REVIEW

Monitoring of human liver and kidney allograft tolerance: a tissue/histopathology perspective

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Summary

Several factors acting together have recently enabled clinicians to seriously consider whether chronic immunosuppression is needed in all solid organ allograft recipients. This has prompted a dozen or so centers throughout the world to prospectively wean immunosuppression from conventionally treated liver allograft recipients. The goal is to lessen the impact of chronic immunosuppression and empirically identify occasional recipients who show operational tolerance, defined as gross phenotype of tolerance in the presence of an immune response and/or immune deficit that has little or no significant clinical impact. Rare operationally tolerant kidney allograft recipients have also been identified, usually by single case reports, but only a couple of prospective weaning trials in conventionally treated kidney allograft recipients have been attempted and reported. Pre- and postweaning allograft biopsy monitoring of recipients adds a critical dimension to these trials, not only for patient safety but also for determining whether events in the allografts can contribute to a mechanistic understanding of allograft acceptance. The following is based on a literature review and personal experience regarding the practical and scientific aspects of biopsy monitoring of potential or actual operationally tolerant human liver and kidney allograft recipients where the goal, intended or attained, was complete withdrawal of immunosuppression.

Introduction

Allograft biopsy evaluation plays a critical role in the emerging field devoted to minimization or complete weaning of immunosuppression from human solid organ allograft recipients. The immediate practical goal of this field is to improve the quality of life and outcomes for allograft recipients by minimizing exposure to the high cost and serious side-effects of chronic immunosuppression, such as hypertension, diabetes, hyperlipidemia, kidney damage, and increased susceptibility to malignancies. Presumably, this can be achieved without sacrificing allograft structure and function consequent to uncontrollable acute or even indolent chronic rejection. A secondary, but equally important, goal is to use allografts as probes to understand cellular and molecular mechanisms associated with immunologic tolerance. The hope is that treatment algorithms might then be devised to routinely induce tolerance to allografts in a large percentage of recipients. These concepts might also be transferable to the related fields of autoimmunity and cancer immunosurveillance.

Since the advent of solid organ transplantation, two general approaches have been used to study clinical allograft acceptance/tolerance: (i) so-called 'spontaneous operational tolerance (SOT)' is a term borrowed from Ashton-Chess et al. [1]. It refers to rare noncompliant recipients and others deliberately removed from immunosuppression who do not develop rejection even long after

the event. SOT recipients are usually identified by trial and error. This approach was pioneered by Starzl who realized that acute rejection was reversible with temporarily increased immunosuppression, with the need for such immunosuppression significantly diminished afterward [2] and (ii) tolerance can also be induced intentionally via hematopoietic macrochimerism, using bone marrow or hematopoietic stem cell transplantation combined with simultaneous [3–5] or delayed kidney transplantation [6–10]. The hematopoietic chimerism approach was based on the original experimental animal observations of Billingham et al. [11]. It was matured in further experimental animal studies and then successfully applied to humans by Sachs, Sykes, and Cosimi, using preconditioning with a nonmyeloablative regimen and major histocompatibility complex (MHC)-matched [3–5] or -mismatched [12] simultaneous bone marrow and living-donor kidney transplantation.

Distinguishing between these approaches has meaning beyond the purpose of understanding how the histopathology literature developed in this field. It also provides insights about the predominant immunologic mechanisms involved in allograft acceptance/tolerance. In experimental animals, tolerance achieved through hematopoietic chimerism is robust, mediated predominantly by deletion [13], and organ-independent. In the first author's experience as a clinical and experimental transplant pathologist, this approach leads to the 'cleanest', or the most normal-appearing allografts. Stable macrochimerism, however, is very difficult to achieve in mismatched humans without graft-versus-host disease [12]. Comparatively, SOT is meta-stable and probably mediated by a combination of deletion, ignorance, and regulation and is organ-dependent. Liver allografts exhibit SOT more frequently than other allografts (see below). SOT allografts are usually not as clean or free from inflammation as allografts in chimerically tolerant recipients. Rather than being totally different, however, the two approaches are qualitatively similar, but differ quantitatively in reference to underlying mechanisms, such as deletion and (micro-)chimerism, that contribute to long-term allograft survival [12,14].

These approaches also fit well with tolerance as recently defined by Girlanda and Kirk [15]. 'True tolerance' refers to the absence of any detectable detrimental immune response as well as the absence of immunocompromise. 'Operational tolerance' refers to the gross phenotype of tolerance in the presence of an immune response and/or immune deficit that has no significant clinical impact. For the histopathologist, the difficult phrase in the operational tolerance definition is, '…that has no significant clinical impact'. This is not so easily determined and will be discussed in greater detail subsequently. For this review, we excluded an evaluation of

pathology material from so-called prope tolerance studies [16,17] and reports of late rejection occurring in patients with low immunosuppression levels because it was difficult to determine what exactly constituted low-level or minimal immunosuppression.

Instead, this review is based on a literature survey and on personal observations from studies in which complete weaning from immunosuppression was the intended goal or provided some insight into the weaning process. It focuses primarily on studies of human SOT; detailed mechanistic studies in experimental animals are beyond the intended scope, except where they serve to illustrate a point relevant to human material. We apologize in advance because many of the histopathologic observations discussed are, because of trial design and material available, anecdotal and descriptive. But currently, that is the state of the field.

All allografts are not created equal

Spontaneous operational tolerance in conventionally treated recipients is, by far, most commonly observed in liver allograft recipients. Most clinical trials that attempt to prospectively wean human recipients from immunosuppression are conducted in liver allograft recipients and these also show the highest rate of success (see below). The traditional and probably the most accurate and authoritative reason given for this success is the so-called 'hepatic tolerogenicity' (reviewed in Benseler et al. [18] and Crispe et al. [19]). This refers to the liver's unique role as an immunologic organ. Examples include: (i) oral tolerance, or the observation that systemic immune responses to any particular antigen are significantly less robust if the antigen is fed orally beforehand; (ii) spontaneous acceptance of fully MHC-mismatched liver allografts without immunosuppression in many animal species, excepting humans and primates; (iii) ability of liver allografts to protect other, extrahepatic, allografts from rejection if the latter are derived from the same donor; and (iv) ability of the liver to protect central immune organs from overstimulation by gut bacteria, bacterial products, and other antigens that normally leak through the intestinal barrier. A detailed discussion of the various mechanisms of hepatic tolerogenicity is beyond the scope of this review. Included are the release of soluble MHC antigens, migratory passenger leukocytes and activation of recipient lymphocytes in secondary lymphoid tissues, microchimerism, hepatic dendritic cell immaturity, activation of naïve T cells and purging of cytotoxic cells within the liver, and stimulation of regulatory T cells (reviewed in Benseler et al. [18] and Crispe et al. [19]).

There are, however, other reasons as to why the liver allograft recipients are more ideal candidates for weaning studies than other conventionally treated solid organ

allograft recipients. First, the vast majority of acute cellular rejection episodes, regardless of severity, are not life- or allograft-threatening, do not produce significant morbidity, and are easily reversible with current immunosuppressive medications [20,21]. Second, reversal of rejection is usually complete: the allografts heal without significant fibrosis, architectural distortion, or loss of function because of robust hepatic regeneration [21–23]. Even the early phases of chronic rejection are reversible in the liver [24]. Therefore, if a liver allograft recipient develops acute or the early chronic rejection during or after weaning, the process is likely to be completely reversible without significant sequelae [21,24]. But there are exceptions and weaning is not risk-free (Table 1).

Table 1. Pre- and postweaning duration, and histopathologic diagnosis in follow-up liver tissue samples from liver allograft recipients withdrawn from immunosuppression.

Study	No. Pts off IS/ attempted	IS time	IS-free time	Histopathologic diagnoses in postweaning biopsy specimens						
				NS* changes	NRH	AR/ CR†	CH	PBC	AIH	Biliary
Starzl *et al.* [14]	6‡	15 years	8.8 years	2		NA	2			
Sandborn *et al.* [29]	0/12	>1 years	0			6/3				
Ramos *et al.* [23]	16/59§	>5 years	3–19 months	7	1	15/0	2			1
	27.1%			12%	2%	25.4%	3.4%			2%
Mazariegos *et al.*¶ [22]; updated in [84]	18/95§	ca. 8 years	0.6–3.5 years	10		21**/3	3	2		3
	19%	1.7–25 years		11%		26%	3.1%	2%		3.1%
Devlin *et al.* [57] and Girlanda *et al.* [61]	5–3/18	>5 years	8–24 months	7	3	4–13/1				2
	28%	5–11 years				22–66%				
Takatsuki *et al.* [52]	24/63	Most >2 years	Mean = 23.5 months			16/0				
	38.1%					(25.4%)				
Pons *et al.* [58]	3/9	62.5 months	17–24 months			2–6††				
	33%	24–105				22–66%				
Tryphonopoulos *et al.* [102]	20/104	>3 years	0.9–3.3 years			70‡‡/2	3			
	19%					69.2%	3%			
Eason *et al.* [103]	1/18	>6 months	<1 year			11/0	4			
	5.5%					61%	22%			
Eghtesad *et al.* [30]	NR/23					13	19			
Tisone *et al.* [31] and Martinez-Llordella *et al.* [33]§§	8+8=16/>34	4.5–5 years average	Yearly Bxs			26/0***	34/34			
	23.4%	>1 year.	Av. = 45.5 months			76.5%	100%			
Koshiba *et al.* [32] and Yoshitomi *et al.* [51]	87/581	Most >2 years	>5 years			8/25†††				
	15%									

AIH, autoimmune hepatitis; AR, acute rejection; CH, chronic hepatitis; CR, chronic rejection; IS, immunosuppression; NR, not reported; NRH, nodular regenerative hyperplasia; NS, nonspecific; PBC, primary biliary cirrhosis.

*Most frequently consists of mild 'nonspecific' portal inflammation and steatosis.

†Vast majority of acute rejection episodes were Banff [104] mild to moderate. Two patients from the Tryphonopoulos *et al.* [102] study developed chronic rejection; one required re-transplantation; three patients in the Mazariegos *et al.* [22] study developed early chronic rejection stabilized by a return to immunosuppression; three patients in the Sandborn *et al.*'s study [29] developed CR and two died; one patient in the study of Devlin [57] and Girlanda *et al.* [61] required re-transplantation because of CR.

‡Pathology results were not available for all patients in this study because some samples were submitted for immunofluorescence and PCR analysis.

§Not all patients were routinely subjected to follow-up biopsies after withdrawal of immunosuppression.

¶Overlaps with the study of Ramos *et al.* [23], but with longer follow-up.

**Seven patients were treated for rejection without biopsy.

††Four patients developed 'portal inflammation' with elevated liver injury test parameters, not necessarily diagnostic of rejection, but were returned to immunosuppression.

‡‡Forty rejection episodes were clinically suspected and 30 were biopsy-proven.

§§Overlapping patient populations.

***Focal ductopenia involving <20% of portal tracts was observed in occasional recipients, but criteria for chronic rejection were felt not to be present.

†††Yoshitomi *et al.* [51] reported decrease in size and increase in number of bile duct and fibrosis in patients, which they attributed to possibly a variant of chronic rejection.

This is in contrast to cardiac allografts, where severe acute cellular rejection might be lethal. In pancreas, lung, and renal allografts significant acute rejection more frequently results in irreversible scarring, architectural distortion, and permanent loss of function. Intestinal allografts can also heal without significant fibrosis, but severe acute rejection is usually more difficult to reverse and accompanied by significant morbidity. Third, liver injury test parameters are more sensitive indicators of injury than are standard function tests for other organs, for example, serum creatinine in kidneys, pulmonary function tests in lung allografts, or symptoms of decreased cardiac output in heart allografts. Finally, liver allografts are more resistant to antibody-mediated rejection than are other solid organ allografts [25].

The reported experience of SOT in conventionally treated liver- and kidney allograft recipients is shown in Tables 1–3. One study from our center [26] that included 50 kidney-, 17 liver-, 14 pancreas-, and 11 intestinal allograft recipients treated with leukocyte-depleting antibodies was not included in these tables because long-term follow-up has not yet been tabulated and the patients were not entirely immunosuppression-free at the time of publication. Spaced weaning leading to a significant reduction in immunosuppression, however, was achievable in a majority of surviving recipients [26]. The kidney cohort in that study overlaps with a series reported subsequently by Shapiro *et al.* [27] with longer follow-up.

