Anti-CD4 Mediates Clonal Anergy during Transplantation Tolerance Induction

By Susan E. Alters, Judith A. Shizuru, Jill Ackerman, Davida Grossman, Karl B. Seydel, and C. Garrison Fathman

From the Department of Medicine, Division of Immunology, S-021, Stanford University School of Medicine, Stanford, California 94305

Summary
Depletion of CD4+ cells using anti-CD4 monoclonal antibodies leads to allograft tolerance. Here we show that anti-CD4-mediated tolerance to pancreatic islets of Langerhans transplanted from an A/J (IEk) donor to a diabetic C57B1/6 (B6) (IE-) recipient occurs in the absence of clonal deletion of the potentially IE-reactive Vα11+ T cells. Instead, a state of clonal anergy is induced in both the CD4+ Vα11+ and CD8+ Vα11+ T cell subsets. This clonal anergy can be partially overcome in vitro by the addition of recombinant interleukin 2.

Materials and Methods
Mice. Adult A/J, B6, and B10.A mice were bred in our departmental animal facility. The transplantation protocol has been described previously (1).

FACS. The relative T cell subset frequencies present in the PBL of anti-CD4-treated and control mice were analyzed by FACS (Becton Dickinson & Co., Mountain View, CA) analysis. PBL were isolated on the indicated days by separation on a Lympholyte M (Cedarlane Laboratories, Ontario, Canada) gradient. CD4+ Vα11+ T cells were analyzed using mAb to Vα11 (RR3-15 [5]), followed by fluoresceinated goat anti-rat (mouse-adsorbed) Ig (Caltag Laboratories, San Francisco, CA). The cells were washed, incubated in 50% normal rat serum to bind to remaining anti-rat Ig, and stained with PE-conjugated anti-CD4 (mAb GK1.5; Becton Dickinson Immunocytometry Systems, San Jose, CA). CD4+ Vα8+ T cells were analyzed using fluoresceinated mAb to Vα8 (F23.1 [10]) followed by PE-conjugated anti-CD4. For anti-CD8 staining, biotinylated anti-Lyt-2 (mAb 53.6.7; Becton Dickinson & Co.) was used followed by avidin-PE (Caltag Laboratories). Cells were analyzed on a modified dual laser FACS with logarithmic amplifiers (Becton Dickinson & Co.).

Anti-TCR Activation Assay. T cell proliferation was induced by receptor crosslinking with mAb specific for Vβ11 (RR3-15 [5]), CD3 (1452C11 [11]), Vβ17 (KJ23 [3]), and Vβ8 (F23.1 [10]). U-bottomed microtiter plates (Flow Laboratories Inc., McLean VA) were coated with the indicated antibody (10 µg/ml) for 2-6 h at 37°C. Plates were washed twice in PBS, and 2 x 10^4 peripheral LN cells were added per well. The plates were incubated for 4 d (CD3) or 5 d (Vβ11, Vβ17, Vβ8) with [3H]thymidine added for the final 12-16 h of culture. For assays on spleen cells, RBC were lysed by hypotonic shock. Where separated cell populations were used, two separated aliquots of cells were stained with either PE-conjugated anti-CD4 mAb GK1.5 (for anti-CD4 depletion) or fluoresceinated anti-CD8 mAb 53.6.7 (for anti-CD8 depletion). The cells were then negatively selected by sorting on a FACStar plus. The resulting CD4-depleted or CD8-depleted populations were >99% pure as determined by FACS analysis.

Results and Discussion
To study the potential mechanisms of transplantation tolerance mediated by depleting regimens of anti-CD4, donor and recipient mice were selected so that the IE antigen was expressed only by the donor tissue. Pancreatic islets of Langerhans were isolated from adult A/J (IE^+) donors and transplanted into the livers of streptozotocin-induced diabetic B6 recipients as previously described (1). B6 mice do not express IE, thus, Vβ11+ T cells, which have been shown to respond to IE allantigens in vivo (12), are present in their periphery. All diabetic mice that were given anti-CD4 antibody at the time they received allogeneic A/J islets showed indefinite acceptance of their graft, as evidenced by persistent normoglycemic
cemia (>200 d). In contrast, diabetic recipients that received allografts without anti-CD4 treatment rejected their islet allografts. 

To determine whether the transplantation tolerance induced under this treatment regimen resulted from clonal deletion of the potentially IE-reactive T cells, we assayed lymphocytes for the expression of a TCR V\_\theta that would have undergone deletion in IE\_+ mice by FACS analysis using a mAb specific for V\_\beta11. mAb to V\_\beta was used as a control since, in contrast to T cells expressing V\_\beta11, T cells expressing V\_\beta8 are not known to be deleted in mice expressing IE. Data presented in Fig. 1 represent the repopulation kinetics of GKL5\_+ cells after treatment with 50 \mu g of the anti-CD4 mAb GKL1.5\_2 a (13) on days -1, 0, and +1 relative to transplant. PBL were isolated on the indicated days, and CD4\_+ V\_\beta11\_+ T cells were analyzed using mAb to V611, followed by fluoresceinated goat anti-rat Ig and PE-conjugated anti-CD4. CD4\_+ V\_\beta8\_+ T cells were analyzed using fluoresceinated mAb to V\_\beta8 followed by PE-conjugated anti-CD4. Due to bound GKL1.5\_2 a, nondepleted CD4\_+ cells are not detected until after day 4. Percentage of cells staining with the indicated antibodies is expressed relative to total CD4\_+ plus CD8\_+ cells. Data represent one of two similar experiments.

