

# Evolution of the Enzyme-Linked Immunosorbent Spot Assay for Post-Transplant Alloreactivity as a Potentially Useful Immune Monitoring Tool

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**Post-transplant monitoring of cellular immunity has the potential to guide alterations in medical therapy. To this end, our laboratory has developed an enzyme-linked immunosorbent spot (ELISPOT) assay for detection of peripheral blood alloimmunity. Peripheral blood lymphocytes (PBLs) from normal volunteers and from renal allograft recipients were tested against donor stimulator cells for their ability to respond in 'one-way' cytokine ELISPOT assays. T cell depletion of donor spleen or PBLs eliminated donor cell cytokine secretion while preserving the ability of these cells to present allo-antigen to responding T cells. Alloreactive IFN- $\gamma$ -producing PBLs derive from the memory T cell pool and are readily detectable in recipients of renal allografts taking immunosuppressant medications. A significant expansion of IFN- $\gamma$ -producing donor-reactive memory PBLs was detectable at 4–6 months post-transplant in those who had experienced an acute rejection episode compared with those with a stable post-transplant course. The data demonstrate the feasibility of repeated post-transplant monitoring of allograft recipients, and provide the foundation for improving the care of human transplant recipients through rational clinical decision-making based on measures of immune function.**

**Key words:** Alloreactivity, cytokine, human, immune monitoring, transplantation

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## Introduction

Despite improvements in short-term renal allograft survival, acute rejection episodes with their concomitant morbidities remain a persistent problem, long-term graft survival has only been modestly improved, and the development of chronic allograft nephropathy remains a dominant cause of graft failure (1). As donor-reactive T lymphocytes are thought to be central mediators of both acute and chronic graft rejection (2), there is significant interest in developing and testing methods for monitoring immune function in transplant recipients that could act as surrogate markers for poor outcomes (3–10). Post-transplant monitoring of cellular immunity might be helpful in this regard and has the potential to permit alterations in medical therapy to prevent rejection before it becomes clinically apparent.

To this end, our laboratory has developed a cytokine enzyme-linked immunosorbent spot (ELISPOT) assay capable of detecting cytokine secretion by individual, antigen-reactive T cells within a population of PBLs (11). Our previous data suggested that this assay detects activated or memory T cells prior to transplantation, and showed that the method holds promise as a predictive marker for post-transplant outcome. Still, there remain a number of technical issues that need to be addressed before this approach can be used as a clinical immune monitoring assay in the post-transplantation period. Under standard conditions of assay performance, recipient PBLs are stimulated with mitomycin C (MMC)-treated or irradiated, donor cells in 'one-way' assays. While such an approach seems to provide frequencies of antigen-primed, donor-reactive T cells in the peripheral blood, this has not been formally demonstrated. Moreover, it is unclear whether the MMC-treated or irradiated donor stimulator cells can, themselves, respond by producing cytokine (in a 'two-way' assay) and thus complicate interpretation of the results. It is further unclear whether using frozen aliquots of donor PBLs or donor spleen cells as stimulators, obtained at the time of transplantation (which is the only feasible method for obtaining and storing donor antigen for subsequent post-transplant monitoring studies), will permit adequate presentation of allo-antigen – freshly isolated donor dendritic cells (DC) may be required as antigen-presenting cells (APCs) in order to optimally and accurately detect frequencies of donor-reactive T cells by ELISPOT. Finally, immunosuppressant medications taken post-transplant may suppress cytokine production and inhibit the ability to detect donor specific reactivity, thus potentially rendering the monitoring approach inconclusive.

In the following sets of experiments we sought to address

these issues and to establish the feasibility of post-transplant immune monitoring by ELISPOT. We demonstrate that post-transplant immune monitoring is indeed feasible, that IFN- $\gamma$  detected in this manner correlates directly with expression of memory cell surface markers, and that by using T-cell-depleted, donor PBLs or donor spleen cells as APCs, we eliminate donor cell cytokine production without sacrificing the ability of these stimulators to present allo-antigen to T cells. Finally we show that this approach can potentially provide meaningful information about the post-transplant donor-reactive immune response in human recipients of renal allografts.

## Methods

### *PBL and spleen cell isolation*

Blood samples were obtained from normal volunteers or from renal allograft recipients followed at the University Hospitals of Cleveland Renal Transplantation Program. PBLs were separated by centrifugation on Isoprep density gradients (Robbins Scientific, Sunnyvale, CA, USA) from heparinized blood collected in green top tubes (11). Whole blood was diluted 1:1 with sterile PBS, aliquotted into 50-mL centrifuge tubes and a volume of Isoprep equal to the original blood volume was under layered using a sterile pipette. The suspension was centrifuged at 2000 r.p.m. (770g) in a swinging bucket rotor in a table-top centrifuge for 20 min at room temperature with no brake. PBLs were isolated from the interface, washed in autologous serum or RPMI + 3% human serum (Gemini Bio-Products, Woodland, CA, USA), recentrifuged at room temperature and counted using acridine orange/ethidium bromide under a UV microscope.

For isolation of spleen cells, sections of donor spleens were placed immediately into RPMI medium, temporarily stored at 4°C, and delivered to the laboratory within 48h of organ harvest. Single cell suspensions of spleen cells in RPMI 3% human serum were produced by mechanical disruption of the tissue using the flat end of a sterile 30–60 cc syringe plunger and passing the disrupted tissue through a stainless-steel screen (no. 60 mesh size). Mononuclear cells were then isolated from this cell suspension by density gradient centrifugation using Isoprep as outlined above.

All studies were performed under the approved guidelines of the Internal Review Boards for Human Studies at the University Hospitals of Cleveland and the Cleveland Clinic Foundation and the Research Subject Protection Program at the University of Minnesota.

### *Antigens*

Purified protein derivative (PPD) was obtained from Evans Medical, Langhurst, UK and used at a final dilution of 1:200. Phytohemagglutinin was obtained from Sigma, St. Louis, MO, USA and was used at a final concentration of 10  $\mu$ g/mL. Mumps soluble antigen was obtained from Bio Whittaker, Walkersville, MD, USA and was used at a final dilution of 1:20.

### *Isolation of naive and memory CD4 T cell subsets*

Naive (CD45RA<sup>+</sup>CD45RO<sup>-</sup>) and memory (CD45RA<sup>-</sup>CD45RO<sup>+</sup>) CD4<sup>+</sup> T cells were isolated from peripheral blood by negative selection using commercially available isolation columns from R & D Systems, Minneapolis, MN, USA. Surface expression of CD45 isoforms on the surface of the resultant purified cells was determined by flow cytometry (11).

