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MONITORING THE PATIENT OFF IMMUNOSUPPRESSION

CONCEPTUAL FRAMEWORK FOR A PROPOSED TOLERANCE ASSAY STUDY IN LIVER TRANSPLANT RECIPIENTS[1]

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The mission of the recently established Immune Tolerance Network includes the development of protocols for the induction of transplant tolerance in organ allograft recipients and the development of assays that correlate with and may be predictive of the tolerant state. The state of clinical organ transplant tolerance seems to already exist in a small minority of conventionally immunosuppressed liver and, more rarely, kidney transplant patients. Immunosuppressive drug therapy has been withdrawn from these patients for a variety of reasons, including protocolized weaning for a uniquely large group of liver patients at the University of Pittsburgh. In this study, we propose to evaluate the validity of a variety of in vitro immunologic and molecular biologic tests that may correlate with, and be predictive of, the state of organ transplant tolerance in stable liver patients off immunosuppression. Only peripheral blood will be available for the execution of these tests. Both adult and pediatric liver graft recipients will be studied, in comparison to appropriate controls. We shall examine circulating dendritic cell (DC) subsets [precursor (p) DC1 and p DC2] including cells of donor origin, and assess both the frequency and function of donor-reactive T cells by ELISPOT and by trans-vivo delayed-type hypersensitivity analysis in a surrogate murine model. Cytokine gene polymorphism and alloantibody titers will also be investigated. It is anticipated that the results obtained may provide physicians with a tolerance assay "profile" that may determine those patients from whom immunosuppressive therapy may be safely withdrawn.

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INTRODUCTION

Although it has been possible using immunosuppressive agents to induce organ transplant tolerance predictably in experimental animals for the past 50 years, no such protocols exist in humans. On the other hand, there are well-documented instances of withdrawal of immunosuppression from human transplant recipients without occurrence of graft dysfunction/rejection. Drug withdrawal may be required for the management of posttransplantation viral infection (Epstein-Barr virus [EBV], cytomegalovirus [CMV], human immunodeficiency virus, hepatitis B or C) or posttransplantation lymphoproliferative disease, or may be the result of patient noncompliance or physician-controlled protocolized weaning. Although such instances of "operational tolerance" (defined herein as stable graft function, in the absence of immunosuppressive therapy) are very rare in kidney recipients, this is a more common finding after liver transplantation. At present, no immunologic or immunogenetic laboratory assays can predict or validate donor-specific tolerance in organ transplantation.

The objective of this proposal (which has been supported by the Immune Tolerance Network [ITN]) is to evaluate, in clinically stable liver transplant recipients off all immunosuppressive therapy, the relevance of selected immunologic tests that may correlate with and be predictive of the tolerant state. We propose to contrast the results obtained with those from control liver transplant patients from whom immunosuppression has also been withdrawn but who have failed weaning and been restored to baseline immunosuppression. An additional control group will be liver recipients maintained continuously on conventional immunosuppression. To assess the potential clinical utility of the tests, we will also conduct a prospective analysis of hepatic allograft recipients undergoing immunosuppressive drug weaning.

The immune response to organ grafts is believed to be initiated by the presentation of alloantigen (alloAg) by donor and self antigen-presenting cells (APC) to host T cells, which differentiate into effector and regulatory cells. Donor dendritic cells (DC), which constitutively express major histocompatibility complex (MHC) and critical T-cell costimulatory molecules, are commonly regarded as the principal instigators of rejection, but evidence also exists for DC tolerogenicity, both in the context of allo- and autoimmunity. In experimental animals, liver transplant tolerance is associated with the persistence of donor DC in host lymphoid

tissue, in the absence of any form of immunosuppressive therapy (*1*). In human heart and liver transplantation, persistence of donor DC has also been observed in the absence of immunosuppression (*2*); in lung recipients, higher levels of donor leukocytes are correlated with better graft survival and with fewer rejection episodes (*3, 4*). Donor DC that can subvert allogeneic T-cell responses in vitro can also prolong the survival of various types of allograft, in some instances indefinitely (*1, 5–7*). We hypothesize (Table 1) that liver and more rare kidney transplant tolerance in humans may be correlated with the persistence of donor DC and with the predominance of a specific donor or recipient DC subset that can be detected in and/or expanded from peripheral blood. We further hypothesize that the quiescent state will correlate with a characteristic host T-cell cytokine response after donor-specific allostimulation. We propose to incorporate studies of recipient genotype for selected cytokines and key costimulatory molecule polymorphisms. The function of alloAg-specific T cells will be tested in vivo in a surrogate mouse delayed-type hypersensitivity (DTH) model, and the presence of donor-specific alloantibodies (alloAbs) will also be investigated. We postulate that the information obtained using these assays (Table 2) will allow us to establish a distinct and reliable laboratory profile, consistent with the "tolerant" state. This information may also delineate the assay(s) most predictive of safe weaning of immunosuppression.

