CONTRASUPPRESSION IN AUTOIMMUNITY

Abnormal Contrasuppression Facilitates Expression of Nephritogenic Effector T Cells and Interstitial Nephritis in kdkd Mice

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The importance of regulatory T cell networks that modulate developing immune responses has been highlighted by a number of recent experimental findings (1–3). Such work has subsequently found application in the development of therapeutic modalities for experimental autoimmune diseases (4–7). Although it is tempting to extrapolate from such studies and ascribe causal relevance to defective immunoregulation in spontaneous autoimmunity (8), it has nevertheless been difficult to provide direct evidence for this hypothesis.

In the present experiments we have used a model of spontaneous autoimmune renal disease to characterize a disease-protective suppressor T cell system, and to investigate additional immunoregulatory defects that are linked to the expression of interstitial nephritis. kdkd mice, a congenic subline of the CBA/Ca strain, spontaneously develop a progressive, ultimately fatal tubulointerstitial nephritis with predictable onset at 8–10 wk of age (9, 10). Previous work from our laboratory (10) has shown that susceptibility to this lesion can be passed to non–disease-prone CBA/Ca mice with kdkd donor cells through radiation bone marrow chimeras. Nephritogenic effector T cells, which both mediate delayed-type hypersensitivity (DTH)[1] to CBA/Ca renal tubular antigens (TBM) and acutely induce interstitial nephritis when transferred under the renal capsule of CBA/Ca mice, have been isolated from the spleen, lymph nodes, and kidneys of nephritic kdkd mice (11). They are antigen-specific, H-2K^k-restricted, Lyt-2$^+$, L3T4$^-$ T cells.

We have also previously shown (10) that disease expression in kdkd mice can be suppressed by the adoptive transfer of T cells from naive CBA/Ca mice. This model, therefore, lends itself to a critical evaluation of the relevance of abnormal immunoregulation to organ-specific autoimmunity. In this report we have further characterized the disease-protective suppressor T cells found both in CBA/Ca

[1] *Abbreviations used in this paper:* DTH, delayed-type hypersensitivity; TBM, soluble renal tubular antigens; GBM, soluble glomerular antigens; CTX, cyclophosphamide; VV$^+$, Vicia Villosa lectin adherent cells; VV$^-$, Vicia Villosa lectin nonadherent cells; Tcs, contrasuppressor T lymphocytes.

and kdkd mice. The T cell suppression in kdkd mice, however, is lost shortly preceding the onset of disease. This loss, interestingly enough, occurs concomitantly with the emergence of tubular antigen-adherent contrasuppressor T lymphocytes. In the presence of these contrasuppressor T cells, autoreactive effector lymphocytes can even be demonstrated in non–disease-prone CBA/Ca mice. Our studies, therefore, support a model in which maintenance of self tolerance, or the development of autoimmunity, is causally linked to the relative dominance of suppression or contrasuppression.

Material and Methods

Animals.   kdkd breeding pairs were obtained from Dr. Mary Lyon (MRC, Harwell, England) and a colony is now maintained by us. CBA/Ca (H-2^k), C57BL/10 (H-2^b), B10.A (H-2^a), B10.BR (H-2^k), and B10.A(5R) (H-2^{i5}) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. B10.A(3R) (H-2^{i3}) mice were the gift of Dr. Carol Cowing (Medical Biology Institute, LaJolla, CA). (CBA/Ca × kdkd)F$_1$ were bred by us. There is not significant alloreactivity between CBA/Ca and kdkd mice, as assessed by mixed lymphocyte reactivity or acceptance of tail skin grafts (10).

Antigens.   CBA/Ca renal tubular and glomerular basement membranes were isolated by a differential sieving technique, sonicated, lyophilized, and stored at −70°C (12, 13). Soluble TBM and glomerular antigen (GBM) were made from these lyophilized membranes by collagenase digestion (14). PPD was purchased from Connaught Laboratories Ltd, Willowdale, Ontario, Canada. Some kdkd mice in selected experiments were also immunized with CFA (13).

Adoptive Transfer Experiments.   Spleen and mesenteric lymph nodes were harvested from donors and made into a single cell suspension. Red blood cells were lysed with Tris-NH$_4$Cl; remaining cells (20–30 × 10^6 cells per recipient) were washed, counted, and intravenously injected using 0.9% NaCl as the vehicle (13). Recipients of cell transfers for DTH assays were pretreated with cyclophosphamide (CTX) 48–72 h before injection to eliminate endogenous suppressor cells (11). In some experiments, cells were depleted of various subpopulations with mAbs and complement before intravenous transfer.

Preparation of Lymphocyte Subpopulations.   Cell suspensions were depleted of T lymphocytes with anti-Thy-1.2 mAb from J1$_j$ hybridoma (15), anti-Lyt-2 mAb (from 3.168.8 hybridoma [16] provided by Dr. Frank Fitch, University of Chicago, Chicago, IL), and anti-L3T4 mAb (from GK-1.5 hybridoma [17, 18] provided by Dr. Fitch) and a mixture of rabbit and guinea pig complement. anti-I-J^k mAb (WF8.C12.8) and anti-I-J^b mAb (WF9.40.5) were provided by Dr. Carl Waltenbaugh (Northwestern University, Chicago, IL) and are also cytotoxic with rabbit and guinea pig complement (19). In our experience, these anti-Thy 1.2, anti-L3T4, and anti-Lyt-2 antibodies (when mixed with exogenous complement) kill ~40, 27, and 16% of pooled lymph node and spleen cells from CBA/Ca or kdkd mice. anti-I-J^b and anti-I-J^k kill ~3–5% of spleen cells of the appropriate H-2 haplotype. In some experiments, *Vicia villosa* adherent (VV$^+$) cells (see below) were further fractionated on TBM or GBM antigen–coated plates (4). In this panning procedure, 2 ml of TBM or GBM antigen at 1 mg/ml was incubated overnight at 4°C on 60 × 15-mm polystyrene plates. Before use, plates were washed several times with RPMI 1640 containing 3% FCS. Cell preparations (20 × 10^6 cells/ml) were added to the plates and incubated at room temperature for 1 h. Nonadherent cells were gently aspirated and saved. Adherent cells were harvested after addition of cold RPMI 1640 with 3% FCS by vigorous pipetting with a Pasteur pipette. Adherent and nonadherent cell fractions were washed, counted, and then admixed with VV nonadherent (VV$^-$) cells before adoptive transfer into CTX-pretreated CBA/Ca recipients.