### *Short-term cell lines*

Short-term alloreactive T cell lines were produced by mixing 5–10  $\times$  10<sup>6</sup> PBLs from a responder with 5–10  $\times$  10<sup>6</sup> irradiated PBLs or spleen cells

obtained from an allogeneic individual in 10 mLs of complete T cell medium [RPMI with 10% de-complemented fetal calf serum (FCS) plus antibiotics and supplemental L-glutamine] in a T25 flask (11). After 1 week, the cells were washed and expanded with IL-2 (1  $\mu$ L/mL, Roche, Mannheim, Germany) for 1 week. The cells were then rewashed and further expanded in the presence of IL-2 for 1 week. The resultant cells were tested for reactivity against the donor in ELISPOT assays. Some cell lines were maintained in long-term culture with recurrent cycles of 1 week stimulation with donor cells (without IL-2), followed by two consecutive, 1 week expansions with IL-2 as above (a 3-week cycle overall).

### *Cryopreservation*

Cryopreservation was performed as a two-step process. The cells were initially pelleted (2000 r.p.m. (770g), 7 min in a swinging bucket rotor in 15-mL tubes) and resuspended in freezing solution I comprised of 60% human serum and 40% RPMI 1640 at room temperature. Freezing solution II, consisting of 80% human serum and 20% DMSO (Sigma), was added at room temperature drop by drop. Cells were placed in a styrofoam box at –80°C for 24–48h, then transferred into liquid nitrogen.

### *Mitomycin treatment and irradiation*

Mitomycin C treatment was performed by incubating PBLs or spleen cells in 50  $\mu$ g/mL mitomycin C (Roche) in PBS for 30 min at 37°C. After treatment cells were washed three times in RPMI 1640 supplemented with 3–5% human serum (11). Viable cells were then counted by trypan blue exclusion. For experiments using gamma irradiation, the cells were exposed to 3000 rads from a cesium source.

### *Preparation of APCs*

T cell depletion of whole blood or single cell suspension of spleen cells was performed with Rosette Sep<sup>TM</sup> (StemCell Technologies, Vancouver, Canada) as recommended by the manufacturer. For whole blood, 15–50  $\mu$ L of the T cell depletion antibody cocktail was added per 1 mL of heparinized blood followed by incubation at room temperature for 20 min. The antibodies bind to the T cells and then form rosettes with the red blood cells. The blood was then diluted 1:1 with PBS-2%FCS and layered over Isoprep. Centrifugation was performed as outlined for isolation of PBLs above. The T cells sediment with the red blood cells and T cell depleted PBLs are obtained. For T cell depletion of spleen cells, 2 mL of antibody cocktail was mixed with single cell suspensions produced from 14g of spleen tissue.

For isolation of DCs from peripheral blood, CD14<sup>+</sup> PBLs were purified by positive selection using MACS magnetic columns (Miltenyi Biotec, Auburn, CA, USA). The isolated cells were cultured for 1 week in cRPMI 1640 supplemented with 5% human serum, 1000 U/mL GM-CSF (R & D Systems) and 1000 U/mL IL-4 (R & D Systems). Medium supplemented with growth factors was replaced after 2–4 d. Prior to use cells were incubated overnight with 200 U/mL TNF $\alpha$  (R & D Systems).

### *Elispot*

Ninety-six-well ELISPOT plates (Cellular Technology Ltd, Cleveland, OH) were coated with 100  $\mu$ L per well of capture antibodies for IFN- $\gamma$  (Endogen, Woburn, MA, USA; 4  $\mu$ g/mL), Granzyme B (Cell Sciences, Norwood, MA, USA; 3  $\mu$ g/mL), IL-2 (R & D Systems; 5  $\mu$ g/mL), IL-10 (PharMingen, San Diego, CA, USA; 5  $\mu$ g/mL), IL-4 (PharMingen; 2  $\mu$ g/mL) or IL-5 (PharMingen; 5  $\mu$ g/mL) in PBS overnight at 4°C. The plates were then blocked with 150  $\mu$ L of PBS-1% bovine serum albumin (BSA, Sigma, St. Louis, MO) per well and washed three times with PBS. Unless otherwise specified 300 000 responder cells per well were stimulated with 300 000 stimulator cells per well. Phytohemagglutinin (PHA; Sigma) was added to selected wells as a positive control at a final concentration of 10  $\mu$ g/mL in cRPMI-5% human serum. Final volume for all assays was 200  $\mu$ L per well. Control wells contained responder cells or stimulators plus medium alone. After an incubation at 37°C for 24h for IFN- $\gamma$ , Granzyme B, IL-2, IL-10 or 48h

for IL-4 and IL-5, the plates were washed three times with PBS, then four times with PBS-Tween (0.025%). Next, 100 $\mu$ L of biotinylated detection antibodies for IFN- $\gamma$  (Endogen; 1  $\mu$ g/mL), Granzyme B (Cell Sciences; 2  $\mu$ g/mL), IL-2 (Endogen; 0.25  $\mu$ g/mL), IL-4 (PharMingen; 2  $\mu$ g/mL) or IL-5 (PharMingen; 2  $\mu$ g/mL) in PBS-Tween-1%BSA were added to the wells overnight at 4°C. On the following day the plates were washed three times with PBS-Tween. Streptavidin-HRP (Dako, Carpinteria, CA, USA) was plated at a 1:2000 dilution in PBS-Tween-BSA for 90 min at room temperature. The spots were developed using 800  $\mu$ L of 3-amino-9-ethylcarbazole (Pierce, Rockford, IL, USA; 10 mg/mL in *N,N*-dimethylformamide) freshly diluted into (24 mL) 0.1 M sodium acetate (pH 5.0), filtered through a 0.45- $\mu$ m filter and mixed with 12  $\mu$ L of H<sub>2</sub>O<sub>2</sub> (200  $\mu$ L/well). After the plates were dried, the resulting spots were counted on a computer-assisted Immunospot image analyzer (Cellular Technology Ltd, Cleveland, OH, USA). The computer-assisted image analyzer has 100% reproducibility when repeatedly counting the same well using single, defined criteria (data not shown). Results are presented as mean values of ELISPOTs detected in triplicate wells containing responder PBLs plus antigen or donor stimulator cells after subtracting the response of wells with responder cells or donor cells alone (< 15 spots per 300 000 cells in each case).

#### Flow cytometry

PE-labeled anti-CD3, PE-anti-CD45RO, FITC-anti-CD45RA, FITC-anti-CD14, FITC-anti-CD19, and control antibodies were purchased from PharMingen. Anti-CD83 was a kind gift from Anat Tambur, Rush Memorial Hospital, Chicago, IL, USA. Flow cytometry was performed as previously described using a Becton Dickinson FACScan (BD Immunocytometry, Bedford, MA, USA) (11). Per sample, 5000–10000 live cells were analyzed.

