American Journal of Transplantation 2003; 3: 689–696
Blackwell Munksgaard



ISSN 1600-6135

# Dendritic Cell Subset Ratio in Peripheral Blood Correlates with Successful Withdrawal of Immunosuppression in Liver Transplant Patients

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**Human dendritic cell (DC) subsets appear to play distinct roles in the induction and regulation of immune responses. While monocytoid DC (DC1) induce T-helper (Th) 1-type responses, plasmacytoid DC (DC2) have been reported to selectively induce Th2 responses. In blood, their precursors (p) can be identified as HLA-DR$^+$ lineage$^-$ cells that are further characterized as CD11c$^+$ CD123$^{-/lo}$ (IL-3R$\alpha^{-/lo}$) (pDC1) or as CD11c$^-$ CD123$^{hi}$ (pDC2) by rare event, flow cytometric analysis. We compared the incidences of pDC1 and pDC2 in peripheral blood mononuclear cell populations isolated from normal healthy controls and from 3 groups of clinically stable liver transplant patients. Group A had been successfully withdrawn from immunosuppression, whereas group B were undergoing prospective drug weaning and on minimal anti-rejection therapy. In group C, drug withdrawal had either failed or never been attempted and patients were on maintenance immunosuppression. Assessment of DC subsets and the pDC2 : pDC1 ratio showed good intra-and interassay reproducibility. Compared with patients in group C, those in groups A and B demonstrated a significantly higher relative incidence of pDC2 and a lower incidence of pDC1 – similar to those values observed in normal healthy controls. Moreover, the pDC2 : pDC1 ratio was significantly higher in patients undergoing (successful) weaning and in those off immunosuppression compared with patients on maintenance immunosuppression.**

**Key words: Dendritic cells, liver, tolerance, transplantation**

**Received 20 September 2002, revised and accepted for publication 8 January 2003**

## Introduction

Organ transplant tolerance can be induced predictably across major histocompatibility complex (MHC) barriers in animals, with or without the use of immunosuppressive agents (1–3). No therapeutic regimens are known to achieve this state in humans, other than in rare instances. Nonetheless, there are well-documented accounts of complete withdrawal of immunosuppression from transplant patients, particularly liver recipients, without the occurrence of graft dysfunction/rejection (4,5). Drug withdrawal may be required for the management of post-transplant viral infection or lymphoproliferative disease, or result from patient noncompliance or physician-controlled weaning. While such instances of operational tolerance are very rare in kidney recipients, the phenomenon is more common following liver transplantation. Although the immunologic basis of liver-induced tolerance has not been elucidated, several mechanisms have been proposed (1,6,7). These include the paradigm of microchimerism (8,9) and the concept that donor-derived antigen (Ag)-presenting cells (APC) may modulate antidonor immunity and promote tolerance induction (10,11). The observation of specific recipient cytokine gene polymorphisms in clinically tolerant liver allograft recipients (12) has provided an impetus for the development of further assays that may reliably predict or validate donor-specific tolerance in human organ transplantation. Development of such a test(s) would be of considerable value in identifying those patients who might be safely weaned off anti-rejection therapy.

The immune response to organ grafts is believed to be initiated by the presentation of alloAg by donor or recipient APC to host Ag-specific T cells. Upon stimulation, these lymphocytes differentiate into effector and regulatory T cells. Bone-marrow-derived dendritic cells (DC) are uniquely well-equipped APC, commonly regarded as the principal instigators of rejection (13,14). Evidence also exists, however, for DC tolerogenicity, in the context both of auto- and alloimmune responses (15–18). These critically important APC circulate as immature DC precursors (p). They exit the blood to reside as immature DC in tissues throughout the body, and traffic via lymph or blood to secondary lymphoid organs following Ag uptake. Blood-borne DC are identified by a distinct surface immunophenotype, that includes lineage negativity, constitutive MHC class II Ag expression and low surface

levels of costimulatory molecules necessary for T-cell activation. In humans, two major subpopulations of blood pDC have been described – monocytoid and plasmacytoid (19–21). Monocytoid DC can be derived from circulating monocytes in response to GM-CSF and IL-4, whereas plasmacytoid DC (the major IFNα-producing cells in blood) (22,23) develop from plasmacytoid T cells after stimulation with IL-3 and CD40L (24). Monocytoid DC, that induce T-helper (Th)1 cell differentiation *in vitro*, and plasmacytoid DC, that promote Th2 cell responses (25), have been termed DC1 and DC2, and may be specialized for the induction of immunity and tolerance, respectively (26).