Serum Treatments.   Sera obtained from >4-mo-old nephritic kdkd mice or normal CBA/Ca mice were heat-inactivated by a 1-h incubation in a 56°C water bath. Spleen cells were sequentially incubated with varying dilutions of serum and exogenous rabbit and guinea pig complement for 1 h at 37°C. Cells were washed twice with PBS before admixture with DTH-reactive cells and adoptive transfer.

Cell Separation Procedure Using VV. Methods were adapted from those described previously by Iverson et al. (20). VV lectin was purchased from E. Y. Laboratories, Inc., San Mateo, CA. ~2–3 ml of stock lectin solution (0.5 mg/ml of VV lectin in citrate saline buffer, pH 5.5) was allowed to incubate on 10-cm Costar tissue culture grade Petri dishes for 60–90 min at room temperature. Dishes were then washed with PBS and incubated with RPMI 1640 with 3% FCS for 20–30 min. 75–100 × 10^6 spleen and lymph node cells were then added to the plates in a volume of 10 ml RPMI 1640 with 5% FCS. Plates were incubated in a 37°C, 5% CO_2 incubator for 45–60 min. Medium containing VV^- cells was removed; plates were washed once with RPMI 1640 + 5% FCS to harvest additional VV^- cells. VV^+ cells were harvested after an addition of 5 ml of 1 mg/ml N-acetyl-D-galactosamine in PBS to the plates (17). Plates were incubated for 10 min at 37°C and VV^+ cells, were removed with a Pasteur pipette. Using this procedure on whole spleen cell preparations (RBCs lysed with Tris-NH_4Cl), total cell recoveries were ~70%. VV^- cells represented 75–80%, and VV^+ cells 20–25% of the recovered population. Viability of recovered cells was 90–95% by Trypan blue exclusion. Where indicated, VV^+ and VV^- subpopulations were incubated with mAbs and complement before reconstitution and adoptive transfer into CTX-pretreated CBA/Ca recipients.

Elicitation and Measurement of DTH Response. 1–2 h after the intravenous adoptive transfer of cells into CTX-pretreated recipients, soluble antigens (TBM or PPD) were injected into one footpad (25 µg of soluble antigen in 25 µl of PBS); the other footpad was injected with PBS alone. Footpad swelling, as an index of DTH, was measured 24 h later using a spring-loaded engineer's micrometer (Schlesinger's for Tools, Brooklyn, NY). The magnitude of swelling was expressed as the mean increment between the antigen-challenged footpad and the PBS-injected footpad in inches × 10^{-3} ± SEM (21). All measurements were cage blind.

Assessment of Renal Disease in kdkd Mice. Kidney tissue was prepared for light microscopy by standard methods for this laboratory (10). The degree of interstitial involvement in aging kdkd mice was quantitatively determined from coded, paraffin-embedded kidneys stained with H and E. Cellular infiltration and tubular damage leading to cortical interstitial destruction was graded on a scale of 0 to 4 (10) where 0 is normal, 0.5 is small focal areas of cellular infiltration; 1 is involvement of <10% of the cortex; 2 is involvement up to 25% of the cortex; 3 is involvement up to 50–75% of the cortex; 4 is extensive damage involving >75% of the cortex. Results from grading by a blinded observer are reported as a mean score ± SEM. In other experiments, some subpopulations of T cells were also placed directly under the kidney capsule (21). To perform these latter transfers, CTX-pretreated CBA/Ca mice were anesthetized, and 10^8 donor lymphocytes in ~0.075 ml of PBS were injected under the kidney capsule with a 30-gauge needle. This volume uniformly lifted the capsule off most of the parenchyma without bleeding. The starting population for these transfers was 10^8 spleen cells of each donor strain. Some groups of animals received the cells remaining after depletion with mAbs + C' or after separation of VV lectin–coated plates. VV^+ and VV^- cells were admixed with CBA/Ca cells or injected alone at the proportions in which they were present in kdkd spleen, i.e., 20–30% and 70–80% respectively. 7 d later, mice in the experimental groups were killed, and the kidneys were longitudinally sectioned with attempted preservation of the subcapsular cell layer. After fixation in 10% buffered formalin, the kidneys were paraffin embedded for staining with H and E. The stained sections were coded for blind reading by two different grading scales. The severity was qualitatively assessed by using a scale modified from previous studies (21): 0, no involvement from the subcapsular cell layer; 0.5, trace pathologic changes of cellular involvement in a focal pattern in the outermost cortical tubular area; 1, superficial, focal peritubular infiltration and tubular atrophy under the transferred cell layer; and 2, focal, deeply extending, heavy cellular infiltrates with peritubular damage and tubular atrophy. The sections were also graded by approximating the tubular layer cell depth of the most advanced infiltrating front of mononuclear cells. Each layer equalled a tubular diameter and was given one point. The data from both methods were expressed as a mean ± SEM.

TABLE I

Antigen-specific Suppression by CBA/Ca Lymphocytes

Donor cell admixture*		Recipient DTH response[‡]	
kdkd	CBA/Ca	CBA/Ca TBM	PPD
+	−	16.3 ± 0.7	16.3 ± 0.9
+	+	6.6 ± 0.3[§]	16.3 ± 0.3
−	−	4.7 ± 0.3[§]	4.3 ± 0.7[§]

* Pooled spleen and lymph node cells from >16-wk-old kdkd mice that had been immunized 2 wk previously with CFA were admixed with equal numbers of CBA/Ca lymphocytes and intravenously injected into CTX-pretreated CBA/Ca recipients.

[‡] Animals were footpad challenged with soluble CBA/Ca TBM and PPD 1–2 h after cell transfer. 24 h later, footpad swelling was measured and expressed as mean increment over control values in inches × 10^{-3} ± SEM.

[§] $p < 0.001$ compared with response with kdkd cells alone.

Statistical Analysis. Differences between experimental groups were determined by Student's t test.