#### Determination of HLA phenotypes

HLA phenotypes were determined by standard, clinically applicable techniques as described. Antigens encoded by HLA class I loci (A and B) were identified by the basic microlymphocytotoxicity assay using local antisera. Class II alleles were determined by sequence-specific priming and PCR.

#### Statistical analysis

Standard statistical methods were used to calculate mean, standard deviation and coefficients of variation. Mean ELISPOT frequencies were compared between groups using the Student's *t*-test. A *p*-value less than 0.05 was considered to be statistically significant.

## Results

#### Optimal preparation of donor stimulator cells

Our overall goal was to determine the optimal approach for monitoring alloreactive, cytokine-producing memory T cells in human peripheral blood. We have previously shown that we can use MMC-treated or irradiated PBLs or spleen cells from a potential transplant donor as sources of allo-antigen to detect IFN- $\gamma$ -producing cells *in vitro* by ELISPOT (11). While such treatment of stimulator cells is an established method for preventing proliferation, we were concerned that irradiated or MMC-treated cells would be capable of secreting cytokines, and thus could confound our ability to interpret results when recipient cells were mixed with stimulators in 'one-way' ELISPOT assays. We therefore performed cytokine ELISPOT assays using untreated spleen cells or PBLs, MMC-treated spleen cells or PBLs and irradiated spleen cells or PBLs in the presence or absence of mitogenic stimulation

with the T cell mitogen, PHA. As shown in Table 1, PHA stimulation of all preparations obtained from either spleen cells or PBLs produced cytokines. Irradiation resulted in lower frequencies of detectable spots for some cytokines when compared with MMC treatment, but overall, the results suggest that neither irradiation nor MMC treatment is ideal for use in 'one-way' stimulation studies in which we propose to detect cytokine production by recipient PBLs in response to donor cells.

As allo-antigen-induced cytokine production derives predominantly from T cells, and non-T cells within the PBLs or spleen are known to act as allogeneic APCs, we tested whether T cell depletion would prevent cytokine production by the donor cells. T cell depletion was readily performed using Rosette Sep (Stem Cell Technologies, Vancouver, Canada). This approach effectively depleted T cells from both peripheral blood and spleen preparations (Figure 1A). CD3<sup>+</sup> cells were reduced from 40% to 70% of the cells in the untreated samples to <2% of the cells following the depletion. To determine the effect of the T cell depletion on cytokine production, we next compared untreated and T cell depleted (TCD) preparations of PBLs and spleen cells after stimulation with PHA (Figure 1B,C). T cell depletion of PBLs reduced the number of PHA-induced IFN- $\gamma$  ELISPOTs by >80% and essentially no IL-2, IL-4 or IL-5 spots were detectable. PHA-induced GrB ELISPOT production was decreased by approximately 50% and IL-10 ELISPOTs were modestly reduced by the T cell depletion of PBLs. T cell depletion of spleen cells was more effective at reducing the number of PHA-induced cytokine secreting cells – essentially no IFN- $\gamma$ , GrB, IL-2, IL-4, IL-5 or IL-10 secreting cells were detectable in this population.

To determine whether TCD cell populations were capable of acting as allogeneic stimulator cells, we tested whether each preparation could elicit recall responses from short-term alloreactive T cell lines in recall ELISPOT assays. PBLs from normal volunteer A (haplotype A1, 23; B70, 49; DR13, 52) were mixed with irradiated allogeneic PBLs from volunteer B (haplotype A2, 28; B13, 14; DR1, 7) for 1 week, expanded with IL-2 for 2 weeks and then tested in recall assays using MMC-treated, irradiated or TCD-stimulator cells from the volunteer B. Figure 2A shows that this AvB cell line does not produce IFN- $\gamma$  if unstimulated, and further confirms that while MMC-treated PBLs produce IFN- $\gamma$  in response to PHA, the same stimulus yields markedly reduced IFN- $\gamma$  production by TCD PBLs. Figure 2A then demonstrates that both the TCD stimulator cells and the MMC-treated stimulator cells from volunteer B can elicit an IFN- $\gamma$ -producing recall response. In fact, the TCD stimulator cells consistently elicited a stronger recall response than the MMC-treated cells, consistent with the enrichment of monocytes and B cells (which are better APCs than T cells) within the stimulator cell population. The AvB cell line did not respond to a third party allogeneic stimulator (data not shown).

We next tested whether such TCD stimulator cells could be

**Table 1:** Mitomycin C (MMC) treatment or irradiation does not prevent cytokine release by spleen cells or PBLs

Spleen cells Cytokine	Untreated Spleen cells		MMC-treated spleen cells		Irradiated spleen cells	
	Control	PHA	Control	PHA	Control	PHA
IFN- $\gamma$	< 5	> 600	< 5	> 600	< 5	425 $\pm$ 16
GrB	< 5	163 $\pm$ 4	< 5	174 $\pm$ 1	< 5	12 $\pm$ 4
IL-2	< 5	> 600	< 5	> 600	< 5	> 600
IL-4	< 5	123 $\pm$ 10	< 5	145 $\pm$ 1	< 5	9 $\pm$ 1
IL-5	< 5	112 $\pm$ 24	< 5	107 $\pm$ 1	< 5	27 $\pm$ 4
IL-10	< 5	23 $\pm$ 1	< 5	15 $\pm$ 1	< 5	< 5

PBLs Cytokine	Untreated PBLs		MMC-treated PBLs		Irradiated PBLs	
	Control	PHA	Control	PHA	Control	PHA
IFN- $\gamma$	48 $\pm$ 26	> 600	60 $\pm$ 2	> 600	< 5	> 600
GrB	< 5	> 600	< 5	> 600	< 5	164 $\pm$ 42
IL-2	6 $\pm$ 1	> 600	8 $\pm$ 1	> 600	< 5	> 600
IL-4	< 5	182 $\pm$ 1	< 5	174 $\pm$ 11	< 5	197 $\pm$ 1
IL-5	< 5	105 $\pm$ 25	< 5	127 $\pm$ 11	< 5	74 $\pm$ 8
IL-10	50 $\pm$ 28	263 $\pm$ 55	18 $\pm$ 11	205 $\pm$ 22	< 5	51 $\pm$ 5

Freshly isolated PBLs or thawed aliquots of frozen spleen cells were plated in ELISPOT wells at a concentration of 300 000 cells per well with and without PHA at 10  $\mu$ g/mL overnight. The results are representative of three individual experiments.

