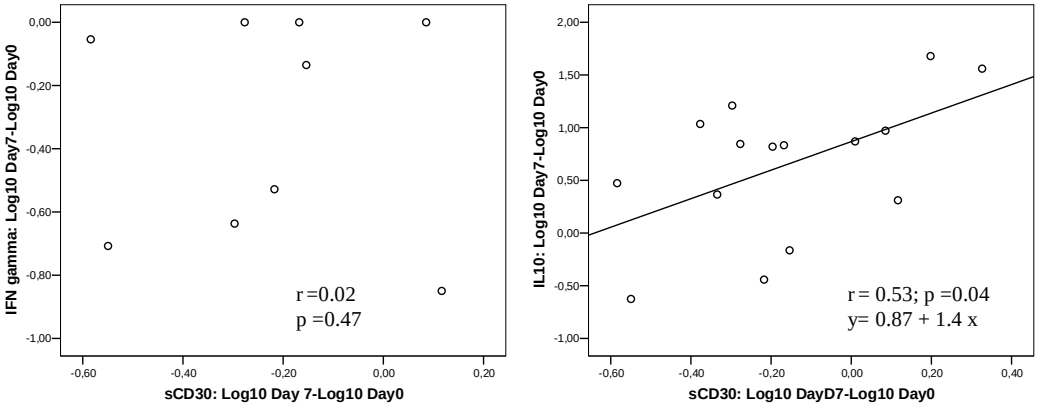
Table 6- Pre-transplantation baseline (day0) and post-transplantation (day7) sCD30 blood levels. Data are geometric mean (SD)

	sCD30 pre-transplant Mean (SD) U/ml	sCD30 post-transplant Mean (SD) U/ml	P value
Pediatric liver transplant	(n=15)	(n=15)	
Patients without rejection (n=6)	102(1.6)	69(1.5)	0.16
Patients with rejection (n=9)	118(1.5)	83(1.6)	0.14
Adult kidney transplant	(n=12)	(n=6)	
Patients without rejection (n=6)	85(1.3)	Not available	
Patients with rejection (n=6)	77(1.6)	35(1.4)	0.02

Table 7- Pre-transplantation baseline (day 0) and post-transplantation (day7) IFN-γ and IL-10 serum levels in the pediatric liver graft group (with and without rejection). Data are geometric mean (SD)

	pre-transplant Mean (SD) pg/ml	post-transplant Mean (SD) pg/ml	P value
IL-10 serum levels (n=15)	11(6.2)	48(2.1)	0.03
IFN-γ serum levels (n=12)	102(15.0)	39(8.8)	0.02

Figure 5- Delta value (in logarithm 10) of serum sCD30 between day 0 and 7 was compared with Th1 (IFN-γ) (r =0.02; p=0.47) and Th2 (IL-10) (r =0.53; p=0.04) cytokines circulating levels at the same day in the liver graft group (with and without rejection).



4.1.5- Discussion

In our study, liver transplant recipients with subsequent rejection had a slightly higher pre-transplantation (day 0) and post-transplantation (day 7) serum sCD30 levels than recipients with subsequent normal graft function; however, considering the small group of patients in the study, this difference did not reach the statistical significance. To our knowledge, monitoring of sCD30 for liver transplant has not yet been published in the literature. The data produced in this limited series of pediatric liver recipients do not suggest however any correlation between sCD30 and early graft acceptance. Similarly in the kidney transplantation group, no difference was found in pre-transplant serum level of soluble CD30 in both groups with graft rejection and normal graft function. The results of the present study are contradictory with those of Susal *et al.* (159). In their study including 3899 patients, they found a high level of pre-transplant sCD30 in the subgroup of patients with poorer graft survival after adult kidney transplantation. Recently, Rajakariar et al also showed that high pre-transplant sCD30 levels are associated with antibody-mediated rejection (C4d staining) which carries a poorer prognosis (179). Nevertheless, we found that the sCD30 levels in patients who developed kidney rejection decreased up to 120% at day 7 post-transplantation. In a similar study, Slavcev et al showed that an important decrease of sCD30 was detected 2 weeks after renal transplantation and patients without rejection had lower sCD30 value compared to patient who experienced rejection episodes (180). Unfortunately, due to unavailability of postoperative blood samples in the kidney recipients without rejection in our study, a comparison of sCD30 levels post-transplantation between graft rejection and normal graft function could not be done. This study also demonstrated that increased level of sCD30 is accompanied by increased circulating IL-10 on day 0 and day 7. Th1 cytokines including IL-2, TNF-α, and IFN-γ mediate cellular immune responses and are pro-inflammatory, whereas, Th2-type cytokines IL-4 and IL-10 have been shown to inhibit the development and function of Th1-cells, to suppress inflammation, and to enhance humoral pathways of the immune response (*75*). Th1-cytokines are mainly involved in allograft rejection, while Th2-type immune response may be graft protective by blocking the graft damaging Th1-type anti-donor response (*37*). But Susal's data (159) have shown an association between rejection and poor graft