Donor-Derived Leukocytes and the Outcome of Organ Transplantation

The postulated role of donor-derived leukocytes in organ transplant tolerance has generated extensive discussion and debate (*8–11*) and provided an impetus for clarification of the possible regulatory function of donor DC in host T-cell responses to alloAgs. The concept that interstitial "passenger leukocytes," in particular DC, are the most important *immunogenic* component of transplanted whole organs has prevailed for many years (*12–15*). Almost a decade ago, Starzl et al. (*16, 17*) used immunohistochemical and molecular biologic techniques to detect donor hematopoietic cells (microchimerism) in blood and in both lymphoid and nonlymphoid tissues of long-surviving, successful human organ allograft recipients. This finding called into question a purely immunogenic role of donor-derived cells, amongst which DC were a consistent, readily detected component. It was proposed that the ability of an organ to be tolerogenic, in the absence or presence of effective immunosuppression, was dependent on its donor leukocyte and not its parenchymal cell component (*16*).

Liver Tolerogenicity and DC

The tolerogenic properties of hepatic allografts have long been recognized [reviewed in (*18*)]. In mice, and in certain rat strain combinations, fully MHC-mismatched liver grafts are accepted without immunosuppression and induce donor-specific tolerance to subsequent heart or skin grafts. Liver allografts are also accepted "spontaneously" between outbred pigs (*19*). In humans, the liver is generally regarded as the least immunogenic of transplanted organs. Its tolerogenic effect seems to result in the long-term acceptance of other organs (e.g., heart or kidney) transplanted simultaneously from the same donor. Cotransplantation of rat heart and liver allografts from the same donor can prevent the development of obliterative arteriopathy in the transplanted heart, a lesion indicative of chronic rejection (*20*). In a tolerant strain combination, reduction of donor hematopoietic cells in the liver by irradiation before transplantation results in graft rejection (*21*). Moreover, the tolerogenic capacity of a leukocyte-depleted liver can be restored after "parking" in a normal donor before transplantation or after administration of donor leukocytes (*22*). These observations suggest that, under certain circumstances, donor hematopoietic cells (or a subset thereof) play a role in long-term graft survival/tolerance induction.

TABLE 1. Hypotheses

"Tolerance" may be correlated with persistence of donor DC and predominance of DC of the "plasmacytoid" lineage (DC2)
The quiescent state may correlate with a characteristic "tolerance-promoting" host T-cell cytokine response after donor-specific stimulation
Functional T-cell hyporesponsiveness with be confirmed by trans-vivo DTH assay

A Crucial Role for Donor DC

The leukocyte lineage(s) that may be involved in the induction of liver tolerance is of crucial relevance in transplant immunology. We have shown that donor-derived DC persist in, and can be propagated from, the bone marrow (BM) of animals that accept fully mismatched liver allografts without immunosuppression. This cannot be achieved in animals that acutely reject heart grafts from the same donor strain (*1*), unless both donor BM cells and immunosuppression are administered (*23*). Recent work of Schlitt's group (*24*), using a heart transplant model, provided evidence that donor hematopoietic cells were essential for tolerance induction, but the identity of these tolerance-promoting cells was not investigated. Further recent evidence of a critical requirement for donor leukocytes (most likely DC) in organ transplant tolerance has come from observations of the donor-specific blood transfusion effect by Josien's group (*25*). Thus, injection of donor-type, but not third-party DC restored tolerance to passenger leukocyte-depleted heart allografts in rats treated with a donor-specific blood transfusion. Our studies revealed for the first time that immature donor DC could induce alloAg-specific T-cell hyporesponsiveness in vitro. More significantly, our work and others' has shown that such immature DC, including liver-derived DC, can prolong allograft (including skin graft) survival; in some instances, indefinite, donor-specific graft survival is induced (*5–7, 26–29*). In addition, there is evidence that persistence of donor liver-derived DC may be important in *long-term* maintenance of transplantation tolerance and in the prevention of chronic rejection in challenge heart allografts (*30*). Collectively, these findings suggest that donor-derived DC have the potential to subvert recipient T-cell responses to alloAg, by as yet ill-defined mechanisms.