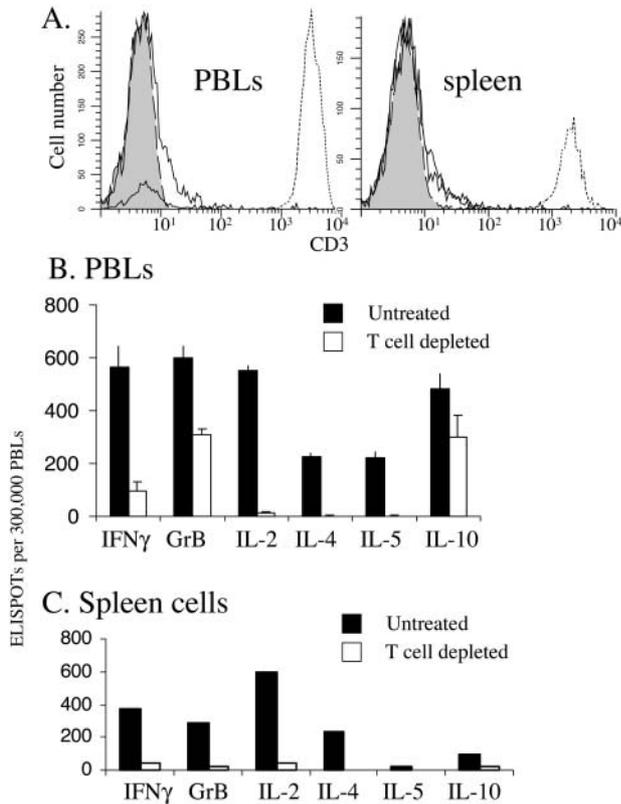
isolated, frozen and later thawed out and used as stimulators, as such an approach would be optimal for studies of post-transplant immune monitoring. PBLs from volunteer C (A1, 24; B7, 57; DR 15, 51) were stimulated with MMC-treated PBLs from volunteer B as outlined above. TCD stimulator cells were then prepared from volunteer B, and were divided into aliquots and frozen. Two weeks later, the short-term CvB cell line was tested in recall ELISPOT assays against freshly isolated, TCD PBLs from volunteer B and against a thawed aliquot of the previously frozen, TCD PBLs. Figure 2B shows that freeze-thawing of TCD preparations does not affect their ability to act as effective allostimulators in this assay. We performed a similar experiment comparing frozen and thawed aliquots of TCD spleen cells with frozen spleen cells that were later irradiated (Figure 2C). Once again the data demonstrate that TCD allogeneic stimulator cells (that do not produce cytokines on their own) are superior allo-stimulator cells compared with similarly prepared, but irradiated cells.

In order to accurately and reproducibly detect alloreactive T cells within the peripheral blood of a transplant recipient, we next needed to determine the optimal concentration of TCD stimulator cells to use in ELISPOT assays. It is generally accepted that dendritic cells are the most potent APCs (12–14), and we therefore further sought to test how well the TCD preparations functioned in comparison to isolated and activated DCs. DCs were isolated from PBLs by positive selection of CD14<sup>+</sup> peripheral blood cells followed by *in vitro* culture for 5 d with GM-CSF and IL-4, and an overnight stimulus with TNF $\alpha$  prior to use. In this manner we isolated a pure population of CD83<sup>+</sup> DCs by flow cytometry (data not shown). Titrations of DC or TCD PBLs were used as stimulators for the short-term CvB cell line in recall IFN- $\gamma$  ELISPOT assays. Figure 3 shows that, as anticipated, DC are the most potent APCs and can elicit a maximal recall response that plateaus at a concentration of 100 000 DCs per well. TCD APCs func-

tioned equally well as stimulators, but required 3–4 times the number of cells to elicit a recall response of equal magnitude to that found using the DCs.

#### ***T cell production of IFN- $\gamma$ by ELISPOT correlates with expression of a memory phenotype***

In order to verify that the secreted IFN- $\gamma$ , the dominant type 1 cytokine produced by human peripheral blood lymphocytes as detected by ELISPOT, is a valid marker of T cell memory we tested PBLs and purified T cell subsets for responses to PPD, a known T cell antigen. Consistent with the previous work (11), PBLs from a Bacillus Calmette–Guérin (BCG)-immunized individual produced IFN- $\gamma$  ELISPOTs in response to PPD with essentially no background spots in the media wells (Figure 4). No response was detectable in PPD-skin-test-negative individuals without a history of *Mycobacterium tuberculosis* infection/exposure or BCG immunization. We next isolated CD4<sup>+</sup> CD45RA<sup>+</sup> CD45RO<sup>-</sup> T cells (a cell surface phenotype consistent with naive T cells) and CD4<sup>+</sup> CD45RA<sup>-</sup> CD45RO<sup>+</sup> T cells [a cell surface phenotype consistent with memory T cells (15)] from the PPD-skin-test-positive individual, by negative selection. Flow cytometry confirmed the purity of each isolated subset (Figure 4B). The isolated T cells were tested in IFN- $\gamma$  ELISPOT assays against PPD using TCD peripheral blood lymphocytes from the same individual as APCs. As shown in Figure 4A, only the memory, CD45RO<sup>+</sup> T cells responded to PPD by producing IFN- $\gamma$ . The detected frequency of the response was ~fourfold higher than that found when unfractionated PBLs were tested, consistent with enrichment of the memory T cells. Notably, these CD45RO<sup>+</sup> T cells did not spontaneously produce IFN- $\gamma$  in the media wells. Nor did they respond to control hen egg white lysozyme, an antigen to which the individual was never exposed (< 5 spots per well, data not shown). In contrast, the CD45RA<sup>+</sup> naive T cells did not respond to PPD but did produce IFN- $\gamma$  in response to PHA, demonstrating their viability.