We hypothesized that circulating levels of these pDC subsets, relative to one another, might reflect the status of liver graft recipients with respect to clinical tolerance and safe withdrawal of immunosuppression vs. rejection risk and dependence on anti-rejection therapy. Rare event, flow cytometric analysis was used to examine the incidences of pDC1 and pDC2 in: (A) stable liver transplant patients off all immunosuppressive therapy, (B) in a group undergoing progressive drug weaning and maintained on minimal immunosuppression, and (C) in patients on maintenance immunosuppression who had failed weaning, or in whom weaning had never been attempted. Normal healthy controls (D) were also examined. The data reveal higher incidences of pDC2 relative to pDC1 in the weaning and tolerant groups compared to patients on maintenance immunosuppression. Moreover, the pDC2 : pDC1 ratio appears to serve as an index of clinical unresponsiveness to the donor that may be of value in determining which liver transplant patients can be weaned safely off immunosuppression.

## Patients and Methods

### *Study population*

Forty clinically stable liver transplant recipients (both children and adults) with normal graft function were eligible for study. All patients provided written informed consent in accordance with a protocol approved by the local institutional review board and the Immune Tolerance Network. The demographics of this population, subdivided into the 3 study groups, are shown in Table 1. The mean age at transplantation was 10.4 ± 16.9 years (range 0.2–60.5 years). Diagnoses at transplantation were biliary atresia (n = 12), metabolic disease (n = 8), autoimmune liver disease (n = 5), cholestatic syndromes (n = 5), acute liver failure (n = 2), and other diagnoses (n = 8). The normal control population (group D) consisted of 13 healthy adults of both sexes, with a mean age at sampling of 34 years (Table 1).

### *Patients off immunosuppression (group A) or undergoing prospective weaning (group B)*

Patients off immunosuppression as described in Table 1 have been free from anti-rejection therapy for a mean of 3.3 years (range of 0.8–7.9 years). They were weaned off drugs by physician-directed protocol (n = 2) as described (5), or emergently for life-threatening infectious disease indications (n = 3), or had self-weaned by noncompliance (n = 1). Initial immunosuppression had consisted of cyclosporine and steroids (n = 3) or tacrolimus and steroid re-cycle (n = 3).

Briefly, the protocol used to achieve drug withdrawal was as follows: prednisone was withdrawn in 50% decrements monthly with corticotrophin stimulation testing to detect adrenal insufficiency if indicated clinically. Calcineurin inhibitors (primarily cyclosporine and when applicable, tacrolimus, were then withdrawn at 1–2-month intervals by 10–25% of the baseline amount. Azathioprine was the final drug withdrawn, when present. For children who presented with acute complications of immunosuppression, such as Epstein-Barr virus (EBV) infection or overt post-transplant lymphoproliferative disease (PTLD), all immunosuppression was stopped immediately. Anti-rejection therapy was resumed when the presenting infection had resolved and with documentation of rejection on biopsy. Patients who did not subsequently require reinstitution of immunosuppression because of normal liver function and/or a severe episode of PTLD were then included in the study group of patients off immunosuppression (group A).

Patients currently undergoing prospective drug weaning (group B) have tolerated uninterrupted drug withdrawal for a mean of 1.3 years. All are on minimal immunosuppression, defined as monotherapy with low drug levels. Immunosuppressive drug dosage and trough levels at the time of blood sampling for this population and the maintenance immunosuppression group (C) are shown in Table 2.

**Table 1:** Demographics of study population

| Group | No. of patients | Sex (M:F) | Variable | Mean (years) | SD (+/–) | Min | Max |
|---|---|---|---|---|---|---|---|
| (A) Off immunosuppression | 6 | 4 : 2 | Age at liver transplant | 6.5 | 5.8 | 0.4 | 16.3 |
| | | | Time post transplant | 9.7 | 6.8 | 2.6 | 18.5 |
| | | | Current age | 16.2 | 9.9 | 3.0 | 25.8 |
| | | | Time off immunosuppression | 3.3 | 3.2 | 0.8 | 7.9 |
| (B) Prospective weaning | 23 | 13 : 10 | Age at liver transplant | 2.4 | 2.6 | 0.2 | 9.0 |
| | | | Time post transplant | 9.2 | 4.6 | 1.3 | 18.2 |
| | | | Current age | 11.6 | 5.3 | 2.0 | 24 |
| | | | Time weaning | 1.3 | 2.3 | 0.1 | 9.7 |
| (C) Maintenance immunosuppression | 11 | 8 : 3 | Age at liver transplant | 29.1 | 23.2 | 1.6 | 60.5 |
| | | | Time post transplant | 5.7 | 6.2 | 0.1 | 17.6 |
| | | | Current age | 34.8 | 24.8 | 2.9 | 65.2 |
| (D) Normal control | 13 | 8 : 5 | Current age | 34.0 | 9.2 | 25.0 | 53.0 |