Results

Specific Suppression of DTH to CBA/Ca TBM by CBA/Ca Lymphocytes. Previous studies from our laboratory (10) have shown that T lymphocytes from CBA/Ca mice can suppress the development of the kdkd interstitial disease. Since our previous work has suggested that kdkd DTH-reactive cells are an important effector mechanism of disease, we tested whether this reactivity would be suppressed by disease-protective CBA/Ca lymphocytes. To address the specificity of a possible suppressive effect, we immunized the kdkd donors of DTH-reactive cells with CFA. As shown in Table I, pooled spleen and lymph node cells from CBA/Ca mice can completely suppress the DTH response to CBA/Ca TBM mediated by kdkd cells in CTX-pretreated CBA/Ca recipients. Previous work from our laboratory (11) has shown that CTX pretreatment of the CBA/Ca recipients is necessary for demonstrating DTH reactivity to CBA/Ca TBM by kdkd lymphocytes. The PPD response is unaffected by the presence of CBA/Ca cells, suggesting that suppression is antigen specific. We have previously demonstrated (11) that the negative DTH response seen after infusion of CBA/Ca cells alone is indistinguishable from that after PBS injection.

Phenotypic Characterization of the CBA/Ca Suppressor Cell. We next characterized the cells mediating this suppression using negative selection techniques. As seen in Table II, the suppression manifested by unfractionated CBA/Ca spleen and lymph node cells is completely abrogated by treating the cells with anti-Thy-1.2 mAb and complement. The T cell subpopulation responsible for suppression was further defined using anti-Lyt-2, anti-L3T4, and anti-I-J antibodies and complement. Whereas pretreatment with anti-Lyt-2 or anti-I-J^k antibodies + C' resulted in loss of suppression, anti-L3T4 or anti-I-J^b + C' had no effect.

Suppression of DTH to CBA/Ca TBM Antigen Is Genetically Dominant and MHC Restricted. Previous work from our laboratory (10) has shown that (CBA × kdkd)F$_1$ mice do not develop the progressive interstitial nephritis of kdkd mice.

TABLE II
Phenotype of CBA/Ca Suppressor Cell

Donor cell admixture*		Recipient DTH response to CBA/Ca TBM[‡]
kdkd	CBA/Ca	
+	−	14.7 ± 2.3
+	+	3.5 ± 1.3[§]
+	C'	3.3 ± 0.9[§]
+	anti-Thy-1.2 + C'	13.0 ± 0.9
+	anti-Lyt-2 + C'	13.4 ± 1.7
+	anti-L3T4 + C'	5.5 ± 0.6[ǀ]
+	anti-I-J[b] + C'	2.8 ± 1.0[§]
+	anti-I-J[k] + C'	14.5 ± 0.7
−	+	3.0 ± 0.7[§]

* Pooled spleen and lymph node cells from >16-wk-old kdkd mice were admixed with equal numbers of CBA/Ca lymphocytes and intravenously injected into CTX-pretreated CBA/Ca recipients. Selected groups of CBA/Ca cells were treated with various mAbs and complement before transfer.

‡ 1–2 h after cell transfer animals were footpad challenged with soluble CBA/Ca TBM in one footpad and PBS in the other. 24 h later, footpad swelling was measured and expressed as mean increment in inches × 10^{-3} ± SEM.

§ p <0.005 compared with kdkd cells alone.
ǀ p <0.01 compared with kdkd cells alone.

TABLE III
H-2 Restriction of CBA/Ca Suppressor Cell

Cotransferred cells*			Recipient DTH response to CBA/Ca TBM[‡]
DTH-reactive cells (kdkd)	Suppressor cells		
	Strain	H-2	
+	—	—	14.8 ± 0.5
+	CBA/Ca	k	6.0 ± 0.6[§]
+	(CBA/Ca × kdkd)F$_1$	k	4.7 ± 0.3[§]
+	B10.BR	k	5.2 ± 0.5[§]
+	B10	b	14.8 ± 0.6
+	B10.A	a	3.2 ± 0.5[§]
−	—	—	4.3 ± 0.3[§]

* Equal numbers of DTH-reactive lymphocytes from >16-wk-old kdkd mice and lymphocytes from strains as indicated were admixed and injected intravenously into CTX-pretreated (B6 × A)F$_1$ recipients. The average DTH response after transfer of CBA/Ca, B10.BR, or B10.A cells alone was 3.1 ± 0.4.

‡ See Table II.

§ p <0.0005 compared with kdkd cells alone.

The existence of a genetically dominant suppressor system is supported by the data in Table III; (CBA × kdkd)F$_1$ lymphocytes suppress the TBM reactivity of kdkd cells as effectively as do CBA/Ca parental cells. We also investigated the genetic requirements for effective interaction between these DTH reactive cells and the suppressor cells. As shown in Table III, B10.BR (H-2^k) and B10.A (H-

TABLE IV

I-J Restriction of Suppression

Cotransferred cells*		H-2 haplotype of suppressor cell							Recipient DTH response to CBA/Ca TBM[‡]
kdkd	Suppressor	K	A_β	A_α	E_β	J	E_α	D	
+	—								16.3 ± 0.3
+	B10	b	b	b	b	b	b	b	16.0 ± 1.2
+	B10.A	k	k	k	k	k	k	d	4.3 ± 0.3[§]
+	B10.A(3R)	b	b	b	b	b	k	d	16.7 ± 0.9
+	B10.A(5R)	b	b	b	b	k	k	d	4.6 ± 0.7[§]
—	—								4.3 ± 0.9[§]

* Cell preparations and antigen challenges were performed as described in Table III.
‡ See Table II.
§ $p < 0.0005$ compared with kdkd cells alone.

TABLE V

Suppression of Disease with Lyt-2⁺, I-J^{k+} CBA/Ca Cells

Donor cells*	Treatment[‡]		Renal Histology of kdkd recipients[§]
—	—		3.0 ± 0.0[∥]
CBA/Ca	C'	(100×10^6)	1.4 ± 0.5
CBA/Ca	anti-L3T4 + C'	(70×10^6)	1.3 ± 0.1
CBA/Ca	anti-Lyt-2 + C'	(84×10^6)	2.6 ± 0.1[∥]
CBA/Ca	anti-I-J^k + C'	(95×10^6)	3.0 ± 0.3[∥]
CBA/Ca	anti-I-J^b + C'	(95×10^6)	1.2 ± 0.4

* Pooled mixtures of spleen and lymph node cells from CBA/Ca mice were transferred into kdkd mice every 10 d beginning at age 6 wk for a total of four cell transfers.
‡ 10^8 pooled CBA/Ca cells were treated with various mAbs and/or complement before transfer. Remaining viable cells were intravenously transferred (approximate numbers in parentheses).
§ kdkd recipients were killed for quantitative assessment of renal disease at 14 wk of age.
∥ $p < 0.05$ compared with recipients of C' treated CBA/Ca cells.