Mechanisms by Which DC May Regulate Immune Reactivity

Published data suggest that DC have the capacity to regulate immune reactivity by a variety of mechanisms (*10, 31–35*). These mechanisms may not be mutually exclusive. We and others have reported that DC whose allostimulatory

TABLE 2. Putative laboratory profile of "tolerant" liver transplant patients and rejectors

Assay	Immunity (rejection)	Tolerance
Dendritic cell type (and origin) (predominant)	DC1	DC2 (including donor)
Trans-vivo DTH	Positive	Reduced
Cytokine secretion (ELISPOT)	IFN-γ; GB	? IL-10; TGF-β; IL-4
Donor-specific Ab prevalence	Present	Absent
Cytokine genotype	IL-10 low	IL-10 high
	TNF-α high	TNF-α low

function is impaired, either by incomplete maturation, selective blockade of costimulatory molecules, the influence of specific cytokines (e.g., interleukin [IL]-10 or transforming growth factor [TGF]-β), or genetic engineering, can induce alloAg-specific T-cell hyporesponsiveness (anergy) or apoptosis in vitro and suppress immune reactivity [reviewed in refs (*31*, *32*, *34*, *36*)].

Expression of molecules associated with the induction of apoptosis [i.e., Fas ligand (CD95L) (*37*, *38*) or nitric oxide (*39*, *40*)] may render DC capable of subverting T-cell responses by promoting activation-induced cell death. Blockade of the B7/CD28 pathway by cytotoxic T lymphocyte Ag 4 (CTLA4) (CD152) Ig significantly increases DC-induced apoptosis of alloactivated T cells (*38*); the apoptosis seems to be mediated, at least in part, via the Fas pathway. Death-inducing ligands on DC other than Fas ligand, such as tumor necrosis factor (TNF) receptor apoptosis-inducing ligand, are being investigated. There is recent evidence that these molecules may be differentially expressed on human DC subsets (*41*).

Immune deviation, i.e., skewing the T helper (Th)1/Th2 cell balance to Th2 cells, has received considerable attention as a mechanism that may underlie tolerance induction. Several groups have shown that DC can induce immune deviation. Thus, Ag-specific suppression of cell-mediated immunity achieved by i.v. administration of Ag-pulsed Langerhans cells or splenic DC, is likely achieved via selective activation of Th2 cells (*42*). DC grown in prostaglandin E_2 are unable to secrete IL-12, so that Th2 cell development is promoted (*43*). IL-10 skews the Th1/Th2 balance to Th2 cells, by blocking IL-12 synthesis by DC (*44*). In transplantation, prolongation of skin graft survival by portal venous immunization with donor DC is associated with Th2 polarization (*45*). In autoimmune disease, a protective effect of autoAg-pulsed DC in experimental allergic encephalomyelitis seems to be mediated by immune deviation (*46*, *47*).

Two Major DC Populations: Myeloid (M)DC and "Lymphoid-Related" (L)DC

DC can be generated from $CD34^+$ hematopoietic progenitors via several pathways (*48*). The hematopoietic growth factors, c-kit ligand and Fms-like tyrosine kinase 3 ligand, promote growth of DC progenitors. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 enhance DC differentiation, whereas TNF and CD40L promote MDC maturation. Monocytes differentiate into MDC in response to GM-CSF and IL-4. In contrast to MDC, so-called "lymphoid-related" (L)DC, identified initially as constitutive DC within the mouse thymus, share a common progenitor with T cells (*49*). The LDC of mouse thymus delete developing T cells with autoreactive potential (*50*, *51*). It has also been suggested that LDC are involved in the maintenance of peripheral tolerance (*48*, *51*, *52*). In mice, LDC can induce T-cell proliferation without concomitant cytokine (IL-2, IL-3, interferon [IFN]-γ, GM-CSF) production (*48*, *53*). They kill $CD4^+$ T cells via Fas (CD95)-mediated apoptosis (*37*). In humans, so-called "plasmacytoid T cells" that express CD4 and MHC class II develop into DC after stimulation with IL-3 and CD40L (*54*). Human MDC, which induce Th1 cell differentiation, and human plasmacytoid DC, which induce Th2 cells, have been termed DC1 and DC2, respectively (*55*, *56*). Due to their in vitro functional properties, it has been suggested (*37*, *57*) that murine LDC may be DC specialized for tolerance induction. Significantly, we have observed that murine LDC of donor origin infused systemically 1 week before organ transplantation can markedly prolong vascularized cardiac allograft survival (*58*). In our proposed studies of tolerant human organ allograft recipients and controls, we will examine whether the presence or predominance of the putatively tolerogenic DC2 subset, which can be propagated from blood (*54*), correlates with the quiescent clinical state and with hypo/unresponsiveness to donor alloAgs.

Characterization of Host Alloimmune Responsiveness

For many years, cellular assays, including mixed leukocyte reactions (MLR), cell-mediated lympholysis, and Th and T cytotoxic cell precursor frequency analyses have been used to monitor the immune response status of organ allograft recipients. The predictive value, sensitivity, and specificity of such assays have often been questioned. Contemporary and developing approaches to evaluation of the hosts' immune status, including preliminary studies, will now be reviewed.

