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Research Article

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Downregulation of T Cell Receptor Expression by CD8⁺ Lymphocytes in Kidney Allografts

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Abstract

Allospecific CD8⁺ T lymphocytes are an important component of the cellular response in allograft rejection. These cells recognize and engage MHC class I antigens, leading to allospecific cytolytic responses and graft rejection. In mouse kidney allografts that survive to 3 wk after transplantation, we noted that the majority of CD8⁺ cells do not express surface α/β T cell receptor α/β (TCR), γ/δ TCR, or CD3. However, these CD8⁺TCR⁻ cells did express surface markers characteristic of T cells, including Thy1.2, CD2, and CD5. In addition, the CD8⁺TCR⁻ cells expressed mRNA for TCR V β gene families, and nearly half stained positive for cytoplasmic V β 8 protein, suggesting that they are T cells that have downregulated α/β TCR protein expression from their cell surfaces. When these surface TCR⁻ cells were isolated from kidney allografts by flow cytometry and cultured in the presence of either allogeneic or syngeneic stimulators, nearly 100% of cells reacquired normal levels of α/β TCR expression with disproportionate usage of V β 8 chains. After recovery of their surface TCR expression, the CD8⁺TCR⁻ population demonstrated strong alloreactivity in culture. These results suggest that the substantial number of CD8⁺TCR⁻ cells found in long-term surviving mouse kidney allografts are α/β -T cells that have downregulated their cell surface expression of TCR. While in other systems this phenotype may identify cells that have engaged antigen, our results indicate that loss of TCR expression by CD8⁺ kidney graft-infiltrating cells may not depend on antigen engagement and that elements in the microenvironment of the kidney graft play a key role in this process. Factors that modulate expression of TCR by graft-infiltrating lymphocytes may have an important role in regulating rejection responses. (*J. Clin Invest.* 1998. 101:2517–2527.) Key words: kidney transplantation • T cell receptor • rejection • minor histocompatibility antigens • transgenic mice

Introduction

Allospecific CD8⁺ T lymphocytes, which accumulate in rejecting organ transplants, are a critical component of the effector limb of the rejection response (1). These cells promote injury and lysis of donor cells through the actions of cytotoxic molecules such as granzyme B and perforin (2). Moreover, expression of granzyme B and perforin strongly correlates with acute cellular rejection in transplant biopsies (3–5), further supporting the critical role of these CD8⁺ T cell functions in the pathogenesis of rejection. The CD8⁺ alloreactive T cell recognizes and engages class I antigens, and its effector functions depend on the molecular interaction of the T cell receptor (TCR)¹ with alloantigens expressed on donor cells. Accordingly, the CD8⁺ T cell response is particularly important in graft rejection when donor and recipient are disparate at MHC class I loci (6–8).

In some in vitro systems, cell surface expression of TCR on individual cells is lost after antigen engagement (9). The loss of TCR from the cell surface impairs the ability of T cells to respond to their cognate antigens by increasing the threshold required for cell activation and to trigger production of cytokines (10). This process has been best characterized using cloned T cell populations with well-defined antigen specificities (9). While a role for modulation of TCR expression in regulating immune responsiveness in vivo has been suggested (11), widespread downregulation of TCR expression among T cells in the course of in vivo immune responses has not been well documented.

In a previous study, we characterized the TCR V β repertoire of CD8⁺ T cells in mouse kidney allografts (12). Unexpectedly, we found that a substantial proportion of graft-infiltrating CD8⁺ cells do not express detectable levels of TCR on their cell surfaces. In the present studies, we demonstrate that the CD8⁺TCR⁻ population consists of CD8⁺ T cells that have downregulated their expression of cell surface TCR proteins. Further, these findings suggest a mechanism through which the alloimmune response may be modulated at the site of inflammation.

Methods

Animals. For the control mouse renal allografts, we transplanted kidneys from [C57BL/6 $\times$ 129/sv] F₁ (H-2^b) mice bred in the Durham Veterans Administration Animal Facility into [BALB/c $\times$ DBA/2J] F₁ (H-2^d) mice purchased from Jackson Laboratories (Bar Harbor, ME). For class I-deficient allografts (class I⁻), we used β_2 -microglobulin-deficient mice [$\beta_2m(-/-)$] (H-2^b) that were generously provided

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1. **Abbreviations used in this paper:** CTL, cytotoxic T lymphocyte; MLC, mixed lymphocyte culture; PE, phycoerythrin; RT-PCR, reverse-transcription PCR; rIL-2, recombinant IL-2; TCR, T cell receptor.

by Dr. Beverly Koller (University of North Carolina) (13). We obtained B6/C-H2bm1/ByJ [bm-1(H-2^b)] mice from Jackson Laboratories. H-Y TCR transgenic mice (14) on a C57BL/6 background were provided by Dr. Barton Haynes (Duke University Medical Center) and were bred in our colony. Transgenic animals were identified by PCR of genomic DNA prepared from tail biopsies. Female transgene-positive and -negative littermates were used as recipients of kidney grafts from male C57BL/6 mice.

Mouse renal transplantation. We performed vascularized kidney transplants in mice as previously described (15). Briefly, the animals were anesthetized with isoflurane and the donor kidney, ureter, and bladder were harvested en bloc, including the renal artery with a small aortic cuff and the renal vein with a small vena caval cuff. These vascular cuffs were anastomosed to the recipient abdominal aorta and vena cava, respectively, below the level of the native renal vessels. Total ischemic time averaged 35–40 min. The donor and recipient bladders were attached dome to dome. The right native kidney was removed at time of transplant and the left native kidney was removed through a flank incision 4 d later. Overall surgical mortality was ~40%.

Mouse heart transplants. Heterotopic mouse cardiac allografts were performed as described by Corry et al. (16) with some modifications. Survival of the grafts was determined by direct palpation through the abdominal wall. Graft-infiltrating lymphocytes were isolated and stained as described below.

Isolation of splenocytes and graft-infiltrating lymphocytes. Splenocytes and graft-infiltrating lymphocytes were isolated from transplant recipients using protocols that have been described previously (17). Cell isolation took place at 3 wk after transplantation, unless otherwise noted. The kidney grafts were minced with razor blades and incubated with 1 mg/ml Type IV collagenase (Sigma Chemical Co., St. Louis, MO) in complete R10 media consisting of RPMI media supplemented with 10% FCS (Gibco-BRL, Gaithersburg, MD), 100 U/ml/100 µg/ml penicillin/streptomycin (Gibco-BRL), 0.05 mM 2-mercaptoethanol (Gibco-BRL), and 2 mM [L]-glutamine (Gibco-BRL), for 30 min at 37°C. We obtained splenocyte suspensions by disrupting the tissue between sterile glass slides. Suspensions were incubated on ice for 5 min to allow debris to settle. The resulting supernatant was centrifuged at 200 g for 10 min at 4°C to pellet the cells. We then harvested the cells by Ficoll centrifugation (Pharmacia, Uppsala, Sweden), and washed and resuspended them in complete RPMI media. Yields ranged from 2–6 × 10⁶ cells per kidney graft and 75–100 × 10⁶ cells per spleen.

Cytofluorometry and cell sorting. We performed staining for cytofluorometry as previously described (17). 1–2 × 10⁶/ml cells were washed and resuspended in PBS/2% FCS/0.02% sodium azide and staining was performed in 100-µl volumes at 4°C using optimal concentrations of antibodies. In most cases, graft-infiltrating cells from individual allografts were analyzed separately. mAbs were coupled to FITC, phycoerythrin (PE), or biotin by standard methods. Binding of biotinylated mAbs was detected using either streptavidin-PE (Vector Laboratories, Burlingame, CA), streptavidin-cychrome (PharMingen, San Diego, CA), or both. In the case of three-color flow analyses, cells were blocked in normal mouse serum at 37°C for 20 min and washed before antibody incubation. The mAbs used were all purchased from PharMingen, except as noted: RM4-5 (anti-mouse CD4), 53-6.7 (anti-mouse CD8α), H57-597 (anti-α/β TCR), GL3 (anti-γ/δ TCR), MR5-2 (anti-V_β8.1, 8.2), F23.1 (anti-V_β8.1, 8.2, 8.3; 18), 30-H12 (anti-Thy1.2), 145-2C11 (anti-CD3), (anti-CD2) and H11-86.1 (anti-CD5). After final washing, cells were fixed with PBS/2% formalin and analyzed within 72 h. Analyses were performed on a Becton Dickinson FACScan (Becton Dickinson & Co., Mountain View, CA). Forward-scatter threshold was set to exclude dead cells and debris from acquisition. Controls for nonspecific staining included cells stained with the appropriately conjugated, irrelevant isotypic antibody. At least 1.5 × 10⁴ cells were analyzed for each antibody combination. Data analysis gates were generated using standard FCS and SSC plots to exclude nonlymphoid cells.