**Table 2:** Immunosuppressive drug dosage and levels at time of peripheral blood sampling

**Prospective weaning (group B)**

| Patient no. n = 23 | Tacrolimus or Cyclosporine (CsA) | |
|---|---|---|
| | Mean daily dose (mg/day) | Trough level (ng/mL) |
| 1001 | 0.2 | ND |
| 1005 | 0.5 | ND |
| 1006 | 0.5 | <2.5 |
| 1010 | 0.5 | 3.0 |
| 1016 | 0.5 | <2.5 |
| 1020 | 0.25 | 1.1 |
| 1022 | 0.5 | <2.5 |
| 1023 | 0.25 | ND |
| 1024 | 1.0 | 3.6 |
| 1025 | 1.5 | <2.5 |
| 1026 | 0.5 | 2.5 |
| 1028 | 25 mg 2 ×/week (CsA) | – |
| 1030 | 1.0 | 2.5 |
| 1031 | 2.0 | 3.8 |
| 1032 | 2.0 | 2.7 |
| 1034 | 50 mg 6 ×/week (CsA) | – |
| 1035 | 1.0 | ND |
| 1038 | 0.5 | <2.5 |
| 1039 | 3.0 | 2.9 |
| 1040 | 160 (CsA) | 153 |
| 1041 | 2.0 | ND |
| 1042 | 0.5 | <2.5 |
| 1043 | 0.5 | ND |

**Maintenance immunosuppression (group C)**

| Patient no. n = 11 | Tacrolimus | | Cyclosporine | | Sirolimus | | Azathioprine | Prednisone |
|---|---|---|---|---|---|---|---|---|
| | Mean daily dose (mg/day) | Trough level (ng/mL) | Mean daily dose (mg/day) | Trough level (ng/mL) | Mean daily dose (mg/day) | Trough level (ng/mL) | (mg/day) | (mg/day) |
| 1007 | 4.0 | 7.4 | – | – | – | – | – | 2.5 |
| 1008 | 2.5 | 7.6 | – | – | – | – | 150 | 15 |
| 1011 | – | – | 350 | 256 | – | – | – | – |
| 1013 | – | – | 150 | 105 | – | – | 50 | 5.0 |
| 1017 | 3.0 | 10.4 | – | – | – | – | – | 7.5 |
| 1018 | 4.0 | 7.2 | – | – | – | – | – | – |
| 1027 | – | – | – | – | 0.14 | <1.0 | – | – |
| 1029 | 14 | 15.3 | – | – | – | – | – | 20 |
| 1036 | 2.0 | ND | – | – | – | – | – | 5.0 |
| 1037 | 6.0 | 3.7 | – | – | – | – | – | – |
| 1044 | 2.0 | 12.4 | – | – | – | – | – | – |

ND = Not detected, – = Not measured.

***Patients on maintenance immunosuppression (group C)***

Patients in this group had either failed drug withdrawal (n = 6) or had never been weaned from immunosuppressive medications because of a concern for rejection and/or disease recurrence (i.e. history of autoimmune hepatitis) or previous rejection (n = 5). One patient was assayed within 14 days of biopsy-proven rejection, while five others had experienced a rejection episode prior to blood sampling (2 weeks–1 month, n = 2; 1.2–8 years, n = 3).

***Isolation of PBMC***

Peripheral venous blood samples from patients and healthy controls were collected in heparinized tubes. The samples were rocked slowly overnight (18 h) at room temperature. Peripheral blood mononuclear cells (PBMC) were then isolated by Ficoll-Paque (Amersham Pharmacia Biotech AB, Uppsala, Sweden) density gradient centrifugation.