survival in patients with high levels of sCD30 and IL-10. An explanation suggested by Susal is that the patients with end stage renal disease are incapable of generating an intact immune response against specific stimuli (159). Only those with high sCD30 may be capable of producing IL-10 that inhibits a nonspecific chronic inflammatory Th1-type immune response and which could be followed by an effective alloimmune response against the graft. In accordance with this observation, Gerli et al has reported that in rheumatoid arthritis, patients with increased sCD30 and synovial $CD30^+$ T cells, secreted high amounts of IL-4, IL-10 and those patients responded well to anti-rheumatic therapy (181, 182). This finding could be in favor of a regulation of Th1 non specific inflammatory response by IL-10 in rheumatoid arthritis. In contrast, Martinez et al recently observed that alloactivated $CD30^+$ T cells could produce Th1 (IL-5 and IFN-γ) as well as Th2 (IL-10) cytokines (*152*). Thus, more studies are needed to clarify the role, origin, and characteristics of $CD30^+$ T cells and sCD30 in alloimmune responses. In the liver graft group, we found interestingly a significant correlation between delta value of sCD30 and IL-10 serum levels (in logarithm10) between day 0 and day 7. This correlation suggests a link between increased serum sCD30 and the increase of IL-10 circulating levels in the first week post-transplantation. However, this parallel increase as found in our limited exploratory study could not be correlated with any benefit in term of early graft acceptance. The mechanism of action of these two parameters on immune response in organ transplantation is controversial. Despite the close relationship between sCD30 and Th2-type immune responses, it is suggested that $CD30^+$ T cell activation may represent a novel graft rejection pathway in which both Th1- and Th2-type cytokines could be involved (179).

4.1.6- Conclusion

In conclusion, our study showed that increased serum sCD30 was correlated with increased IL-10 circulating levels, but not with IFN-γ levels in the post-transplantation period. Pre-transplantation sCD30 did not predict the occurrence of early post-transplant acute rejection and sCD30 at day 7 post-transplantation could not be correlated with acute rejection in liver graft recipient. Accordingly, the monitoring of sCD30 could be a tool to assess immunological risk in renal transplant but did not appear to be a valuable mean for early immunological monitoring in liver allograft recipients in the small group

of patients analysed in this study. Further immunological investigation will be required in order to better delineate the role of sCD30, IL-10 and IFN-γ serum monitoring in solid-organ transplant recipient.

4.2- Pre- and post-transplant monitoring of granzyme B enzyme-linked immunosorbent assay in pediatric liver recipients

4.2.1- Introduction

4.2.1.1- Granzyme B enzyme-linked immunosorbent assay in organ transplants

The ability to consistently induce robust, sustained, donor-specific tolerance would offer many benefits to transplantation recipients (183). Although the progress of new immunosuppressive drugs in recent years has led to a spectacular improvement in short term results, it has also led to a limited improvement in long-term graft survival rates in organ transplantation. Furthermore, overimmunosupression is usually associated with severe side effects, whereas low doses of IS are frequently associated with a risk of acute rejection (184, 185). The major goal in organ transplantation is to define the optimal immunosuppressive load, according to the individual immunological profile of each transplant patient.

In this context, predictive tests would be useful in order to evaluate the patient's immunological risk of early rejection. Recently, ELISPOT has been introduced as an assay that can detect an immune response against donor antigens presented either by the direct pathway (recipient T cells cultured with donor stimulators) or the indirect pathway (recipient T cells cultured with recipient antigen-presenting cells plus donor cell lysates or pulsed with peptides derived from donor cells) (*84*). Some studies suggest that the use of the IFN-γ ELISPOT assay as a surrogate measurement for CTL and NK responses may be indicative of the immunological status of transplant recipients (*37*). However, the IFN-γ ELISPOT assay may not be an accurate measure of cytotoxic lymphocytes since non-cytotoxic cells can also secrete IFN-γ. Since GrB is exclusively present in the granules of CTL and NK cells and is a key mediator of the granule exocytosis-mediated cytolytic pathway (163, 186, 187), it may constitute an excellent candidate marker for immunological monitoring in transplantation by the ELISPOT method (177). Pre- and