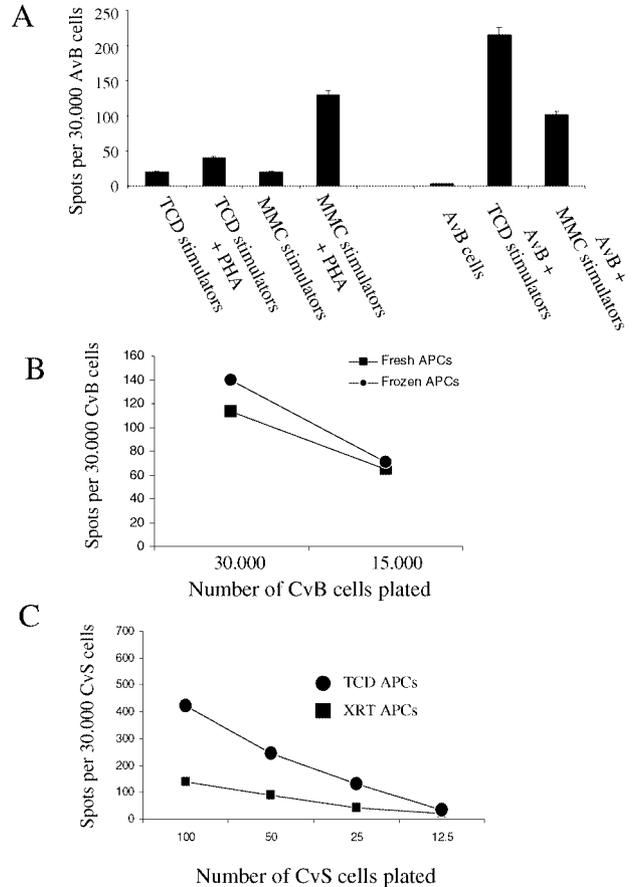
**Figure 1: T cell depletion markedly reduces cytokine production by stimulator spleen cells and peripheral blood lymphocytes.** (A) Samples were stained with FITC anti-CD3 (unfilled) or isotype control antibody (gray) before (dashed line) and after (solid line) T cell depletion and analyzed by flow cytometry. (B–C) Ficoll-isolated or T cell depleted PBLs (B) or spleen cells (C) were tested in cytokine ELISPOT assays for cytokine production in response to PHA. Results represent average values of duplicate wells counted by computer-assisted image analysis (< 15% variability between wells) after subtraction of spontaneous production of cytokine in the absence of stimulus (< 20 spots per well). The experiments were each repeated twice with similar results.

In further control experiments, TCD APCs, TCD APCs plus PPD and TCD APCs plus PHA did not produce IFN- $\gamma$  (data not shown), confirming that the detected responses were indeed derived from the T cells. In sum, the data clearly demonstrate that IFN- $\gamma$  production by ELISPOT in this short-term (< 24h) ELISPOT assay is one functional marker of memory, but not naive, T lymphocytes.

#### **Effect of freezing responder cells on their ability to respond to allo-APCs**

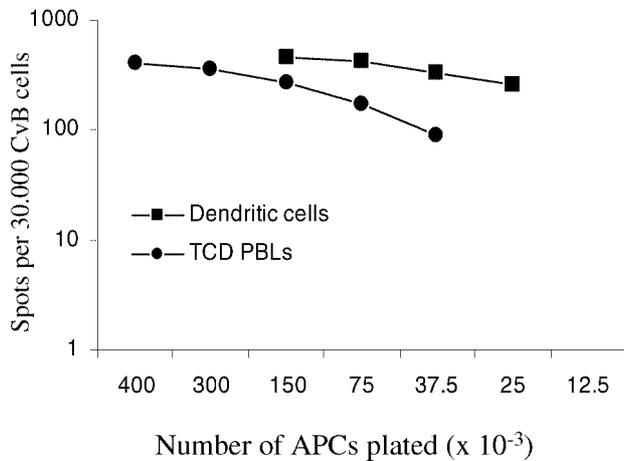
Ideally, it would be best to study donor-reactive immune responses in real time using freshly isolated PBL samples from transplant recipients. For practical reasons (i.e. a limited supply of donor samples, inability to perform all assays as soon as the sample becomes available, performance of retrospective analyses) it may be desirable to use frozen PBLs in recall assays. It is therefore of critical importance to know whether freezing the recipient PBLs affects their ability to respond to

#### **Post-Transplant ELISPOT Monitoring**

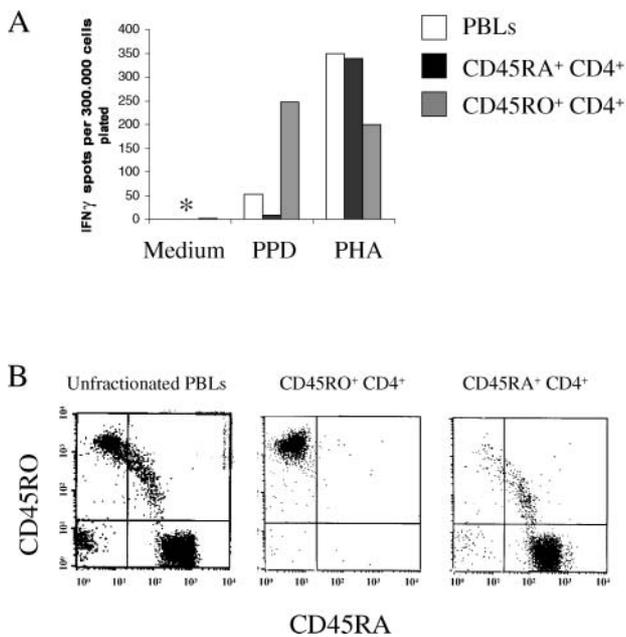


**Figure 2: T cell depleted PBLs act as allo-antigen presenting cells.** Short-term alloreactive T cell lines (AvB, CvB and CvS) were produced by *in vitro* culture of PBLs from normal volunteers with irradiated stimulator cells from HLA disparate donor volunteers. (A) AvB T cells were tested in IFN- $\gamma$  ELISPOT recall assays against mitomycin C (MMC)-treated PBL stimulators or T cell depleted (TCD) PBL stimulators obtained from the same individual (volunteer B) as the original stimulator donor. (B) CvB T cells were tested in IFN- $\gamma$  ELISPOT recall assays against fresh or frozen and subsequently thawed T cell depleted stimulator cells obtained from the same individual (volunteer B) as the original stimulator donor. There was no significant difference in the recall responses for the two different types of stimulator cell preparations. (C) CvS T cells were tested in IFN- $\gamma$  ELISPOT recall assays against frozen aliquots of T cell depleted spleen cells or frozen aliquots of spleen cells (not T cell depleted) that were irradiated after thawing. The frequency of recall ELISPOTS was higher when T cell depleted stimulator cells were used in the assay ( $p < 0.05$ ). All experiments were repeated at least once with similar results.

donor antigens. In an effort to address this question, we isolated PBLs from three normal volunteers and tested them immediately against three different HLA-disparate, TCD spleen samples in IFN- $\gamma$  ELISPOT assays. Simultaneously, we froze aliquots of the same PBLs, thawed them several days later and performed another set of ELISPOT assays against the same stimulators. As shown in Figure 5, freeze-thawing had a minimal effect on the frequency of detected responses. In the overwhelming majority of cases the results using fresh



**Figure 3: T cell depleted PBLs can function as well as dendritic cells in terms of the maximum recall ELISPOT response elicited.** Purified dendritic cells and purified T cell depleted PBLs were isolated from volunteer B and used as stimulators for a recall IFN- $\gamma$  ELISPOT assays with CvB T cell line. At the highest concentration of T cell depleted stimulator cells, there was no statistically significant difference in the frequency of recall ELISPOTs when compared with dendritic cells. The results are representative of two independently performed experiments.