***Immunofluorescence staining and flow cytometric analysis***

PBMC were suspended at a concentration of $2.5 \times 10^6$/mL in cell staining buffer (CSB) [phosphate-buffered saline (PBS) supplemented with 1% v/v fetal calf serum (FCS) and 0.1% w/v sodium azide]. The cells were incubated in normal goat serum (Gibco-BRL) to block nonspecific binding of antibodies to Fc receptors. They were then aliquoted ($0.2–0.5 \times 10^6$) into polystyrene tubes and washed (× 1) with CSB. The cells were stained for

30 min on ice with FITC-conjugated anti-CD3 (clone SP340), CD14 (clone M5E2), CD19 (clone J4.119) and CD20 (clone 2H7), Cy-Chrome conjugated anti-HLA-DR (clone G46-6), PE-conjugated anti-CD123 (IL-3Rα, clone 7G3), and APC-conjugated anti-CD11c (clone S-HCL-3). All antibodies except anti-CD19 (Beckman Coulter Corporation, Hialeah, FL, USA) and anti-CD11c (BD Sciences Immunocytometry Systems, San Jose, CA, USA) were purchased from BD PharMingen (San Diego, CA, USA). Stained cells were washed (× 1) in CSB, suspended in PBS and fixed in 4% v/v paraformaldehyde to a 1% final concentration. Four-color flow cytometric analysis of 50 000–250 000 cells was performed using a Coulter Epics Elite ESP flow cytometer (Beckman Coulter) in conjunction with EXPO32$^{tm}$ software (Applied Cytometry Systems, Sheffield, UK). Cells that were negative for CD3, CD14, CD19 and CD20 (lineage$^-$) and HLA-DR$^+$ were gated and analyzed for CD11c and CD123 expression. Precursors (p) of monocytoid (pCD1) and plasmacytoid DC (pDC2) were identified as lineage$^-$, HLA-DR$^+$, CD11c$^+$, CD123$^{-/lo}$ and lineage$^-$, HLA-DR$^+$, CD11c$^-$, CD123$^{hi}$, respectively. The incidence of each subset was expressed as a percentage of lineage$^-$ HLA-DR$^+$ PBMC; in addition, the pDC2:pDC1 ratio was determined for each subject at the time of sampling.

### *Statistical analysis*

Results are expressed as arithmetic means ± 1 SD. The Mann–Whitney U-test was used to compare differences between means. 'p' values $< 0.05$ were considered to be significant. Coefficient of variation was determined to assess assay reproducibility. Linear regression analysis using the 'least squares' method was employed to examine the correlation between the pDC2 : pDC1 ratio and length of time off immunosuppression.

## Results

### *Identification of pDC subsets in peripheral blood*

PBMC were isolated from normal healthy controls and from liver recipients successfully withdrawn from immunosuppression (group A), those undergoing drug weaning (group B), and from patients in whom drug withdrawal had failed or had never been attempted (maintenance immunosuppression; group C). Lineage$^-$ HLA-DR$^+$ cells were identified on the basis of four-color flow cytometric analysis. Subpopulations of CD11c$^-$ CD123$^{hi}$ lin$^-$ HLA-DR$^+$ (pDC2) and CD11c$^+$ CD123 $^{-/lo}$ lin$^-$ HLA-DR$^+$ (pDC1) cells were further delineated. Representative data from a normal healthy adult control and from individual patients in groups A–C are shown in Figure 1. For each individual, the incidence of circulating pDC1 expressed as a percentage of lin$^-$ HLA-DR$^+$ PBMC was greater than that of pDC2. A third, minor population (in relation to each pDC subset) of lin$^-$ HLA-DR$^+$ CD11c$^-$ CD123$^-$ cells was also present consistently in each group.

### *Assay reproducibility*

To determine intra-assay variability, DC analysis was performed 3 times concurrently on blood samples obtained