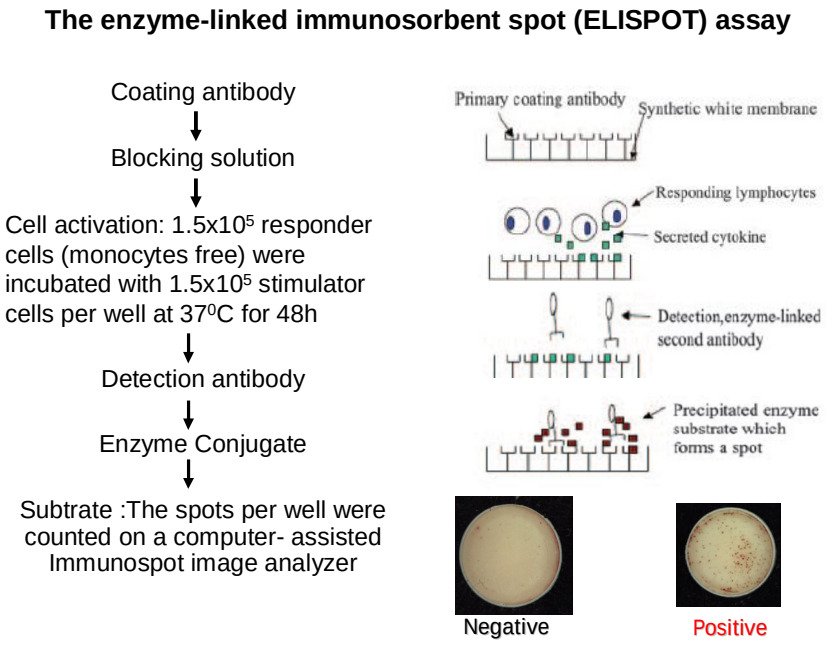
post-transplantation monitoring of GrB secretion in recipients might be helpful as a marker of lack of alloresponsiveness which can result in tolerance and has the potential to permit changes in medical therapy to prevent rejection before it becomes clinically apparent.

4.2.1.2- Enzyme-Linked Immunosorbent Assay

ELISPOT assay can be performed with cells activated *in vivo* or *in vitro* against a specific antigen. When there is an *in vitro* activation, this assay is a hybrid that combines features of a MLR and an ELISA assay in that responder/recipient T cells are cultured with inactivated stimulator/donor or third-party cells in tissue culture plates coated with an antibody that is specific for the cytokine of interest (many cytokines have been studied, including IFN-γ, IL-2, IL-4, IL-5, and IL-10. After a fairly brief culture period, the cells are washed away and the bound cytokine is detected, using labeled secondary antibodies and an automated plate reader (Figure 6). Because of the short culture period, each spot that is detected represents a cell that had been primed to the stimulating antigen(s) *in vivo* (effector or memory T cells). Thus, this assay measures the frequency of previously activated or memory T cells that respond to donor antigens by producing a selected cytokine rather than the total amount of cytokine that is produced and secreted into supernatants (as measured using an ELISA). This is an important advantage because cytokines are captured immediately upon secretion from cells, whereas cytokines that are secreted in supernatants may be subject to breakdown or dilution or may be used up by other cells. Another unique feature of this assay is that it is capable of distinguishing between recipient T cells that are responding to donor antigens that are presented *via* either the direct or the indirect pathways of antigen presentation. For detection of directly presented donor antigens, recipient T cells are cultured with whole, inactivated donor cells. For detection of recipient T cells that are responding to indirectly presented donor antigens, recipient T cells are cultured with recipient APC plus donor proteins in the form of cell lysates or peptides (*90*). Distinguishing between these two pathways of antigen presentation may be important because early antidonor responses after transplantation tend to be dominated by direct responses, whereas late responses tend to occur more frequently *via* the indirect pathway (188). A recent publication that used the ELISPOT assay to compare whole peripheral blood leukocytes or CD25-depleted peripheral blood

leukocytes in stable renal transplant patients (thus hyporeactive to donor antigens) demonstrated evidence of regulation in half of studied individuals (188). In addition, the assay has been optimized for use of frozen cells that can be easily stored. Although no studies to date have reported the use of this assay to identify functionally tolerant humans after transplantation, the frequency of IFN-γ producing cells has been reported to correlate with the incidence of acute and chronic rejection as well as allograft function after renal transplantation (189-191). Obtaining and storing sufficient numbers of donor cells to perform the assay repeatedly is a practical limitation, particularly in recipients of deceased-donor organs. Nevertheless, on the basis of these promising results in small cross-sectional studies, large, multicenter, prospective studies that also include "tolerant" patients are under way to evaluate the ability of this assay to predict rejection and tolerance in transplant recipients (*49*).