2ᵃ) cells suppressed the DTH response of kdkd cells to CBA/Ca TBM as effectively as CBA/Ca (H-2ᵏ) cells, implying that background genes are not critical for the suppressive effect.

Using intra-MHC recombinant mice as suppressor cell donors, we examined more precisely the requirements for functional homology. Table IV shows that expression of I-J^k is required for functional suppression as B10.A(3R) (I-J^b) cells do not suppress.

Suppression of Histologic Disease by Lyt-2⁺, I-J^{k+} CBA/Ca Lymphocytes. We next evaluated the effect of depletion of various T cell subsets on disease suppression by CBA/Ca lymphocytes. kdkd mice received serial cell transfers starting at 6 wk of age, before the onset of nephritis. As seen in Table V, treatment of the CBA/Ca lymphoid cells with anti-Lyt-2 or anti-I-J^k and C' greatly diminished the disease suppression seen with C' treated CBA/Ca cells. In these experimental groups, the histologic renal disease was as severe as that of untreated kdkd mice,

TABLE VI

Age-dependent Suppressor Cell Function in kdkd Mice

Age of cotransferred kdkd cells*				Recipient DTH response to CBA/Ca TBM‡
6 wk	8 wk	10 wk	16 wk	
−	−	−	+	17.3 ± 0.3
+	−	−	+	5.6 ± 1.2§
−	+	−	+	17.6 ± 1.9
−	−	+	+	16.3 ± 0.7
+	−	−	−	6.7 ± 0.9§
−	+	−	−	17.3 ± 0.9
−	−	+	−	14.3 ± 2.3
−	−	−	−	4.3 ± 0.3§

* Pooled spleen and lymph node cells from kdkd mice of various ages were admixed and cotransferred or injected alone as indicated into CTX-pretreated CBA/Ca recipients. + and − designate cell populations cotransferred.

‡ See Table II.

§ p <0.0005 compared with 16-wk-old kdkd cells alone.

i.e., involvement of 50–75% of the renal cortex with cellular infiltration and tubular damage at 14 wk of age. Cells treated with anti-L3T4 or anti-I-J^b and C′ were as effective in disease suppression as cells treated with C′ alone; in these groups mononuclear cell infiltrates were patchy and limited to 10–15% of the cortical area.

Loss of Demonstrable Suppressor Cell Function in kdkd Mice with Aging. To examine the relevance of these suppressor cells to the spontaneous interstitial nephritis of kdkd mice, we further explored earlier observations regarding suppressor cells in prenephritic, young kdkd mice (11). Unfractionated spleen and lymph node cells from 5-wk-old kdkd mice, which do not display DTH reactivity to CBA/Ca TBM, suppress the TBM-specific DTH reactivity of lymphocytes from 16-wk-old kdkd mice in the acute adoptive transfer assay (11). To ascertain whether the loss of this suppressor cell activity was temporally correlated with the onset of tubulointerstitial disease at approximately age 10 wk, we harvested spleen and lymph node cells from kdkd mice at 6, 8, and 10 wk of age and examined their ability to elicit or suppress a DTH response to TBM in CTX-pretreated CBA/Ca recipients. As shown in Table VI, unfractionated lymphocytes from 6-wk-old kdkd mice suppress the reactivity of cells from 16-wk-old mice and demonstrate no reactivity to TBM when transferred alone. No suppressor activity is demonstrable in unfractionated cell preparations from 8- or 10-wk-old kdkd mice; these cells, however, do demonstrate DTH reactivity to CBA/Ca TBM. Thus, several weeks preceding the earliest histologic evidence of interstitial disease, DTH-reactive cells are demonstrable in kdkd mice.

Mechanism of Suppressor Cell Bypass in kdkd Mice. Our studies to this point suggest that the functional loss or inactivation of a tubular antigen-specific suppressor mechanism is kinetically related to the development of interstitial nephritis in kdkd mice. We therefore addressed several possible mechanisms by which this loss of suppression may have occurred. Preliminary studies revealed that functional suppression by CBA/Ca cells was unaffected by preincubation

TABLE VII

Contrasuppressor Cells in Nephritic kdkd Mice

kdkd donor cells*	Number of cells transferred[‡]	Recipient DTH response to CBA/Ca TBM[§]
Unfractionated	15×10^6	16.7 ± 1.0[‖]
VV$^+$	3×10^6	4.8 ± 0.9
VV$^+$	12×10^6	6.0 ± 0.5
VV$^-$	12×10^6	4.3 ± 0.5
VV$^+$ + VV$^-$	15×10^6	16.1 ± 1.0[‖]
VV$^-$ (anti-I-J^k + C′)	12×10^6	14.8 ± 0.5[‖]
VV$^-$ (anti-I-J^k + C′)	6×10^6	11.3 ± 0.7[¶]
VV$^+$ + VV$^-$ (anti-I-J^k + C′)	3×10^6 (VV$^+$)	10.7 ± 0.6[¶]
	6×10^6 (VV$^-$)	
VV$^-$ (anti-I-J^b + C′)	12×10^6	4.7 ± 0.3
VV$^-$ (C′)	12×10^6	3.3 ± 0.3

* Pooled spleen and lymph node cells from >16-wk-old kdkd mice were fractionated on VV lectin–coated plates. Nonadherent cells were further treated, where indicated, with anti-I-J mAbs and/or complement.

[‡] Numbers of cells transferred reflect their relative percentage in starting population of pooled spleen and lymph node cells and/or counts preceding treatment with mAbs and complement.

[§] See Table II.

[‖] $p < 0.0005$ compared with VV$^-$ cells transferred alone.

[¶] $p < 0.005$ compared with VV$^-$ cells transferred alone.