Sorting was performed on a FACStar Plus (Becton Dickinson & Co.) using unfixed cells. Lymphocytes isolated from kidney grafts were stained with anti-CD8 and anti-α/βTCR antibodies and were sorted into CD8⁺α/βTCR[−] and CD8⁺α/βTCR⁺ subsets. Using this approach, we routinely obtained 1–2 × 10⁵ CD8⁺ cells from each rejecting kidney graft.

Mixed lymphocyte culture. Suspensions of splenocytes from naive C57BL/6 × 129/sv (H-2^b) mice were prepared and cultured with irradiated (3,000 rad) splenocytes from the donor strain [(DBA/2J × BALB/c)F₁; H-2^d] in R10 media as described above. Recombinant IL-2 (Hoffman-LaRoche, Inc., Nutley, NJ) was added at 19 U/ml. After 1 wk, cells were harvested by Ficoll-Hypaque centrifugation (Organon Teknika, Durham, NC) and resuspended in media (1 × 10⁵/ml) with irradiated stimulators (1 × 10⁶/ml). Cytofluorometry was performed weekly.

Culture of graft-infiltrating lymphocytes. Graft-infiltrating cells from 3-wk kidney allografts were isolated, stained, and sorted as described above. Sorted cells were washed once with R10 media and resuspended at a concentration of 1 × 10⁵ cells/ml. These cells were cultured with irradiated stimulators (1 × 10⁶/ml) in R10 media supplemented with 19 U/ml rIL-2. Cells were harvested at 7-d intervals by Ficoll centrifugation and restimulated in the same manner. Flow cytometry was performed weekly.

Assay for T cell cytotoxicity. T cell cytotoxicity was quantitated by neutral red uptake assay (19) using bone marrow macrophage targets as previously described (17). Briefly, macrophages were generated in vitro from bone marrow cells obtained from the femurs of BALB/c (H-2^d), C57BL/6 (H-2^b), and class I[−] (H-2^b) mice. Macrophages (3.5 × 10⁴/well) were incubated in triplicate with CD8⁺ cells, beginning with effector:target ratios of 5:1. After 18 h incubation at 37°C, 0.03% neutral red dissolved in PBS was added to each well. After 15 min at 37°C, excess neutral red was removed by washing twice with PBS and then cell-associated dye was released from cells by treatment with 0.5 M acetic acid/0.5% SDS. We measured neutral red uptake, attributed to dye ingestion by viable macrophages remaining in the monolayer, spectrophotometrically at a 550-nm wavelength with a Titertek Multiskan Plate Reader (Flow Laboratories, McLean, VA). We calculated lysis calculated as follows:

$$\% \text{ CD8-mediated lysis} = (\text{OD}_{\text{control}} - \text{OD}_{\text{experimental}}) / \text{OD}_{\text{control}} \times 100,$$

where OD_{experimental} is the mean absorbance produced by residual viable macrophages after incubation with T cell effectors. OD_{control} is the mean absorbance produced by identically prepared monolayers in the absence of effectors. Assays were performed on cells sorted on three separate occasions.

Production of IFN-γ by CD8⁺ T cells. IFN-γ production by allostimulated CD8⁺ T cells in cytotoxic T lymphocyte (CTL) assays was assessed using a sandwich ELISA as previously described (20). The hybridoma cell line R4-6A2 producing mAb to IFN-γ was obtained from the American Tissue Culture Collection (Rockville, MD) (21). Immulon-2 ELISA plates (Dynatech, Chantilly, VA) were pretreated overnight at 4°C with 10 µg/ml solution of this mAb to IFN-γ in PBS and then blocked with PBS/0.05% Tween/3% BSA (Sigma Chemical Co.). IFN-γ standards and tissue culture supernatants were plated in triplicate in PBS/0.05% Tween/1% BSA (Sigma Chemical). After washing, bound IFN-γ was detected with a rabbit anti-mouse IFN-γ antibody (1.25 µg/ml) (20) followed by a peroxidase-conjugated goat anti-rabbit IgG (Sigma Chemical Co.). Both reagents were dissolved in PBS/0.05% Tween and each well was washed four times after each incubation. After addition of o-phenylenediamine (1 mg/ml; Sigma) and 0.003% hydrogen peroxide in citrate buffer pH 5.0, the absorbance at 450 nm of each well was measured at 15 and 30 min using a Titertek Multiskan Plate Reader (Flow Laboratories).

RNA isolation. Total RNA was isolated from sorted cell populations using the guanadinium thiocyanate-phenol-chloroform extraction method (22). The RNA obtained was resuspended in diethylpyrocarbonate-treated water and stored at −70°C.

PCR analysis of cytokine and V β gene expression in sorted cells. We determined mRNA expression for cytokines using reverse transcriptase-polymerase chain reaction (RT-PCR) (23). cDNA was generated from the entire RNA preparation using 1 μ g oligo-dT primer (Promega Biotec, Madison, WI), 300 U MMLV-reverse transcriptase (Gibco-BRL), in 1 $\times$ RT buffer containing 3 mM MgCl₂, 10 mM DTT (Gibco-BRL), 40 U RNasin (Promega Biotec), and 0.5 mM dATP, dCTP, dGTP, and dTTP (Promega Biotec), incubated at 42°C for 60 min. For PCR, we amplified cDNA in a final volume of 50 μ l containing 1 μ M of each primer, 3 mM MgCl₂, 2.5 U Taq Polymerase (Promega Biotec), 50 mM KCl, 10 mM Tris, and 0.25 mM deoxynucleotide mix. PCR conditions were adjusted for each primer pair. In general, conditions were 45 s denaturation at 95°C (1 min for V β expression), 1 min annealing at 60°C, and 90 s (1 min for V β expression) extension at 72°C. cDNA from both CD8⁺TCR⁺ and CD8⁺TCR⁺ populations were PCR amplified for 35 cycles, except when amplified for β -actin expression, in which amplification was 20 cycles. We chose the number of cycles so that the amount of product amplified was proportional with the amount of specific mRNA in the original preparation. The PCR reaction mixture was electrophoresed on 2% agarose gels and products identified after ethidium bromide staining. The PCR primers to detect cytokines were obtained from Stratagene (La Jolla, CA) unless as noted: IL-2: 5'-GTCAACAGCGCACCCACTTCAAGC-3' (sense primer 99–122) and 5'-GCTTGTTGAGATGATGCTTTGACA (antisense primer 526–549); IL-4: 5'-ACGAGATGGATGTGCCAACGTC-3' (sense primer 203–226) and 5'-CGAGTAATCCATTTGCATCATGC-3' (antisense primer 458–481); IFN- γ : 5'-TACTGCCACGGCACAGTCATTGAA-3' (sense) and 5'-GCAGCGACTCCTTTTCCGCTTCT-3' (antisense); Granzyme-B: 5'-CCCAGGCGCAATGTCAAT-3' (sense) and 5'-CCAGGATAAGAACTCGA-3' (antisense) (Operon Technologies, Alameda, CA); β -Actin: 5'-GTGGGCCGCTCTAGGCACCA-3' (sense primer 105–124) and 5'-CGGTTGGCCTTTAGGGTTTCAGGGGG-3' (antisense primer 325–349).

For analysis of TCR V β gene expression, a V β -specific oligomer and an oligomer downstream from the CB region, which recognizes both CB1 and CB2, were used. The sequences of the specific murine TCR β V and TCR β C primers are shown below and details of the PCR are similar to those described previously for the analysis of human T cell populations (24): V β 2: 5'-GCCACACGGGTCACTGATACGGAGCTGAGG-3'; V β 8: 5'-CAAAACACATGGAGGCTGCA-3'; V β 14: 5'-GACCAATTCATCCTAAGCACGCAGAAGCTG-3'; V β 15: 5'-ACTGTGAATCAGCAATCAA-3'; CB: 5'-GCCAAGCACACGAGGGTAGCC-3'.

