Brief Definitive Report

Anti-CD4 Mediates Clonal Anergy during Transplantation Tolerance Induction

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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 (IE^k) donor to a diabetic C57Bl/6 (B6) (IE^-) recipient occurs in the absence of clonal deletion of the potentially IE-reactive $V_\beta11^+$ T cells. Instead, a state of clonal anergy is induced in both the $CD4^+V_\beta11^+$ and $CD8^+V_\beta11^+$ T cell subsets. This clonal anergy can be partially overcome in vitro by the addition of recombinant interleukin 2.

T cells play a central role in the rejection of allografted tissue. We have previously demonstrated that administration of depleting regimens of an anti-CD4 mAb at the time of transplantation allows indefinite survival of islet (1) and heart (2) allografts. The mechanisms involved in this induced transplantation tolerance are not understood.

Several groups have shown that murine TCR V_β gene segments (i.e., $V_\beta5$, $V_\beta11$, and $V_\beta17$) may encode reactivity with the class II MHC antigen IE; therefore, mice expressing IE generate self-tolerance by deleting the majority of $V_\beta5^+$, $V_\beta11^+$, and $V_\beta17^+$ T cells from the periphery (3–6). Adult-induced tolerance may not be due to clonal deletion (7) but may instead be mediated by clonal anergy (8) or suppression (9). Data presented in this report begin to address the role of these peripheral mechanisms in anti-CD4-induced transplantation tolerance.

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. $CD4^+V_\beta11^+$ T cells were analyzed using mAb to $V_\beta11$ (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). $CD4^+V_\beta8^+$ T cells were analyzed using fluoresceinated mAb to $V_\beta8$ (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 $V_\beta11$ (RR3-15 [5]), CD3 (1452C11 [11]), $V_\beta17$ (KJ23 [3]), and $V_\beta8$ (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 2×10^5 peripheral LN cells were added per well. The plates were incubated for 4 d (CD3) or 5 d ($V_\beta17$, $V_\beta11$, $V_\beta8$) with [^{3}H]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^k) donors and transplanted into the livers of streptozotocin-induced diabetic B6 recipients as previously described (1). B6 mice do not express IE, thus, $V_\beta11^+$ T cells, which have been shown to respond to IE alloantigens 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 normogly-

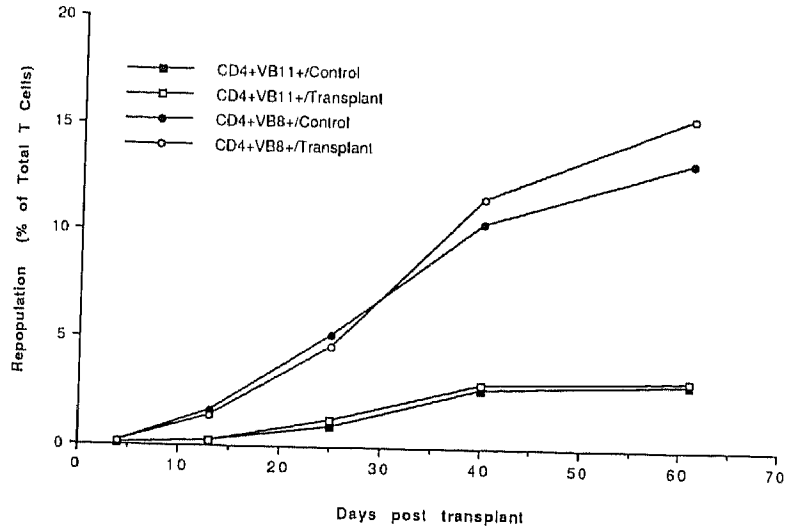

Figure 1. Repopulation of $CD4^+V_\beta11^+$ (□) and $CD4^+V_\beta8^+$ (○) T cells in anti-CD4-treated nontransplanted (*filled*) vs. transplanted (*open*) mice. Mice were injected intravenously three times with 50 μg GK1.5γ2a mAb on days −1, 0, and +1 relative to transplant. PBL were isolated on the indicated days, and $CD4^+V_\beta11^+$ T cells were analyzed using mAb to $V_\beta11$, 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 GK1.5γ2a, 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.

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_β that would have undergone deletion in IE^+ mice by FACS analysis using a mAb specific for $V_\beta11$. mAb to $V_\beta8$ 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 $GK1.5^+$ cells after treatment with 50 μg of the anti-CD4 mAb GK1.5γ2a (13) on days −1, 0, and +1 relative to transplant, a dose that depletes ~90% of the $CD5^+CD8^-$ cells (14). The kinetics of repopulation of the $CD4^+V_\beta11^+$ cells are comparable in both the anti-CD4-treated nontransplanted group and the anti-CD4-treated transplanted group (Fig. 1), indicating that the presence of an islet allograft from an IE^+ donor did not influence the repopulation kinetics of $V_\beta11^+$ T cells in an IE^- recipient. Furthermore, there was no significant reduction in the frequency of $V_\beta11^+$ (or $V_\beta5^+$; data not shown) T cells of allografted animals 6 mo post-treatment when compared with untreated B6 mice (Table 1). These data indicate that anti-CD4-mediated transplantation tolerance is not due to clonal deletion of potentially IE-reactive T cells.