Much of the data used to construct these tables are difficult to verify because individually tolerant recipients are often reported more than once and the same patients are not easily traced among the studies. But, even so, the relative ease with which liver allograft recipients can be completely weaned from immunosuppression as compared with kidney allografts recipients is obvious, especially if one compares the ratio of SOT/total transplants. SOT has been reported in the global literature in at least 49 kidney allograft recipients versus 148 liver allograft recipients (Tables 1–3). These numbers, however, are probably significantly lower than the actual number of SOT recipients who are either unknown and/or unreported.

Weaning trial designs

Most prospective 'weaning trials' have been conducted in liver allograft recipients. The various trials were similar in design and comprised primarily of conventionally treated and immunologically stable liver allograft recipients more than 2 years after transplantation, without technical complications, evidence of rejection or significant allograft pathology (Table 4). The clinical perspective, including the details of initial immunosuppression, which differed somewhat among the studies, has been expertly reviewed elsewhere [28]. Most trials weaned immunosuppression slowly over a period of months. Attempts at weaning earlier after transplantation were reported in recipients treated with lymphocyte-depleting antibodies at the time of transplantation [26].

It is difficult to contest the premise of weaning trials that less immunosuppression without rejection is desirable. But only the study of Sandborn *et al.* [29], who attempted to wean Cyclosporine, included contemporaneous matched controls maintained on conventional immunosuppression to determine whether the withdrawal from immunosuppression was indeed beneficial overall. One study from our center included comparison to a historic control group [30]. Other studies compared immunosuppressant-dependent (failed weaning) with immunosuppressant-free (successful weaning) recipients [31–33]. In general, no specific molecular mechanistic hypothesis was being tested in these weaning trials other than the one that microchimerism and long-term allograft acceptance under immunosuppression are conducive to immunosuppression-free allograft acceptance [34]. Therefore, the data collected differed somewhat among the studies. It would be more ideal, in conventionally treated recipients, to compare a 'weaning' group with a maintenance immunosuppressive therapy group and include both potentially positive and negative endpoints, such as incidence of acute and chronic rejection and development of graft fibrosis over a period of time, incidence and severity of immunosuppression-related complications (renal failure, diabetes, cardiovascular disease, malignancies) and cost of medications.

The data on majority of the SOT kidney allograft recipients have been derived from anecdotal reports based on individual patients that were either noncompliant or who had anti-rejection medication withdrawn because of immunosuppression-related complications (Table 2). Again, no specific hypothesis was being tested in these reports other than the possibility that immunosuppression weaning might be possible. In contrast, studies attempting to induce tolerance through macrochimerism were all conducted prospectively and tested the hypothesis that hematopoietic chimerism would lead to allograft tolerance in outbred humans (Table 3). The approaches included: (i) using a nonmyeloablative preparatory regimen and simultaneous MHC-matched [3–5] or -mismatched [12] bone marrow- and living-related kidney transplants; (ii) delayed renal transplantation after successful bone marrow transplantation from the same living-related donor using myeloablative therapy [6–10]; and (iii) MHC-mismatched renal transplantation after total lymphoid irradiation, lymphoid depletion, and donor hematopoietic stem cell infusion [35,36].

Table 2. Retrospective studies of 'spontaneous' kidney allograft acceptance/tolerance.

Study	Pts	IS time	IS free	Graft routine histopathology follow-up	IPEX and other allograft tissue studies
Owens et al. [54]	4/6	3–180 months	8–52 months	Acute rejection occurred in 2/6 patients 8–18 months after weaning from IS; no histopathology descriptions of rejecting or stable allografts	NA
Uehling et al. [55]	1/5	0.2–1	0.5–2.5	Two patients developed severe rejection after several months off IS; two returned to IS and one remained off IS; no histopathology description of any rejecting or stable allografts	Not done
Hussey [105]	1/8	NA	40 months	Most patients either developed severe rejection or returned to immunosuppression; no histopathology description of any rejecting or stable allografts	
Zoller et al. [56]	6/48	958 ± 792 days	394 ± 645 days	21/48 experienced graft failure (most within a few months) and only six continued to do well after IS cessation; no histopathology description of any rejecting or stable allografts	NA
Starzl et al. [64,66] and Randhawa et al.* [63]	7	2–36 years	0.3–>12 years	Three patients subjected to Bx: one tolerant and two patients on minimal steroids showed mild patchy interstitial lymphocytic inflammation forming occasional small clusters, but without tubulitis or vascular injury. Mild arterial nephrosclerosis, focal glomerular lobular accentuation and global glomerulosclerosis involving up to 10% of glomeruli	In testable cases, lymphoid infiltrates were of recipient origin; endothelium and tubules were primarily of donor origin; one case showed recipient-type cells in the mesangium
Fischer et al. [106] associated with pregnancy	1	9 years	9 years	No pathology	NA
Burlingham et al. [68,69]	1	2 years	7–8 years	Pre weaning biopsy showed focal infiltrate and fibrosis, but no tissue damage; F/U biopsy 7 years subsequently showed focal infiltrates but no tubulitis or evidence of acute rejection. Biopsy at ca. 8 years showed acute rejection; details not given	Not done
Christensen et al. [107]	1	3 years	3 years	No pathology	NA
VanBuskirk et al. [108], Xu et al. [67]	2	>1.5 years	10.3 and 18 years	Biopsies obtained from two SOT patients after F/U of 10.3–18 years. Both showed lymphoid aggregates and scattered interstitial mononuclear cell infiltrates without tubulitis, allograft vasculopathy, interstitial fibrosis, or tubular atrophy	Numerous $CD4^+/TGF-\beta1^+/CD25^{+/-}/FoxP3^-$ in the interstitium; $TGF-\beta1^-/FoxP3^+/CD25^+$ cells mainly in lymphoid aggregates
Roussey-Kesler et al. [53] and Brouard et al. [109]	10	1–13 years	1–20 years	Biopsies from two recipients: (i) after 13 years of SOT renal dysfunction prompted a biopsy that showed grade I CAN with mild nephroangiosclerosis without significant lymphoid infiltration or specific changes suggestive of chronic rejection (C4d-) and (ii) after 7 years of SOT renal dysfunction prompted dialysis and a biopsy that was negative for acute rejection, but showed focal fibro-edema associated with mild mononuclear infiltration, and double contours in GBM and moderate arteriolar hyalinosis (not shown) diagnosed as grade Ib CAN with allograft glomerulopathy (C4d-)	Not done
Newell et al. [110]	16	13 ± 10 years	>1 year	No pathology	NA

CAN, chronic allograft nephropathy; F/U, follow-up; GBM, glomerular basement membrane; IPEX, immunoperoxidase tissue staining; IS, immunosuppression.
*Two of the three patients were maintained on very low (7.5 and 10 mg, respectively) of prednisone daily.

Table 3. Prospective studies of 'induced' kidney allograft acceptance/tolerance.

Study	Pts	IS time	IS free	Allograft findings/histology, if available	IPEX and other allograft tissue studies
MHC-mismatched cadaveric renal Tx (Strober et al. [35]) or living-related kidney Tx (Scandling et al. [36]) after TLI, lymphoid depletion and donor hematopoietic cell infusion	4	ca. 3 years	2–5.8 years	Obstructed ureter prompted a Bx at 10 m after IS withdrawal showed normal glomeruli and blood vessels and an occasional focus/cluster of interstitial mononuclear cells. A subsequent episode of obstruction was followed by increased creatinine and a second biopsy showed diffuse mononuclear-cell infiltrate consistent with either chronic obstruction or rejection. Another patient had a 'normal' Bx 20 m after withdrawal of IS. Most recent patient [36] had normal function at 28 months, but no biopsy was reported	Not done
Simultaneous MHC matched (Spitzer et al. [3], Buhler et al. [4], Fudaba et al. [5]) and MHC mis-matched (Kawai et al. [12]) living-related renal and BMTx using non-myeloablative regimen	6 m + 4/5 mm	9–14 months	2–5.3 years	MHC matched donors: not reported in any detail MHC mismatched donors: one allograft lost to antibody-mediated rejection. One developed anti-donor HLA class II antibodies 2 months after complete IS withdrawal with C4d deposits in the allograft and segmental duplication of the GBM in some glomeruli. Biopsies from the three other grafts reported as normal and/or showing transient mononuclear cell infiltrates after IS withdrawal	MHC mismatched donors: intragraft levels of FoxP3 mRNA were about six times higher in the stable IS-free group than in the stable-with-IS group, whereas the granzyme B mRNA levels were similar. Therefore, the ratio of FoxP3:granzyme B might be important
Delayed living-related renal Tx after successful BMTx from the same donor: Sayegh et al.* [6], Butcher et al.* [7] Helg et al.* [8], Jacobsen et al.† [9], Sorof et al.† [10]	8	0–2 years	0–2.5 years	No pathology	NA
alemtuzumab depletion without (Kirk et al. [37])or with deoxyspergualin (Kirk et al. [38]) in living donor Tx	7 + 5	0	18–32 days	Atypical rejection developed in all 12 patients in both studies characterized by macrophages-rich infiltrates before T-cell extravasation, which initially distended interstitial capillaries, but subsequently became diffuse involving most of the interstitial capillaries, the interstitium, and tubules during clinical rejection	IPEX staining for CD3, CD4 CD8, CD20, CD45RO CD68, CD56, perforin, granzymeB, and HLA-DR showed macrophage-rich infiltrates during rejection with few CD45RO⁺ cells, no increased NK cells, and upregulation of HLA-DR on tubules; increased transcripts associated with macrophage/monocyte function and chemokines. C4d staining was negative in the second study [38]

Table 3. continued

Study	Pts	IS time	IS free	Allograft findings/histology, if available	IPEX and other allograft tissue studies
Thymoglobulin or Campath pretreatment followed by Post-Tx tacrolimus monotherapy and spaced weaning (Shapiro et al. [27]) in cadaveric Tx	191	3–6 months	NA	45% of recipients developed rejection during weaning. Weaning was unsuccessful in about one-third of Thymoglobulin-treated recipients. Two-thirds were on reduced/spaced tacrolimus therapy after a follow-up of 24–39 months. Seventy-four per cent of the Campath-treated patients were on spaced weaning after 12–18 months of follow-up. Protocol biopsies were not performed, but patient and graft survival and the rate of CAN progression was similar to historic, conventionally treated controls	NA

BMTx, bone marrow transplant; GBM, glomerular basement membrane; IPEX, immunoperoxidase tissue staining; IS, immunosuppression; MHC, major histocompatibility complex; TLI, total lymphoid irradiation; Tx, transplant; m, MHC matched donors; mm, MHC mismatched donors.
Kidney transplants after MHC-identical* or parent to offspring (haploidentical)† BMT from the same donor.

Three other prospective kidney trials are included in Table 3. Two by Kirk *et al.* [37,38] used alemtuzumab leukocyte depletion both without [37] and with coexistent deoxyspergualin therapy [38], but without other immunosuppressants in related and unrelated living donors. Shapiro *et al.* [27] used thymoglobulin or alemtuzumab depletion plus tacrolimus monotherapy with fully mismatched cadaveric donors. These studies differed in the timing and dosage of alemtuzumab. Kirk *et al.* [37,38] did not use any baseline immunosuppression except deoxyspergualin in his second study [38], whereas Shapiro *et al.* [27] relied on tacrolimus monotherapy, which was weaned shortly after transplantation. They were also, however, testing the hypothesis that depletion of the recipient immune system would create favorable conditions for the development of tolerance [37,38] and donor leukocyte migration might positively contribute to this process [34] through the induction of chimerism.

Clinical and detailed histopathologic observations

This section will follow the observations during enrollment and follow-up of patients participating in immunosuppression minimization trials.