Cytokine Gene Polymorphism

Polymorphisms for many human cytokine genes that influence inflammation and immune responsiveness have been described. These have been demonstrated in a variety of disease conditions, including insulin-dependent diabetes mellitus, lupus erythematosus, and sepsis (*59–61*). In a recent review, Bidwell et al. (*62*) catalogued approximately 70 known cytokine polymorphisms. Perrey et al. (*63–71*) reported that for transplant rejection, the important polymorphisms lie in the promoter regions of IL-10 and TNF-α, in the first intron of the IFN-γ gene, and in the signal sequence of TGF-β. For instance, a mutation in position −308 (A) in the promoter region of the TNF-α gene is associated with increased TNF-α production, whereas a mutation in position −1082 (G) in the IL-10 gene corresponds with lower IL-10 production. Preliminary observations of adult renal and heart graft recipients suggest that high TNF-α and low IL-10 producers do seem to experience more recurrent and steroid-resistant acute rejection (*64*).

At our institution, pediatric heart transplant recipients have shown a similar association. TNF-α high and IL-10 low genotypes are significantly associated with a risk of recur-

rent graft rejection. Polymorphisms of IFN-γ and TGF-β may also affect graft outcome. High IFN-γ and TGF-β genotype is associated with the development of fibrosis and chronic rejection in lung transplant recipients (*66, 67, 70*). High TGF-β status has been found in two cases of peripheral kidney transplant tolerance (*72*). We have identified IFN-γ low and IL-6 high genotype to be a significant risk factor for the development of posttransplantation lymphoproliferative disease in patients with primary EBV infection (before transplantation recipient EBV negative/donor EBV positive) (*73*).

Preliminary results on 9 pediatric liver transplant patients off immunosuppression, 2 patients on minimal immunosuppression (on the weaning protocol) and 31 patients on continuous immunosuppression show that an IL-10 intermediate genotype was associated significantly ($P<0.01$) with the successful weaning protocol, whereas other cytokines (TNF-α low and IL-6 high) showed a trend. We need to study more patients to increase statistical power. Interestingly, the two patients currently maintained on minimal tacrolimus immunosuppression exhibit a similar cytokine genotype pattern to the patients who were successfully weaned. In our proposed study, identification of specific genetic factors for adverse graft outcome in patients that reject upon drug tapering, and a unique genotype associated with stable allograft outcome in patients off immunosuppression, would be of major benefit to the clinician and the patient. This would enable a move away from "protocol" immunosuppression to the identification of stable patients that could be safely weaned off all immunosuppressive agents.

CTLA4 and CD40 Polymorphism

In the presence of T cell receptor ligation of the MHC/peptide complex, the interaction of CD28 with B7 molecules provides the potent costimulatory signal necessary for T-cell activation and cytokine production. The critical contribution of this costimulatory pathway in the T-cell response to both auto- and alloAgs is now well established (*74, 75*). This has been further emphasized by the therapeutic benefits of costimulatory blockade demonstrated in several transplant and autoimmune disease models (*76–82*). Recent data indicate that CTLA4 (CD152), a CD28 homologue, serves as a negative regulator of the immune response, inhibiting T-cell proliferation and IL-2 production (*83, 84*). Furthermore, there is evidence to support the requirement for CTLA4 in the induction and maintenance of tolerance in allo- and autoimmune models (*85, 86*). The CTLA4 gene is situated on chromosome 2q33. Three polymorphisms have been identified—a dinucleotide $(AT)_n$ repeat polymorphism in the 3'-untranslated region of intron 3, a threonine to alanine (A→G) substitution at position 49 in exon 1, and the third in the promoter region of the CTLA4 gene (−318 relative to the ATG start codon) (*87–89*). This raises the possibility that these polymorphisms may have functional significance in the propensity of an individual to develop autoimmune conditions and to reject an allograft. An association has been demonstrated between the initial two CTLA4 polymorphisms and several autoimmune diseases (90–92); the third polymorphism has not been investigated extensively.