Immunofluorescence staining and confocal microscopy. We performed FACS as described above, using FITC-conjugated H57-597 (anti α/β TCR) and PE-conjugated 53-6.7 (anti-mouse CD8 α) (PharMingen). Sorted cells were washed with PBS, resuspended in R10 media plus 19 U/ml recombinant IL-2 and maintained at 37°C, 5% CO₂ overnight. TCR⁺ cells did not re-express surface TCR after overnight incubation (see Fig. 6 B). Sorted cells were then washed twice in PBS and resuspended at 1 $\times$ 10⁵ cells/ml in fresh PBS. Cytospin preparations of sorted cells or T cell lines were prepared using a Cytospin 3 Cytocentrifuge and single-circle, coated Cytoslides (Shandon Lipshaw, Pittsburgh, PA). On average, 75,000–100,000 cells were applied to each slide. Cells were immediately fixed in acetone:methanol (1:1) at –20°C for 5 min. Slides were washed three times in PBS/1%BSA/1% Tween-20 (Sigma Chemical Co.) and blocked with heat-inactivated normal rabbit serum (1:5; Sigma Chemical Co.) at room temperature for 30 min. After washing in PBS/1%BSA/1% Tween-20, cells were stained with F23.1 (anti-V β 8.1, 8.2, 8.3) for 30 min at room temperature and secondary-stained with FITC-conjugated polyclonal rabbit anti-mouse IgG (Cappel, Durham, NC). Cells were mounted in vectashield, turned upside down, and analyzed by a LSM 410 inverted scanning confocal microscope (Zeiss Inc., Thornwood, NY) equipped with an argon/xenon laser and Laser Scan 3.5 software. Magnification was 630-fold unless otherwise indicated.

Statistical analysis. Data are presented as the mean $\pm$ SEM. Statis-

tical significance was assessed using ANOVA and the Student's unpaired *t* test or the Wilcoxon rank-sum test as appropriate.

Results

Substantial numbers of CD8⁺ α/β TCR⁺ cells can be isolated from long-surviving mouse kidney allografts. Fig. 1 shows a two-color cytofluorometry plot of CD8 versus α/β TCR staining in graft-infiltrating cells isolated from mouse kidney allografts at 3 wk after transplantation. At 1 wk, > 90% of CD8⁺ cells express α/β TCR (12). However, at 3 wk, a significant fraction of CD8⁺ T cells (63.9 $\pm$ 2.9%; 2.41 $\pm$ 0.58 $\times$ 10⁶ cells) do not express detectable levels of α/β TCR on their cell surface. In contrast, the majority (> 85%) of infiltrating CD4⁺ cells in these grafts continue to express α/β TCR at both 1 and 3 wk after transplant (data not shown). To look for the presence of CD8⁺TCR⁺ cells in other donor-recipient combinations, kidneys from H-2^d donors were transplanted into H-2^b recipients (Table I). In this combination, a significant proportion (62.1 $\pm$ 8.9%; *P* = NS vs. H-2^b $\times$ H-2^d) of CD8⁺ cells did not express surface α/β TCR. To evaluate the role of class I antigens on the accumulation of these cells within the graft, we analyzed kidneys transplanted across an isolated MHC class I disparity (*bm-1* kidneys into C57BL/6 recipients) and kidney allografts from β_2 -microglobulin-deficient donors (class I[–]). In both cases, a substantial and equivalent proportion of CD8⁺ α/β TCR⁺ cells were detected (56.0% and 49.1 $\pm$ 9.5%, respectively; *P* = NS compared with H-2^b $\times$ H-2^d).

To determine whether a minor histocompatibility difference would be sufficient to induce the accumulation of CD8⁺TCR⁺ cells, we transplanted male C57BL/6 kidneys into female mice and analyzed the surface phenotype of the infiltrating cells. Compared with the other strain combinations tested with varying degrees of MHC donor-host disparity, relatively few CD8⁺TCR⁺ cells were detected in the H-Y disparate grafts (16.1 $\pm$ 5.6%; *P* < 0.0001 compared with H-2^b $\times$ H-2^d).

The CD8⁺ α/β TCR⁺ graft-infiltrating cells express T cell lineage markers. To further evaluate the origin and lineage of these CD8⁺TCR⁺ cells, we analyzed expression of other cell surface proteins using three-color cytofluorometry. These results are summarized in Fig. 2. Consistent with the absence of surface α/β TCR, these cells were also surface CD3⁺ (89%). Further, they did not express γ/δ TCR (98.6%). The CD8⁺ α/β TCR⁺ cells were, however, strongly Thy1.2⁺ and also expressed CD2 and CD5. This phenotypic profile is most consistent with a thymus-derived T cell lineage.

Table I. Expression of α/β TCR by CD8⁺ Cells Infiltrating Kidney Allografts 3 wk after Transplantation

Donor $\times$ Recipient	Number of allografts	% of CD8 ⁺ cells not expressing α/β TCR
H-2 ^b $\times$ H-2 ^d	16	63.9 $\pm$ 2.9
H-2 ^d $\times$ H-2 ^b	3	62.1 $\pm$ 8.9
Class I (H-2 ^b) $\times$ H-2 ^d	6	49.1 $\pm$ 9.5
<i>bm-1</i> $\times$ C57BL/6 (H-2 ^b)	2	56.0
C57BL/6 male (H-Y antigen ⁺) $\times$ C57BL/6 female	4	16.1 $\pm$ 5.6*

**P* < 0.0001 compared with control H-2^b $\times$ H-2^d allografts.

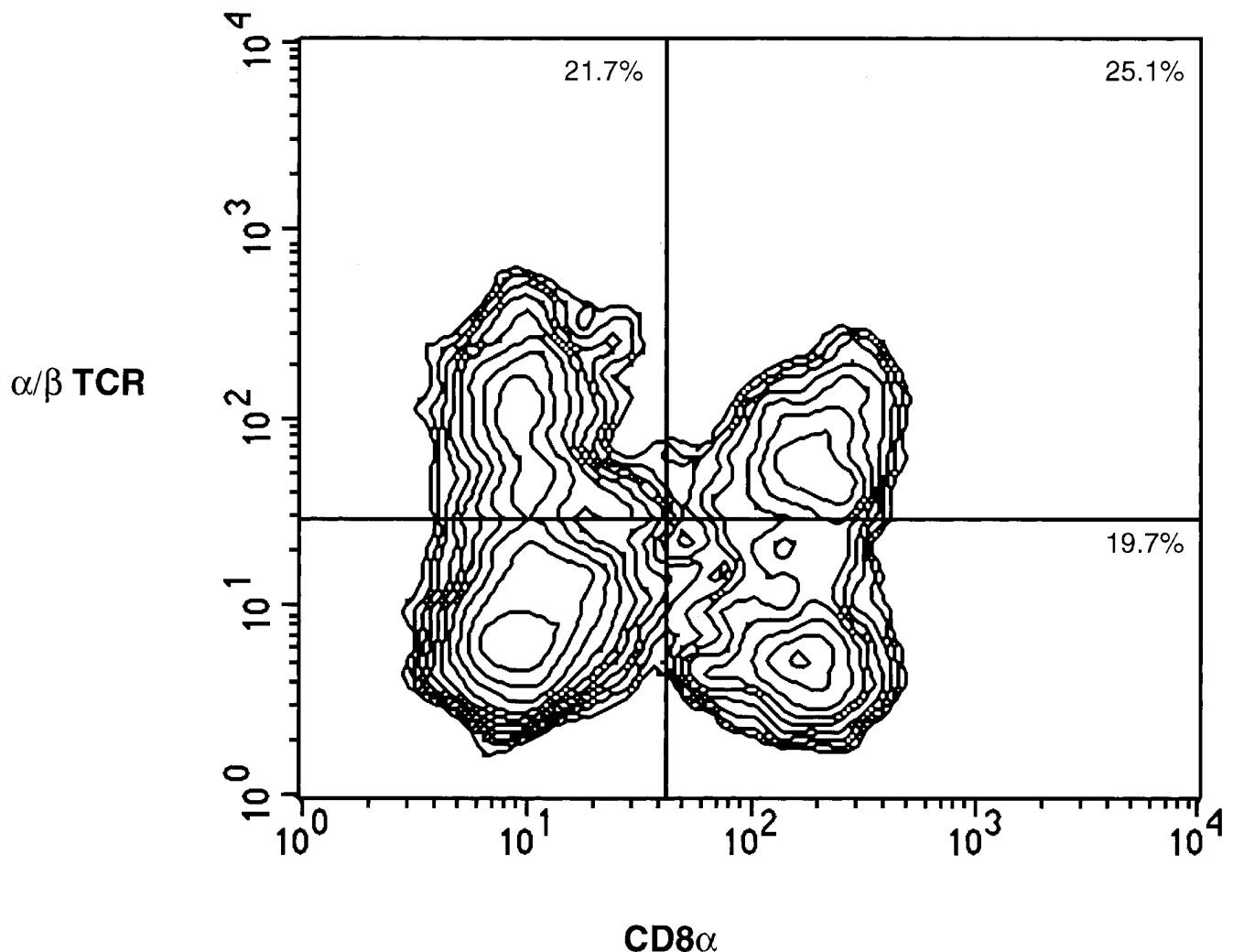


Figure 1. Analysis of α/β TCR expression on $CD8^+$ cells in the kidneys of transplanted mice. This is a representative geographic plot of staining for CD8 (horizontal axis) and α/β TCR (vertical axis). Data are shown for cells infiltrating kidney allografts, 3 wk after transplantation. The percentage of $CD8^+$ cells that express α/β TCR is 56.0%. Fluorescence intensity for both CD8 and α/β TCR is shown on a four-decade log scale. 15,000 cells were analyzed.