TABLE VIII

Contrasuppression Is Mediated by Lyt-2$^+$, I-J^{k+} T Cells

kdkd donor cells*	Treatment of VV$^+$ cells	Recipient DTH response to CBA/Ca TBM[‡]
VV$^+$ + VV$^-$	—	16.0 ± 0.5
VV$^+$ + VV$^-$	C′	17.0 ± 1.0
VV$^+$ + VV$^-$	Anti-Thy-1.2 + C′	4.0 ± 0.5[§]
VV$^+$ + VV$^-$	Anti-L3T4 + C′	17.3 ± 0.3
VV$^+$ + VV$^-$	Anti-Lyt-2 + C′	4.3 ± 0.6[§]
VV$^+$ + VV$^-$	Anti-I-J^k + C′	4.0 ± 0.8[§]
VV$^+$ + VV$^-$	Anti-I-J^b + C′	16.0 ± 0.8
	—	3.3 ± 0.7[§]

* Pooled spleen and lymph node cells from >16-wk-old kdkd mice were fractionated on VV lectin–coated plates. Adherent cells (VV$^+$) were further treated, where indicated, with mAbs and/or complement.

[‡] See Table II.

[§] $p < 0.0005$ compared with untreated VV$^+$ and VV$^-$ cells.

with heat-inactivated serum from nephritic kdkd mice + C′ (data not shown), suggesting these mice do not develop easily demonstrable antibodies to suppressor T cells. Therefore, we next investigated the possibility that the suppressor mechanism was bypassed by an emergent contrasuppressor (Tcs) network. To pursue this hypothesis we used the observation from other antigenic systems that contrasuppressor cells adhere to the VV lectin (20, 22–24). As shown in Table VII, separation of pooled spleen and lymph node cells from nephritic kdkd mice on VV-coated plates resulted in two cell populations (VV$^+$ and VV$^-$), neither of

TABLE IX

Contrasuppressor Cells Are Specifically Adherent to TBM Antigens

kdkd donor cells*		Recipient DTH response to CBA/Ca TBM[‡]
VV[+] (Number added)	VV[−]	
Unfractionated	+	$17.0 \pm 0.8^{§}$
TBM adherent (2 × 10⁶)	+	$18.0 \pm 1.3^{§}$
TBM adherent (1 × 10⁶)	+	$17.3 \pm 0.3^{§}$
TBM adherent (1 × 10⁵)	+	4.3 ± 0.6
TBM nonadherent (3 × 10⁶)	+	4.3 ± 0.3
GBM adherent (3 × 10⁶)	+	3.7 ± 0.7
GBM nonadherent (3 × 10⁶)	+	$16.7 \pm 1.0^{‖}$
—	−	4.0 ± 0.5

* Pooled spleen and lymph node cells from >16-wk-old kdkd mice were fractionated on VV lectin–coated plates. VV adherent cells were further fractionated on either TBM or GBM coated plates. Resultant cell populations were admixed with 12 × 10⁶ VV nonadherent cells per recipient and intravenously transferred into CTX-treated CBA/Ca recipients.
[‡] See Table II.
[§] $p <0.0005$ compared with negative control.
[‖] $p <0.005$ compared with negative control.

which could mediate a DTH response to CBA/Ca TBM. Reconstitution of these two populations restored demonstrable DTH reactivity in the adoptive-transfer assay. Since our previous data had shown that the CBA/Ca suppressor cells were I-J[k+], we treated VV^{−} cells with anti-I-J mAbs and/or C'. VV^{−} cells treated with anti-I-J[k] + C' demonstrated DTH reactivity to CBA/Ca TBM, whereas treatment with anti-I-J[b] + C', or C' alone did not facilitate functional expression of DTH reactivity in the VV[−] subpopulation. VV^{+} cells did not function to augment the DTH response of an intermediate number of anti-I-J[k] + C'–treated VV^{−} cells, suggesting that they have no function in the absence of suppressor cells. The data in Table VII also show that in defined preparations of spleen and lymph node cells from nephritic kdkd mice, DTH effector cells (VV[−]), suppressor cells (VV[−]), and contrasuppressor cells (VV[+]/Tcs) can all be shown to be functionally present.

Phenotypic Analysis of VV[+]/Tcs Cells Mediating Contrasuppression. We next analyzed the surface markers of the Tcs in our system by negative selection with mAbs and complement. In Table VIII it can be seen that treatment of the VV^{+} cells with anti-Thy-1.2, anti-Lyt-2, or anti-I-J[k] and complement results in elimination of contrasuppressor function, whereas anti-L3T4 or anti-I-J[b] have no effect. Thus contrasuppressor effector function in this system is mediated by Lyt-2^{+}, I-J[k+] T cells.

Antigen Specificity of the Tcs in Nephritic kdkd Mice. Since we are unaware of other parenchymal antigens to which kdkd lymphoid cells spontaneously exhibit reactivity in a DTH assay, we could not test antigen specificity of the Tcs cells in that manner. Instead we examined whether Tcs cells would specifically adhere to TBM antigen. ~40% of the VV[+]/Tcs spleen cell population was adherent to TBM-coated plates. As shown in Table IX, contrasuppressor function was enriched in the TBM adherent population. VV^{+} cells nonadherent to TBM exhibited no contrasuppressor function, even when admixed with VV^{−} cells at a

TABLE X

T_{DTH} but not T_{Cs} Are Present in CBA/Ca and Young kdkd Mice

Donor cells*		Recipient DTH Response to CBA/Ca TBM‡
VV⁺	VV⁻ (treatment)	
16 wk kdkd	16 wk kdkd	13.0 ± 0.5§
5 wk kdkd	16 wk kdkd	3.7 ± 0.3
CBA/Ca	16 wk kdkd	4.0 ± 0.0
16 wk kdkd	5 wk kdkd	16.0 ± 0.3§
5 wk kdkd	5 wk kdkd	4.0 ± 0.5
—	5 wk kdkd (C′)	3.3 ± 0.6
—	5 wk kdkd (anti-I-Jᵏ + C′)	15.3 ± 0.3§
—	5 wk kdkd (anti-I-Jᵇ + C′)	3.7 ± 0.3
16 wk kdkd	CBA/Ca	4.0 ± 0.0
16 wk kdkd (6 × 10⁶)	CBA/Ca	16.3 ± 0.6§
—	CBA/Ca (C′)	4.0 ± 0.5
—	CBA/Ca (anti-I-Jᵏ + C′)	16.0 ± 0.5§
—	—	3.7 ± 0.6

* Pooled spleen and lymph node cells from kdkd mice of varying age or CBA/Ca mice were separated on VV lectin–coated plates. VV⁻ cells were further treated where indicated. 3×10^6 VV⁺ cells (except where indicated) were admixed with 12×10^6 VV⁻ cells and transferred as previously described.
‡ See Table II.
§ $p < 0.0005$ compared with negative control.

quantity (3×10^6) equal in number to the VV⁺ starting population. On the other hand, contrasuppressor function was present only in the VV⁺/Tcs cell population nonadherent to GBM antigen, implying that the relevant Tcs cells are not nonspecifically adherent to plastic or other protein antigens. Other cell types in the VV⁺ population probably display nonspecific binding since ~35–40% of the VV⁺ spleen cells adhere to GBM plates as well, despite functional Tcs activity only in the GBM nonadherent population.