Circulating Allospecific Cytokine-Producing Th cells

Despite the essential role ascribed to alloreactive T cells in transplant rejection, there are no clinically useful methods of measuring the frequency of allospecific T cells in a given individual. Such functional information might help to tailor immunosuppressive therapy for high versus low risk recipients and potentially eliminate graft failure in selected patients. Both $CD4^+$ and $CD8^+$ T cells can mediate allograft rejection through direct cytotoxicity and/or via the induction of cytokine-induced inflammation in the target organ (*93–95*). In particular, the Th1 cytokine IFN-γ has been strongly linked to rejection in animals and humans (*93–96*). This suggests that the ability to detect IFN-γ-producing alloreactive T cells may provide a useful measure of the strength of the donor-specific response. Recently, Heeger et al. (*95, 97–99*) have developed a highly sensitive cytokine ELISPOT assay capable of characterizing the frequency of allospecific T cells in short-term culture. The frequency of peripheral blood lymphocytes induced to secrete IFN-γ in response to allogeneic stimulators reflects the number of memory cells directed against that donor and does not seem to be influenced by the number of human leukocyte antigen (HLA) mismatches between responder and stimulator. Furthermore, in living-related and cadaveric kidney allografts, the lowest frequency of donor-specific IFN-γ spots correlates with good graft outcome, whereas the highest responders experience rejection episodes. Conversely, increased production of IL-5, a Th2 cytokine, combined with low production of IFN-γ, is seen in stable renal patients' peripheral blood mononuclear cells (PBMC) stimulated with mitogens (*99*).

Surrogate In Vivo Analysis of T Cell-Mediated Responses to Donor AlloAgs

Transplant patients may be at risk of becoming sensitized to donor Ags if injected with donor Ag during traditional skin tests. Carrodeguas et al. have described an alternative method for human DTH testing that involves the transfer of human PBMC plus Ag into the pinnae or footpads of naive SCID mice. This induces a measurable DTH-like swelling response, referred to as the "trans-vivo DTH response." As proof of principle, data were obtained during trans-vivo DTH studies with alloAgs, CMV, or tetanus toxoid (*100*). In general, human T cells must be colocalized with Ag and APC to produce swelling. Such responses are Ag-specific and require previous Ag sensitization. This assay offers a simple and reliable clinical monitoring device and provides a model to study in vivo mechanisms of human DTH responses.

The assay can also be applied to identify patients that exhibit alloAg-triggered regulation of DTH as a marker of allograft acceptance. There are three potential patterns of DTH response in transplant recipients:

1. Positive DTH to alloAg and recall Ag in patients who require immunosuppression and have had multiple episodes of rejection.
2. Negative DTH to donor alloAg and down-regulation of DTH response to recall Ag when both Ags are coadministered (donor Ag-linked nonresponsiveness).
3. Negative DTH to donor Ag without inducing the regulatory response, namely the donor Ag-linked nonresponsiveness to recall Ags.

Quantitation of Anti-Donor MHC Class I and II AlloAbs

Most studies on HLA-specific Abs in transplantation have been performed on kidney transplant patients. There is cur-

rently no doubt that these Abs are important in acute and chronic rejection (*101–104*). Sundaresan et al. (*105*) have reported a correlation between the development of bronchiolitis obliterans (OB) in lung transplant recipients and HLA-specific Abs. Virtually all patients with detectable Abs develop OB at about 2 years after transplantation, whereas 60% of patients without Abs remain free of OB at 3 years after transplantation. The development of HLA-specific Abs is also associated with a higher incidence of chronic rejection in heart transplant patients (*106, 107*). These findings support the concept that alloAbs are an inherent component of the allograft immune response leading to rejection. In our proposed study, we shall ascertain the relation between clinical tolerance to liver grafts and circulating alloAb levels.

Significance for Immune Tolerance

Based on this conceptual framework, we propose to apply a spectrum of relevant immunologic assays to stable liver allograft recipients off all immunosuppression and to compare results with appropriate control groups. The information accumulated, using minimally invasive, relatively infrequent assays that are easy to perform and interpret, will provide a "roadmap" for clinicians, to help identify subjects from whom immunosuppressive therapy may be safely withdrawn.

DESCRIPTION OF WORK

The Study Populations

Presently, 27 pediatric and 16 adult (total 43) liver transplant patients off all immunosuppression are on file at the University of Pittsburgh Medical Center and can be considered for study. Donor HLA type is known for virtually all of the pediatric patients and for many of the adults. Where corresponding recipient HLA type is not presently available, this will be determined by DNA analysis. No stored donor cells are available for these patients. Thus, donor-specific responses will be elicited with donor-matched cells, purified alloAg, or synthetic allopeptides. We have also identified a total of 33 control pediatric (n=9) and adult (n=24) liver transplant recipients from whom all immunosuppression was withdrawn but who subsequently exhibited rejection and were restored to baseline immunosuppression. None of these patients underwent retransplantation. Many of these subjects have been followed for many years (*108, 109*). An additional control group will be pediatric and liver transplant patients (n=38, approximately) with a history of rejection and receiving conventional immunosuppression, who have never been weaned off immunosuppressive agents. We shall also perform the proposed tests *prospectively*, in a separate small population of liver graft recipients undergoing protocolized weaning. The latter group will comprise four to six patients per year.

Scale and Distribution of Proposed Investigations

A total of 30–50 ml of blood will be obtained per clinic visit (two visits per patient), except in cases where patient age/weight is limiting. PBMC and serum from all patients will be analyzed for immune response gene polymorphism and anti-donor HLA Abs, respectively. Subgroups of patients will also be tested for trans-vivo DTH or ELISPOT+DC analyses.