Figure 6- Illustration of the basic principles of the ELISPOT assay



4.2.2- Objectives of study

The first aim of this study was to analyze the detailed kinetics of GrB-producing cells before and during the early post-transplantation period. We also investigated the pre- and post-transplantation secretion of GrB in pediatric liver recipients who developed acute rejection compared to patients with graft acceptance. We hypothesized that GrB ELISPOT could be a marker for recipient alloreactivity for immunological monitoring in organ transplantation.

4.2.3- Patients and methods

Patients

Twenty-eight pediatric patients (median age: 2.1 years, range: 4 months - 13 years) who received an orthotopic LT between 1999 and 2004 at the Pediatric Liver Transplant Program at Saint-Luc University Clinics, Brussels, Belgium, were included in this study.

Ten patients received a cadaveric transplant (CD) and 18 patients received a living related liver transplant (LRLT). Thirteen of our patients (CD =6, LRLT = 7) received an immunosuppressive regimen consisting of steroid-free, basiliximab and tacrolimus. The other patients were treated with steroid and tacrolimus (CD =4, LRLT = 11). The indications for LT were essentially biliary atresia (n=13), familial cholestasis (n=4), hepatic malignancies (n=4), metabolic diseases (n=1) and miscellaneous indications (n=6).

PBMC from these 28 pediatric liver recipients were stimulated with donor PBMC (in the case of LRLT) or donor spleen cells (in the case of CD) or with third-party cells HLA class II mismatched with patient and donor. Cryopreserved PBMC and spleen cells were thawed for use in the GrB ELISPOT test. In this group, 14 patients had biopsy-proved acute rejection within six months of transplantation (CD =6, LRLT = 8).

Recipient responder cells:

PBMC from recipients at baseline (day 0) and days 7, 14, 28 post-transplantation were isolated from whole blood by Ficoll (GE Healthcare Bio sciences AB, Uppsala, Sweden) density gradient centrifugation and frozen in liquid nitrogen with dimethyl sulfoxide cryoprotectant (Merck, Darmstadt, Germany).

Donor stimulator cells:

Donor cells were isolated by standard Ficoll density gradient preparation from the peripheral blood of the living donors (n=18) and from donor spleen in the case of CD (n=10). For isolation of spleen cells, section of donor spleens were placed immediately into RPMI medium, temporarily stored at $4^{o}C$, and delivered to the laboratory within 48h of organ harvest. Single cell suspensions of spleen cells in RPMI 5% human serum were produced by mechanical disruption of tissue. Aliquots of stimulator cells were frozen and stored in liquid nitrogen ($-196^{o}C$) with dimethyl sulfoxide cryoprotectant (Merck, Darmstadt, Germany). Stimulator cells were used after irradiation with 25Gy.

Thawing

Cryopreserved PBMC and spleen cells were stored at $-196^{o}C$ until thawing to set up the assay. Cryopreserved cells were thawed in waterbath and slowly diluted with warm culture medium RPMI (Invitrogen) containing 5% fetal bovine serum (FBS) and 1% penicilline-streptomycin, 1% L-glutamine. Viable cells were counted using an optical microscope in the presence of trypan blue stain 0.4% (Invitrogen) (data not shown).

The enzyme-linked immunospot (ELISPOT) assay

The ELISPOT kit (BD Biosciences, CA, USA) includes plates to be coated with 100 µl per well of capture antibodies for GrB (1 mg/ml final concentration) in PBS (Invitrogen) and stored overnight at $4^{0}C$. The plates were washed once and then blocked with 200 µl of RPMI containing 10% FBS and 1% penicillin-streptomycin, 1% L-glutamine per well and incubated for 2 hours.

After thawing, recipient cells were cultured in a plastic bottle at $37^{0}C$ overnight, to remove monocytes by adherence. In young and small pediatric patients we were unable to collect a sufficient volume of blood to obtain the number of cells usually used in ELISPOT assay ($3x10^{5}$ responder cells), particularly with respect to the T lymphopenia commonly observed during the early post-transplant period. The GrB ELISPOT technique therefore was adapted here by using $1.5x10^{5}$ T responder cells per well. Cell suspensions were then measured at $1.5x10^{5}$ cells/ ml for every recipient and stimulator.