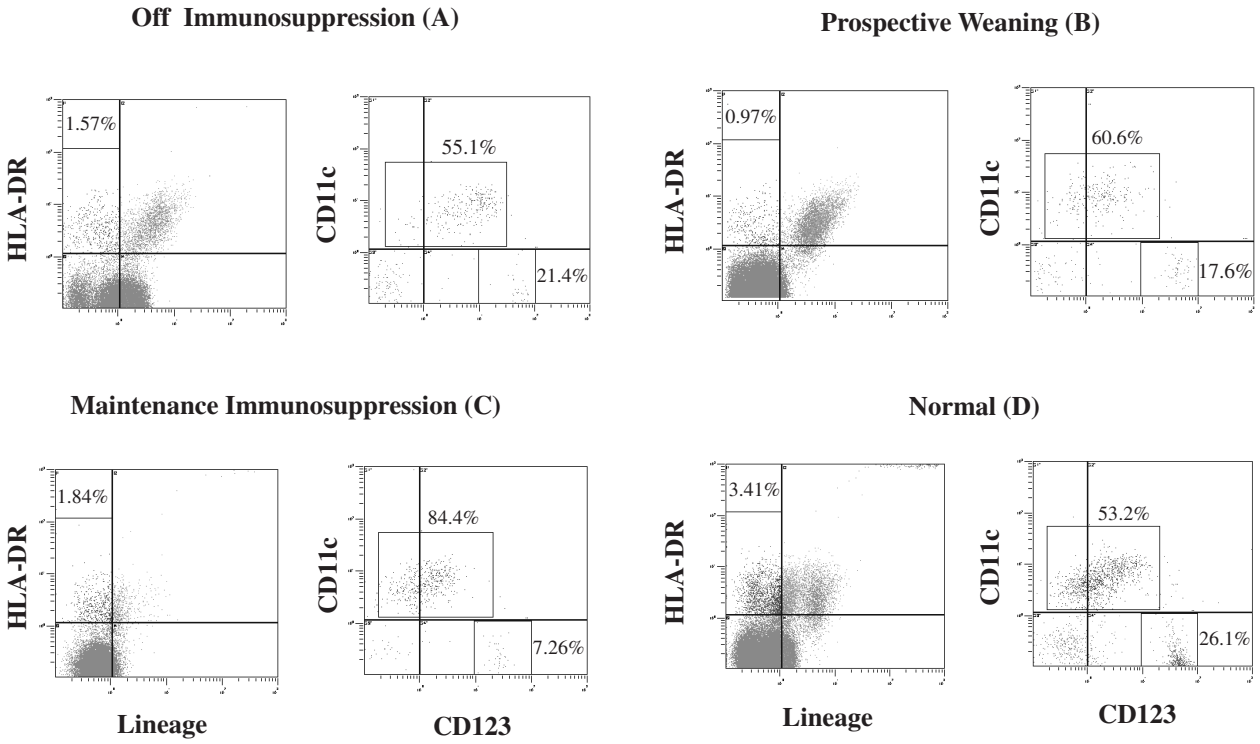


**Figure 1: Identification of pDC subsets in peripheral blood of liver transplant recipients and normal healthy controls by 4-color, rare event, flow cytometric analysis.** Lin$^-$ HLA-DR$^+$ cells were further characterized as either CD11c$^+$ CD123$^{-/lo}$ (IL-3Rα$^-$) (pDC1) or CD11c$^-$ CD123$^{hi}$ (pDC2). Lin$^-$ HLA-DR$^+$ cells represented a small percentage of PBMC (consistently $< 4\%$). The incidences of pDC1 and pDC2 are expressed as percentages of lin$^-$ HLA-DR$^+$ cells. Representative data from one patient in each of group A (off immunosuppression), group B (prospective weaning; low-dose immunosuppression) and in group C (maintenance immunosuppression) are shown, in addition to data from a normal healthy control (group D).

from three normal healthy adults. As shown in Table 3, the coefficient of variation (CV) for % pDC1, % pDC2 and % pDC2 : % pDC1 was consistently $\leq$10%. Similarly, interassay reproducibility was confirmed for blood samples obtained at different intervals from the same subjects (% pDC2 : % pDC1 consistently <6 %), Table 4.

### *Patients off immunosuppression and undergoing prospective weaning exhibit higher relative incidences of pDC2 compared with those on maintenance immunosuppression*

The incidences of pDC subsets for all subjects in each study group, together with mean values, are shown in Figure 2. In addition, individual values and mean ratios of % pDC2 : % pDC1 are presented. The pDC2 population was increased significantly (approximately 4-fold, from $\sim$4.7 to $\sim$20% of the HLA-DR$^+$ lin$^-$ population) in group A (off immunosuppression) and the weaning group (group B) compared with the maintenance immunosuppression group (group C). Conversely, the percentage pDC1 was significantly lower in groups A and B compared with C. When the data for pDC1 and pDC2 subsets for each subject were combined to render the pDC2 : pDC1 ratio, patients in groups A and B exhibited a 3–5-fold significantly higher mean value (similar to normal controls) than those in group C.