Pre weaning clinical profiles and biopsies

In most, but not all, prospective SOT liver and kidney allograft immunosuppression minimization trials, 'pre weaning' biopsies are obtained. The liver injury test parameters and serum creatinine are usually normal or near-normal, but minor abnormalities are not uncommon. The purposes of the biopsy are to: (i) exclude any histopathologic rejection-related activities or other findings, such as significant fibrosis, that might exclude the patient from the trial and (ii) document any other baseline inflammatory and/or structural changes present before withdrawal so that they can be compared with findings in subsequent biopsies. The rationale for these biopsy-based exclusions is as follows. Low-level subclinical rejection is likely to significantly worsen after weaning and any additional insult on an already structurally compromised allograft would likely lead to failure. In addition, any changes to allograft structure might represent a heretofore unrecognized manifestation of rejection, or a beneficial effect of immunosuppression withdrawal.

Most 'pre weaning' liver allograft biopsies are obtained several years after transplantation and show changes that are typical of protocol biopsies obtained at that time. These biopsies are often difficult to interpret and the subject of a recent Banff consensus document [39]. Nearly 75% of biopsies obtained from adult recipients surviving more than 1 year 'with abnormal liver tests' will show

Table 4. Design of immunosuppression withdrawal trials after liver transplantation and time until rejection, if it occurred.

Study	Inclusions/exclusion criteria	Time until rejection
Sandborn *et al.* [29]	Adult, cadaveric donors >12 months s/p Tx Normal liver biopsy within 3 months of weaning Serum creatinine >2.1 mg/dl or creatinine clearance <35 ml/min	6 months (1–21 months)

Ramos *et al.* [23]	Adult, cadaveric donors, >5 years post-transplant;
	  >2 years without rejection
	History of medical compliance
	Immunosuppression related complications
	Primary physician cooperation
	Absence of rejection or severe necro-inflammatory
	  disease on liver biopsy
		6.5–22.5 m Average = 15 months

Mazariegos *et al.** [22]	Same as above	0.2–42 months
Devlin *et al.* [57] and Girlanda *et al.* [61]	Adult, cadaveric donors Side effect of immunosuppression	(range, 10–176 days) Three weeks after withdrawal of immunosuppression
Takatsuki *et al.* [52]	Pediatric, living-related donor ≥2 year post-Tx; nl. graft function; ≥1 year rejection-free Evidence of medical compliance Cooperative local physician for follow-up	Median = 9.5 months (1–63 months)
Pons *et al.* [58]	Adult cadaveric donors >2 years after Tx	NA

Tryphonopoulos *et al.* [102]	Adult, cadaveric donors
	Compared BM infusion group to controls to determine
	  if infusion augmented chimerism and whether
	  augmented chimerism increased tolerance
	>3 years post-Tx; stable liver function tests; rejection-free 12 months
	Recipients with autoimmune disorders excluded
		17.8 (no BM)–23.9 (BM) months

Eason *et al.* [103]	Adult and pediatric, presumed cadaveric >6 months post-transplant without rejection Tacrolimus monotherapy with trough levels <5 ng/ml Normal liver function tests; no recurrent disease	NA
Tisone *et al.* [31]	Adult, cadaveric donors HCV RNA serum positivity 12 months after transplant; absence of advanced disease Biopsy proven HCV recurrence with normal graft function Treatment compliance	0.5–8 months, except for one patient at month 43
Koshiba *et al.*† [32]	Pediatric, living-related donor Pediatric patients; >2 years s/p Tx; >1 year rejection-free Normal liver function Parental permission	NA

*Overlaps with the population of Ramos *et al.* [23].
†Overlaps with the population of Takatsuki *et al.* [52].

histopathologically significant abnormalities [40–45], which are usually attributable to recurrent disease or biliary tract strictures [40–45]. The percentage is significantly less in pediatric recipients because recurrent disease is much less common. However, unexplained chronic hepatitis/inflammation is seen in a high percentage of pediatric recipients at some centers and this might represent a form of late rejection [46,47]. In addition, nearly 25% of biopsies from long-surviving 'asymptomatic adult recipients with normal liver tests' will show significant abnormalities if the original disease is one that commonly recurs, such as hepatitis C virus(HCV), steatohepatitis, primary biliary cirrhosis, or autoimmune hepatitis [40–45] and in up to 11% of recipients the pathology findings were judged to be of clinical significance [48].

Other minor histopathologic abnormalities occur in about two-thirds of long-term biopsies, even without recurrent disease, in asymptomatic recipients with normal or near-normal liver tests [40–45]. Common findings are portal venopathy and nodular regenerative hyperplasia; thickening and hyalinization of small hepatic artery branches [43,49], 'nonspecific' portal and lobular inflam-

mation [43–45,50], and Ito cell hyperplasia [48]. A higher percentage of split and liver donor allografts also show architectural changes compared to whole cadaveric organs (A. Demetris, unpublished observation). The pathogenesis, significance, long-term consequences, and impact of weaning on these otherwise unexplained, long-term histopathologic findings are in need of further study. It is important in drug minimization trials that changes associated with long-term engraftment are not confused with variants of rejection after weaning, which reinforces the need for pre weaning biopsies.

It is worth emphasizing that original disease recurrence is a significant problem in adult liver transplantation [39], accounting for about 50% of all episodes of allograft dysfunction occurring more than 1 year after transplantation. In contrast, biliary atresia is the indication for the vast majority of pediatric liver transplants. As this disease does not recur after transplantation, interpretation of the results of pediatric weaning studies, such as those from the Kyoto group [32,51,52], are less complicated from the perspective of recurrent disease.

In kidney allografts, interpretation of baseline pathology changes is generally much less complicated and the findings are usually attributable to age and hypertension, calcineurin toxicity, and/or diabetes-related changes, such as patchy interstitial fibrosis/tubular atrophy, mild mononuclear interstitial inflammation, and arterial and arteriolonephrosclerosis of varying severity. In contrast to liver allografts, recurrence of the original disease is much less common and rarely impacts the decision to continue with weaning. For weaning studies, however, theoretically the pre weaning biopsy should show no evidence of active tubulitis or other obvious rejection-related changes and negative C4d stains [26]. Weaning has been attempted, however, in recipients showing borderline changes when the serum creatinine levels were near-normal and/or stable [26]. It is very difficult, however, on the basis of routine light microscopy to absolutely and reliably distinguish between borderline changes and nonspecific inflammation associated with aging and arterial and arteriolonephrosclerosis. Some of these patients did not develop more severe rejection after weaning [26], but characterization of the infiltrates with formal long-term follow-up studies are needed to determine the impact of this decision.

Clinicopathologic observations during and shortly after weaning

Allograft biopsies were obtained in most weaning trials only when there was an elevation in liver injury test parameters for liver allografts or serum creatinine for kidney allografts. However, at the Liver Sessions 2007 Banff consensus meeting, which was devoted to late allograft dysfunction and weaning of immunosuppression, all participating hepatologists and surgeons agreed that protocol follow-up biopsies should be mandatory, or at least strongly encouraged, in such trials. Arguments raised by Roussey-Kesler *et al.* [53] not to conduct protocol biopsies in stable SOT kidney allograft recipients included the possibility that minimal histopathology findings might be misleading and result in an unjustified return to immunosuppression. In addition, the need to conduct serial biopsies to monitor possible progression of subtle findings carries a risk of morbidity. And weaned stable allograft recipients might not want to undergo biopsy evaluation. Counter-arguments to these reasonable points of concern are that the biopsy findings and interpretation should be viewed similar to any other laboratory test result and incorporated into the entire clinicopathologic profile. In addition, much can be learned from the biopsy material, particularly as there is evidence to suggest that in humans the allograft plays an important role in the maintenance of tolerance.

Elevation of the liver injury test parameters in liver allograft recipients or serum creatinine in kidney allograft recipients is not uncommon and usually occurs within the first several months during or after drug withdrawal [22,23,27,52,54–57]. In liver allograft recipients, however, elevated liver injury test parameters did not distinguish between those who developed acute rejection and those who did not [22,23,57]. This is because biopsies obtained for elevated liver injury test parameters showed a variety of changes, including recurrence and/or exacerbation of underlying chronic viral or autoimmune hepatitis, primary biliary cirrhosis, biliary tract complications, steatohepatitis, nodular regenerative hyperplasia, and nonspecific 'lobular reactive changes' (Table 1). This illustrates that immunosuppression also prevents immunologically mediated liver injury other than rejection. In addition, mildly elevated liver injury test parameters occasionally returned to normal without therapeutic intervention after a biopsy had largely excluded acute rejection as the cause of the dysfunction [22,23].

Persistent significant elevations of liver injury test parameters (3X baseline values), however, usually signal the development of a clinically relevant problem. Of the various enzyme measurements comprising the standard liver injury test profile, gamma-glutamyl transpeptidase (GGTP) elevations were felt to be the most specific and sensitive for rejection in two studies from two different studies/centers [23,52]. In contrast to its value in the early post-transplant period, total serum bilirubin was a relatively insensitive marker for acute rejection developing after weaning [22,23,52,57].

When acute rejection was identified as a cause of liver allograft dysfunction during or after weaning, in our experience, the histopathologic appearance of most, but not all cases, was typical of that reported for late onset acute rejection (reviewed in [39]). Most of these episodes were graded as mild, but occasional cases of moderate to severe symptoms were reported (Table 1). In several liver studies, however, even when biopsy analysis had excluded other causes of allograft dysfunction associated with a clinical diagnosis of acute rejection, the histopathologic findings were not always typical of those reported for acute rejection [22,23,57,58]. There are at least four possible reasons for this observation: (i) acute rejection occurring late after transplantation (>1 year) in liver allografts differs from that occurring earlier within the first several months of transplantation, even in conventionally immunosuppressed recipients (reviewed in [39]); (ii) the composition of both the allograft and the recipient immune system are different early versus late time points after transplantation; (iii) immunosuppressive regimens used before weaning, such as lymphocyte-depleting antibodies, can alter the histopathologic appearance of acute rejection after weaning; and (iv) understandable clinical anxiety about elevated liver injury test parameters occurring in conjunction with weaning might trigger therapeutic intervention before characteristic histopathologic changes have time to develop.

Portal and/or perivenular inflammation is almost invariably observed. The major histopathologic differences between acute liver allograft rejection occurring after weaning versus 'typical early acute rejection' include less inflammatory bile-duct damage and more interface and lobular necro-inflammatory activity in the former. These differences cause the biopsies obtained after weaning to resemble hepatitis, which in turn, results in some diagnostic difficulties for the pathologist [22,23,57,58]. Increased interface and disease activity is also seen after weaning in patients with an original disease of autoimmune hepatitis [57] and primary biliary cirrhosis [23], some of whom develop new onset autoimmune hepatitis. In addition, early and rapid weaning of immunosuppression in HCV$^+$ recipients treated with lymphocyte-depleting antibodies can 're-arm' the immune system [30]. This manifests histopathologically as an aggressive hepatitis with rapid progression of fibrosis [30]. The important message from the above observations is that rejection is not the only cause of allograft dysfunction that occurs after weaning (Table 1).

No standardized reliability studies have been conducted on biopsy samples obtained in the setting of immunosuppression weaning to determine if pathologists agree in their interpretation. This is because of the rarity of such samples and the anxiety associated with clinical decision-making process. Such studies, however, are important and will be needed, particularly to define changes that might signal a need to return to immunosuppression versus ones that are probably benign and nonprogressive. In the meanwhile, in the first author's experience, reliance on standardized criteria is suggested [39].

Acute and chronic kidney allograft rejection occurring during or after weaning, in our experience, has not differed significantly enough from that usually seen in conventionally treated immunosuppression patients to cause diagnostic difficulties for the histopathologist. Development of anti-donor antibodies in the peripheral circulation and C4d deposits in the kidney, however, usually signals a need for returning to immunosuppression if the deposits are accompanied by histopathological evidence of significant tissue injury. Recurrence of the original disease can also be the primary cause of allograft kidney dysfunction after weaning, but the incidence is much less common than in liver allografts (unpublished observation).