DTH Effector Cells, but not Tcs Cells, Are Present in CBA/Ca and Prenephritic kdkd Mice.   We reasoned that if this autoimmune tubulointerstitial nephritis was related to an abnormally active Tcs cell that overrides suppression, contra-suppression should be predictably absent in non–disease-prone CBA/Ca mice and prenephritic kdkd mice. This hypothesis is supported by the data in Table X. VV⁺ cells from either 5-wk-old prenephritic kdkd mice or CBA/Ca mice failed to override the suppressor cells in the VV⁻ cell population from older nephritic kdkd mice. However, when VV⁺/Tcs cells from nephritic (16 wk) kdkd mice were admixed with VV⁻ cells from 5-wk-old kdkd mice, DTH reactivity was demonstrable. This finding suggested that DTH effector cells are present in the peripheral lymphoid organs of 5-wk-old kdkd mice, but in the absence of an expanded Tcs cell population their expression is normally masked by suppression. In studies confirming this hypothesis, we showed that 5-wk-old kdkd VV⁻ cells, when treated with anti-I-Jᵏ + C′, could mediate a DTH response whereas cells treated with anti-I-Jᵇ and/or C′ alone were unreactive in the acute adoptive-transfer assay. We next examined whether DTH effector cells were also present in non–disease-prone CBA/Ca mice. As seen in Table X, although no DTH

Table XI

Nephritogenic Effector Cells in Non–disease-Prone CBA/Ca Mice

Donor cells* (treatment)	Histology after subcapsular transfer[‡]	
	Severity (0–2 scale)	Maximum depth (tubular diameters)
CBA/Ca (C′)	0.2 ± 0.1	0.7 ± 0.6
CBA/Ca (anti-I-J^k + C′)	2.0 ± 0.0[§]	13.0 ± 1.1[§]
CBA/Ca (anti-I-J^b + C′)	0.2 ± 0.1	0.3 ± 0.3
CBA/Ca + kdkd VV$^+$ cells	1.7 ± 0.2[§]	14.8 ± 3.3[§]
CBA/Ca + kdkd VV$^-$ cells	0.3 ± 0.2	0.5 ± 0.4
kdkd VV$^+$ cells alone	0.0	0.0

* Donor cells were harvested from CBA/Ca or >16 wk kdkd spleens. Where indicated, cells were treated with mAbs and/or complement or separated on VV-coated plates. Remaining cells (starting population of 10^8 spleen cells per recipient) were resuspended in 0.075 ml PBS and placed under the renal capsule of CTX-pretreated CBA/Ca mice.
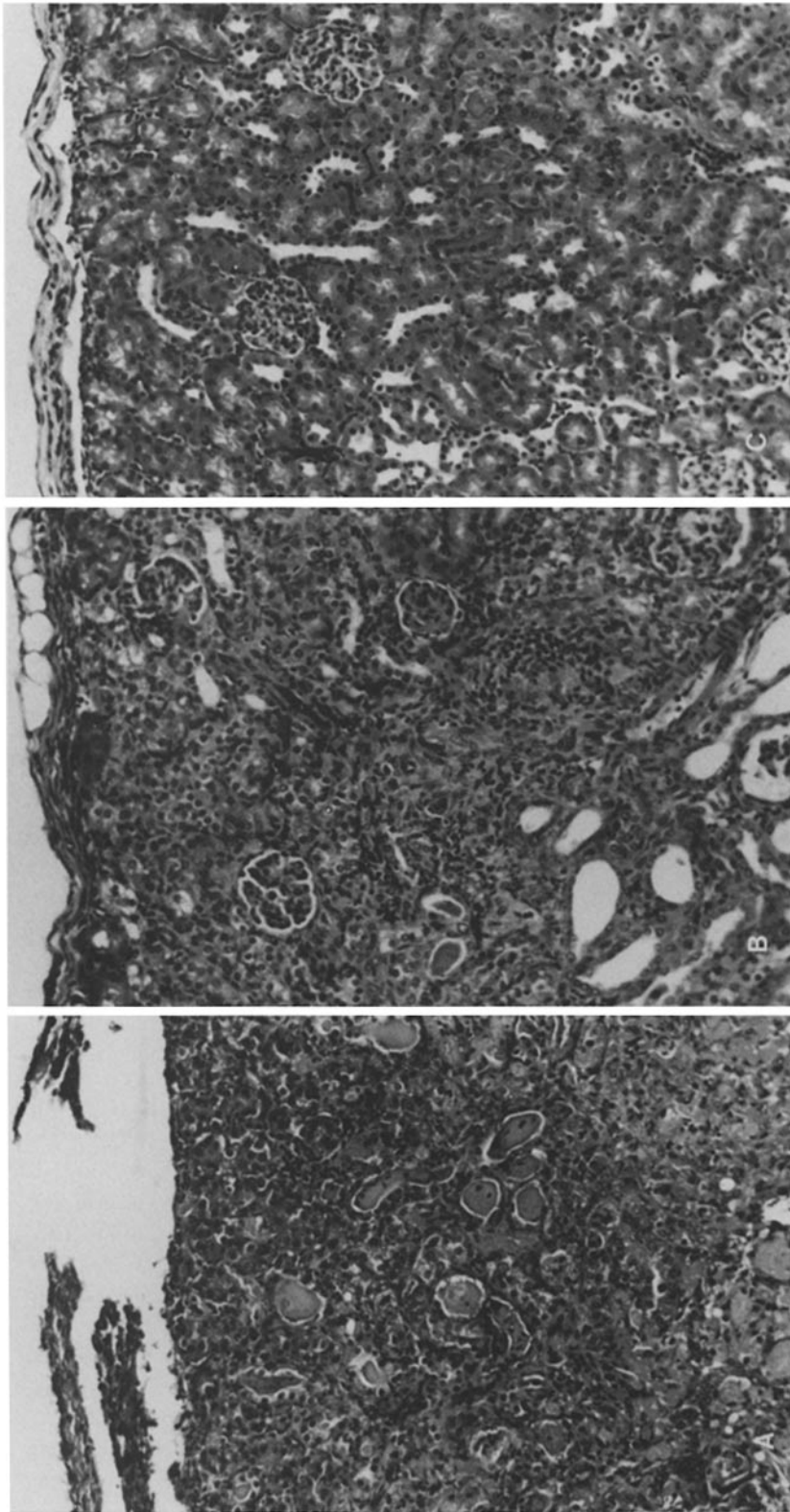
‡ Kidneys were harvested for histologic grading 7 d after cell transfer.