We next asked whether T cells expressing the relevant V\_\beta gene segments could be activated in response to anti-TCR specific crosslinking (Fig. 2). As expected, LN cells from normal B6 mice, B6 mice transplanted with A/J islets, and normal B10.A mice showed good proliferation in response to immobilized mAb to CD3, a determinant found on all T cells. In contrast, LN cells from the transplanted mice showed a greatly reduced response, relative to normal B6 mice, when stimulated with immobilized mAb to V\_\beta11. The response of LN cells from eight of nine transplanted mice approximated that of the B10.A mice, which have clonally deleted most V\_\beta11\_+ T cells, whereas the response of one of the transplanted mice, while about fivefold greater than that of the B10.A, was still \approx 50\% that of the control B6 mouse (data not shown). This decreased response is specific for the V\_\beta11\_+ population, as the response of V\_\beta8\_+ T cells stimulated with mAb to V\_\beta8 was comparable in normal B6 and in transplanted mice (Fig. 2).

To determine if the unresponsiveness seen in the transplanted mice was found in either or both the CD4\_+ V\_\beta11\_+ or CD8\_+ V\_\beta11\_+ T cell subsets, CD4-depleted and CD8-depleted (>99\% pure) populations were obtained from spleen cells of transplanted and normal B6 mice by cell sorting and were used in the anti-TCR-mediated activation assay. The results with unseparated spleen cells (Table 2.A) were similar to those with LN cells (Fig. 2); the V\_\beta11\_+ T cell population from the transplanted mice showed little or no response to anti-V\_\beta11 stimulation. When CD4-depleted and CD8-depleted spleen cell populations from the transplanted mice were used in the anti-TCR activation assay, both displayed minimal reactivity to anti-V611 compared with the response
Table 2. Anti-\( \gamma_1 \) Response of CD4-depleted (− CD4) and CD8-depleted (− CD8) Cells

| Responder | No. of cells | \( \gamma_1 \) | \( \gamma_1 \) | CD3 |
|-----------|--------------|--------------|--------------|-----|
| A B6      | \( 4 \times 10^5 \) | 7,776        | 67,499       | ND  |
| Transplant| \( 2 \times 10^5 \) | 3,402        | 26,405       | ND  |
| B B6 (− CD8) | \( 2 \times 10^5 \) | 5,349        | 22,173       | 54,295 |
| Transplant (− CD8) | \( 2 \times 10^5 \) | 5,897        | 7,189        | 38,086 |
| B6 (− CD4) | \( 2 \times 10^5 \) | 1,908        | 20,217       | 68,058 |
| Transplant (− CD4) | \( 2 \times 10^5 \) | 5,716        | 6,232        | 58,491 |
| C B6 (− CD8) + B6 (− CD4) | \( 2 \times 10^5 \) each | 7,671 | 53,100 | 44,060 |
| Transplant (− CD8) + transplant (− CD4) | \( 2 \times 10^5 \) each | 7,093 | 23,274 | 49,816 |
| D B6 (− CD8) + transplant (− CD4) | \( 2 \times 10^5 \) each | 8,156 | 20,067 | 37,170 |
| Transplant (− CD8) + B6 (− CD4) | \( 2 \times 10^5 \) each | ND   | 16,986 | ND   |

T cell proliferation was induced by receptor crosslinking as described in Fig. 2. For experiments using separated cells, two separated aliquots of cells were stained with either PE-conjugated anti-CD4 mAb GK1.5 (for anti-CD4 depletion) or fluoresceinated anti-CD8 mab 53.6.7 (for anti-CD8 depletion) and negatively selected by cell sorting. The resulting CD4-depleted or CD8-depleted populations were >99% pure as determined by FACS analysis. Data are expressed as the mean of triplicate wells in total cpm. Data represent one of three similar experiments.

Mixing CD4-depleted cells from a responsive B6 mouse with CD8-depleted cells from a transplanted mouse, or CD8-depleted cells from a responsive B6 mouse, indicated that neither population from the transplanted mouse could suppress the response of cells from a normal mouse (Table 2 D).

Previously, it has been reported that T cell clones that have been rendered anergic do not produce IL-2 in response to TCR occupancy despite the expression of functional IL-2 receptors (15). We have also shown that rIL-2 can partially reconstitute the ability of \( \gamma_1 \) T cells of transplanted mice to respond to immobilized anti-\( \gamma_1 \) (Fig. 2), indicating that one defect of the unresponsive \( \gamma_1 \) T cell population may include the inability to produce IL-2.

The induced peripheral transplantation tolerance described...
here is similar to the clonal anergy described previously (16-18) in several respects: (a) clonal deletion of the potentially IE-reactive Vg11+ T cells did not occur; (b) the Vg11+ T cells from the tolerant B6 mice showed a greatly reduced response relative to normal B6 mice when stimulated with immobilized mAb to Vg11; and (c) the unresponsiveness could be partially overcome in vitro by the addition of rIL2. Furthermore, the results presented here demonstrate that the unresponsiveness to anti-Vg11 activation is found in both the CD4+ Vg11+ and CD8+ Vg11+ T cell subsets. Finally, our mixing experiments show that suppression of one subset of tolerant cells by the other did not occur. Taken together, these results indicate that depleting regimens of anti-CD4 mediate transplantation tolerance due to the induction of clonal anergy to alloantigen.

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Address correspondence to Dr. C. Garrison Fathman, Department of Medicine, Division of Immunology, S-021, Stanford University, School of Medicine, Stanford, CA 94305-5111.

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