Cytokine mRNA expression by $CD8^+$ graft-infiltrating cells. To begin to assess the functional nature of the $CD8^+TCR^-$ cells, we defined their profile of cytokine mRNA expression using reverse transcription (RT)-PCR. $CD8^+$ lymphocytes were isolated from mouse kidney allografts and sorted into α/β TCR $^+$ and α/β TCR $^-$ populations as described in Methods. RNA was isolated from the individual cell populations and expression of relevant mRNA species was assessed using semi-quantitative RT-PCR. PCR products were analyzed on 2% agarose gels. A representative set of ethidium-stained gels is depicted in Fig. 3. In the $CD8^+\alpha/\beta$ TCR $^-$ cells, mRNA for IL-2, IFN- γ , and granzyme-B were easily identified, while the IL-4 PCR product was more difficult to detect. Thus, $CD8^+\alpha/\beta$ TCR $^-$ cells express a profile of cytokine mRNAs consistent with an activated $CD8^+$ T cell phenotype.

TCR V β genes are expressed in $CD8^+\alpha/\beta$ TCR $^-$ cells. Based on the cell surface markers on these cells and their expression of various cytokines, we hypothesized that $CD8^+\alpha/\beta$ TCR $^-$ cells might be T cells that had downregulated their cell surface

expression of α/β TCR proteins. To further investigate this possibility, we assayed their expression of mRNA for three representative TCR V β genes by RT-PCR. As shown in Fig. 4, the $CD8^+TCR^-$ cells expressed mRNAs for all of the V β genes tested and the qualitative profile of V β mRNA expression was similar to the $CD8^+TCR^+$ population. This result confirms the α/β TCR lineage of these cells and provides additional evidence suggesting that these cells have downregulated surface expression of TCR proteins. Interestingly, V β 8 expression appeared to predominate in both the surface TCR $^+$ and TCR $^-$ populations (Fig. 4). This is consistent with previous studies demonstrating that V β 8.3 is overexpressed by alloreactive $CD8^+$ cells in this donor-recipient combination (12).

Subcellular localization of V β 8 in $CD8^+TCR^-$ cells. To determine whether intracellular TCR protein might be present within the TCR $^-$ cells, we performed immunostaining of freshly isolated TCR cells, using F23.1, an mAb that recognizes V β 8 proteins. Stained cells were analyzed by confocal laser scanning microscopy. We used two *Listeria monocyto-*

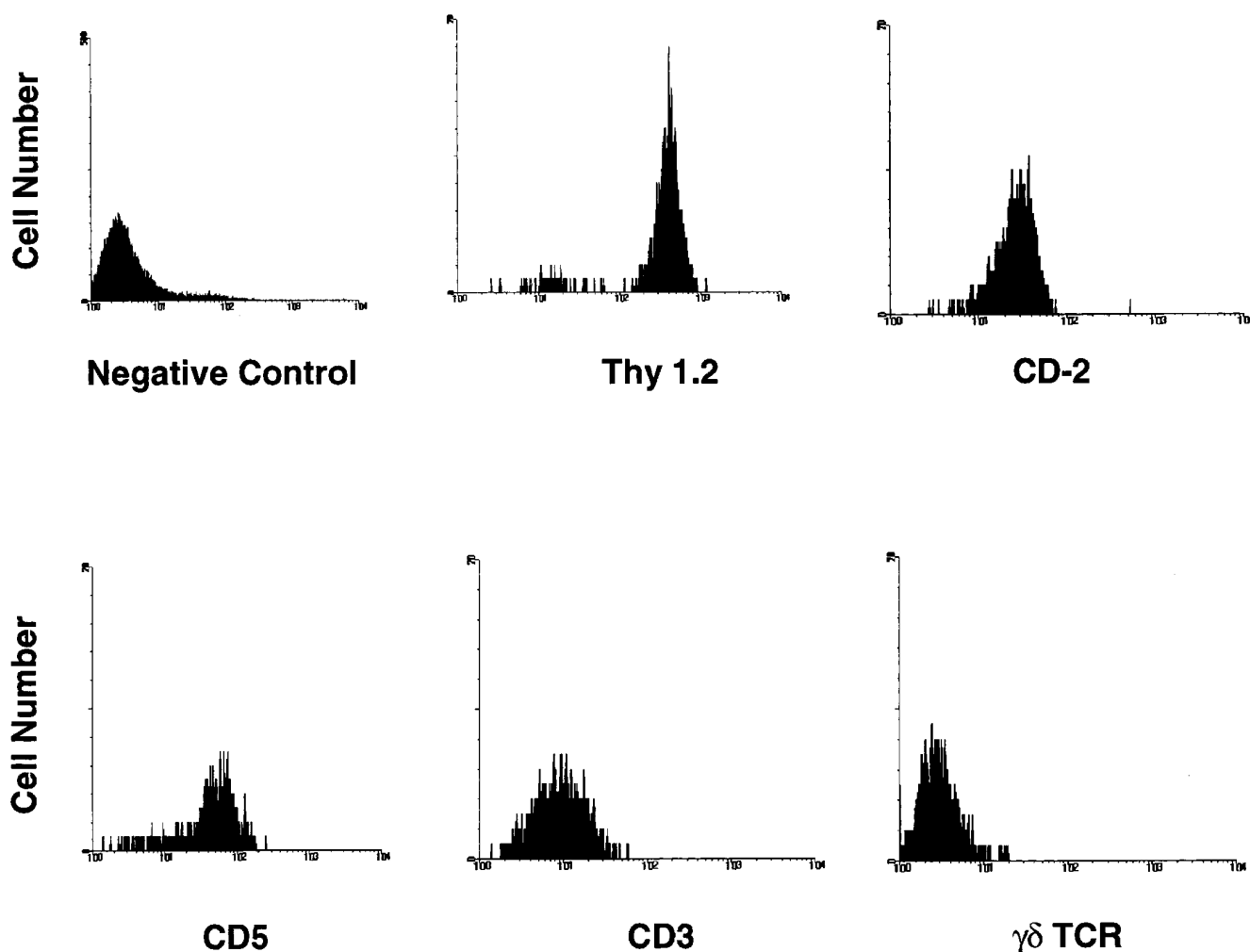


Figure 2. Phenotypic analysis of $CD8^+ \alpha/\beta TCR^-$ cells infiltrating kidney allografts, using three-color cytofluorometry, as described in Methods. Lymphocytes isolated from kidney allografts 3 wk after transplantation were stained with FITC-anti-CD8, PE-anti- $\alpha/\beta TCR$ and biotinylated antibodies for a number of cell surface proteins. In these panels, histograms were generated for the expression of the relevant cell surface protein (horizontal axis) vs. cell number (vertical axis), after gating on $CD8^+ \alpha/\beta TCR^-$ cells. Fluorescence intensity for each protein is shown on a four-decade log scale and event number is shown in linear scale. These analyses are representative of data obtained from lymphocytes from six allografts.

genes-immune $CD8^+$ T cell clones as controls. As a negative control, we used clone 8, a line that exclusively expresses $V\beta 5.2$ and as a positive control, we used clone 37, that exclusively expresses $V\beta 8$ (25). Fig. 5 A shows the $V\beta 5.2^+$ clone 8 stained with F23.1. There is no detectable fluorescence. In contrast, $V\beta 8^+$ clone 37 cells stained strongly positive (Fig. 5 B), demonstrating both cell surface and intracellular granular fluorescence that was predominantly perinuclear. Virtually every cell was highly fluorescent. Staining of freshly isolated $CD8^+ TCR^-$ cells is shown in Fig. 5, C and D. The majority of these cells stain positively with anti- $V\beta 8$ antibody, while some cells are clearly $V\beta 8^-$. Furthermore, the frequency of $V\beta 8$ expression in $CD8^+ TCR^-$ cells was similar to the frequency of $V\beta 8$ expression that we demonstrated for $CD8^+ TCR^+$ cells (12). The intensity of staining is clearly greater than in the negative control (Fig. 5 A), but less intense than in the positive control (Fig. 5 B). As Fig. 5 D shows, $V\beta 8$ staining was localized to perinuclear areas, frequently along one pole of the cell.