§ p <0.001 compared with recipients of C′-treated CBA/Ca cells.

response was seen when VV$^-$ CBA/Ca cells were admixed with 3×10^6 VV$^+$ cells from 16-wk-old kdkd mice, a response was present when 6×10^6 VV$^+$ 16-wk kdkd cells were used. A DTH effector population could also be demonstrated with VV$^-$ CBA/Ca cells after treatment with anti-I-J^k and complement. These latter cells did not mediate DTH to GBM, or solubilized liver antigens (data not shown), suggesting a genetic predisposition in CBA/Ca mice to tubulointerstitial disease (25).

Non–Disease-prone CBA/Ca Mice Harbor Nephritogenic Effector Cells. In our previous studies (11) of kdkd mice, we have shown that the Lyt-2$^+$ T cells mediating DTH to CBA/Ca TBM can also acutely induce renal injury when transferred beneath the renal capsule of CTX-pretreated CBA/Ca mice. Since DTH effector cells are demonstrable in CBA/Ca mice under the conditions described above, we next tested whether nephritogenic effector cells could also be demonstrated in CBA/Ca spleens under appropriate conditions. As shown in Table XI, CBA/Ca spleen cells treated with C′ alone or anti-I-J^b and C′ effect minimal renal damage in CTX-pretreated syngeneic recipients. CBA/Ca cells depleted of suppressor cells with anti-I-J^k and C′, or unfractionated CBA/Ca spleen cells reconstituted with kdkd contrasuppressor cells (VV$^+$) result in a severe, deeply infiltrating histologic lesion (Table XI and Figure 1, *A* and *B*), which is indistinguishable from that seen after subcapsular transfer of unfractionated kdkd lymphoid cells (11). kdkd VV$^+$ cells alone did not display nephritogenic effector function when injected at the same number (30×10^6) as that admixed with CBA/Ca cells.

A critical control group in these subcapsular transfer studies was cotransfer of unfractionated CBA/Ca spleen cells with kdkd VV$^-$ cells. Although both of these cell populations contain potential nephritogenic effector cells, no significant renal damage was observed (Fig. 1C), presumably attributable to functional suppression by both CBA/Ca and VV$^-$ kdkd cells and absence of contrasuppres-

FIGURE 1. Subcapsular cell transfers. CBA/Ca spleen cells treated with anti-I-J^k + C′, or unfractionated CBA/Ca spleen cells combined with VV$^+$ or VV$^-$ kdkd spleen cells were separately placed under the renal capsule of CTX-pretreated CBA/Ca mice. After 7 d, the kidneys were harvested and sectioned for histologic grading. (A) anti-I-J^k + C′–treated CBA/Ca spleen cells; significant tubular destruction and infiltration of the interstitium with mononuclear cells can be observed. (B) CBA/Ca spleen cells admixed with VV$^+$ kdkd spleen cells; deranged interstitial architecture and mononuclear cell infiltration similar to A. (C) CBA/Ca spleen cells admixed with VV$^-$ kdkd cells; no significant abnormalities were observed. These sections were indistinguishable from normal CBA/Ca kidneys (Original magnification, × 200).

sion. These findings again highlight the importance of interacting immunoregulatory cells in determining the expression of nephritogenic effector cells.

Discussion

We have been interested in studying immunoregulatory mechanisms as they relate to the expression of experimental autoimmune interstitial nephritis. The present studies extend earlier observations from our laboratory that suggested a role for abnormal immunoregulation in the spontaneous nephritis of kdkd mice. Unfractionated, but not T-depleted, spleen cells from naive CBA/Ca mice suppress the development of interstitial nephritis in kdkd mice (10). Spleen cell preparations, or renal-eluted lymphocytes, from diseased kdkd mice contain a population of cells that exhibit reactivity to CBA/Ca TBM in a DTH assay. Since these DTH-reactive cells are phenotypically identical to those mediating interstitial damage in acute subcapsular transfer studies (11), we have used these DTH-reactive cells as functional markers of nephritogenic effector cells and have used this DTH assay, in conjunction with histologic studies, to characterize disordered immunoregulatory mechanisms. Indeed, our present studies further validate the use of the DTH response to characterize the nephritogenic immune response, as the CBA/Ca T cells that are disease-protective also suppress the DTH response. These suppressor cells are antigen specific, and I-J restricted. Both disease suppression and suppression of the DTH response are mediated by an $Lyt-2^+$, I-J^{k+}, $L3T4^-$ subpopulation of the CBA/Ca splenic T cells. Since susceptibility to this renal lesion is transmitted as an autosomally recessive trait, we had previously proposed (10) that the disease might result from the absence of a genetically dominant suppressor T cell system. This hypothesis required reexamination after the demonstration of a suppressive effect by unfractionated spleen cell populations from young, prenephritic, kdkd mice (11). As shown in the present studies, loss of such suppression by unfractionated kdkd spleen cells temporally correlated with the onset of disease. Although we could find no evidence for a lymphocytotoxic antibody to explain this apparent loss of suppression, separation of spleen cells on VV-coated plates revealed the presence of functional Tcs cells in nephritic kdkd mice. These Tcs cells are $Lyt-2^+$, I-J^+, and specifically adherent to solubilized TBM antigens. They appear to functionally bypass the suppressor cells, since in the absence of Tcs lymphocytes, kdkd spleen cells do not exhibit DTH reactivity unless suppressor cells are eliminated with anti-I-J^k and complement. Using such separation procedures, we were able to demonstrate DTH-reactive cells and nephritogenic effector cells both in prenephritic kdkd mice and non–disease-prone CBA/Ca mice. An expanded population of Tcs cells, however, was only demonstrable in nephritic kdkd mice.