The leukocyte-depleting alemtuzumab studies of Kirk *et al.* [37,38] nicely illustrate how the treatment strategy can influence the histopathologic findings. In the first study, no other baseline immunosuppression was used [37]. The histopathologic findings during clinical rejection that subsequently developed in all recipients, several weeks after transplantation, were not typical of early acute cellular rejection in conventionally treated renal allograft recipients. Instead, chemokine and macrophage function-rich transcripts were detected in needle biopsies early after transplantation, accompanied by margination of macrophages in interstitial capillaries on day 14. This occurred before the onset of significant T-cell infiltration and clinically evident rejection. Macrophage infiltrates became more diffuse at the onset of clinical dysfunction involving most of the interstitium, capillaries, and tubules. Small numbers of CD45RO$^+$ (memory) T cells were limited to the areas of macrophage infiltration, and tubulitis, when seen, was macrophage-predominant. Treatment with corticosteroids, OKT3, and sirolimus monotherapy reversed these episodes. A follow-up study added deoxyspergualin to the regimen in an attempt to inhibit macrophage function [38]. However, neither the clinical results nor the histopathologic features of rejection were significantly different from the study using alemtuzumab alone [37] and C4d stains to monitor for antibody-mediated rejection were negative in the second study [38].

A general consensus in most kidney- and liver-weaning studies is that biopsy monitoring to determine the cause of dysfunction after weaning is an absolute necessity. Close clinicopathologic correlation, however, is even more important. In our experience, unbalanced emphasis on either the histopathologic findings or clinical profile can

adversely impact either the scientific validity of the study and/or patient safety. The usual situation is that the pathologist is the worrier, whereas the clinicians are more reassured by stable liver injury test parameters or creatinine, although the reverse can also occur. As the truth is often somewhere in between the two viewpoints, one should override the other only when findings are obvious, or there is evidence of a clear trend over a period of time. Long-term follow-up often provides a clear indication of whether the chosen approach was correct or not.

Correlation between preweaning biopsy findings and outcome

Baseline biopsy findings in some liver studies proved to be associated with successful weaning when compared with biopsies from unsuccessfully weaned patients. Significant variables included: (i) less portal inflammation, overall; (ii) less CD3^{+} and CD8^{+} but more CD45RO^{+} lymphocytes within the lobules [59]; (iii) more advanced portal fibrosis in HCV^{+} recipients [31]; and (iv) an increase of potentially regulatory FoxP3^{+} T cells within the allografts of pediatric recipients [32,60]. Unsuccessful weaning, conversely, was associated with significantly more chronic portal inflammation on hematoxylin and eosin (H&E) stains and decreased CD45RO^{+} as well as increased CD8^{+} lymphocytes in the lobules [59]. These observations suggested that chronic portal inflammation and lobular CD8^{+} cells might represent a latent form of rejection held in check by medications, which manifests itself clinically after the removal of immunosuppression.

A worry is that seemingly 'tolerant' patients might actually be experiencing low-grade chronic rejection. For example, in one liver series, five patients were categorized initially as showing SOT [57]. During longer follow-up, however, one recipient developed acute rejection requiring reinstitution of immunosuppression, another required retransplantation for chronic rejection, and a third resumed immunosuppression because of a kidney transplant [61]. We appear to have observed similar occurrences in liver allograft recipients, but more characterization of tissue samples is needed (A. Demetris, unpublished observations). Similar findings have been reported in SOT kidney allograft recipients, so it is prudent to continue to closely follow seemingly tolerant patients.

Studies examining associations between pre weaning kidney biopsy findings and postweaning outcome have not been reported.

Clinicopathologic observations in stable SOT recipients

Many centers do not sample the allografts of SOT recipients if they are 'clinically stable'. Thus the number of

tissue samples from SOT liver and kidney allografts who remain off of immunosuppression after the biopsy, is considerably smaller than the total number of SOT recipients. Instead, biopsies are obtained only when indicated by elevated liver injury test parameters or serum creatinine. Consequently, the number of reported protocol tissue samplings in stable SOT recipients, whom remain immunosuppression-free after biopsy, is exceeding small. In total, 'more or less' protocol biopsies were obtained from eight SOT kidney recipients and from six chimeric-bone marrow plus kidney recipients and reported in the literature (Tables 2 and 3). The total number of liver allograft biopsies from SOT is more difficult to tabulate because different reports frequently contain overlapping patient populations (Table 1). The number appears to be between 100 and 200. This is somewhat disappointing because protocol biopsies from SOT patients can provide clinically and scientifically useful information.

There have been three studies, two liver- [14,58,62] and one kidney transplant [63,64] that have characterized the donor/recipient phenotype of cells infiltrating and comprising SOT allografts. In liver allografts, the vast majority of hepatocytes, bile ducts cells, and large vessel endothelia remain of donor origin, as do the tubular epithelial cells and endothelial cells of kidney allografts [63,64]. A majority of infiltrating leukocytes, however, were of recipient origin [14,58,62–64]. But donor hematopoietic cells can also be detected amidst the interstitial inflammation in some nearly SOT kidney allografts on low-dose immunosuppression [26]. The significance of persistent donor hematopoietic cells within the allograft and whether it predicts subsequent acceptance has not been studied in any detail. In SOT liver allografts, some replacement of sinusoidal lining cells can be seen. But it is difficult to distinguish between Kupffer's cells and endothelial cells and the level of sinusoidal cell replacement did not correlate with the ability to wean immunosuppression [58].

No long-term follow-up biopsies were conducted in the SOT kidney allografts, and because of the small numbers, it is difficult to draw any conclusions regarding any association with weaning. At this time, however, the evidence suggests that recipient replacement of donor epithelial or endothelial cells within the allograft is not a substantial mechanistic contributor to the development of SOT. Whether persistence of donor hematopoietic cells, including dendritic cells (DC) in the interstitium of allografts is associated with acceptance, as in experimental animals [65] is being actively investigated in our SOT tissue samples.

The Kyoto group conducted protocol biopsies in 14 pediatric living-donor liver allograft recipients who had been weaned from all immunosuppression. These biopsies

were compared with biopsies from control liver allograft recipients maintained on chronic immunosuppression [51]. There was more extensive portal fibrosis, ductular reactions, more CD8^{+} cells and decreased luminal diameter of bile ducts in SOT immunosuppression-free recipients [32,51]. The authors worried that the changes observed in the SOT recipients might represent a subtle, heretofore unrecognized, variant of chronic rejection [32,51]. Some of their concern is warranted because of the significant fibrosis and increased CD8^{+} lymphocytes is similar to that reported by Wong *et al.* [59], above. The mean follow-up in the Kyoto SOT group, however, was several years longer than that in their control group. This raises some questions about the etiology of these changes, which are not entirely typical of either early or late, acute or indolent chronic rejection. As the influence of longer term engraftment, regardless of immunosuppression, needs to be considered, more follow-up and detailed characterization of the changes are needed in this cohort.

Tisone *et al.* [31] studied the effect of immunosuppression weaning in HCV[+] recipients and conducted protocol biopsies at 1 month after completion of weaning and yearly thereafter. Interestingly, successfully weaned patients initially showed more advanced fibrosis in baseline biopsies than immunosuppression-dependent HCV[+] recipients. After weaning, fibrosis failed to progress significantly, or actually regressed, in patients removed from immunosuppression [31]. In contrast, the immunosuppression-dependent HCV[+] recipients showed fibrosis progression typical of conventionally treated HCV[+] recipients. Thus, complete weaning of immunosuppression showed a beneficial effect on HCV-induced fibrosis progression in one patient subset [31]. They also mentioned that focal ductopenia, a histopathologic finding of concern for early chronic rejection, was occasionally observed in protocol biopsies from the SOT patients. It was, however, always limited to less than 20% of the portal tracts, which is of uncertain significance. Once again, however, longer follow-up is needed in this cohort to make sure that early chronic rejection does not occur. But it is reassuring that this group did not show significantly elevated GGTP levels (a biochemical marker of ductopenia) as compared with the immunosuppression-dependent controls [31].

A common finding reported in SOT kidney allografts is that of patchy interstitial inflammation that is often arranged into small nodular aggregates [63,64,66,67] (Tables 2 and 3). Some biopsies have been reported as normal. Other findings include mild arterial nephrosclerosis, focal global glomerulosclerosis, grade 1 chronic allograft nephropathy with mild 'nephroangiosclerosis', moderate arteriolar hyalinosis and double contours of the glomerular basement membrane indicative of transplant glomerulopathy (Table 2). Most of these findings, however, are largely nonspecific from a light microscopic perspective and are commonly encountered in aged and/or hypertensive or diabetic kidneys and those with calcineurin toxicity. A possible exception is some of the transplant glomerulopathic changes, which might signal a form of antibody-mediated injury.

Xu *et al.* [67] characterized the patchy tubulointerstitial lymphocytic infiltrates in two SOT kidney allografts after 10.3 and 18 immunosuppression-free years. They found the interstitial infiltrates to be enriched with CD4^{+}/transforming growth factor (TGF)-$\beta1^+$/CD25[±]/FoxP3^{−} adaptive regulatory T cell (T_{reg}) and lymphoid aggregates enriched with TGF-β1-/FoxP3^{+}/CD25^{+} natural T_{reg}. Several years earlier, Burlingham *et al.* [68] reported a SOT kidney allograft recipient that showed similar findings (i.e. patchy interstitial infiltrates without damage) in a pre weaning biopsy. The patient remained stable during follow-up and a biopsy after 7 immunosuppression-free years was unchanged. The serum creatinine, however, gradually increased from 1.6 to 1.8 to 2.0 mg/dl and the patient eventually developed biopsy-confirmed acute rejection 9.7 years after transplantation [68,69].

Roussey-Kesler *et al.* [53] reported 10 SOT kidney allograft recipients after 9.4 ± 5.2 immunosuppression-free years. Most of these patients had interrupted weaning of immunosuppression over a long period of time and donor age was younger than donors used in the general transplant population. One patient, after 13 years of SOT, developed renal dysfunction. A biopsy showed grade I chronic allograft nephropathy with mild nephroangiosclerosis without significant lymphoid infiltration or specific changes suggestive of chronic rejection. C4d staining was negative and no anti-HLA antibodies were detected in the circulation. Renal function also deteriorated progressively in another patient, requiring dialysis. An allograft biopsy in this patient performed after 7 immunosuppression-free years, showed grade Ib chronic allograft nephropathy with allograft glomerulopathy, but without C4d staining.

The most impressive and carefully documented series of biopsies from tolerant kidney allograft recipients were reported by Kawai *et al.* [12]. They induced tolerance using combined bone marrow and kidney transplants from MHC single-haplotype mismatched living-related donors with a nonmyeloablative preparative regimen. Of the five patients enrolled in that trial, one allograft was lost to antibody-mediated rejection. One other developed anti-donor HLA class II antibodies 2 months after complete immunosuppression withdrawal. Biopsies from this patient showed C4d deposits and segmental duplication of the glomerular basement membrane in some glomeruli. The patient was not returned to immunosuppression because of uncertainty about the significance of the

relatively minor changes that did not worsen over a period of time. Protocol biopsies from the three other grafts obtained from between 666 and 1135 days after transplantation and from about 400 to 1000 days after withdrawal of all immunosuppression were reported as normal and/or showing transient mononuclear cell infiltrates; C4d stains were negative. Intra-graft levels of FoxP3 mRNA were about six times higher in the stable immunosuppression-free group than in the stable-with-immunosuppression group, whereas the granzyme B mRNA levels were similar. Therefore, the ratio of FoxP3:granzyme B might be an important marker of a favorable T_{reg}–$T_{effector}$ ratio and allograft acceptance.

Lessons learned and common characteristics of spontaneously/operationally tolerant allografts

Common clinical characteristics of successful weaning that emerge from the review of SOT liver- and kidney allografts include living-related allografts, immunologically stable/noninflamed allografts, and long survival, in situ, under conventional immunosuppression with gradual weaning of immunosuppression over months to years (Tables 1–3). Conversely, early and abrupt weaning of immunosuppression, nonrelated cadaveric allografts, or previously inflamed allografts are more likely to experience rejection after weaning. The 'take home' messages reported in the liver trials are shown in Table 5. These observations/lessons are beginning to point toward immunologic processes associated with graft acceptance, and eventually, these will translate into molecular pathways. But currently, the field is in its infancy.