$1.5x10^{5}$ responder cells were stimulated with $1.5x10^{5}$ donor cells per well and tested in duplicate. Phytohemagglutinin (Remel, Europe) was used as mitogen stimulator to verify viability and function of the PBMC at a final concentration of 10µg/ml in RPMI 5% FBS. All recipient cells were also stimulated by the third party cells consisting of

HLA cells class II mismatched with patient and donor. Final volume for all assays was 200 µl per well. Control wells contained only responder cells or stimulator cells plus medium alone. After incubation at 37^0C for 48h, the plates were washed twice with deionized water (soaking for 4 minutes at each washing step) and then three times with PBS-Tween-20 (0.05%). Next, 100 µl of biotinylated anti-human GrB (2µg/ml) in the dilution buffer (PBS containing 10% FBS) were added to the wells for 2 hours of incubation at room temperature. The plates were washed three times with PBS-Tween-20 (0.05%) (soaking for 2 minutes at each washing step). Streptavidine-HRP was plated at 1:100 in dilution buffer for 1 hour at room temperature. The plates were washed four times with PBS-Tween-20 (0.05%) (soaking for 2 minutes at each washing step) and then twice with PBS. The spots were visualized using 100 µl /well of AEC Substrate Reagents (BD Biosciences, CA, USA). Plates were developed for 30 minutes at room temperature in the dark and reaction stopped by rinsing the plates with distilled water. The membranes were air-dried and spots per well were counted on a computer-assisted Immunospot image analyzer (AID ELISPOT Reader systems). A number < 5 spots per well was considered negative in the control wells. Results were calculated as mean values of spots detected in duplicate wells containing responder cells plus donor or third-party cells after subtracting the response of wells with donor or third-party cells alone.

Statistical analysis

ELISPOT frequencies were reported as geometric mean and standard deviation because of lognormal distributions. Data were log-transformed before statistical analyses. Time trends were tested using ANOVA for repeated measurements. If significant, paired Student t tests were used to compare levels at days 0 and 7, 14, 28. Unpaired Student t tests were used to compare patients with rejection with those without rejection. All tests were two tailed and a p-value <0.05 was considered as statistically significant.

4.2.4- Results

Dependent on the availability of recipient materials, recipient population study included 14 patients who developed acute rejection within 6 months post-transplantation and 14 patients without such occurrence.

Table 8 shows the detailed data of kinetic mean ELISPOT frequencies cells spontaneously producing GrB, and GrB-producing cells stimulated by donor or third-party antigens at baseline pre-transplant and days 7, 14, 28 post-transplant.

Overall, the reactivity to donor cells was found to be similar to the reactivity to third-party cells in pre-transplantation (5(3.3) spots versus 5(3.1) spots, respectively: p=0.73). However, after transplantation, the reactivity to donor cells was higher than the reactivity to third-party cells at day 7, 14 and reached a significant difference at day 28 post-transplant (25(2.5) spots versus 9(3.8) spots, respectively: p=0.05) (Figure 7).The kinetic line of GrB in Figure 7 appeared similar in all 3 assays: spontaneous release of GrB by PBMC in the absence of stimulator cells (control wells, GrB-producing cells without stimulation *ex-vivo*), PBMC stimulated by donor cells and PBMC stimulated by third-party cells.

Next, frequencies of GrB-producing donor-reactive cells were compared between the patients with and without rejection within six months post-transplant. At baseline, no difference of GrB value was found in patients with acute rejection compared to patients with normal graft function (day 0: 4(3.9) spots versus 5(2.9) spots, respectively p=0.65). At day 7 post-transplantation, GrB monitoring showed an increase in all patients. In particular, patients who developed acute rejection had a higher value than those without rejection within the first six months, but the differences observed did not reach statistical significance (day 7: 15(4.9) spots versus 10(4.0) spots, respectively: p=0.55), whereas frequencies of GrB in the rejection group showed a significant increment (up to 360%) at day 7 from baseline value (15(4.9) spots versus 4(3.9), respectively p=0.04) (Figure 8). In contrast, in the group without rejection, no significant increment (up to 200%) in GrB production occurred between day 7 and day 0 (10(4.0) versus 5(2.9), respectively: p=0.15). At day 14 post-transplantation, interestingly, a decrease was observed in all recipients whatever the sub group, with or without rejection. Similarly, GrB values did not differ significantly between the rejection versus normal group (day 14: 11(3.0) spots versus 8(6.0) spots, respectively: p=0.60). At day 28 post-transplantation, frequencies of GrB rose again in both groups. Similarly, no such significant differences were found between the normal and rejection groups (day 28: 23(2.4) spots versus 21(2.6) spots, respectively: p=0.85). However, comparing day 0 and day 28, frequencies of GrB showed

a significant increase both in the rejector group (4(3.9) spots versus 21(2.6) spots, respectively: p=0.01) and in the non-rejector graft group (5(2.9) spots versus 23(2.4), respectively: p=0.01).