Since the initial description of contrasuppression as an activity inhibitory of T cell suppression, and not attributable to excess helper activity (26), many studies (reviewed in 27) have verified the potent role of contrasuppression in influencing the expression of an immune response. Our observations regarding the counterbalancing effects of Ts and Tcs in this model of spontaneous autoimmune disease are strikingly similar to those in models of induced immune responses to specific antigens (23, 24, 28–30). The relative insensitivity of anti-SRBC plaque-forming B10 spleen cells to antigen-specific suppressor factors, for example, is dramati-

cally altered by interfering with the contrasuppressor circuit in vitro (28). Tcs cells also display important regulatory effects in vivo; their intravenous transfer with TNP-labeled peritoneal exudate cells (TNP/PEC) results in sensitization to TNP instead of the tolerance typically displayed with TNP/PEC injected alone (23). Tcs cells are also required for the adoptive transfer of contact hypersensitivity, unless Ts cells in the recipient are temporally inactivated by CTX (20). This finding is similar to that of the present study; however in our system, to demonstrate contrasuppressor-independent adoptive transfer of DTH, suppression must be abrogated both in the recipient and in the transferred inoculum of cells.

A critical aspect of our observations concerning the evolution of Tcs cells in kdkd mice is that their activity correlates with expression of disease; contrasuppressor cells are demonstrable in nephritic mice but not in prenephritic mice nor in non–disease-prone CBA/Ca mice. Similar findings have been described (31) in the induced-immune response to type III pneumococcal polysaccharide. In this system, only immunogenic doses of type III pneumococcal polysaccharide activate Tcs cells; animals rendered tolerant or genetically unresponsive to this immunogen do not harbor detectable Tcs (31).

Phenotypic characterization of the Tcs in our model in part parallels that in other antigenic systems (26, 28–30). In models of induced immune responses, $Lyt-1^+$, 2^-, $Lyt-1^+$, 2^+, and $Lyt-1^-$, 2^+ T cells have been variably shown (22) to participate in the contrasuppressor network. The effector Tcs cells in kdkd mice appear to be $Lyt-2^+$, $L3T4^-$, T cells which bear I-J determinants and specifically bind to TBM antigens. Several investigators have described (22, 24) effector Tcs as $Lyt-1^+$, 2^- cells, in systems where the target cell of contrasuppression was an $Lyt-1^+$ cell. Phenotypic similarity between effector Tcs cells and their targets may reflect similar genetic restrictions. We have interpreted the directed binding to tubular antigen by our Tcs cells as being supportive of antigen specificity. More definitive data would be obtained by assessing contrasuppressor function with cells demonstrating DTH reactivity to other parenchymal antigens. We are not aware, however, of other self-antigens to which kdkd mice exhibit DTH reactivity. Others have found (30) that, although the inducer Tcs cells and their soluble factor (TcsF) are antigen specific, the soluble factor in the effector limb of the contrasuppressor network is antigen nonspecific.

Investigations (29) into the mechanism of contrasuppression have supported a model of competitive interaction between effector TcsF and TsF at the level of the helper cell. Helper cells preincubated with TcsF become resistant to suppression, whereas pretreatment with TsF results in resistance to contrasuppression. Coculture with TcsF and TsF together is reported (29) to result in net suppression. Our present studies and others (31) suggest that such resistance, generated in in vitro culture conditions, is not irreversible and can be overcome. That is, Tcs cells, Ts cells, and DTH-reactive cells all functionally coexist in the peripheral lymphoid organs of kdkd mice. Exposure of such DTH-reactive cells (or associated Th cells) to Tcs cells clearly does not preclude demonstrating functional suppression of these cells in vivo (Table VII). Likewise, despite the dominant net suppression seen in naive CBA/Ca mice, DTH-reactive cells in this population can respond after admixture of increasing numbers of Tcs cells. The

finding that effective contrasuppression of CBA/Ca spleen cells requires relatively more contrasuppressor cells than kdkd spleen cells do, suggests that the suppressor cell population is larger in CBA/Ca mice and that the net effect is critically dependent on relative numbers of interacting immunoregulatory T cells (Tables IX and X).

The broader significance of our findings will lie in their general applicability to autoimmune disease. The observed correlation between abnormal contrasuppression and disease activity is unlikely to be unique to tubulointerstitial nephritis; clearly effector cells reactive to other organ-specific antigens in non-diseased animals might induce disease under conditions of accentuated contrasuppression or diminished suppression. Earlier work (32) in a model of autoimmunity induced by polyclonal B cell activators, for example, has correlated autoantibody production with abnormal contrasuppression.

A major question raised by the present work is the nature of the genetic defect, transmitted as an autosomal recessive trait (9, 10), that leads to expansion of the contrasuppressor population. Recent experiments have described the regulation of Tcs cell activation by Ts cells (level 2 suppressors). Thymic migration of the level 2 suppressors probably occurs subsequent to that of Tcs lymphocytes (33), perhaps explaining enhanced autoimmunity after neonatal thymectomy in some models (32). The antigen specificity of such level 2 suppressors is undefined; they may be nonspecific in function. A genetically determined defect in contrasuppressor regulation on chromosome X (9) may well underlie the hereditary tubulointerstitial nephritis of kdkd mice.

Summary

We have used the murine model of spontaneous autoimmune interstitial nephritis in kdkd mice to examine the importance of abnormal immunoregulation in the expression of disease. T cells from naive congenic CBA/Ca mice suppress both histologic renal injury in the kdkd strain as well as the DTH reactivity to CBA/Ca renal tubular antigens mediated by lymphocytes from nephritic kdkd mice. These antigen-specific suppressor T cells are $Lyt-2^+$, $L3T4^+$, $I-J^{k+}$, genetically dominant and $I-J^k$ restricted. Unfractionated spleen cells from young, prenephritic kdkd mice also demonstrate such suppressor function. Shortly preceding disease onset, however, net suppression is functionally bypassed by emergent contrasuppressor T cells. These regulatory cells are also $Lyt-2^+$ and $I-J^{k+}$, and adhere both to the Vicia Villosa lectin and CBA/Ca TBM. By admixing these contrasuppressor cells with spleen cells from non–disease-prone CBA/Ca mice we were able to demonstrate the presence of DTH-reactive and nephritogenic effector cells in the latter population. Such nephritogenic effector cells could also be simply demonstrated after depletion of the suppressor cells with anti-$I-J^k$ mAbs and complement. These findings support a role for contrasuppressor cells in the abrogation of tolerance to parenchymal self-antigens.

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