Our experimental approach with respect to individual assays will be as follows.

Gene Polymorphism

For each patient, we will analyze the polymorphic variants for TNF-α, IL-10, IFN-γ, IL-6, IL-4R, and TGF-β. We will determine the genotype of the recipient as a high or low producer for the individual cytokine. DNA extraction and subsequent amplification will be carried out according to standard methods developed for molecular typing for HLA polymorphisms (*110*). The allelic polymorphism of interest will be identified by polymerase chain reaction (PCR) with sequence-specific primers. Commercially available kits will be purchased from One-Lambda Inc. (CA).

CTLA4/CD40 Gene Polymorphism

Patients will be genotyped for the candidate polymorphisms, including the three identified polymorphisms in CTLA4 and a fourth polymorphism in CD40.

DC Assays

These are summarized in Table 3. As shown in Figure 1, rare $CD4^+$ IL-$3R\alpha^+$ HLA-DR^+ lin^- [precursor (p)DC2] can be identified in normal human peripheral blood by four-color flow cytometric analysis. We are confident that these cells, and also $CD4^+$ $CD11c^+$ HLA-DR^+ lin^- MDC (or pDC1), can be flow sorted and subjected to PCR analysis for the sex-determining region of the Y chromosome (where the donor is male and the recipient female) or for mismatched donor (HLA) alleles. Previously, we have shown, using immunocytochemical and molecular biologic techniques, that DC of donor origin (donor MHC class I/II^+) can be identified in cell populations expanded from the BM or blood of liver allograft recipients (*1, 111, 112*).

To generate DC1, blood monocytes are cultured with GM-CSF and IL-4 for 5 days in RPMI-1640 with 10% fetal calf serum; the resulting immature DC1 can then be matured by exposure for 24 hr to CD40L or anti-CD40 mAb (*55*). To generate DC2, $CD4^+$ $CD11c^-$ lin^- plasmacytoid cells are isolated (>98% purity) from peripheral blood after immunomagnetic bead depletion of $CD3^+$, $CD14^+$, $CD19^+$, $CD20^+$, and $CD56^+$ cells and sorting (*54*), then cultured with recombinant human IL-3 (±CD40 ligation) for 5–7 days.

Frequency of AlloAg-Specific Cytokine-Secreting Cells

We propose to use the ELISPOT assay to evaluate the frequency of alloreactive $CD4^+$ or $CD8^+$ PBMC-producing Th1 cytokines [IFN-γ and the cytotoxic effector molecule granzyme B (GB) and the Th2 cytokine IL-5] both in our study and control liver transplant recipients. We have chosen these mediators based on the excellent results obtained by Heeger et al. with renal transplant recipients (*93–96, 98, 99*). Currently, Heeger et al. are evaluating the regulatory cytokines IL-10 and TGF-β1, and when these assays are reproducible, we will include them in our panel. We will stimulate the patient's PBMC with donor-specific class II and class I allopeptides. As specificity controls, we will use a panel of various peptides; as a positive control, we will use phytohemagglutinin (PHA) stimulation, and CMV or tetanus toxoid Ag will be used to test the memory response (these are soluble Ags like the allopeptides presented by self-APC). Stable liver transplant recipients off immunosuppression should have a low frequency of allostimulated IFN-γ- or GB-producing cells. We also anticipate a low ratio of PHA-