These data confirm the qualitative RT-PCR analysis and demonstrate that $CD8^+ TCR^-$ cells not only express $V\beta 8$ mRNA, but contains $V\beta 8$ proteins in intracellular compartments.

Isolation and culture of $CD8^+ \alpha/\beta TCR^-$ graft-infiltrating cells. To test whether the $CD8^+ \alpha/\beta TCR^-$ phenotype would be maintained in vitro, we cultured $CD8^+ \alpha/\beta TCR^-$ cells under several conditions. Shown in Fig. 6 A is a representative post-sort analysis of $CD8^+ \alpha/\beta TCR^-$ cells demonstrating the purity of the population of cells that were typically obtained (96–98% $CD8^+ \alpha/\beta TCR^-$). Cells were first cultured in the presence of 20 U/ml rIL-2 alone. After 48 h, the majority of cells remained $\alpha/\beta TCR^-$ (Fig. 6 B). Under similar culture conditions, TCR^+ cells maintained TCR expression (data not shown), and after 3–4 d in culture with IL-2 alone, there was a significant reduction in the number of viable cells. We also cultured the $CD8^+ \alpha/\beta TCR^-$ cells with IL-2 plus irradiated, allogeneic splenocytes isolated from mice of the original (H-2^d) donor strain. After 1 wk in culture under these conditions (Fig. 6 C), the

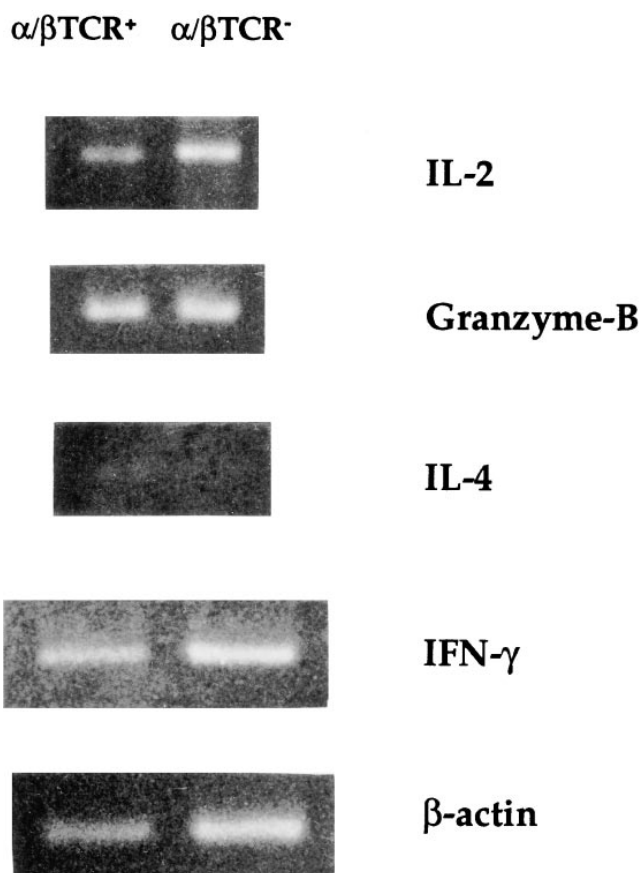


Figure 3. Cytokine mRNA expression in CD8⁺ T cells infiltrating kidney allografts. Total RNA was obtained from α/β TCR⁺ and α/β TCR⁻ cells isolated by FACS, and subjected to RT-PCR as described in Methods. PCR products for each cytokine analyzed and β -actin were sized-fractionated on ethidium-stained 2% agarose gels. This figure is representative of results obtained in two separate sorting experiments.

number of viable cells had nearly doubled and virtually 100% of cells expressed α/β TCR. To determine whether exposure to alloantigen was required for re-expression of TCR, we cultured CD8⁺TCR⁻ cells with IL-2 plus irradiated, syngeneic splenocytes for 5 d. Cell growth varied in these cultures. In two instances, cell viability was reduced by 60–65%, while on one occasion, cells expanded 4.5-fold. However, in all instances, > 95% of viable cells cultured in this fashion expressed TCR (Fig. 7). Thus, allogeneic stimulation is not required for recovery of surface TCR expression in culture.

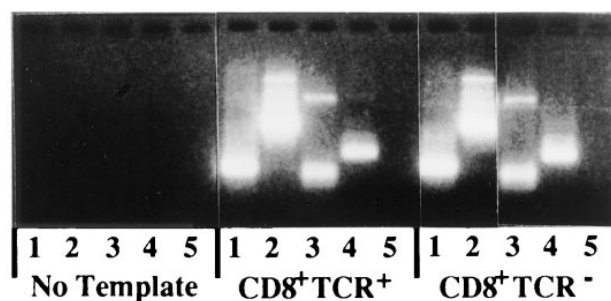
Allospecific functions of the CD8⁺ α/β TCR⁻ graft-infiltrating cell population. To directly examine alloreactivity and function of CD8⁺ α/β TCR⁻ graft infiltrating cells, we measured cytotoxicity and IFN- γ production. As Fig. 8 A shows, the CD8⁺ α/β TCR⁻ cells that had reacquired expression of α/β TCR readily lysed donor (H-2^b) targets, but showed minimal response against syngeneic (H-2^d) cells or class I deficient targets (H-2^b). As demonstrated in Fig. 8 B, substantial amounts of IFN- γ were produced after exposure to donor H-2^b targets, whereas, only modest amounts of IFN- γ were detected after exposure to syngeneic H-2^d or class I⁻ targets. Thus, the graft-infiltrating CD8⁺ α/β TCR⁻ cells that upregulate TCR expres-

sion after removal from the kidney graft are highly alloreactive.

TCR surface V β expression after culture of CD8⁺ α/β TCR⁻ cells. We next evaluated the V β repertoire of cultured CD8⁺ α/β TCR⁻ cells that had re-acquired TCR expression. Sorted CD8⁺ α/β TCR⁻ cells were expanded for 1 wk in culture in the presence of irradiated donor splenocytes and IL-2, harvested by Ficoll centrifugation, and stained with monoclonal antibodies against α/β TCR and V β 8. In two experiments, 59.1% of these cells expressed V β 8, and in particular, V β 8.3 (40.0%). Compared with the V β repertoire of splenocytes from allograft recipients (38.1% V β 8 and 12.1% V β 8.3, respectively), this represents a marked increase in TCR V β 8-bearing cells. This over representation of V β 8 and V β 8.3 was also seen in CD8⁺ α/β TCR⁺ cells freshly isolated from kidney allografts (12).

TCR expression is maintained on most CD8⁺ cells in other alloimmune responses. To determine whether loss of TCR expression on CD8⁺ cells observed in kidney allografts occurs in the course of other alloimmune responses, we examined the surface phenotypes of cells in long-term mixed lymphocyte cultures (MLC) and of graft-infiltrating cells isolated from cardiac allografts. After 1 and 3 wk of stimulation in the MLC,

Exp 1



Exp 2

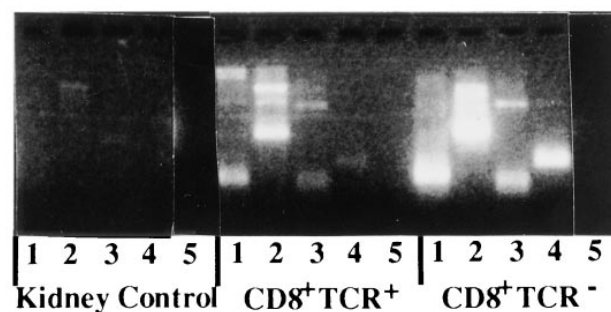


Figure 4. TCR β V (V β) mRNA expression in sorted CD8⁺ α/β TCR⁻ cells isolated from kidney allografts. CD8⁺ α/β TCR⁻ and CD8⁺ α/β TCR⁺ populations were sorted into separate pools and RNA was extracted. cDNA from an equal number of cell equivalents was aliquoted into separate reactions containing different V β -specific primers and a C β -primer. Data are shown for RNA extracted from a control BALB/c kidney not undergoing graft rejection. For each sample primers were specific for β V2 (lane 1), β V8 (lane 2), β V14 (lane 3), β V15 (lane 4), or no murine-specific primers (lane 5). Figure shows data from two separate experiments.