Problems with early abrupt weaning and the advantage of relatively long allograft residence under immunosuppression and slow weaning are all probably related to the immunologic interface between the donor and recipient. Early after transplantation, in conventionally treated recipients, this interface is an activated and contentious one because: (i) the massive migration of donor hematolymphoid cells and cellular debris (danger-associated molecules) from the allograft floods the recipient lymphoid tissues [70,71] and (ii) tissue damage from preservation-related injury [72] fosters recipient leukocyte migration and retention within the allograft. The migration of donor leukocytes and debris, particularly from liver allografts, floods recipient lymphoid tissues with innate activation signals that can have both positive and negative effects, such as activation and partial deletion of donor-reactive lymphocytes and/or development of allospecific memory cells [14,34,73,74]. This probably explains why more immunosuppression is needed to prevent rejection early after transplantation and why it is more difficult to wean immunosuppression at this time [2,14].

The immunologic barrier is overcome or subverted, however, while using the combined bone marrow or hematopoietic stem cell and kidney transplant approach [12]. Part of the early success in this pioneering trial is likely related to the relatively harsh conditioning regimen; but it is also nonmyeloablative, and weaning from immunosuppression has been rapid and deliberate. As compared with other trials using more conventional immunosuppression, this approach also shows a higher overall rate of success, but currently it can be applied to only a limited subset of patients. Nevertheless, the high rate of success, convincing demonstration of donor-specific nonreactivity, and 'cleanliness' of the allografts [12], in our opinion, suggest that deletion has occurred in these patients, at least early after transplantation. And deletion results in more robust tolerance. As macrochimerism was only observed transiently in these patients [12], it will be interesting to determine whether the deletion, donor-specific nonreactivity and allograft cleanliness persist long-term.

Preservation injury eventually heals, donor passenger leukocyte migration diminishes, and most, but not all, hematolymphoid cells within the allograft are eventually replaced by recipient ones. And the recipient immune system is no longer the same as is was before transplantation. In SOT, however, the allograft also contributes significantly to acceptance because the organ (liver versus kidney) and prolonged exposure under treatment enhances the ability to ultimately wean immunosuppression. However, the role of the allograft in SOT is not well understood and is evolving. Speculations include: (i) provision of a stromal niche for donor hematopoietic stem cells [75] and maintenance of microchimerism [65]; (ii) provision of donor antigen needed to stimulate adaptive T_{reg} cells, which causes them to locate there [67,76]; (iii) a unique micro-environment in the case of the liver variably dampens a number of different immune responses [18,19,77]; (iv) a sink for alloreactive cells slowly mediating chronic rejection; or (v) some combination of the above.

Other nonrejection-related insults, such as recurrence of the original disease and technical complications associated with inflammation, can either sustain or re-activate the contentious allograft/recipient immunologic interface. This, in turn, can predispose to rejection, even in seemingly SOT allografts. Examples include diminished ability to wean immunosuppression in patients with autoimmune hepatitis or primary biliary cirrhosis in liver allografts [22,23] and triggering of apparent rejection after an episode of obstructive uropathy [35] (Table 3). Also, HCV-negative liver allografts that are inflamed at the time of weaning are more prone to rejection. This is probably related to the alterations of leukocyte trafficking

Table 5. 'Take home' messages of the liver immunosuppression minimization trials.

Study	Take home messages
Starzl *et al.* [14]	Micro-chimerism is frequently observed in long-term liver allograft survivors
	Not all recipients require long-term maintenance immunosuppression
	'Tolerant' recipients/accepted allografts and show inflammation/hepatitis, not attributable to rejection
Sandborn *et al.* [29]	Renal toxicity of cyclosporine is improved
	High percentage (ca. 50%) developed acute rejection; 2/12 (17%) developed chronic rejection
Ramos *et al.* [23]	Enzyme elevations typically occurred about 150 days into the weaning process, but not all associated with rejection
	Close monitoring needed; liver injury test parameters not adequate monitor, but weaning is safe: no allografts failures or permanent damage
	Weaning should not be attempted until 5–10 years after transplantation; micro-chimerism not necessarily associated with acceptance
	Acute/chronic rejection had typical presentation; sometimes preceded by 'nonspecific lobular changes'
Mazariegos *et al.* [22]	Close physician surveillance during weaning with frequent assessment of liver function; weaning should not be abrupt/quick
	LFTs not a good discriminator of rejectors versus tolerant; but patient should be biopsied and returned to immunosuppression, if needed
	Cyclosporine-treated recipients more resistant to weaning than those treated with tacrolimus or azathioprine
Devlin *et al.* [57], Wong *et al.* [59], and Girlanda *et al.* [61]	Close physician surveillance as required; transient rise in liver injury test parameters not always indicative of rejection – can spontaneously resolve
	Acute rejection that develops does not always show histopathologic features of 'classic' acute cellular rejection
	Microchimerism not statistically associated with graft acceptance
	Successful drug withdrawal correlated with nonimmune mediated liver diseases, HLA matching, low incidence of early rejection
	Ability to wean associated with less portal inflammation, less CD8$^+$ lymphocytes and more lobular CD45RO$^+$ lymphocytes
Takatsuki *et al.* [52]	Weaning can be attempted in a majority of recipients; successful in up 38.1% of living-related donor liver recipients
	Liver injury test parameters were not significantly different in the rejection versus weaned groups
	Mechanisms of graft acceptance unclear
Pons *et al.* [58]	'Tolerance'/graft acceptance observed in 33% of recipients
	Sinusoidal endothelial cell chimerism was frequent, but not necessary for graft acceptance
	Portal inflammation without endothelialitis or bile duct damage might represent either 'latent' rejection or 'immunologic activation' associated with graft acceptance
Tryphonopoulos *et al.* [102]	Bone marrow infusion increases the level of microchimerism, but does not significantly increase the percentage of patients that can be weaned from immunosuppression
	About 20% of stable liver allograft recipients can be weaned from all immunosuppression
Eason *et al.* [103]	Clinical 'tolerance'/graft acceptance can be achieved in a minority of recipients
	Weaning from immunosuppression can be risky
Tisone *et al.* [31] and Martinez-Llordella *et al.* [33]	Univariant analysis: longer F/U after Tx, treatment with ribavirin, less steroids, more advanced architectural distortion/fibrosis on entry biopsy, and lower first week cyclosporin blood levels associated with ability to wean.
	Multivariate analysis: low cyclosporine trough levels during those first post-transplant week and initial steroid free immunosuppression independently associated with ability to wean
	'Tolerance'/graft acceptance associated with lower fibrosis progression/regression after weaning
	Differential expression of genes in circulating blood mononuclear cells associated with: (i) IL-2 signaling; (ii) pro-inflammatory, oxidative stress, apoptosis, etc. associated with HCV; (iii) upregulation of Vδ1$\gamma\delta$, NK receptors and TGF-β signaling; and (iv) increased percentage of FoxP3$^+$, increased Vδ1$\gamma\delta$: Vδ2$\gamma\delta$ ratio CD4$^+$/CD25$^+$/CD62L^{high}
Koshiba *et al.* [32], Yoshitomi *et al.* [51], and Li *et al.* [60]	Recipients of living-related donors can be successfully weaned more frequently than mismatched cadaveric allografts
	Baseline biopsies show increased infiltration by CD4$^+$/CD25^{high} and peripheral blood shows increased ratio of Vδ1/Vδ2 ratio as compared with normal individuals
	Graft acceptance resembles successful pregnancy in that Vδ1$\gamma\delta$ T cells express very high IL-10 levels
	Tolerant grafts showed more portal fibrosis, ductular reactions, and decreased luminal diameter of bile ducts as compared with those maintained on immunosuppression; might be a variant of late onset rejection

through the organ, which diminishes immunologic ignorance.

It is not surprising that recipients of living-related allografts are more easily weaned from immunosuppression than nonrelated cadaveric organs. They are usually better MHC-matched than cadaveric organs and generally experience less severe ischemic/preservation-related injury. And if the donor is the mother, oral exposure to maternal antigens through breast feeding might positively contribute to tolerance induction. Clearly, more work is needed in studying the relationship between innate and adaptive immunity in triggering rejection in stable SOT allografts.

Several studies showed the presence of mononuclear infiltrates in SOT kidney and liver allografts (Tables 1–3). Many completely normal nonallograft kidney and livers show similar findings. But most transplant pathologists intuitively react with some level of concern because inflammation is so frequently associated with tissue damage and formation of aggregates and/or germinal centers in tissues is a time-tested marker of chronic inflammation. Yet Xu and Burlingham [67] have reported, in humans, how these infiltrates might represent a 'protective' response in the allograft. Their observation of a T_{reg}-rich infiltrate supports the hypothesis that peripheral allograft tolerance involves T_{reg}-dominance in the T_{reg}–$T_{effector}$ ratio homeostasis, as in experimental animals [76,78,79]. Their observation is also consistent with the finding that T_{reg} localize in allograft tissue and at sites of inflammation [76]. A higher T_{reg}–$T_{effector}$ ratio was also observed in tolerant kidney allografts studied by Kawai *et al.* [12] and increased T_{reg} were noted in the liver allografts of tolerant pediatric recipients, but a T_{reg}–$T_{effector}$ ratio was not reported [32].

It should also be noted, however, that nodular lymphoid aggregates have also been used to distinguish chronically rejecting organs from seemingly tolerant ones in experimental animal studies [65,80]. But perhaps the quantity, composition or function of the lymphocytes/nodules differ between tolerance and chronic rejection, as in the peripheral circulation [81,82]. Or perhaps the two processes, tolerance and chronic rejection, are closely related and differ only in the severity and pace of the response in relationship to the lifespan of the recipient: A 65-year-old liver allograft recipient that is slowly developing chronic rejection over a period of 20 years might be better off considered tolerant rather than returned to maintenance immunosuppression. Regardless, better characterization and comparison to similar infiltrates in normal nontransplant tissues and stable allograft recipients on immunosuppression is needed. These seemingly benign infiltrates in tolerated organs appear to be related to the well-recognized affinity of adaptive T_{reg} for allograft tissue and sites of inflammation [76,78,79]. But as TGF-β secretion plays an important role in their function [76] it will be important to determine whether regulation itself might produce pathology/fibrosis. T lymphocytes showing a regulatory phenotype, and producing significant TGF-β, were recently shown to be associated with IgG4-cholangiopathy, a fibrosing condition of bile ducts [83] that can affect other organs.

Another common characteristic of SOT in liver- and kidney allografts is that it appears to be meta-stable and to evolve over a period of time. Seemingly minor perturbations can trigger clinically significant acute rejection episodes, even in patients who have been off all suppression for many years. At least one study, however, suggests that the instability decreases with time [84]. In addition, it is not entirely surprising that some apparently well-tolerated human allografts show features of chronic rejection after longer follow-up. This occurred in several renal allografts and at least one liver allograft recipient (Tables 1–3). And as we already know that liver injury test parameters and serum creatinine are not sensitive markers of tissue injury, some method of follow-up by protocol will benefit patient management and contribute to an understanding of mechanisms associated with allograft acceptance. The first author would certainly advocate for protocol biopsies, even in stable SOT patients, at least until we understand the process better.

Roles of the pathologist, features of interest within tolerated allografts, and sampling/testing recommendations

The pathologist will be asked to play two roles in this emerging field of immunosuppression minimization. The first, and most important, will be a clinical one in monitoring allograft acceptance and 'helping in decision-making, but not unilaterally deciding,' as to whether a particular recipient needs to be returned to immunosuppression or not. To successfully play this role, the pathologist has to be able to distinguish all of variants of antibody- and cell-mediated rejection that might require a return to immunosuppression from changes associated with long-term engraftment, recurrent disease, and technical complication where immunosuppression might not be indicated. Furthermore, there are likely to be findings of uncertain significance and these will require follow-up over a period of time. As with any new pathology endeavor, limiting biopsy analysis and interpretation to one or a small group of pathologists with a specific interest in immunosuppression minimization will decrease observer variation.

Thus, at a minimum, samples that should be obtained in any weaning study include: (i) indicated biopsies to

determine the cause of any allograft dysfunction before weaning; (ii) protocol biopsies immediately before weaning in stable recipients; (iii) indicated biopsies in recipients who develop any significant evidence of graft dysfunction after weaning; and (iv) protocol biopsies in stable recipients after weaning. The schedule for, and even whether to obtain, protocol biopsies in stable recipients off all immunosuppression is controversial. But at least one sample after 1, 3, and 5 immunosuppression-free years is reasonable, in the first author's opinion. But defensible arguments can be made for more or less frequent sampling. It is ideal to also have available donor and/or postreperfusion biopsies to determine whether early events, such as significant donor disease or preservation/reperfusion injury affect the ability to wean subsequently.