**Figure 4: IFN- $\gamma$  production by ELISPOT represents a marker of antigen primed T cells.** (A) Unfractionated PBLs, naive CD45RA<sup>+</sup> CD4<sup>+</sup> T cells or memory CD45RO<sup>+</sup> CD4<sup>+</sup> T cells were isolated from a PPD-reactive normal volunteer and tested in IFN- $\gamma$  ELISPOT recall assays against PPD or PHA as a positive control. Purified CD4<sup>+</sup> T cell subsets were assayed in the presence of T cell depleted syngeneic PBLs as APCs (these cells did not produce cytokine with or without stimulation with PHA, data not shown). \* <math>< 5/300,000</math> ELISPOTs detected for each cell population. (B) Flow cytometry demonstrating the purity of the isolated T cell subsets.



**Figure 5: Responder cells can be frozen without loss of allo-reactivity.** Responder PBLs isolated from three different volunteers were either directly tested in IFN- $\gamma$  ELISPOT assays against a panel of allogeneic stimulator cells or were frozen and thawed 1 week later and tested in IFN- $\gamma$  ELISPOT assays against the same panel of allogeneic stimulator cells.

and frozen responder cells differed by <math>< 20\%</math>, although larger differences were detected in some responder stimulator combinations. The data suggest that it would be best to evaluate serial responses from the same individual using either only freshly isolated PBLs, or only frozen PBLs if possible.

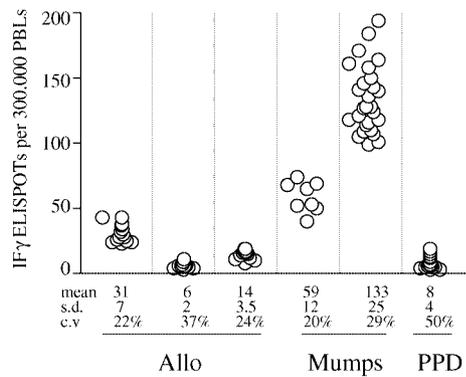
**Assay reproducibility**

In order to be able to state with assurance that alterations in detected ELISPOT frequencies are attributable to clinical events, it is necessary to understand the inter-assay and intra-assay variability. To address this, PBLs from three normal individuals were repeatedly tested against allogeneic TCD stimulator cells, mumps antigen or PPD. The PBLs were obtained on two or three different days and tested in triplicate or quadruplicate by up to six different investigators and counted by computer-assisted image analysis. The results in Figure 6A show that the intrawell variability is low with an average coefficient of variation of ~20%. As might be anticipated, the highest statistical variability was noted in wells with <math>< 10</math> ELISPOTs per well, a situation in which small differences in absolute numbers (i.e. two vs. four detected spots) have a large influence on the calculated coefficient of variance (> 100%) but are likely to have little clinical significance. Similar reproducibility was noted using PBLs from stable renal allograft recipients taking immunosuppressant medications in response to allogeneic stimulators and protein antigens (Figure 6B).

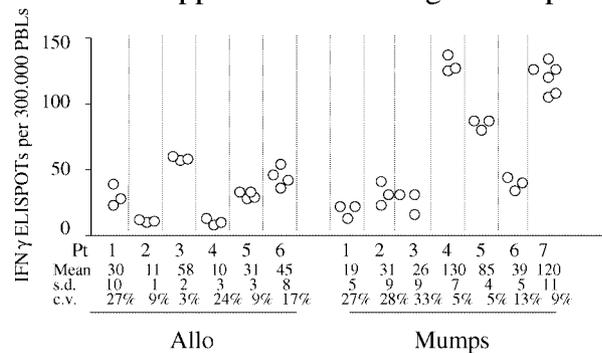
**Detection of alloreactive T cell immunity after renal transplantation**

Having established optimal conditions for detecting alloreactive T cells in PBLs and demonstrating that IFN- $\gamma$  production as assessed by this method is a measure of T cell memory, we next determined the feasibility of performing post-transplantation immune monitoring in recipients of renal allografts. As part of a prospective, NIH-funded immune monitoring trial to evaluate donor reactive immunity as a predictor of short- and long-term outcome, we collected post-transplant PBL samples from renal allograft recipients with clinical follow-up

A Normal volunteers



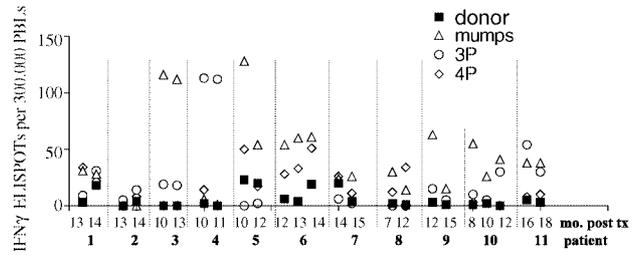
B Immunosuppressed renal allograft recipients



**Figure 6: Well-to-well variability of the ELISPOT assay.** (A) PBLs obtained from one normal volunteer were tested against three different allostimulators cells (left), PBLs from two additional volunteers were tested against mumps antigen (middle), and PBLs from one volunteer were tested against PPD (right). Blood was obtained from each volunteer on at least two different days and tested by two to six different technicians in triplicate or quadruplicate. (B) PBLs from seven different renal allograft recipients were obtained on at least two different days and tested in multiple ELISPOT wells for responses to allogeneic, TCD stimulator cells, mumps antigen or PPD as indicated. Patient 7 in panel B was only tested against mumps antigen. For both panels, each point represents the number of spots detected in a single well (n = 8–28 for each response in panel A and n = 3–6 for each response in panel B). Mean, standard deviation (SD) and coefficient of variance (c.v.) are listed for each set of responses tested.

of 2–18 months. Maintenance immunosuppression regimens varied, but all initially included corticosteroids and either cyclosporine or tacrolimus, and sirolimus or mycophenolate mofetil. All patients were recipients of first renal transplants.