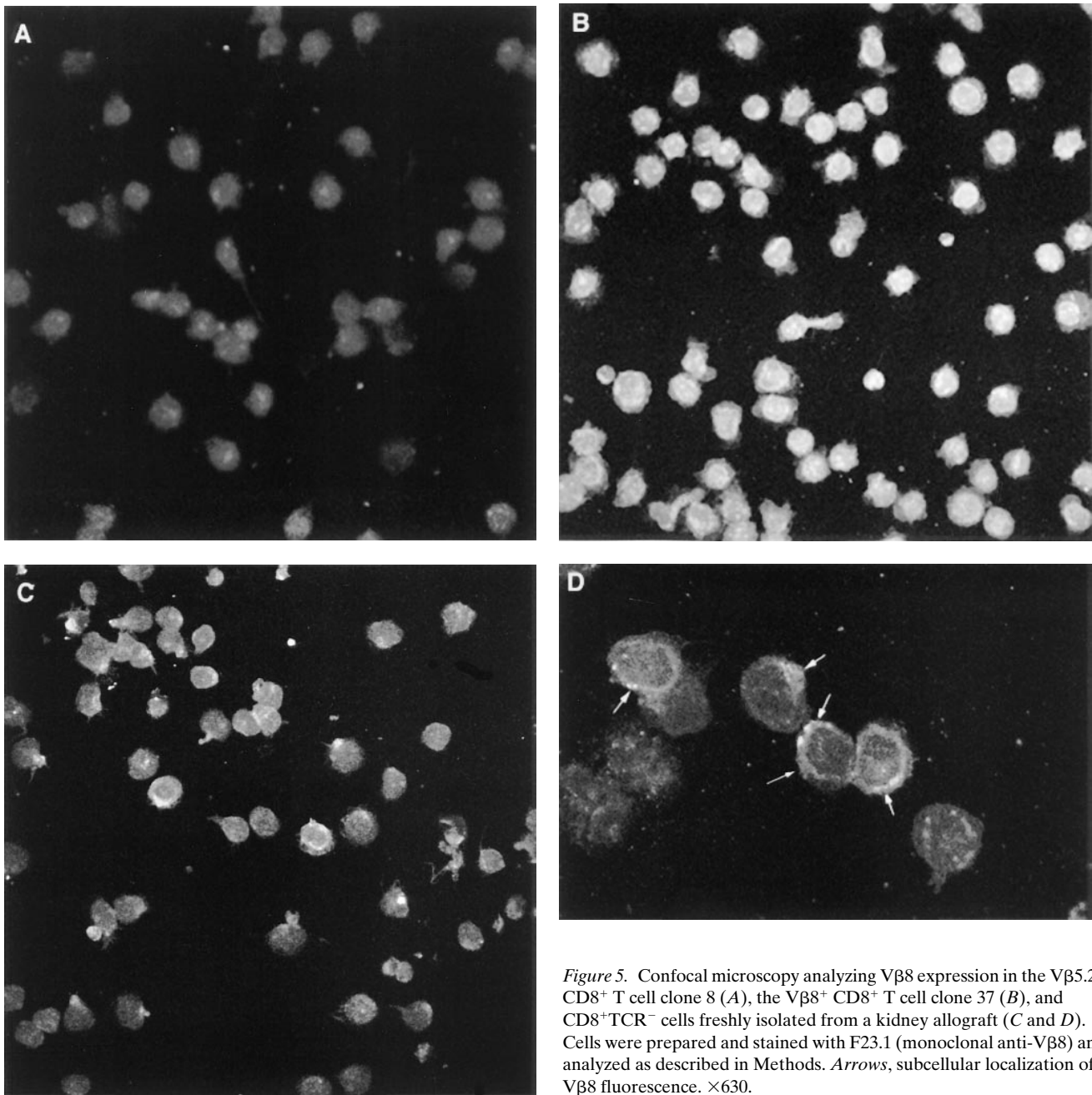


Figure 5. Confocal microscopy analyzing V β 8 expression in the V β 5.2⁺ CD8⁺ T cell clone 8 (A), the V β 8⁺ CD8⁺ T cell clone 37 (B), and CD8⁺TCR⁻ cells freshly isolated from a kidney allograft (C and D). Cells were prepared and stained with F23.1 (monoclonal anti-V β 8) and analyzed as described in Methods. Arrows, subcellular localization of V β 8 fluorescence. $\times 630$.

CD8⁺ responding cells were almost entirely α/β TCR⁺ (89.9 and 98.8%, respectively). The extent of TCR downregulation in cardiac allograft rejection was examined in hearts from *bm-1* donors transplanted into C57BL/6 recipients. In this combination across an isolated MHC class I difference, most cardiac allografts survive for many weeks without immunosuppression and thus we could evaluate the infiltrating CD8⁺ cells at time points after transplant (3 wk) that were equivalent to those we had studied in the kidney grafts. The majority of CD8⁺ T cells isolated from these cardiac allografts were TCR⁺ ($84.8 \pm 2.4\%$), in contrast to the *bm-1* kidney allografts, in which only 44% of cells were TCR⁺.

Increased numbers of alloreactive cells in the recipient T cell repertoire does not enhance TCR downregulation. To further

address the issue of antigen engagement in loss of TCR expression, we performed additional experiments in which kidneys from male C57BL/5 mice were transplanted into female H-Y TCR transgenic mice (14). As described above, significant TCR downregulation is not seen in this combination when wild-type females are used (Table I). In the H-Y transgenic mice, the transgene consists of re-arranged TCR genes from a clone that recognizes H-Y peptides in the context of class I MHC from H-2^b (14). In female transgenic mice, > 85% of peripheral CD8⁺ T cells bear the H-Y-specific TCR (14) and therefore, in this transplant combination, represent antigen-specific, alloreactive cells. When infiltrating lymphocytes were isolated and analyzed for TCR expression, the majority of the CD8⁺ cells in these H-Y disparate grafts expressed