We next asked whether T cells expressing the relevant V_β 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 ~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-$V_\beta11$ compared with the response

Table 1. *Analysis of Transplanted Mice for Clonal Deletion of $V_\beta11^+$ T Cells*

Stain	Strain of Mouse		
	B10.A	B6	Transplant
$CD4^+V_\beta11^+$	0.07 ± 0.03	3.0 ± 0.6	2.6 ± 0.2
$CD4^+V_\beta8^+$	14.3 ± 3.3	15.9 ± 2.5	13.4 ± 1.4
$CD8^+V_\beta11^+$	1.8 ± 0.1	3.3 ± 0.7	3.6 ± 0.4
$CD8^+V_\beta8^+$	12.6 ± 3.4	9.0 ± 1.0	9.5 ± 0.4

B6 mice transplanted with A/J islets coincident with anti-CD4 administration were analyzed 6 mo after being transplanted. Age-matched B6 mice serve as a positive control and B10.A mice give an indication of the frequency of $V_\beta11^+$ T cells expected when clonal deletion has occurred. Staining was done as in Fig. 1, except that for CD8 staining, biotinylated anti-CD8 mAb was used followed by avidin-PE. Percentages represent the mean and SEM from five to six mice relative to total $CD4^+$ plus $CD8^+$ T cells.

Table 2. *Anti-$V_\beta 11$ Response of CD4-depleted (−CD4) and CD8-depleted (−CD8) Cells*

	Responder	No. of cells	Proliferation		
			$V_\beta 17$	$V_\beta 11$	CD3
				cpm	
A	B6	4×10^5	7,776	67,499	ND
		2×10^5	3,402	26,405	ND
	Transplant	4×10^5	8,370	7,259	ND
		2×10^5	8,057	6,955	ND
B	B6 (−CD8)	2×10^5	5,349	22,173	54,295
	Transplant (−CD8)	2×10^5	5,897	7,189	38,086
	B6 (−CD4)	2×10^5	1,908	20,217	68,058
	Transplant (−CD4)	2×10^5	5,716	6,232	58,491
C	B6 (−CD8) + B6 (−CD4)	2×10^5 each	7,671	53,100	44,060
		10^5 each	7,093	23,274	49,816
	Transplant (−CD8) + transplant (−CD4)	2×10^5 each	7,702	8,651	41,688
		10^5 each	5,361	5,521	48,368
D	B6 (−CD8) + transplant (−CD4)	2×10^5 each	8,156	20,067	37,170
	Transplant (−CD8) + B6 (−CD4)	2×10^5 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.

of these populations from normal B6 mice (Table 2 *B*). Mixing CD4-depleted cells from a responsive B6 mouse with CD8-depleted cells from a transplanted mouse, or CD4-depleted cells from a transplanted mouse with 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 $V_\beta 11^+$ T cells of transplanted mice to respond to immobilized anti-$V_\beta 11$ (Fig. 2), indicating that one defect of the unresponsive $V_\beta 11^+$ T cell population may include the inability to produce IL-2.

The induced peripheral transplantation tolerance described

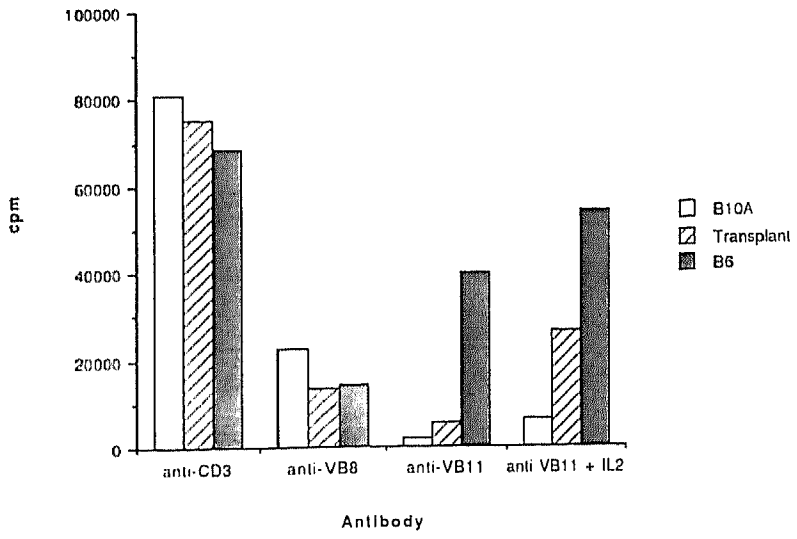

Figure 2. Transplanted mice show a poor response to specific anti-$V_\beta 11$ stimulation. T cell proliferation was induced by receptor crosslinking with mAb specific for $V_\beta 17$ (negative control), $V_\beta 11$, $V_\beta 8$, and CD3 (positive control). Peripheral LN cells (2×10^5) were added per well and the plates incubated for 4 d (CD3) or 5 d ($V_\beta 17$, $V_\beta 11$, $V_\beta 8$) with [^{3}H]thymidine added for the final 12–16 h of culture. For experiments with IL-2, 50 μl of rIL-2 (50 U/ml; Cetus Corp.) was added to the appropriate wells. Data are expressed as the mean of triplicate wells in Δ cpm with the background (cpm for $V_\beta 17$) subtracted. Data represent one of nine similar experiments.

here is similar to the clonal anergy described previously (16–18) in several respects: (*a*) clonal deletion of the potentially IE-reactive $V_\beta11^+$ T cells did not occur; (*b*) the $V_\beta11^+$ T cells from the tolerant B6 mice showed a greatly reduced response relative to normal B6 mice when stimulated with immobilized mAb to $V_\beta11$; and (*c*) the unresponsiveness could be partially overcome in vitro by the addition of rIL-2. Furthermore, the results presented here demonstrate that the unresponsiveness to anti-$V_\beta11$ activation is found in both the $CD4^+V_\beta11^+$ and $CD8^+V_\beta11^+$ 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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