Figure 7 shows the results of studies performed 7–18 months post-transplant in 11 stable renal allograft recipients (unchanged renal function, as determined by serum Cr level, compared with the nadir Cr value at 0.5–3 months post-transplant). PBLs from each patient were tested in IFN-γ and GrB ELISPOT assays against donor, third party (3P) spleen cells (defined as having the same number of mismatches

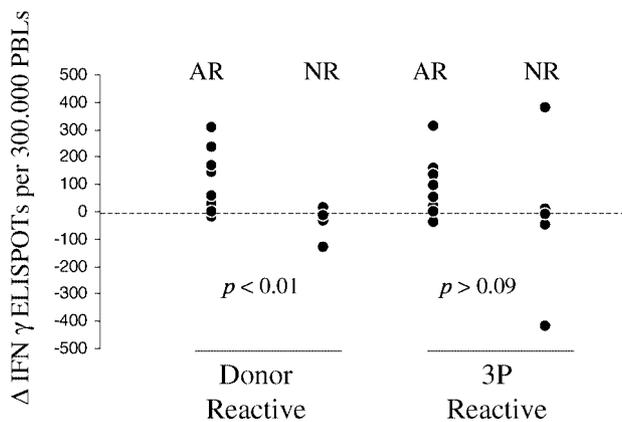


**Figure 7: Post-transplant immune responses in stable renal allograft recipients.** Frequency of donor-, 3P-, 4P- and mumps-reactive PBLs producing IFN-γ in 11 renal allograft recipients with stable renal function (no change in serum creatinine between nadir at 0.5–2 months post-transplant and 12 months post-transplant) tested on at least two different time points 7–18 months post-transplant. The results represent mean values of triplicate wells.

with the recipient as the donor sample), fully mismatched fourth party (4P) spleen cells, and mumps antigen (IFN-γ only). PBLs from all individuals responded strongly to PHA (> 250 spots per 300 000 cells, not shown) confirming viable cells in each case. The data illustrate several relevant points. First, donor reactive PBLs were low but detectable in each individual post-transplant with frequencies between 1 and 45/300 000 IFN-γ producers. Second, in most individuals, the frequency of 3P-reactive and 4P-reactive PBLs was higher than the frequency of donor reactive responses, but there was clearly some variability. Third, nine of the 11 patients had detectable anti-mumps immunity demonstrating our ability to detect recall immunity by memory T cells in response to a relevant infectious agent despite ongoing immunosuppression. Finally, each stable patient was tested on at least on two separate occasions within a 5-month time period during which time the individuals were taking unaltered doses of immunosuppressive medications and had no clinically detectable illnesses. Notably, each patient demonstrated persistently lower responses to donor antigens compared with responses to 3P, 4P or mumps antigens suggesting adequate immunosuppression and donor-specific hyporesponsiveness.

**Expansion of donor reactive PBLs in patients with a previous acute rejection episode**

We next hypothesized that an episode of acute graft rejection would result in expansion of the memory or effector, IFN-γ-producing, donor-reactive T cell repertoire. To evaluate this possibility, we performed a cross-sectional analysis of the donor reactive peripheral blood immune response in renal allograft recipients with and without an episode of biopsy-proven acute cellular rejection. We identified nine renal transplant recipients (from the cohort of 67) who had experienced an acute rejection episode within the first 4 months after the transplantation and from whom we had stored single aliquots of PBLs obtained 5–8 months post-transplantation. PBLs obtained from 13 transplant recipients without acute rejection over the same 5–8 month time period were studied as controls. The recipients' PBLs obtained pre- and post-transplantation were tested in IFN-γ ELISPOT assays in response to



**Figure 8: Acute rejection results in expansion of donor reactive PBLs.** Stored PBLs from nine renal transplant recipients 5–8 months post-transplantation that had experienced an acute rejection episode within the first 4 months following the transplantation were assayed in IFN- $\gamma$  recall ELISPOT assays against donor stimulators. PBLs from 13 transplant recipients without acute rejection over the same 5–8 month time period were studied as controls. The recipients' PBLs obtained pre- and post-transplantation were tested in IFN- $\gamma$  ELISPOT assays in response to donor and third party (3P) stimulator cells. The results are expressed as the change in frequency of IFN- $\gamma$ -producers between the pre-transplant sample and the 5–8 month post-transplant sample. Positive numbers therefore imply an expansion of alloreactive PBLs over time while negative numbers reflect a diminution in the frequency of alloreactive PBLs.

donor and 3P stimulator cells. The results were expressed as the change in frequency of IFN- $\gamma$ -producers between the pre-transplant sample and the 5–8 month post-transplant sample (Figure 8). Positive numbers therefore imply an expansion of alloreactive PBLs over time while negative numbers reflect a diminution in the frequency of alloreactive PBLs. Donor-reactive PBLs from patients with a previous acute rejection episode expanded in frequency over time, while donor-reactive PBLs from those without acute rejection did not ( $p < 0.01$  between groups). Third party-reactive responses generally followed the donor-reactive responses. The third party-reactive responses did not differ statistically between the stable group and the group that experienced acute rejection, although this was largely the result of two outliers (Figure 8). For each individual in both groups, the responder cells produced  $> 150$  IFN- $\gamma$  ELISPOTs per 300,000 cells plated in response to the mitogen PHA (data not shown) confirming cell viability.

## Discussion

A technique capable of repeatedly and easily providing a reliable and clinically meaningful measurement of donor-reactive immunity post-transplantation could be of significant benefit to the transplant community. Pharmaceutical agents in use are largely dosed empirically or on the basis of classic pharmacokinetic algorithms, and their use is accompanied by significant co-morbidities related to either excessive im-

munosuppression or to other actions of the drug. As a result, it would be desirable to use the minimum dose required to prevent acute and chronic rejection. In fact physicians would prefer to taper or even eliminate the use of certain medications to prevent long-term morbidities (7,16). The risk of empiric immunosuppressive drug minimization is the development of acute rejection. Nonetheless, it is notable that such rejection episodes, while increased in frequency, only occur in a minority of the individuals who undergo single drug withdrawal post-transplantation (e.g. corticosteroids or cyclosporine) (7,16). Tailored therapy for each patient, based on a functional measure of the donor-reactive immune response, would clearly be preferable to empiric reduction in therapy in all patients. In addition, despite the effectiveness of present therapies in prolonging graft survival, acute rejection episodes remain a clinical problem. If it were possible to detect an incipient rejection episode prior to clinical recognition (i.e. elevation in serum creatinine) it might be possible to treat pre-emptively and thus prevent the morbidities associated with acute rejection. It is further possible that alterations in immune function may be associated with or even predict those patients at highest risk of developing chronic allograft rejection. If so, early therapeutic interventions might prevent or delay the onset of graft failure in these high-risk individuals. Finally, there is a strong interest in developing immune-based protocols capable of inducing tolerance to the donor organ (17). Achievement of clinical tolerance is dependent at least in part on having a reliable measure of immune function capable of determining whether the immune response specific for the donor, but not other antigens is effectively controlled (18).