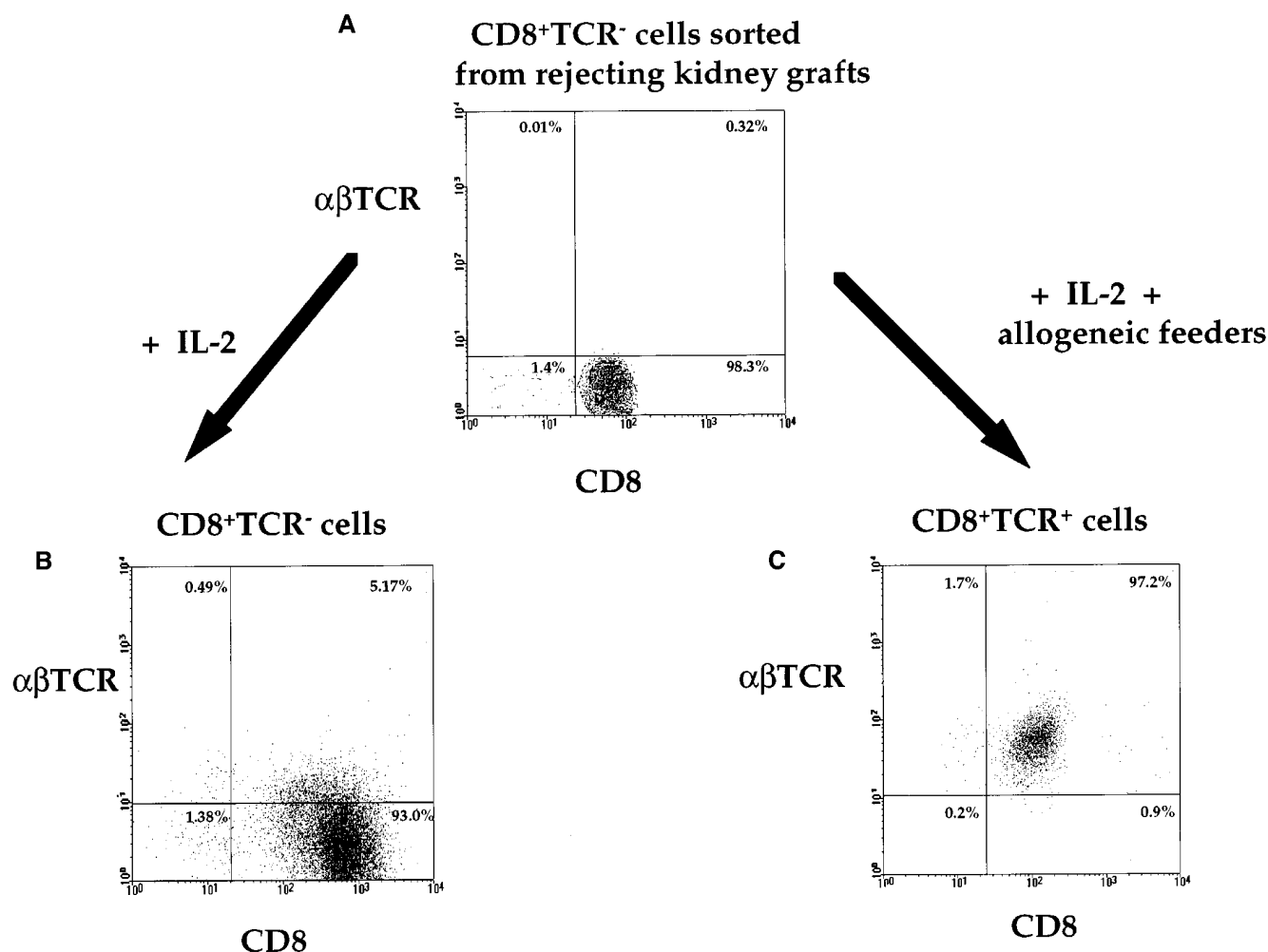


Figure 6. Regulation of α/β TCR expression in culture by graft infiltrating CD8⁺ T cells. CD8⁺ T cells were sorted by FACS into α/β TCR⁺ and α/β TCR⁻ populations. In each panel, a representative dot plot with staining for CD8 on the horizontal axis and for α/β TCR on the vertical axis is shown. (A) Postsort analysis of α/β TCR⁻ cells. An average of $\geq 95\%$ purity of cell populations was obtained. (B) Effects of IL-2 on TCR expression in vitro. CD8⁺ α/β TCR⁻ cells were cultured with 20 U/ml recombinant IL-2 and then stained with FITC-anti-CD8 and PE-anti- α/β TCR. (C) TCR expression is upregulated after in vitro stimulation with alloantigen. Sorted CD8⁺ α/β TCR⁻ cells were cultured for one week in the presence of 20 U/ml rIL-2 and 15-fold allogeneic splenocytes and stained as described in Methods.

TCR ($77.2 \pm 5.9\%$) and most expressed the antigen-specific, transgene-encoded TCR ($62.1 \pm 7.4\%$). Furthermore, the percentage of CD8⁺TCR⁺ cells in the transgenic recipients was virtually identical to that seen in H-Y disparate grafts transplanted into nontransgenic females ($83.9 \pm 5.6\%$; $P = \text{NS}$). Therefore, despite substantial skewing of the T cell repertoire toward alloreactive cells in the transgenic mice, TCR downregulation was not enhanced.

Discussion

In this current study, we describe reversible loss of TCR expression on a large number of CD8⁺ T cells at the site of an ongoing in vivo immune response. In other systems, TCR downregulation occurs after antigen engagement (9). Thus, the CD8⁺TCR⁻ phenotype might identify allospecific cells that have encountered antigen. The possibility that these may represent antigen-activated cells is supported both by their cyto-

kine profile and their high alloreactivity upon recovery of TCR expression. In other models, decreased cell surface expression of TCR has been correlated with impaired antigen recognition and related immune functions (10, 11, 26). Downregulation of the TCR-CD3 complex can be induced by exposure to antigen and may be specifically triggered by antigen engagement by the TCR (9, 27). Thus, downregulation of TCR expression may be a normal adaptive mechanism within the immune system that limits antigen-specific responses.

While the role of antigen engagement in modulating TCR expression has been demonstrated in various in vitro systems, our data suggest that loss of TCR expression on infiltrating cells in kidney grafts is largely antigen independent. For example, while donor and recipient are disparate for both class I and II MHC antigens in our experiments, substantial downregulation of TCR is observed only in the CD8⁺ graft-infiltrating cell population; the majority of CD4⁺ cells retain TCR expression. Second, while previous studies have suggested that only a

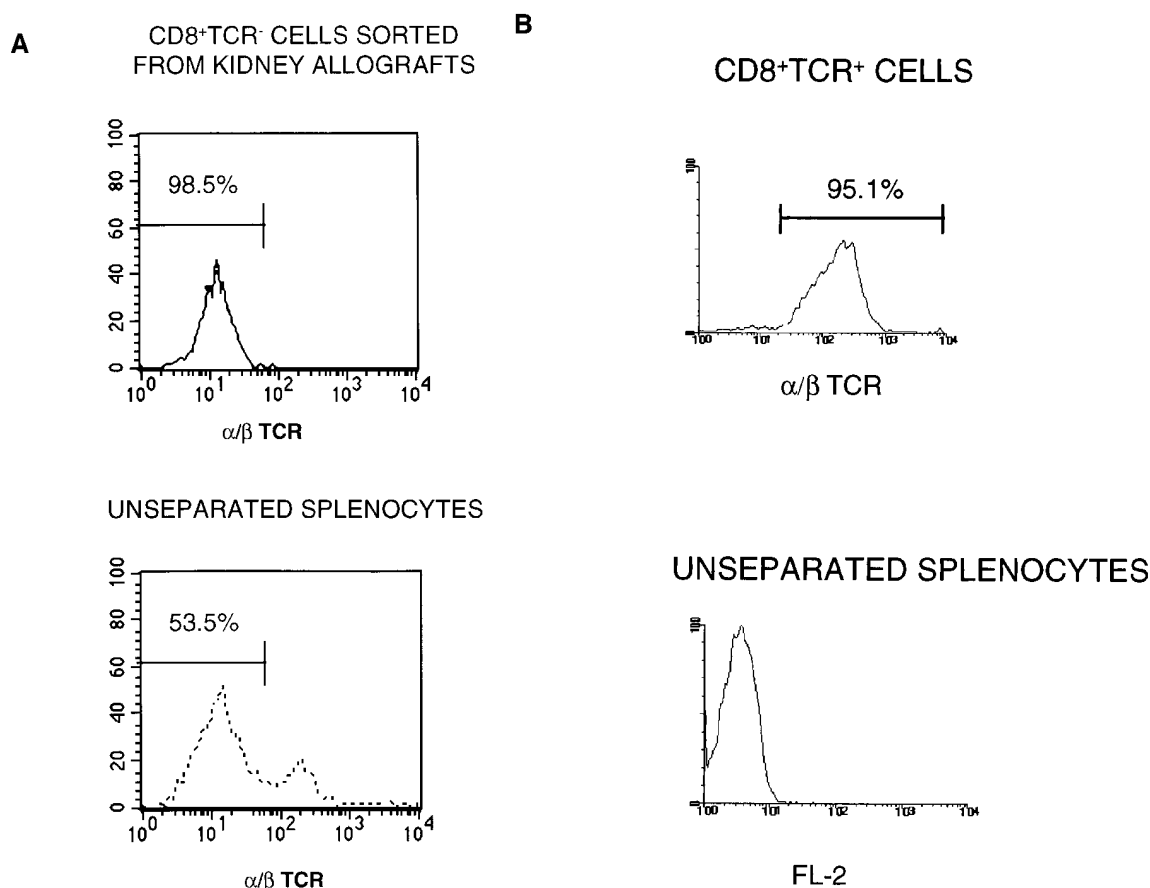


Figure 7. TCR expression in CD8⁺α/βTCR⁻ cells cultured in the presence of syngeneic antigen-presenting cells. Graft-infiltrating CD8⁺ T cells were sorted into TCR⁺ and TCR⁻ populations and cultured as described in Methods. (A) Histogram of α/βTCR staining of TCR⁻ cells (*top*) after FACS, demonstrating an average of > 97% purity. Shown for comparison is staining of nonseparated splenocytes from a naive mouse containing ~ 46% TCR⁺ cells (*bottom*). (B) Single-color histogram of TCR expression in cultured TCR⁻ cells. After 5 d in culture, TCR⁻ cells were purified by Ficoll centrifugation and stained with PE-conjugated anti-TCR antibody and 0.1 μg/ml propidium iodide (*top*). Shown for comparison is the background fluorescence using a PE-labeled isotype control antibody on unseparated splenocytes (*bottom*). 10,000 viable cells were analyzed. Fluorescence intensity is shown on a four-decade log scale and event number shown in linear scale.