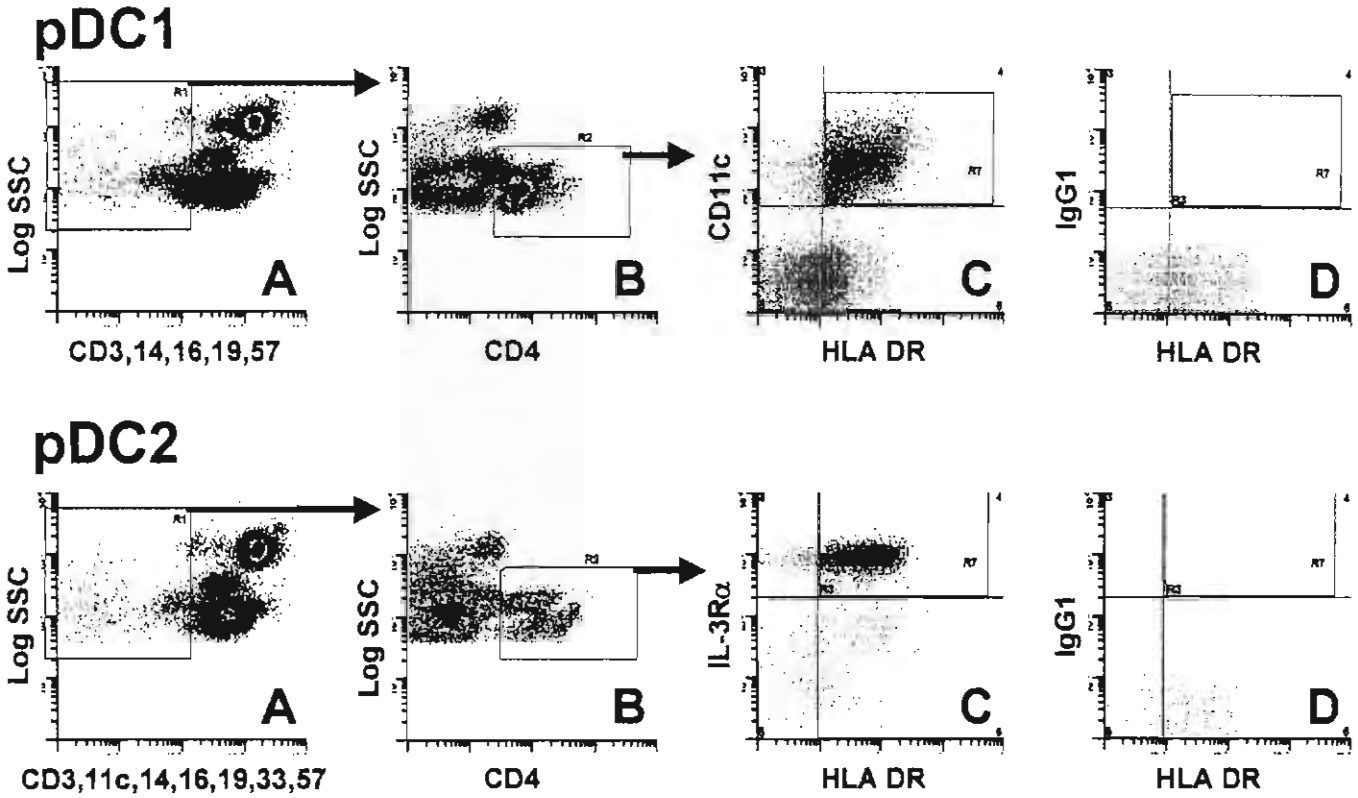

FIGURE 1. Detection of $CD4^+$ lin^- $CD11c^+$ HLA-DR^+ pDC1 and $CD4^+$ lin^- IL-$3R\alpha^+$ ($CD123^+$) HLA-DR^+ pDC2 in the peripheral blood of a healthy human subject. Panels A show the gating strategy used to identify lineage negative events. The lineage negative events are then projected onto a plot of CD4 versus log side scatter (panels B); events with intermediate or low side scatter and $CD4^+$ expression are included. The subsequent panels (C) show events compound gated with lineage negative events and projected onto a bivariate scatter plot of CD11c versus HLA-DR or IL-$3R\alpha^+$ versus HLA-DR. pDC1 (blue) cells were identified as $CD4^+$ lin^- $CD11c^+$ HLA-DR^+. pDC2 (red) were identified as $CD4^+$ lin^- IL-$3R\alpha^+$ ($CD123^+$) HLA-DR^+. Panels D show background staining with IgG1 instead of CD11c or IL-$3R\alpha$ (negative control).

TABLE 3. DC analyses

Plasmacytoid (pre-DC2) versus monocytoid (pre-DC1)
Relative and absolute numbers
? DC of donor origin (PCR for SDR of Y-chromosome or donor HLA alleles)
In freshly isolated cells
In in vitro propagated cells

induced IFN-γ/IL-5. In contrast, the liver transplant recipients who failed the weaning protocol and rejected their allograft should have high IFN-γ and GB frequencies and a high IFN-γ/IL-5 ratio. We will perform these assays on freshly collected blood samples to avoid false-negative results associated with freezing or low numbers of viable cells.

Trans-vivo DTH

Human→SCID mouse trans-vivo DTH to test donor-reactive T cell responses will be performed as described (*100*). It has already been observed that several transplant patients successfully withdrawn from immunosuppression exhibit the tolerance phenotype [Drs. Burlingham and Orosz, personal communication 2000; and VanBuskirk et al. (*113*)]. Each test requires two mice, $30-40 \times 10^6$ patient PBMC, and target (allo) Ag. Where donor cells are not available, class I and class II "null" EBV-transformed LCL expressing HLA Ags corresponding to the mismatched class I of the donor or surrogate EBV-LCL that share class I with the donor will be used, because these are sufficient to trigger the immune regulation of DTH. Alternatively, allopeptide (as described above) will be tested (Table 4). We will correlate the results (two tests per patient) with those of the DC and ELISPOT assays. Preliminary data obtained in two stable liver transplant recipients maintained on low-dose immunosuppression and shown in Table 5 indicate that a combination of ELISPOT and trans-vivo DTH assays may prove valuable in predicting the "tolerant" or "rejector" state. In patient 1, a pattern of stable donor-specific hyporeactivity in MLR (for 4 years) (data not shown) correlates with a lack of IFN-γ-producing cells (ELISPOT) in response to donor but significant responses to unrelated third-party panel cell and PHA stimulation. In addition, this patient regulates the EBV DTH responses in the presence of sonicated donor Ag. By contrast,