### *Higher pDC2 : pDC1 ratios are observed with length of time off immunosuppression*

When the pDC2 : pDC1 ratios in the six clinically tolerant patients (group A) were plotted in relation to length of time off immunosuppression, a significant correlation was noted, with much higher ratios being observed in those patients between 6 and 8 years off all anti-rejection therapy compared with those off drugs for shorter periods (Figure 3).

**Table 3:** Assessment of intra-assay reproducibility

| Subject | Test | % pDC2 | % pDC1 | % pDC2 / % pDC1 |
|---|---|---|---|---|
| A | 1 | 7.60 | 57.7 | 0.132 |
| | 2 | 8.31 | 53.0 | 0.157 |
| | 3 | 7.82 | 49.4 | 0.158 |
| | Mean | 7.91 | 53.4 | 0.149 |
| | SD | 0.36 | 4.15 | 0.015 |
| | CV % | 4.6 | 7.8 | 9.9 |
| B | 1 | 12.7 | 77.4 | 0.163 |
| | 2 | 11.0 | 78.3 | 0.141 |
| | 3 | 10.8 | 79.5 | 0.135 |
| | Mean | 11.5 | 78.4 | 0.146 |
| | SD | 1.04 | 1.02 | 0.015 |
| | CV % | 9.1 | 1.3 | 10.1 |
| C | 1 | 2.86 | 87.4 | 0.034 |
| | 2 | 2.58 | 86.5 | 0.030 |
| | 3 | 2.92 | 87.2 | 0.033 |
| | Mean | 2.79 | 87.1 | 0.032 |
| | SD | 0.18 | 0.46 | 0.002 |
| | CV % | 6.5 | 0.5 | 6.4 |

The assay was performed 3 times concurrently on the same blood sample from 3 normal healthy adults.
CV = coefficient of variation.

**Table 4:** Assessment of interassay reproducibility

| Subject | Test (interval) | % pDC2 | % pDC1 | % pDC2 / % pDC1 |
|---|---|---|---|---|
| A | 1 | 8.63 | 82.9 | 0.104 |
| | 2 (9 weeks) | 9.27 | 82.9 | 0.112 |
| | Mean | 8.95 | 82.9 | 0.108 |
| | SD | 0.45 | 0.06 | 0.006 |
| | CV % | 5.1 | 0.1 | 5.2 |
| B | 1 | 14.08 | 62.0 | 0.227 |
| | 2 (14 weeks) | 9.12 | 40.0 | 0.228 |
| | Mean | 11.6 | 51.0 | 0.228 |
| | SD | 3.51 | 15.53 | 0.001 |
| | CV % | 30.2 | 30.5 | 0.3 |
| C | 1 | 9.79 | 84.5 | 0.116 |
| | 2 (38 weeks) | 8.79 | 82.0 | 0.107 |
| | Mean | 9.29 | 83.2 | 0.112 |
| | SD | 0.71 | 1.79 | 0.006 |
| | CV % | 7.6 | 2.1 | 5.7 |

The assay was performed on blood samples obtained at different intervals from 3 normal healthy adults.
CV = coefficient of variation.

## Discussion

These findings reveal that circulating pDC2 are more prevalent, relative to pDC1, in stable liver transplant patients off immunosuppression (group A) or in those undergoing successful drug withdrawal (group B) compared to patients on maintenance immunosuppression (group C). Before alluding to the possible biologic significance of this observation and its potential application, several points need to be emphasized in relation to data interpretation. First, although all three patient groups were similar with respect to the time post transplant at which they were studied (6–10 years), they differed in terms of age distribution. The fact that there are more pediatric than adult liver recipients off immunosuppression in our study population is reflected in a younger population of patients off immunosuppression, both at the time of transplant and currently. Nonetheless, normal adult controls with a similar mean age to patients in the rejection group also differ from the latter in their pDC subsets, and are in fact similar to patients off immunosuppression or undergoing prospective weaning, even though those two patient populations are younger. Second, patients in groups B and C were receiving systemic anti-rejection therapy at the time of sampling although, clearly, the level of immunosuppression was considerably higher in the maintenance