minority (< 1%) of infiltrating cells in rejecting kidney grafts are truly antigen specific (28), we find that > 50% of the CD8⁺ of graft infiltrating cells have lost TCR expression. Further, the absence of normal expression of class I antigens on the graft (for example, β₂-microglobulin-deficient donor grafts) does not significantly reduce the proportion of CD8⁺ T cells that downregulate TCR expression. When the CD8⁺TCR⁻ cells are removed from the graft and cultured in the presence of alloantigen, TCR expression returns. Thus, alloantigen in the culture environment does not inhibit recovery of TCR expression, nor does it cause an appreciable loss of TCR once expression has been re-acquired. Finally, in our experiments using H-Y TCR transgenic mice, we find no enhancement of TCR downregulation in H-Y-disparate kidney allografts when the majority of graft-infiltrating CD8⁺ cells bear alloantigen-specific, transgene-encoded TCRs. Accordingly, our data indicate that factors other than TCR engagement mediate this effect.

As TCR downregulation was not seen in long-term MLC or cardiac allografts and the TCR⁻ phenotype is lost when the cells are removed from the graft, factors that are present or enriched in the microenvironment of a renal allograft may medi-

ate this process. Previous studies have demonstrated biochemical pathways for TCR downregulation that are not antigen dependent and do not require direct stimulation through the TCR. For example, brief exposure to phorbol esters causes downregulation of TCR expression in human T cell lines (29–31) and reduced TCR expression after PMA exposure is associated with phosphorylation of CD3γ and CD3δ (30). PMA, in the presence of calcium ionophore or PHA, can activate T cells independent of TCR (32) and this activation triggers cytokine gene expression (33). However, a precise relationship between T cell activation by PMA or other non-TCR-dependent pathways, and downregulation of TCR expression has not been established.

Several previous studies have described TCR downregulation in association with tolerance induction. For example, Zanders et al. (27) found that T cell clones could be rendered tolerant through exposure to large concentrations of peptide antigen, and that this state of specific tolerance was associated with loss of CD3 expression from cell surfaces. In the thymus, Schneider et al. have also described downregulation of TCR after exposure to a tolerogenic signal (34). Downregulation of

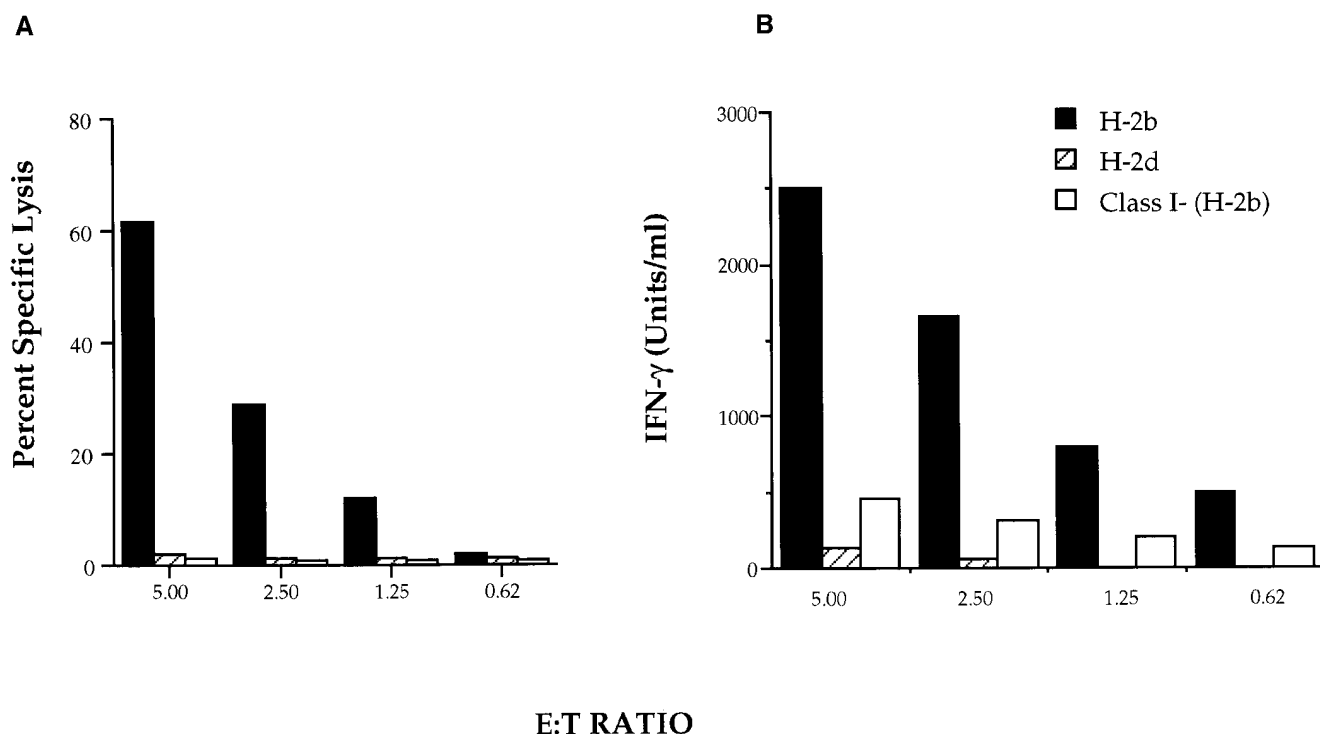


Figure 8. Cytotoxic activity and IFN- γ production by CD8 $^{+}$ α/β TCR $^{-}$ cells that had upregulated surface TCR expression after culture with allogeneic splenocytes. (A) CTL activity was measured by neutral red uptake assay using bone marrow macrophage targets. This assay is representative of three experiments. (B) IFN- γ produced by CD8 $^{+}$ α/β TCR $^{-}$ CTLs was measured by ELISA as described in Methods. This assay is representative of three experiments. The macrophage targets were prepared from allogeneic [C57BL/6 $\times$ 129/sv] F $_1$ (H-2 b) (closed bars), syngeneic [BALB/c $\times$ DBA/2J] F $_1$ (H-2 d) (hatched bars), and class I $^{-}$ (H-2 b) (open bars) mice.

TCR has also been demonstrated in a model of extrathymic tolerance induction to class I alloantigens using TCR transgenic mice. In that study, the CD8 $^{+}$ population was primarily affected and downregulation of the clonotypic TCR was observed in lymphoid organs (11). In contrast, in our study we find that the loss of TCR expression, while also primarily involving CD8 $^{+}$ cells, occurs only at the site of inflammation: within the kidney allograft. As originally reported by Russell and associates, some mice develop donor-specific tolerance after receiving a kidney allograft with no other immunomodulatory interventions (35). Furthermore, in contrast to skin and cardiac allografts, which are rejected within 1–2 wk, mouse kidneys transplanted across a complete H-2 mismatch survive for prolonged periods without immunosuppression (15, 17, 35). Despite their prolonged survival, these grafts develop histological features of rejection and marked reduction in their glomerular filtration rates. As we have previously demonstrated, 1 wk after transplantation, when the acute cellular response is first manifested, most of the CD8 $^{+}$ cells in the graft also express TCR (12). Only at later time points, 3 wk and beyond, are we able to detect the TCR $^{-}$ cells. While we have no evidence to directly link our observation of widespread downregulation of TCR expression on graft-infiltrating cells to the prolonged survival of mouse kidney allografts, it is interesting to note that significant numbers of CD8 $^{+}$ TCR $^{-}$ cells are only seen in long-surviving (> 3 wk) allografts.

In summary, we have identified in long-term surviving kidney allografts a sizable proportion of CD8 $^{+}$ T cells that have reversibly lost expression of α/β TCR proteins. While the

mechanism for this downregulation is uncertain, our data do not support a major role for antigen engagement and suggest that factors in the microenvironment of the kidney graft play a key role in this process. As the loss of TCR expression is associated with impaired function and responsiveness, this modulation of TCR expression may play a role in negatively regulating the intragraft immune response.

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