Routine light and histochemical microscopic examination, appropriate to the organ, is mandatory because it provides a plethora of information and because it is fast and inexpensive and based on abundant empirical data. We attempt to preserve as much tissue as possible for future experimental studies and routinely obtain H&E stains alone in liver allografts and H&E, Methenamine–silver–trichrome (MST) combination, PAS, and C4d stains in kidney allografts. Fibrosis can be reliably assessed by polarization microscopy. Beyond these tests, more sophisticated (experimental) analyses must balance the various limitations such as: sample size, potential yields of testing modalities; and research interests of the investigator and the field.

Beyond basic general diagnostic considerations, major histopathologic features of focus should include the overall tissue architecture, severity and composition of inflammatory infiltrates, development and/or progression of fibrosis and parenchymal cell atrophy and obliterative arteriopathy. The latter features are more easily followed in kidney than in liver allografts and are important, albeit not entirely specific, histopathologic markers of chronic allograft rejection. Routine tissue monitoring for C4d deposition in conjunction with circulating anti-donor antibodies is an absolute necessity in kidney allografts. In liver allografts, C4d deposits are infrequent and their clinical significance is much less certain unless there is sinusoidal deposition, which is rare. Most centers do not routinely obtain C4d stains for liver allograft recipients, but it is probably prudent to do so for any cause of unexplained allograft dysfunction or when anti-donor antibodies are detected in the circulation.

Any noticeable progression of routine histopathologic findings over a period of time, such as interstitial fibrosis and parenchymal cell atrophy, especially if it is accompanied by laboratory-validated deterioration of function, should prompt a thorough re-evaluation of the immunosuppression management policy. This recommendation

includes caveats of intra- and inter-observer variation, sampling issues, and whether the rate of deterioration is relevant to the clinical setting. For example, a minimal or very slow progression of allograft fibrosis over a period of time without immunosuppression might be the result of sampling issues or be an acceptable alternative for a diabetic-prone elderly allograft recipient.

The second scientific role of the pathologist complements and extends the clinical one. Immunostaining, *in situ* hybridization, and various nucleic acid and protein expression arrays can be used to gain a functional understanding of the routine histopathology findings. Specific areas of interest would include evidence of injury and a response to injury in endothelial and parenchymal cells and the phenotype and activation/maturational status of various leukocyte populations, including organ-resident DC and various T-cell subsets. But assay selection should be balanced by considerations of tissue availability and potential significance and impact of any result(s). Recent development of multiplex staining in tissue sections has helped to conserve tissue by staining for multiple antigens in the same section (Fig. 1).

For example, livers and kidney (and allografts) usually show a relatively low rate of cellular stress and regeneration, as determined by immunohistochemical staining, and deviations from controls/normal might be a reason for concern. But any experimental result should not significantly influence the clinical decision-making process,
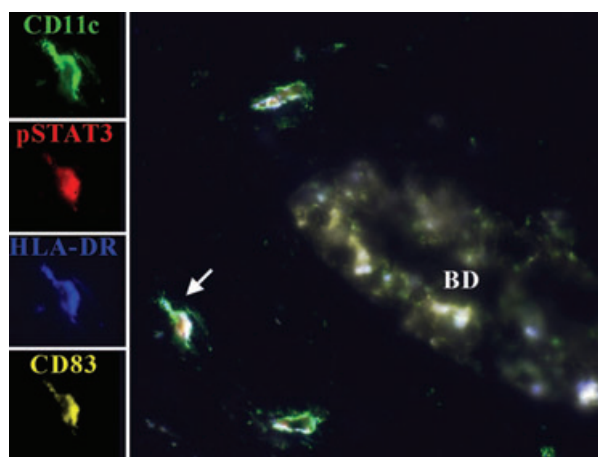

Figure 1 Nanoparticle colloidal semiconductor quantum dot (Qdots)-labeled monoclonal antibodies can be used for multiplex staining of tissue sections to monitor dendritic cell maturation status using markers such as CD11c (green; mDC), CD83 (yellow), HLA-DR (blue) and phosphorylated STAT3 (red; pSTAT3). This is an example from a 'tolerant' human liver allograft recipient. The individual colors of the cell highlighted by the arrow are shown in the left panels. Protein expression from Qdot immunofluorescence images is also quantifiable using NIH ImageJ analysis software. BD, bile duct.

unless scientifically validated. Evidence of injury and response to injury in endothelial and parenchymal cells might be monitored using markers of caspase activation, apoptosis, proliferation, and senescence-related changes, such as Ki67, PCNA, TUNEL, caspase 3, p16, p21, heme oxygenase-1, and increased expression of DNA repair enzymes. Beyond C4d deposits, one might look for immunohistochemical evidence of subtle endothelial injury. This might manifest as upregulation of anti-apoptotic molecules bcl-2, bcl-xl, or stress-induced hemoxygenase-1 HO-1 [85–89], CD46 [90,91], the complement regulatory proteins CD55 [92] and CD59 [93,94]; or pAKT [85,95,96] and Phospho-S6 Ribosomal Protein (Ser235/236) [86,97] or reduced expression of ICAM-1 and VCAM-1 [85–89], complement component 3 receptor-alpha [91,98], and complement component 5a receptor [91,98].

Our group is particularly interested in the donor versus recipient origin and activation/maturational status of organ-resident DC as they occupy an important niche within the immune system as monitors of the environment and translators of innate-into-adaptive immunity (Figs 1 and 2). In the first author's experience, well-tolerated allografts almost invariably contain residual donor DC [65]. And DC are especially good at triggering rejection [99] and tolerogenic pathways [100]. At a very basic level, however, we do not know whether the composition and maturational/activation status of interstitial leukocytes and DC in tolerated allografts resembles normal organs. And this will likely provide information about the mechanisms of allograft acceptance. Considering previous studies on the importance of naïve and memory T cells

[59] and γδ-T cells [32,33] the composition of resident allograft leukocytes will certainly be of interest, as will expression of immunomodulatory cytokines such as TGF-β and interleukin (IL)-10.

The position of the liver within the body, immediately downstream of the intestines, also appears to be an important contributor to the tolerogenic properties [19] of the liver and might help explain why it is the liver allograft recipients who are able to be more easily withdrawn from immunosuppression and display SOT [77]. Our group has been interested in hepatic STAT3 activity (pSTAT3) [77], which is higher in the liver than in other commonly transplanted organs. Bacteria and bacterial products normally leak through the intestines into the portal venous blood and this stimulates Kupffer's cells to produce IL-6, which in turn, upregulates hepatic STAT3 activity. Activated or phosphorylated STAT3 inhibits hepatic myeloid and plasmacytoid dendritic cell maturation [77]. The critical role of IL-6 is illustrated in normal IL-6-deficient mice livers, which harbor DC that are significantly more mature than DC in normal wild-type mice livers. Depletion of gut commensal bacteria in the wild-type decreased hepatic pSTAT3 levels and caused hepatic dendritic cell maturation [77]. Activated STAT3 has also recently been recognized as a key modulator of tumor immunity [101] being involved in several aspects of tumor immunology including inhibition of DC maturation and expansion of T_{reg} within neoplasms (reviewed in [101]). Thus, the normal physiologic state of the liver might resemble a tumor microenvironment [77,101] and in turn, this might enable recipients to be weaned from immunosuppression without triggering a rejection reac-
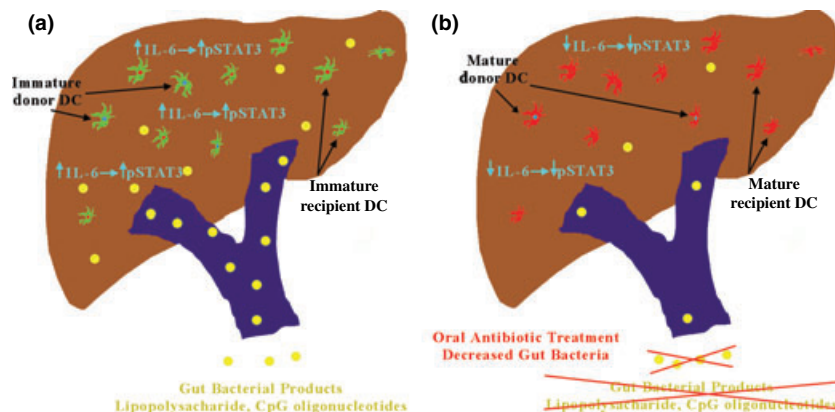

Figure 2 A model whereby intrahepatic IL-6-mediated activation of STAT3 (pSTAT3) stimulated by gut-derived bacterial products in the portal blood inhibits liver DC maturation. (a) In an allograft with gut-bacterial product stimulated IL-6/STAT3 activity, both passenger donor DC (green cells with blue nuclei) and infiltrating recipient DC (green cells with red nuclei) are immature with subsequent reduced migration to secondary lymphoid tissues. This might help explain the persistence of donor DC within tolerant allografts. (b) When hepatic IL-6/STAT3 activity is reduced, by decreasing gut bacteria with oral antibiotics, for example, the DC maturational threshold is reduced and both donor and recipient liver DC are more mature (red cells). These mature DC have increased migration from the organ and are more potent stimulators of allo-reactivity. Subsequently, the liver DC compartment would be repopulated with recipient DC.

tion. Clearly, molecular mechanisms beyond STAT3 are likely involved in the complex process of liver allograft acceptance, but pre-existing mechanisms to prevent an over-reaction to gut-derived antigens likely contribute significantly to the process.

Tolerance in humans induced via hematopoietic macrochimerism, even if transient, appears to be deletional and robust, at least early after transplantation [12], but might evolve towards relatively less stable regulatory pathways subsequently after transplantation [12]. SOT in conventionally treated allograft recipients can be studied in more patients and appears to rely less on deletion and more on active regulation. Therefore, study of the regulatory characteristics of lymphocytes within SOT allografts has, and will continue, to gain popularity. As many of these studies will likely involve study of FoxP3 expression it is worthwhile to note that most human T cells express FoxP3 during early stages of T-cell activation [76]. Therefore, studies using this marker alone to define T_{regs} should be interpreted with caution. Expression of the IL-7 receptor (CD127) might be helpful in this regard, as $CD4^+CD25^+CD127^{+low}$ cells include threefold more $FoxP3^+$ T cells than the classic $CD4^+CD25^{hi}$ subset, but show equivalent regulatory activity [76].

Perspective and future studies

One of the most remarkable observations made during compilation of this article was the realization that tissue samples from SOT liver- and kidney allograft recipients were scarce. This is not only attributable partially to the infrequency with which SOT patients are identified, but also to the fact that clinicians are hesitant to perform biopsies on otherwise seemingly stable SOT recipients. As mentioned before, clinicians might be misled by insignificant histopathologic curiosities. In addition, biopsies are invasive and not without risk of morbidity, and even mortality albeit rarely. In our opinion, the benefits of protocol biopsies in this situation outweigh their risks. It is crucial, however, that the tissue samples be used wisely. The issue of how to use them is not always an easy decision.

The choice of controls for SOT tissue studies can be problematic, especially for liver allografts because of the high incidence of recurrent disease. Normal age-matched control liver tissue, stable allograft recipients on immunosuppression, stable allograft recipients on immunosuppression with the same recurrent disease, and recipients that fail weaning attempts are possible comparison groups for SOT patients. Each one has advantages and drawbacks.

The advent of array technology and discovery science often pits those who practice 'discovery' science against those who practice 'mechanistic/hypothesis testing' science. Both have advantages and shortcomings. The essence of hypothesis testing is to associate a specific cell or pathway or system with a specific phenomenon. Key interventional experiments that change the potentially critical component are then conducted to determine whether the relationship holds up, as expected/hypothesized. The major problem, however, is how to identify the critical cell, pathway, or system that ultimately controls complex biologic phenomena like immunologic tolerance to human allografts. One could expend significant resources studying an unimportant cell, pathway, or system. In addition, interventional experiments in humans are usually delayed until the final stage of hypothesis testing. Moreover, they are expensive and often difficult to interpret.