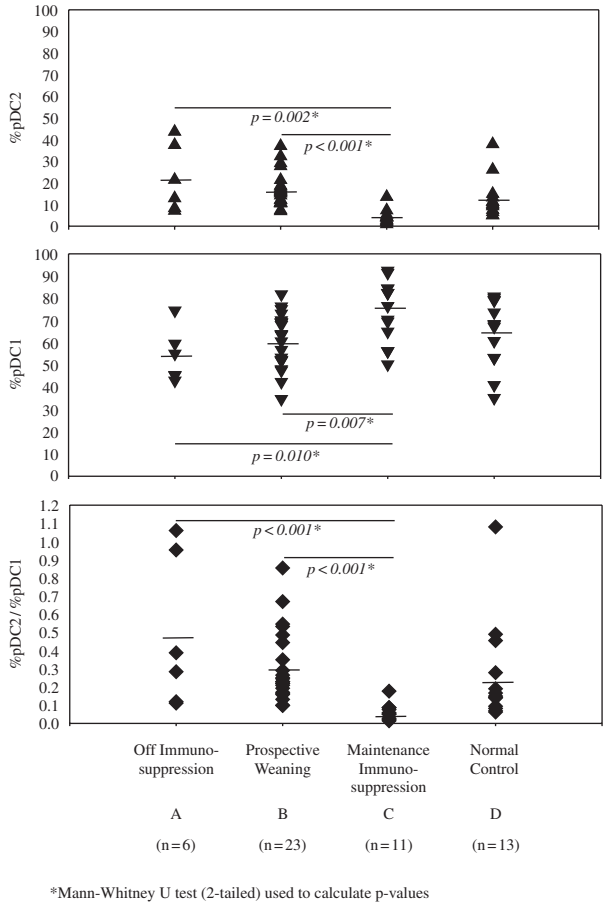


**Figure 2: Incidences of pDC1 and pDC2 and the resulting pDC2 : pDC1 ratio in the three groups of liver transplant patients (A–C) and healthy controls (D).** Values are expressed as percentage of $lin^-$ HLA-$DR^+$ PBMC. Data were generated following mAb staining and rare event, flow cytometric analysis as depicted in Figure 1. Individual patient or control values, arithmetic means and significances of differences between the patient groups are shown.

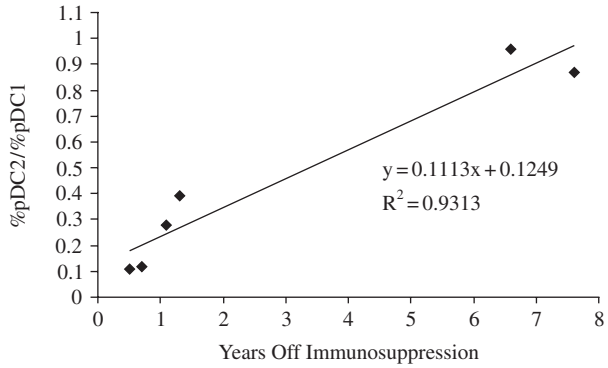


**Figure 3: pDC2 : pDC1 ratios in the six clinically tolerant liver transplant patients plotted in relation to time off immunosuppressive therapy.**

immunosuppression group (C). Since these latter patients exhibited a significantly reduced relative incidence of pDC2, and a lower pDC2 : pDC1 ratio compared with those receiving either no or very low immunosuppression, a possible interpretation of this result is that anti-rejection therapy may account for the observed alteration in DC subset profile.

Although there is evidence that calcineurin inhibitors (cyclosporine) (27), corticosteroids (28) or antiproliferative agents (29) can affect the maturation of *in vitro*-generated DC, there are no reports of which we are aware that any immunosuppressive drug differentially affects the *in vivo* production of DC subsets from hemopoietic progenitors. Nor is there evidence that the drugs used to suppress rejection affect the trafficking of DC subsets to or from the blood, or their *in vivo* survival. Indeed, our findings in murine models suggest that this is not the case, at least with respect to systemic cyclosporine or rapamycin administration (Hall, Coates, Taner, Hackstein and Thomson, unpublished observations). The recent characterization of the murine counterparts of human plasmacytoid DC (30,31) will allow formal testing of this issue.