Table 8- Pre-transplant baseline (day 0) and post-transplant (days 7, 14, 28) GrB ELISPOT frequencies in pediatric liver recipients (with and without rejection within six months after transplantation). Data are expressed as geometric means and standard deviations.

	Recipients without rejection (n=14)	Recipients with rejection (n=14)	P value
GrB-producing cells (spontaneous release)			
Day 0	4(2.4)	4(2.8)	0.72
Day 7	5(3.7)	8(2.8)	0.38
Day 14	7(3.3)	3(4.6)	0.29
Day 28	9(2.3)	13(2.9)	0.35
GrB-producing cells stimulated by donor cells			
Day 0	5(2.9)	4(3.9)	0.65
Day 7	10(4.0)	15(4.9)	0.55
Day 14	8(6.0)	11(3.0)	0.60
Day 28	23(2.4)	21(2.6)	0.85
GrB-producing cells stimulated by third-party cells			
Day 0	8(3.6)	4(2.6)	0.18
Day 7	6(4.2)	10(4.2)	0.47
Day 14	8(4.2)	4(4.3)	0.43
Day 28	10(4.6)	8(3.2)	0.79

Figure 7- Kinetic frequencies of ELISPOT GrB-producing cells in all recipients at baseline and days 7, 14, 28 post-transplant. Curves showed spontaneous release, and release after stimulation by donor cells or by third-party cells (comparison at day 28 between the stimulation by donor cells versus third-party cells showed statistical significance with p<0.05). Data are expressed as geometric means and standard deviations.

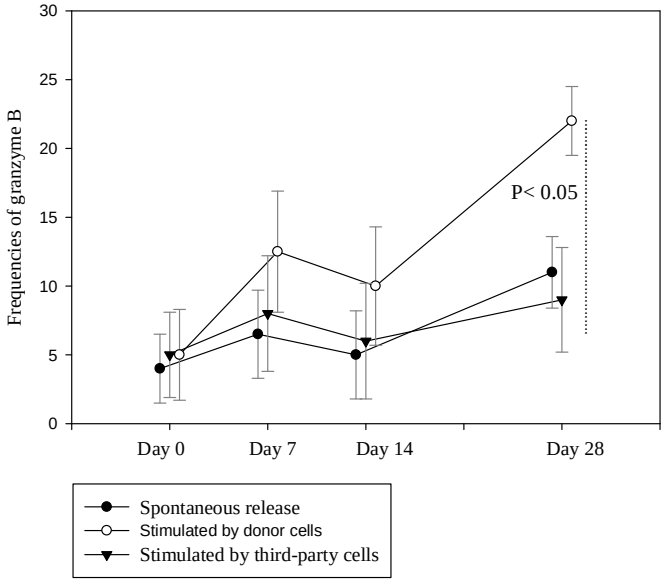
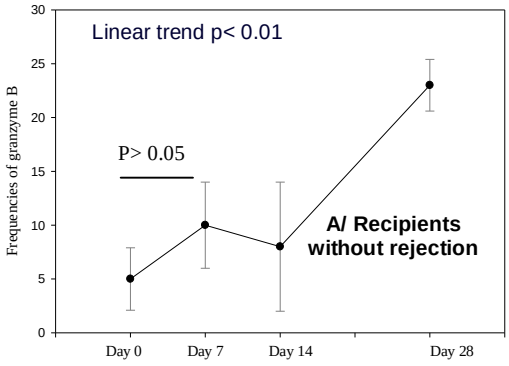
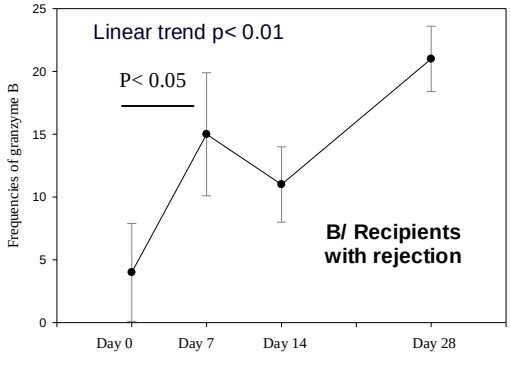


Figure 8- Changes in GrB ELISPOT stimulated by donor cells at predefined intervals, within one month of transplantation in A (group of patients without rejection) and B (rejection group within six months after transplantation). Data are expressed as geometric means and standard deviations.





4.2.5- Discussion

The ELISPOT assay has the advantage of detecting cytokine release in response to antigens at a single cell level, thereby permitting direct calculation of secreting cell frequencies. This assay method has been used as an immunological tool in several clinical scenarios, such as for patients with melanoma, CMV infection, multiple myeloma and transplantation (192). In allograft immune monitoring, the IFN-γ ELISPOT is the most popular ELISPOT assay and is often used as a marker either for CTL or for rejection, especially in kidney transplantation (*85*). To our knowledge, the role of GrB ELISPOT in LT has never previously been studied.