'Discovery science', in contrast, has recognized that array technology and bioinformatics are reducing biologic phenomena to a 'closed', albeit very complex, systems. No assumptions are made about the particular importance of one cell, molecule, or signaling system over another. Instead, expression array analyses are conducted on populations that exhibit a phenomenon and prominent genes, proteins, pathways, or systems emerge [33]. Single nucleotide polymorphism arrays also have the potential to contribute significantly to our understanding of tolerance. Genetic tendencies certainly contribute to the development of certain original diseases that lead to transplantation and are likely to also contribute to the ability to wean immunosuppression. This discovery approach also has drawbacks. Not all components of biologic systems are amenable to array analyses. For example, mRNA expression arrays are only quantitative and some protein arrays do not account for the activation/phosphorylation status of proteins (e.g. STAT3), which can significantly affect function. It is not a trivial task to identify nodal points in complex systems that ultimately control or significantly influence the phenomenon being observed. A particular gene or protein might be one of the most up- or downregulated quantitatively during the process, but might not be an important nodal regulator.

In the end, it is our opinion, that the best approach to the study of tolerance in tissue samples will be a combination of both the approaches. The 'shotgun' criticism currently applied to many array studies will eventually give way to 'targeted' or focused arrays through hypothesis testing that measure only key parameters associated with the biologic process of interest.

References

1. Ashton-Chess J, Giral M, Brouard S, Soulillou JP. Spontaneous operational tolerance after immunosuppressive drug withdrawal in clinical renal allotransplantation. *Transplantation* 2007; **84**: 1215.

2. Starzl TE, Marchioro TL, Waddell WR. The reversal of rejection in human renal homografts with subsequent development of homograft tolerance. *Surg Gynecol Obstet* 1963; **117**: 385.

3. Spitzer TR, Delmonico F, Tolkoff-Rubin N, *et al.* Combined histocompatibility leukocyte antigen-matched donor bone marrow and renal transplantation for multiple myeloma with end stage renal disease: the induction of allograft tolerance through mixed lymphohematopoietic chimerism. *Transplantation* 1999; **68**: 480.

4. Buhler LH, Spitzer TR, Sykes M, *et al.* Induction of kidney allograft tolerance after transient lymphohematopoietic chimerism in patients with multiple myeloma and end-stage renal disease. *Transplantation* 2002; **74**: 1405.

5. Fudaba Y, Spitzer TR, Shaffer J, *et al.* Myeloma responses and tolerance following combined kidney and non-myeloablative marrow transplantation: *in vivo* and *in vitro* analyses. *Am J Transplant* 2006; **6**: 2121.

6. Sayegh MH, Fine NA, Smith JL, Rennke HG, Milford EL, Tilney NL. Immunologic tolerance to renal allografts after bone marrow transplants from the same donors. *Ann Intern Med* 1991; **114**: 954.

7. Butcher JA, Hariharan S, Adams MB, Johnson CP, Roza AM, Cohen EP. Renal transplantation for end-stage renal disease following bone marrow transplantation: a report of six cases, with and without immunosuppression. *Clin Transplant* 1999; **13**: 330.

8. Helg C, Chapuis B, Bolle JF, *et al.* Renal transplantation without immunosuppression in a host with tolerance induced by allogeneic bone marrow transplantation. *Transplantation* 1994; **58**: 1420.

9. Jacobsen N, Taaning E, Ladefoged J, Kristensen JK, Pedersen FK. Tolerance to an HLA-B,DR disparate kidney allograft after bone-marrow transplantation from same donor. *Lancet* 1994; **343**: 800.

10. Sorof JM, Koerper MA, Portale AA, Potter D, DeSantes K, Cowan M. Renal transplantation without chronic immunosuppression after T cell-depleted, HLA-mismatched bone marrow transplantation. *Transplantation* 1995; **59**: 1633.

11. Billingham RE, Brent L, Medawar PB. "Actively acquired tolerance" of foreign cells. *Nature* 1953; **172**: 603.

12. Kawai T, Cosimi AB, Spitzer TR, *et al.* HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med* 2008; **358**: 353.

13. Ildstad ST, Sachs DH. Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of allografts or xenografts. *Nature* 1984; **307**: 168.

14. Starzl TE, Demetris AJ, Trucco M, *et al.* Cell migration and chimerism after whole-organ transplantation: the basis of graft acceptance. *Hepatology* 1993; **17**: 1127.

15. Girlanda R, Kirk AD. Frontiers in nephrology: immune tolerance to allografts in humans. *J Am Soc Nephrol* 2007; **18**: 2242.

16. Calne R, Moffatt SD, Friend PJ, *et al.* Prope tolerance with induction using Campath 1H and low-dose cyclosporin monotherapy in 31 cadaveric renal allograft recipients. *Nippon Geka Gakkai Zasshi* 2000; **101**: 301.

17. Calne RY. Prope tolerance – the future of organ transplantation from the laboratory to the clinic. *Transpl Immunol* 2004; **13**: 83.

18. Benseler V, McCaughan GW, Schlitt HJ, Bishop GA, Bowen DG, Bertolino P. The liver: a special case in transplantation tolerance. *Semin Liver Dis* 2007; **27**: 194.

19. Crispe IN, Giannandrea M, Klein I, John B, Sampson B, Wuensch S. Cellular and molecular mechanisms of liver tolerance. *Immunol Rev* 2006; **213**: 101.

20. Wiesner RH, Demetris AJ, Belle SH, *et al.* Acute hepatic allograft rejection: incidence, risk factors, and impact on outcome. *Hepatology* 1998; **28**: 638.

21. Demetris AJ, Ruppert K, Dvorchik I, *et al.* Real-time monitoring of acute liver-allograft rejection using the Banff schema. *Transplantation* 2002; **74**: 1290.

22. Mazariegos GV, Reyes J, Marino IR, *et al.* Weaning of immunosuppression in liver transplant recipients. *Transplantation* 1997; **63**: 243.

23. Ramos HC, Reyes J, Abu-Elmagd K, *et al.* Weaning of immunosuppression in long-term liver transplant recipients. *Transplantation* 1995; **59**: 212.

24. Demetris A, Adams D, Bellamy C, *et al.* Update of the International Banff Schema for Liver Allograft Rejection: working recommendations for the histopathologic staging and reporting of chronic rejection. An International Panel. *Hepatology* 2000; **31**: 792.

25. Demetris AJ, Murase N, Nakamura K, *et al.* Immunopathology of antibodies as effectors of orthotopic liver allograft rejection [Review]. *Semin Liver Dis* 1992; **12**: 51.

26. Starzl TE, Murase N, Abu-Elmagd K, *et al.* Tolerogenic immunosuppression for organ transplantation. *Lancet* 2003; **361**: 1502.

27. Shapiro R, Basu A, Tan H, *et al.* Kidney transplantation under minimal immunosuppression after pretransplant lymphoid depletion with Thymoglobulin or Campath. *J Am Coll Surg* 2005; **200**: 505; Quiz A559.

28. Lerut J, Sanchez-Fueyo A. An appraisal of tolerance in liver transplantation. *Am J Transplant* 2006; **6**: 1774.

29. Sandborn WJ, Hay JE, Porayko MK, *et al.* Cyclosporine withdrawal for nephrotoxicity in liver transplant recipients does not result in sustained improvement in kidney function and causes cellular and ductopenic rejection. *Hepatology* 1994; **19**: 925.

30. Eghtesad B, Fung JJ, Demetris AJ, *et al.* Immunosuppression for liver transplantation in HCV-infected patients: mechanism-based principles. *Liver Transpl* 2005; **11**: 1343.

31. Tisone G, Orlando G, Cardillo A, *et al.* Complete weaning off immunosuppression in HCV liver transplant recipients is feasible and favourably impacts on the progression of disease recurrence. *J Hepatol* 2006; **44**: 702.

32. Koshiba T, Li Y, Takemura M, *et al.* Clinical, immuno-logical, and pathological aspects of operational tolerance after pediatric living-donor liver transplantation. *Transpl Immunol* 2007; **17**: 94.

33. Martinez-Llordella M, Puig-Pey I, Orlando G, *et al.* Multiparameter immune profiling of operational tolerance in liver transplantation. *Am J Transplant* 2007; **7**: 309.

34. Starzl TE, Demetris AJ, Murase N, Ildstad S, Ricordi C, Trucco M. Cell migration, chimerism, and graft acceptance. *Lancet* 1992; **339**: 1579.

35. Strober S, Dhillon M, Schubert M, *et al.* Acquired immune tolerance to cadaveric renal allografts. A study of three patients treated with total lymphoid irradiation. *N Engl J Med* 1989; **321**: 28.

36. Scandling JD, Busque S, Dejbakhsh-Jones S, *et al.* Tolerance and chimerism after renal and hematopoietic-cell transplantation. *N Engl J Med* 2008; **358**: 362.

37. Kirk AD, Hale DA, Mannon RB, *et al.* Results from a human renal allograft tolerance trial evaluating the humanized CD52-specific monoclonal antibody alemtuzumab (CAMPATH-1H). *Transplantation* 2003; **76**: 120.

38. Kirk AD, Mannon RB, Kleiner DE, *et al.* Results from a human renal allograft tolerance trial evaluating T-cell depletion with alemtuzumab combined with deoxyspergualin. *Transplantation* 2005; **80**: 1051.

39. Banff Working G, Demetris AJ, Adeyi O, *et al.* Liver biopsy interpretation for causes of late liver allograft dysfunction. *Hepatology* 2006; **44**: 489.

40. Berenguer M, Rayon JM, Prieto M, *et al.* Are posttransplantation protocol liver biopsies useful in the long term? *Liver Transpl* 2001; **7**: 790.

41. Burra P, Mioni D, Cecchetto A, *et al.* Histological features after liver transplantation in alcoholic cirrhotics. *J Hepatol* 2001; **34**: 716.

42. Berenguer M, Aguilera V, Prieto M, *et al.* Delayed onset of severe hepatitis C-related liver damage following liver transplantation: a matter of concern? *Liver Transpl* 2003; **9**: 1152.

43. Pappo O, Ramos H, Starzl TE, Fung JJ, Demetris AJ. Structural integrity and identification of causes of liver allograft dysfunction occurring more than 5 years after transplantation. *Am J Surg Pathol* 1995; **19**: 192.

44. Sebagh M, Rifai K, Feray C, *et al.* All liver recipients benefit from the protocol 10-year liver biopsies. *Hepatology* 2003; **37**: 1293.

45. Slapak GI, Saxena R, Portmann B, *et al.* Graft and systemic disease in long-term survivors of liver transplantation. *Hepatology* 1997; **25**: 195.

46. Evans HM, Kelly DA, McKiernan PJ, Hubscher S. Progressive histological damage in liver allografts following pediatric liver transplantation. *Hepatology* 2006; **43**: 1109.

47. Shaikh OS, Demetris AJ. Idiopathic posttransplantation hepatitis? *Liver Transpl* 2007; **13**: 943.

48. Abraham SC, Poterucha JJ, Rosen CB, Demetris AJ, Krasinskas AM. Histologic abnormalities are common in

49. Gane E, Portmann B, Saxena R, Wong P, Ramage J, Williams R. Nodular regenerative hyperplasia of the liver graft after liver transplantation. *Hepatology* 1994; **20**: 88.

50. Rosenthal P, Emond JC, Heyman MB, *et al.* Pathological changes in yearly protocol liver biopsy specimens from healthy pediatric liver recipients. *Liver Transpl Surg* 1997; **3**: 559.

51. Yoshitomi M, Koshiba T, Sakashita H, *et al.* Requirement of protocol biopsy before and after complete cessation of immunosuppression following living-donor liver transplantation. *Am J Transplant* 2006; **6**: 173.

52. Takatsuki M, Uemoto S, Inomata Y, *et al.* Weaning of immunosuppression in living donor liver transplant recipients. *Transplantation* 2001; **72**: 449.

53. Roussey-Kesler G, Giral M, Moreau A, *et al.* Clinical operational tolerance after kidney transplantation. *Am J Transplant* 2006; **6**: 736.

54. Owens ML, Maxwell JG, Goodnight J, Wolcott MW. Discontinuance of immunosuprression in renal transplant patients. *Arch Surg* 1975; **110**: 1450.