Our findings pose questions about the immunobiologic significance of the elevated relative incidence of pDC2 in successful weaning and tolerant patients compared with those with a history of rejection. Conceivably, the elevated incidence of pDC2 may indicate a role of these cells in maintenance of the tolerant/unresponsive state. There is evidence that, in contrast to freshly isolated pDC1 (20,21), pDC2 freshly isolated from normal human blood fail to induce proliferation in allogeneic T cells (24,32). Such immature DC that fail to induce proliferation can promote Ag-specific T-cell hyporesponsiveness (33) and the generation of T regulatory cells (34,35). Interestingly, umbilical cord blood that, unlike peripheral blood, contains immature DC2 and not DC1 (36), induces a relatively low incidence of acute graft-vs.-host disease when used as a source of allogeneic stem cells. Based on their capacity to selectively induce Th2 cell responses (25), it has been suggested that the DC2 subset has potential to promote transplantation tolerance (37). The capacity of human peripheral blood pDC2 to induce Ag-specific T-cell anergy (38) also suggests that pDC2 may be involved in the induction/maintenance of peripheral T-cell tolerance. Variation in the pDC2 : pDC1 ratio in blood and in the graft (of which the blood ratio may be reflective) could have important consequences for antidonor reactivity and graft outcome. At present, it is only possible to speculate on the extent to which donor-derived DC may contribute to these postulated regulatory effects (10,39). Conceivably, pDC2 that can be mobilized selectively in living donors in response to G-CSF (37) may be of potential therapeutic value for the induction of tolerance to solid organ grafts.

Clearly, further study is required to evaluate the potential of pDC subset analysis as an index of antidonor

(un)responsiveness and predictor of safe weaning from immunosuppressive therapy. We have embarked upon prospective sequential analysis of pDC subsets in liver transplant patients selected for weaning. This will allow us ascertain the predictive value of the assay. As we have demonstrated, assessment of pDC subsets has proved highly reproducible upon repeated analysis of normal healthy individuals. In addition, data are being accumulated to establish whether the results obtained with the 'DC assay' correlate with *in vitro* tests of antidonor T-cell responsiveness and its regulation, in particular *trans*-vivo delayed-type hypersensitivity analysis (40) and ELISPOT quantitation of specific donor-reactive T-cell responses (41).

From a clinician's standpoint, it is of note that the findings in the tolerant group were similar whether or not drug withdrawal was accomplished slowly, by physician-directed protocol, or emergently, because of infectious disease indications. If verified subsequently during the course of prospective weaning, these findings could allow for more rapid drug tapering and thus minimization of drug-related toxicities, such as hypertension or nephrotoxicity, that are difficult to resolve once established.

In general, there was good homogeneity within each study group. In group A, the range of % pDC2 : % pDC1 ratios was 0.1–1.1, with a mean 7 times higher than that of the maintenance immunosuppression group. The two children (1014 and 1019) with the lowest ratios exhibited values 2-fold higher than the maintenance immunosuppression group (mean ratios of 0.12 vs. 0.06, respectively). These patients have now been off immunosuppression for 1.1 and 0.9 years, respectively. Patient 1014 has evidence of a clinically occult EBV viremia, while patient 1019 was removed from immunosuppressive therapy because of myelodysplastic syndrome. The influence of these acute processes upon the results are unknown. Repeated values over time will be of great interest. It is also notable that there appears to be a significant relationship between time off immunosuppression and the DC subset ratio. Thus the two patients with the highest ratios in the tolerant group (Figure 2) also have been off drugs for the longest period of time (6.6 and 7.6 years) (Figure 3). The impact of time post transplant and time off immunosuppression can also be evaluated by sequential sample analyses.

In conclusion, this investigation indicates that plasmacytoid DC precursors (pDC2) are more prevalent in the circulation of liver transplant patients successfully withdrawn from immunosuppression, as well as in those stable patients on low levels of anti-rejection therapy undergoing physician-controlled drug weaning, compared to those patients requiring ongoing maintenance immunosuppression. Further studies to extend these findings and to more fully characterize tolerant liver allograft recipients are ongoing in a larger patient population.

## Acknowledgments

This research was performed as a project of the Immune Tolerance Network, a 7-year clinical research project headquartered at the University of California San Francisco and supported by the National Institute of Allergy and Infectious Diseases, the National Institute of Diabetes, and Digestive and Kidney Disease and the Juvenile Diabetes Research Foundation. We are grateful to Drs Holger Hackstein and Igor Dvorchik for expert statistical analyses and advice. We also acknowledge the invaluable assistance of clinical coordinators Kathy Iurlano RN, BSN, CCTC, Lynn Seward RN, and Amy Smith RN, CCTC in patient enrollment.

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