This study compared stimulation by donor or third party cells in GrB ELISPOT assay. Reactivity to donor cells was found to be higher than the reactivity to third-party cells at day 28 post-transplantation. This could be explained by the fact that recipient cells were stimulated by donor antigens not only in the direct pathway but also in the indirect pathway of allorecognition in the early post-transplant period. Later on, reactivity to the donor cells coud be reinforced by the presence of a large number of memory T cell one month after transplantation. Memory T cells have a response to a foreign antigen (recall response) greater in magnitude and faster than naïve T cell response. Memory T cells generate a considerable number of effector T cells that induce cytolytic activity and produce GrB within hours of antigenic restimulation (193).

Kinetic frequencies of GrB ELISPOT were analysed at predefined intervals within one month of transplantation to evaluate the predictive potential of the assay for early rejection. In this study, GrB-producing cells increased significantly at day 7 post transplant in patients with rejection. This observation was in accordance with a previous study in our pediatric recipients where routine liver-graft biopsies on day 7 after transplantation showed histological signs of acute rejection in 94% of the children given a steroid-free immunosuppressive regimen and in 74% of the recipients treated with steroids (*33*). This finding could explain why the GrB value was high at day 7 in the rejector groups in our study at that time. At day 14, GrB secretion fell, then rose again during week 3. On average it reached a second higher value around day 28 in all recipients. This finding was also detected in IFN-γ monitoring. Nickel et al. found that in post renal transplant, IFN-γ-producing cells increased and peaked at week 4 and declined

during the following weeks corresponding to when immunosuppressive doses were usually reduced (*88*).

In control wells where GrB was produced by PBMC without *in vitro* stimulation, the kinetic line of GrB appeared similar, but slightly lower than in the group where PBMC were stimulated by donor or third party cells. This finding indicated that recipient lymphocytes might be activated *in vivo* through recognition of the donor antigens after transplantation and could secrete GrB even without being stimulated in the *in vitro* assay.

According to literature, monitoring of IFN-γ ELISPOT has been widely used in renal transplant. Van Besouw et al found that the IFN-γ ELISPOT assay is superior to the GrB ELISPOT assay as surrogate marker for CTL activity after third-party stimulation (*85*). Some studies demonstrated that IFN-γ could be a predictive marker for the risk of rejection when tested before transplantation (86-89, 194), and some authors confirmed the good correlation between IFN-γ-producing lymphocytes both with acute rejection (89, 189, 190) and with chronic rejection (*49*) in post-transplant episodes. In liver transplant recipients, the IFN-γ ELISPOT technique has been used to assess hepatitis C virus eradication and Epstein-Barr virus cytotoxic T cells (195). For GrB, only the role of gene expression in rejection following organ transplantation has usually been investigated. In human allograft biopsy, GrB expression seems to be related to acute rejection in renal (165, 166), hepatic (167, 168) and intestinal transplant (169). In peripheral blood, the genetic marker of GrB has been detected, predicting kidney (170, 171) and pancreas transplant rejection (172). Furthermore, Clement et al have reported identifying GrB in bronchoalveolar lavage specimens when evaluating lung rejection (173) and Li et al have claimed that measuring of mRNA encoding cytotoxic proteins in urinary cells offers a non invasive means of diagnosing acute rejection of a renal allograft (174). For the GrB ELISPOT technique, some authors have demonstrated that GrB ELISPOT assays could be an alternative to the standard 51Cr-release assay for measuring granule-mediated cytotoxicity (163), monitoring in cancer vaccine trials (175) or in kidney transplantation (85, 176). They have also shown that GrB could be a more specific candidate marker than IFN-γ for measuring the cytotoxic capacity of immune effector cells such as NK cells (85, 176, 177), but not yet, as far as we know, for exploring allogenic responsiveness in LT.