Despite the clear need for immunologic monitoring of transplant recipients, there are a paucity of studies describing approaches that provide information with which to make clinically useful decisions. Our data provide evidence that ELISPOT monitoring of peripheral immunity post-transplantation has that potential. ELISPOT detection of cytokine-secreting T cells occurs at single cell resolution and at frequencies as low as 1 cell per 300,000 bystander cells (11), making the assay sufficiently sensitive to detect immune responses even in immunosuppressed individuals. The present data provide strong evidence that antigen-specific production of IFN- $\gamma$  as detected in this short-term assay represents the fingerprint of a previously primed T cell. Antigen-specific IFN- $\gamma$ -producing T cells were restricted to the subpopulation expressing a cell surface phenotype of CD45RO<sup>+</sup>, CD45RA<sup>-</sup>, consistent with being a primed or memory T cell (15), and not a naïve cell. The data therefore suggest that IFN- $\gamma$ -producing cells as detected by ELISPOT in patients with allografts, represent cells that have been sensitized to the graft antigens, providing an *ex vivo* reflection of the evolving *in vivo*, donor-reactive immune response. We have not detected donor-reactive IL-4, IL-5 or IL-10 production in any patient studied thus far (data not shown).

In this report we first demonstrated procedures for optimal detection of donor-reactive immunity by ELISPOT as a clinical

tool for post-transplantation monitoring. TCD donor PBLs or spleen cells can be rapidly and efficiently prepared and frozen in aliquots. The procedure largely eliminates the cytokine-producing potential of the cells, yet maintains the cells' ability to present allo-antigen to responding recipient PBLs. The result is that TCD stimulators elicit a one-way, responder anti-stimulator immune response. The results further demonstrate that freeze-thawing does not diminish the allo-antigen presenting potential of the stimulators thus permitting this approach to be used for clinical immune monitoring. Spleen cells or PBLs from transplant donors can be prepared and frozen at the time of transplant and thawed for use in recall assays post-transplant without further treatment.

Previous work from our laboratory has already shown that pre-transplant evaluation of donor-reactive immunity can provide supplemental information to the HLA match and antibody cross-match for prediction of post-transplant outcome (11). In the present studies we extended these findings to demonstrate that donor-reactive immunity can be detected post-transplant, even in the context of potent immunosuppression. The pattern of immune reactivity for stable renal allograft recipients > 1 years post-transplant may represent a clinically useful finding. These patients seem to be adequately immunosuppressed (no donor reactive immunity) but respond to mumps or 3P or 4P antigens, consistent with donor-specific hypo-responsiveness, and consistent with reports by other laboratories (19,20). Patients with this immune reactivity pattern represent a candidate study group for selective immunosuppression minimization or withdrawal. The continued use of post-transplant monitoring could then be employed to determine whether such patients develop donor-reactive immunity as the therapy is altered. Our data further demonstrate expansion of donor-reactive PBLs in patients who experienced a clinical cellular rejection episode compared with patients who had stable renal function and no evidence of rejection over the same time period. Expansion of a donor-reactive T cell repertoire is indeed anticipated in response to a transplant rejection, but has not been clearly demonstrated previously in human graft recipients. We do not yet know whether serial monitoring of donor reactive immunity will reliably predict acute rejection or chronic allograft dysfunction, as these studies are ongoing.

Use of donor stimulator cells in this assay is one measure of the primed, total (both direct and indirect) T cell alloimmune response. T cells from the recipient PBLs can directly recognize donor MHC-peptide complexes on donor cells and it is likely that the majority of the responses detected in this assay represent direct reactivity as this is the dominant allo-antigen recognition pathway post-transplantation. While we cannot be certain that the approach detects all of the primed cells specific for the stimulators, it is reassuring that use of TCD stimulator cells, under controlled conditions using short-term cell lines, can elicit the same frequency of responses as purified and activated dendritic cells (the most potent APCs known).

In addition to eliciting a response for directly primed T cells,

the recipient APCs found within the recipient PBLs can theoretically process and present donor antigens to indirectly primed T cells (21). It remains unclear at this time, however, whether the use of donor TCD stimulator cells is an adequate approach for preferentially detecting T cells primed through the indirect pathway. Studies in animal models and humans suggest that panels of synthetic overlapping peptides (i.e. derived from the donor HLA molecules) may be a better approach for specifically evaluating indirect alloreactivity (22,23). Recent studies by Najafian and colleagues have in fact demonstrated that the use of synthetic donor HLA-derived peptides mixed with recipient PBLs can elicit indirect responses in transplant recipients with poor renal function (10). Studies comparing the different antigenic preparations in selected individuals are ongoing. Nonetheless, one practical advantage of using donor cells as opposed to synthetic peptides as a source of allo-antigen is that the expense involved in synthesis of large numbers of peptides can be avoided.

It should be noted that a number of other assays are presently being evaluated for efficacy in post-transplant immune monitoring. Proliferation to donor cells in one-way mixed lymphocyte responses and detection of alloreactive helper or cytotoxic T cells by limiting dilution analysis have been used by clinical and research laboratories for many years (24,25). The labor-intensiveness and time-consuming nature of these assays, along with inconsistent correlations with transplant outcomes in some studies have prevented their broad acceptance as reliable immune monitoring tools. More recent work by Suthanthiran and colleagues have shown that quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of message for T cell-derived inflammatory mediators (i.e. granzyme B, perforin, transforming growth factor beta) from allograft biopsies, peripheral blood cells or urine cells obtained from transplant recipients can provide supplemental diagnostic information regarding the presence or absence of acute or chronic rejection (9). Preliminary work by other groups also suggests that cellular phenotyping by flow cytometry (4) or delayed type hypersensitivity (26) can be used to evaluate allo-antigen-sensitized T cells that have developed post-transplantation. Intriguing data by Nickerson and colleagues suggest that specific urine spectrographic patterns of samples taken from transplant recipients can act as strong correlates of rejection (5). We feel that ELISPOT approach, with its high sensitivity, its ability to characterize both frequency and cytokine profile, and its ability to detect allo-antigen-primed T cells offers some advantage over many of these other assays, but the use of several complementary approaches in combination is the most likely approach that will yield clinically useful information.

As transplantation medicine moves into the 21st century, it is possible to envision an era in which serial immunologic monitoring analyses will rationally guide the day-to-day care of transplant recipients. Drug doses could be individually optimized, pre-emptive therapies could be instituted for incipient acute rejection episodes, and early treatment for prevention

of chronic allograft dysfunction could be started in high-risk individuals, all based on the results of reliable studies of peripheral alloreactive immunity. The implications of such an approach, including the cost savings and avoidance of side-effects related to use of the minimum amount of immunosuppressive therapy, while maximizing benefit to the allograft recipient, are enormous. Whether ELISPOT-based immune monitoring, in conjunction with other approaches, will provide this type of information remains to be formally determined through ongoing prospective trials. Still, the data presented herein demonstrate the feasibility of repeated post-transplant monitoring of allograft recipients, and the results provide the foundation for improving the care of human transplant recipients in which rational clinical decisions are based on measures of immune function.

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