55. Uehling DT, Hussey JL, Weinstein AB, Wank R, Bach FH. Cessation of immunosuppression after renal transplantation. *Surgery* 1976; **79**: 278.

56. Zoller KM, Cho SI, Cohen JJ, Harrington JT. Cessation of immunosuppressive therapy after successful transplantation: a national survey. *Kidney Int* 1980; **18**: 110.

57. Devlin J, Doherty D, Thomson L, *et al.* Defining the outcome of immunosuppression withdrawal after liver transplantation. *Hepatology* 1998; **27**: 926.

58. Pons JA, Yelamos J, Ramirez P, *et al.* Endothelial cell chimerism does not influence allograft tolerance in liver transplant patients after withdrawal of immunosuppression. *Transplantation* 2003; **75**: 1045.

59. Wong T, Nouri-Aria KT, Devlin J, Portmann B, Williams R. Tolerance and latent cellular rejection in long-term liver transplant recipients. *Hepatology* 1998; **28**: 443.

60. Li Y, Wu Y, Sakaguchi S, Wood K, Pirenne J, Koshiba T. Presence of regulatory T cells within tolerant graft of human liver and intestinal transplantation. *Am J Transplant* 2006; **6**: 1056.

61. Girlanda R, Rela M, Williams R, O'Grady JG, Heaton ND. Long-term outcome of immunosuppression withdrawal after liver transplantation. *Transplant Proc* 2005; **37**: 1708.

62. Starzl TE, Demetris AJ, Trucco M, *et al.* Systemic chimerism in human female recipients of male livers. *Lancet* 1992; **340**: 876.

63. Randhawa PS, Starzl T, Ramos HC, Nalesnik MA, Demetris J. Allografts surviving for 26 to 29 years following living-related kidney transplantation: analysis by light microscopy, in situ hybridization for the Y chromosome, and anti-HLA antibodies. *Am J Kidney Dis* 1994; **24**: 72.

64. Starzl TE, Demetris AJ, Trucco M, *et al.* Chimerism and donor-specific nonreactivity 27 to 29 years after kidney allotransplantation. *Transplantation* 1993; **55**: 1272.

65. Demetris AJ, Murase N, Ye Q, *et al.* Analysis of chronic rejection and obliterative arteriopathy. Possible contributions of donor antigen-presenting cells and lymphatic disruption. *Am J Pathol* 1997; **150**: 563.

66. Starzl TE, Murase N, Demetris AJ, *et al.* Lessons of organ-induced tolerance learned from historical clinical experience. *Transplantation* 2004; **77**: 926.

67. Xu Q, Lee J, Jankowska-Gan E, *et al.* Human CD4+CD25low adaptive T regulatory cells suppress delayed-type hypersensitivity during transplant tolerance. *J Immunol* 2007; **178**: 3983.

68. Burlingham WJ, Grailer AP, Fechner JH Jr, *et al.* Microchimerism linked to cytotoxic T lymphocyte functional unresponsiveness (clonal anergy) in a tolerant renal transplant recipient. *Transplantation* 1995; **59**: 1147.

69. Burlingham WJ, Jankowska-Gan E, VanBuskirk A, Orosz CG, Lee JH, Kusaka S. Loss of tolerance to a maternal kidney transplant is selective for HLA class II: evidence from trans-vivo DTH and alloantibody analysis. *Hum Immunol* 2000; **61**: 1395.

70. Larsen CP, Morris PJ, Austyn JM. Migration of dendritic leukocytes from cardiac allografts into host spleens. A novel pathway for initiation of rejection. *J Exp Med* 1990; **171**: 307.

71. Demetris AJ, Murase N, Fujisaki S, Fung JJ, Rao AS, Starzl TE. Hematolymphoid cell trafficking, microchimerism, and GVH reactions after liver, bone marrow, and heart transplantation. *Transplant Proc* 1993; **25**: 3337.

72. Avihingsanon Y, Ma N, Pavlakis M, *et al.* On the intraoperative molecular status of renal allografts after vascular reperfusion and clinical outcomes. *J Am Soc Nephrol* 2005; **16**: 1542.

73. Sun J, McCaughan GW, Gallagher ND, Sheil AG, Bishop GA. Deletion of spontaneous rat liver allograft acceptance by donor irradiation. *Transplantation* 1995; **60**: 233.

74. Bishop GA, Sun J, DeCruz DJ, *et al.* Tolerance to rat liver allografts. III. Donor cell migration and tolerance-associated cytokine production in peripheral lymphoid tissues. *J Immunol* 1996; **156**: 4925.

75. Sakamoto T, Ye Q, Lu L, Demetris AJ, Starzl TE, Murase N. Donor hematopoietic progenitor cells in nonmyeloablated rat recipients of allogeneic bone marrow and liver grafts. *Transplantation* 1999; **67**: 833.

76. Kang SM, Tang Q, Bluestone JA. CD4+CD25+ regulatory T cells in transplantation: progress, challenges and prospects. *Am J Transplant* 2007; **7**: 1457.

77. Lunz JG III, Specht SM, Murase N, Isse K, Demetris AJ. Gut-derived commensal bacterial products inhibit liver dendritic cell maturation by stimulating hepatic interleukin-6/signal transducer and activator of transcription 3 activity. *Hepatology* 2007; **46**: 1946.

78. Torrealba JR, Katayama M, Fechner JH Jr, *et al.* Metastable tolerance to rhesus monkey renal transplants is correlated with allograft TGF-beta 1+CD4+ T regulatory cell infiltrates. *J Immunol* 2004; **172**: 5753.

79. Hara M, Kingsley CI, Niimi M, *et al.* IL-10 is required for regulatory T cells to mediate tolerance to alloantigens *in vivo. J Immunol* 2001; **166**: 3789.

80. Baddoura FK, Nasr IW, Wrobel B, Li Q, Ruddle NH, Lakkis FG. Lymphoid neogenesis in murine cardiac allografts undergoing chronic rejection. *Am J Transplant* 2005; **5**: 510.

81. Louis S, Braudeau C, Giral M, *et al.* Contrasting CD25hiCD4+ T cells/FOXP3 patterns in chronic rejection and operational drug-free tolerance. *Transplantation* 2006; **81**: 398.

82. Baeten D, Louis S, Braud C, *et al.* Phenotypically and functionally distinct CD8+ lymphocyte populations in long-term drug-free tolerance and chronic rejection in human kidney graft recipients. *J Am Soc Nephrol* 2006; **17**: 294.

83. Zen Y, Fujii T, Harada K, *et al.* Th2 and regulatory immune reactions are increased in immunoglobin G4-related sclerosing pancreatitis and cholangitis. *Hepatology* 2007; **45**: 1538.

84. Mazariegos GV, Sindhi R, Thomson AW, Marcos A. Clinical tolerance following liver transplantation: long term results and future prospects. *Transpl Immunol* 2007; **17**: 114.

85. Narayanan K, Jendrisak MD, Phelan DL, Mohanakumar T. HLA class I antibody mediated accommodation of endothelial cells via the activation of PI3K/cAMP dependent PKA pathway. *Transpl Immunol* 2006; **15**: 187.

86. Narayanan K, Jaramillo A, Phelan DL, Mohanakumar T. Pre-exposure to sub-saturating concentrations of HLA class I antibodies confers resistance to endothelial cells against antibody complement-mediated lysis by regulating Bad through the phosphatidylinositol 3-kinase/Akt pathway. *Eur J Immunol* 2004; **34**: 2303.

87. Badrichani AZ, Stroka DM, Bilbao G, Curiel DT, Bach FH, Ferran C. Bcl-2 and Bcl-XL serve an anti-inflammatory function in endothelial cells through inhibition of NF-kappaB. *J Clin Invest* 1999; **103**: 543.

88. Salama AD, Delikouras A, Pusey CD, *et al.* Transplant accommodation in highly sensitized patients: a potential role for Bcl-xL and alloantibody. *Am J Transplant* 2001; **1**: 260.

89. Hancock WW, Buelow R, Sayegh MH, Turka LA. Antibody-induced transplant arteriosclerosis is prevented by graft expression of anti-oxidant and anti-apoptotic genes. *Nat Med* 1998; **4**: 1392.

90. Atkinson JP, Liszewski MK, Richards A, Kavanagh D, Moulton EA. Hemolytic uremic syndrome: an example of insufficient complement regulation on self-tissue. *Ann N Y Acad Sci* 2005; **1056**: 144.

91. Halloran PF, Afrouzian M, Ramassar V, *et al.* Interferon-gamma acts directly on rejecting renal allografts to prevent graft necrosis. *Am J Pathol* 2001; **158**: 215.

92. Lin SS, Hanaway MJ, Gonzalez-Stawinski GV, *et al.* The role of anti-Galalpha1-3Gal antibodies in acute vascular rejection and accommodation of xenografts. *Transplantation* 2000; **70**: 1667.

93. Colvin RB. Antibody-mediated renal allograft rejection: diagnosis and pathogenesis. *J Am Soc Nephrol* 2007; **18**: 1046.

94. Colvin RB, Smith RN. Antibody-mediated organ-allograft rejection. *Nat Rev Immunol* 2005; **5**: 807.

95. Boulday G, Haskova Z, Reinders ME, Pal S, Briscoe DM. Vascular endothelial growth factor-induced signaling pathways in endothelial cells that mediate overexpression of the chemokine IFN-gamma-inducible protein of 10 kDa *in vitro* and *in vivo*. *J Immunol* 2006; **176**: 3098.

96. Mohanakumar T, Rhodes C, Mendez-Picon G, Flye WM, Lee HM. Antiidiotypic antibodies to human major histocompatability complex class I and II antibodies in hepatic transplantation and their role in allograft survival. *Transplantation* 1987; **44**: 54.

97. Lepin EJ, Zhang Q, Zhang X, *et al.* Phosphorylated S6 ribosomal protein: a novel biomarker of antibody-mediated rejection in heart allografts. *Am J Transplant* 2006; **6**: 1560.

98. Lautenschlager I, Hockerstedt K, Meri S. Complement membrane attack complex and protectin (CD59) in liver allografts during acute rejection. *J Hepatol* 1999; **31**: 537.

99. Demetris AJ, Qian S, Sun H, *et al.* Early events in liver allograft rejection: delineation of sites of simultaneous intragraft and recipient lymphoid tissue sensitization. *Am J Pathol* 1991; **138**: 609.

100. Demetris AJ, Murase N, Starzl TE. Donor dendritic cells in grafts and host lymphoid and non-lymphoid tissues after liver and heart allotransplantation under short term immunosuppression. *Lancet* 1992; **339**: 1610 (letter).

101. Yu H, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. *Nat Rev Immunol* 2007; **7**: 41.

102. Tryphonopoulos P, Tzakis AG, Weppler D, *et al.* The role of donor bone marrow infusions in withdrawal of immunosuppression in adult liver allotransplantation. *Am J Transplant* 2005; **5**: 608.

103. Eason JD, Cohen AJ, Nair S, Alcantera T, Loss GE. Tolerance: is it worth the risk? *Transplantation* 2005; **79**: 1157.

104. Anonymous. Banff schema for grading liver allograft rejection: an international consensus document. *Hepatology* 1997; **25**: 658.

105. Hussey JL. Letter: discontinuance of immunosuppression. *Arch Surg* 1976; **111**: 614.

106. Fischer T, Schobel H, Barenbrock M. Specific immune tolerance during pregnancy after renal transplantation. *Eur J Obstet Gynecol Reprod Biol* 1996; **70**: 217.

107. Christensen LL, Grunnet N, Rudiger N, Moller B, Birkeland SA. Indications of immunological tolerance in kidney transplantation. *Tissue Antigens* 1998; **51**: 637.

108. VanBuskirk AM, Burlingham WJ, Jankowska-Gan E, *et al.* Human allograft acceptance is associated with immune regulation. *J Clin Invest* 2000; **106**: 145.

109. Brouard S, Dupont A, Giral M, *et al.* Operationally tolerant and minimally immunosuppressed kidney recipients display strongly altered blood T-cell clonal regulation. *Am J Transplant* 2005; **5**: 330.

110. Newell K, Burlingham W, Marks V, *et al.* Preliminary analysis of the ITN registry of tolerant kidney transplant recipients. *Transplantation* 2006; **82**: 1055.