In our study, the only difference revealed was that GrB increased up to 360% at day 7 from baseline in the rejection group, whereas this increment was 200% only in the group without rejection. However, comparing day 0 and day 28, kinetic of GrB frequencies showed a significant increase in both groups with or without rejection. By time-period, there was no significant difference in frequencies of GrB-producing cells discovered in either group. In previous studies, the GrB ELISPOT has been shown to be a surrogate marker for CTL activity. However a limitation of the GrB ELISPOT assay is that it measures degranulation, not direct target cell lysis. As such, degranulation may not always equate to cell death if the target cells contain serpin proteinase inhibitor 9, a protein that inhibits the proteolytic activity of GrB, or if effector cells are perforin deficient. Moreover, the GrB ELISPOT assay also does not measure cytotoxicity mediated by FasL pathway. Furthermore, in rat liver grafts, Ogura et al demonstrated that in the absence of $CD8^+$ CTL, $CD4^+$ T cells may induce cytotoxicity and graft rejection. $CD4^+$ CTL mediated cytotoxicity is usually associated with Fas-mediated killing, and high levels of both FasL and GrB were detected in the $CD8^+$ T cells-deleted graft (177, 196). In renal transplantation, van Besouw et al reported that the activated CTL exert their effector function not only by exocytosis of GrB and perforine by the cytotoxic granules, but also that FasL and cytokines (TNF-α) exert cytotoxic effects through cognate receptors, such as Fas, expressed by target cells, all leading to cell death of the target cells (*85*). GrB ELISPOT was introduced to test for cytotoxicity in renal rejection but compared with kidney allografts, liver allografts have long been considered to have different immunological behaviors. This means that in LT, the induction of donor-specific tolerance is facilitated and hyperacute rejection or graft loss due to chronic rejection are rarely observed (197). Furthermore, the study cohort of Navarro et al indicated that unlike renal allografts, HLA matching or mismatching had no clinically significant impact on liver graft survival (198). We speculated therefore that according to the literature GrB ELISPOT assay could be considered as the best test for CTL in kidney transplant but it could be only of value as an early rejection biomarker for pediatric liver transplantation if a kinetic analysis can be done.

The secretion of GrB in every patient from baseline to one month post-transplant in our study could be dependent on the role of effector and memory T cells in the

alloreactivity response of recipients through recognition of donor antigens after transplantation. Further research should be encouraged, to identify the role of GrB ELISPOT assay associated with detecting memory and regulatory T cells, in order to establish an immunological profile for tolerant recipients in the long term post-transplantation.

4.2.6- Conclusion

In general, frequencies of GrB ELISPOT pre-transplantation did not predict the occurrence of early post-transplant acute rejection and GrB values at days 7, 14 and 28 could not be correlated with acute rejection in pediatric liver recipients. However, a kinetic study of GrB ELISPOT increment between baseline and day 7 could be helpful to predict or confirm early rejection in the small group of liver allograft recipients analyzed in this study.

5- GENERAL CONCLUSION AND FUTURE PROSPECTS

Monitoring of immune responses, potential minimization of IS, and clinical tolerance induction constitute factors that may contribute to optimization of outcome of pediatric clinical transplantation. One of the greatest limitations is our incomplete understanding of clinical tolerance, and the difficulty to detect its presence in individual recipients. In this context, finding predictive tests not only would be useful in order to evaluate the patient's immunological risk of early rejection but would also enable prediction of lack of alloresponsiveness which could be an indicator of tolerance or potentially allow changes in medical therapy.

In this thesis, we endeavor to identify the role of sCD30 and GrB ELISPOT in pediatric organ transplantation; however, although results of these assays did not reveal sensitive and specific markers in detecting early rejection. Kinetic monitoring of GrB ELISPOT variation was found helpful during this study to predict or confirm early prediction of rejection in the liver allograft recipients. Furthermore, in our analysis of the literature, we face several major challenges as we try to identify relevant immunological assays. First, numerous assays may be as predicting rejection, but in practice were inconsistent and also failed reliably to distinguish tolerant from non tolerant patients. Moreover, each assay has different advantages and disadvantages. It may therefore be necessary to evaluate multi-assays in each patient at different times in order to try to establish an immunological profile for tolerant recipients in the long-term. Next, considering that immune response in transplanted recipients may change in pre and post-transplantation, monitoring assays need to be performed over time with analysis of the detailed kinetics to ensure that tolerance is not lost. Finally, infants present a relative naïve immune system, but they have the full capacity to develop allogeneic responsiveness to donor antigens. In clinical research, to the best of our knowledge, no specific assay for use only in the field of pediatric organ transplantation has been developed. Moreover, restriction in blood volume samples limits *in vitro* assays in infants. In the future, the value of peripheral blood transcriptional profiling could be a new tool to identify recipients who are immunotolerant without pharmacological IS. Validation of these findings in prospective IS weaning trials would thereby open the door to the possibility of avoiding immunosuppressive drugs in suitable recipients highly

likely to be tolerant. Accordingly, a multidisciplinary approach including assessment of dynamic profiles in multicenter studies should be established, so that ideal strategies in the immunological monitoring of kidney and liver transplant in pediatric recipients can be implemented.

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