TABLE 4. Sources of donor Ag for use in ELISPOT/ trans-vivo DTH assay

Sonicated donor cells
Sonicated surrogate donor HLA-matched cells (PBMC or splenocytes)
Self EBV-transformed B cells together with donor Ag
HLA-coated beads

TABLE 5. Alloreactive T-cell responses in stable liver transplant recipients

Patient	ELISPOT (IFNγ-secreting cells/3.10^5 PBMC)			Trans-vivo DTH (mm^{-2})	
	Responder	Responder+Donor	Responder+third-party	EBV	EBV+donor
1	47±21	28±4	121±15	65	30
2	45±15	89±16	95±40	110	100

Patient 1 exhibits donor-specific hyporeactivity in MLR; patient 2 does not exhibit donor-specific hyporeactivity in MLR. Both patients are currently maintained on low-dose immunosuppression.

patient 2, who shows no donor-specific hyporeactivity in MLR, exhibits IFN-γ responses to donor as well as third-party cells and does not regulate the DTH response to EBV. By extending these studies to "tolerant" and "rejecting" liver transplant patients, we hope to validate these assays that may predict the tolerant state.

We will test the induction of donor alloAg-triggered nonresponsiveness to EBV by injecting the footpad of naive SCID mice with (i) patient's PBMC (8×10^6) plus EBV antigen (positive DTH) or autologous EBV-LCL or (ii) patient's PBMC + donor-derived EBV-LCL or surrogate EBV-LCL that express donor HLA class I Ag. If the recipient was allosensitized, but developed regulatory cells, we anticipate detecting inhibition of the EBV-related DTH response in the presence of donor HLA class I. This inhibition can be reversed by neutralizing Abs to TGF-β or IL-10 (*113*) and is restricted to the class I mismatch of the donor-recipient pair.

Patients who do not have donor-specific DTH, but cannot inhibit recall responses may have deleted donor-specific T cell clones, or they may have avoided allosensitization due to augmented immunosuppression. The establishment and maintenance of donor Ag-linked non-responsiveness can be used as a marker for patients entered the weaning protocol since the regulated phenotype can be expressed in patients on immunosuppression.

Quantitation of AlloAb

Sensitization against HLA Ags will be determined using the sensitive ELISA test. The Lambda Antigen Tray (LAT) is based on the principle of presenting purified HLA antigens, instead of cells, as alloAb targets. Purified class I and II HLA Ags are bound directly on Terasaki trays. The specific binding of Ab from the test sample with any of these Ags is detected by subsequent incubation with alkaline phosphatase-conjugated Ab that recognizes only human IgG. A quantitative measure of the extent of reaction is obtained by spectrophotometric determination, after the addition of the appropriate enzyme substrate for the development of color. Qualitative assessment of Ab specificity is performed by analysis of the LAT reactivity pattern, using a code sheet provided by the manufacturer.

In our laboratory, we have access to a sophisticated computer program (Multiscreen) for assigning Ab specificity patterns towards private HLA antigens and public epitopes (defined from cross-reacting groups) that are associated with critical amino acid residues (assigned from molecular sequence information) (*114*). Multiscreen is particularly powerful for the analysis of sera from highly sensitized patients with panel-reactive antibody values over 90% (*114*). The HLA Ab specificity patterns revealed by the different screening assays will be compared with the HLA typing information of the transplant donor to determine whether donor-specific HLA allosensitization has occurred.

Biopsy Pathology

Protocol graft core biopsy analysis will be undertaken. Liver biopsy specimens will be subjected to histopathologic analysis that may aid in characterization of the "tolerant" state. Subject to tissue availability, immunohistochemistry will be performed and material (including isolated DNA) cryopreserved and banked for future investigation.

Microarray/Tetramer Assays

In our study, core support will be provided by the ITN to utilize Network microarray and tetramer facilities. An Affymetrix machine/chips that can be used for microarray studies has recently been obtained. In addition, the Network has funded a group of investigators to develop appropriate MHC class II multimers for study. Full collaboration with the NIAID Tetramers Facility for class I tetramers and ITN advisors is also envisaged. PBMC samples from our study patients will be cryopreserved for these important future analyses.

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