

# In Goodpasture's Disease, CD4<sup>+</sup> T Cells Escape Thymic Deletion and Are Reactive with the Autoantigen $\alpha$ 3(IV)NC1

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**Abstract.** Goodpasture's disease is characterized by rapidly progressive glomerulonephritis and pulmonary hemorrhage, in association with circulating and deposited anti-glomerular basement membrane antibodies that recognize the  $\alpha$ 3 chain of type IV collagen [ $\alpha$ 3(IV)NC1] (known as the Goodpasture antigen). Unlike many other autoimmune diseases, recurrences are rare. In experimental models and human studies, both humoral and cellular mechanisms have been demonstrated to be involved in disease pathogenesis. However, there are few data on the characteristics of the autoreactive T cells or the mechanisms of tolerance to the autoantigen in human patients. It was demonstrated, using immunohistochemical analyses and reverse transcription-PCR, that the Goodpasture antigen is

expressed in normal human thymus. Using limiting dilution analyses, the frequencies of circulating autoreactive T cells in patients and control subjects were assessed. During acute disease, there were increased frequencies of CD4<sup>+</sup> T cells reactive with  $\alpha$ 3(IV)NC1 (ranging from 1:6300 to 1:65,000), which decreased with time. There was a significant difference between patients during their acute disease phase and control subjects with respect to the frequency index for  $\alpha$ 3(IV)NC1-specific CD4<sup>+</sup> T cells ( $P < 0.05$ , Mann Whitney  $U$  test). The decrease in autoreactive CD4<sup>+</sup> T-cell numbers during recovery may be the reason why recurrences are infrequent and may explain the loss of pathogenic autoantibodies with time, because of a lack of T-cell help.

Goodpasture's disease is characterized by rapidly progressive glomerulonephritis, often accompanied by pulmonary hemorrhage. It is associated with circulating glomerular basement membrane (GBM)-specific antibodies, which deposit in a linear manner along the GBM and give rise to the characteristic renal biopsy findings. The autoantigen has been characterized as the  $\alpha$ 3 chain of type IV collagen [ $\alpha$ 3(IV)NC1] (1,2), which has limited tissue distribution (3) and to which the autoantibody response is highly restricted (4). Unlike other autoimmune conditions, the disease rarely exhibits a relapsing-remitting course (5). Without treatment, autoantibody levels decrease to normal in a period of 2 to 3 yr (6).

Both humoral and cellular immune responses are implicated in disease pathogenesis. Several lines of evidence suggest the pathogenicity of antibodies. First, passive transfer of anti-GBM antibodies induces nephritis in monkeys, sheep, and rodents (7–9). Second, antibody levels are broadly correlated with disease activity in clinical studies (10,11). Third, disease recurrence has been documented when renal transplantation has been undertaken in the presence of circulating antibodies (11).

However, data from experimental models indicate that cell-mediated immunity is both necessary (12) and sufficient (13) to induce disease and that anti-T-cell therapy is effective in preventing disease (14). T cells are implicated in the pathogenesis of human disease by the presence of intraglomerular T cells in renal biopsy samples (15,16) and by the strong association of the disease with the HLA class II alleles DR15 and DR4 (17). Furthermore, the class-switched autoantibodies (predominantly IgG1 and IgG4) suggest that T-cell help is required for disease initiation (18). There are few published data on the nature of the autoreactive T cells. Our group previously reported proliferation of peripheral blood mononuclear cells (PBMC) from patients with Goodpasture's disease in response to affinity-purified human GBM. However, one-half of the control subjects also exhibited some proliferation in response to GBM (19). Another group generated GBM-specific T-cell clones from patients; however, the clones were CD8<sup>+</sup> and HLA class I-restricted, making them less relevant to the study of the T cells involved in human disease (20).

Recent data indicated that a number of autoantigens, including "sequestered antigens" and those with restricted tissue expression (such as myelin basic protein, glutamic acid decarboxylase, and insulin), are expressed in human thymic tissue (21). These findings suggested that central deletion of autoreactive T cells contributed to the T-cell tolerance to tissue-specific autoantigens. They also suggested that autoreactive T cells that escaped this selection would be of low avidity and perhaps regulated in health by peripheral mechanisms of tolerance (22). Alternatively, thymic dysfunction in presenting these antigens might lead to loss of

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central tolerance. We sought the presence of the Goodpasture antigen (GA) in human thymic tissue using immunohistochemical analysis with a monoclonal antibody (mAb) directed against the GA and using reverse transcription (RT)-PCR with  $\alpha 3(IV)NC1$ -specific primers. We proceeded to measure the frequency of autoreactive T cells directed against  $\alpha 3(IV)NC1$  in patients at different disease time points and in control subjects. Our results suggest that central tolerance may operate to eliminate autoreactive T cells against the GA but some CD4<sup>+</sup> T cells do escape this regulation and are observed at an elevated frequency in acute disease.

## Materials and Methods

### *Expression of GA in Human Thymus*

Human thymic tissue was obtained from children (age, 1 wk to 13 mo) undergoing routine cardiac surgery and was immediately snap-frozen in liquid nitrogen. Sections were cut and maintained at  $-20^{\circ}\text{C}$ . Staining was performed using the P1 mAb directed against  $\alpha 3(IV)NC1$ , which was produced as described previously (23). The second-layer antibody was rabbit anti-mouse Ig (Dako, Bucks, UK), and detection was performed using the avidin-biotin complex immunoperoxidase technique (Dako). Briefly, the sections were incubated with the primary antibody at  $4^{\circ}\text{C}$  overnight, followed by incubation with the second-layer antibody at room temperature for 1 h; development was performed with the avidin-biotin complex counterstained with hematoxylin. Negative control experiments were performed with isotype-matched mAb. The positive control was a section of renal tissue from a patient with Goodpasture's disease.

### *RT-PCR for $\alpha 3(IV)NC1$*

Whole thymus was homogenized, and RNA was extracted using RNazol B (Biogenesis, Poole, Dorset, UK). Thymic explants were used to obtain epithelial cell cultures, from which RNA was also extracted. Briefly, thymic fragments were treated with collagenase (Sigma, Poole, Dorset, UK) and plated onto collagen-coated tissue culture flasks. Epithelial cell purity was confirmed with cytokeratin staining.

Five micrograms of total RNA was reverse-transcribed with Superscript II (Gibco-BRL, Paisley, Scotland), using oligo(dT) (Gibco-BRL) as a primer. For amplification with a set of  $\alpha 3(IV)NC1$ -specific primers, 5% of the reaction product was used. Primers were as follows: forward, GCCCGATCCATGGGATTGCCAGGTTTG; reverse, TCCCCTCGAGCCTCGAGTTCTGCTGTCT. The following PCR conditions were used: 1 min at  $94^{\circ}\text{C}$ , 1 min at  $60^{\circ}\text{C}$ , and 1 min at  $72^{\circ}\text{C}$  for 35 cycles. A negative control experiment was performed with no reverse transcriptase in the reaction mixture.

### *Antigen Production*

Recombinant  $\alpha 3(IV)NC1$  was produced in COS-7 cells by using DEAE-dextran-mediated transfection, according to previously published methods (24,25). The  $\alpha 3(IV)NC1$  plasmid DNA was cloned into a pFLAG CMV-1 expression system (Kodak, Rochester, NY), and the secreted material was purified in a single affinity step on an anti-FLAG-agarose column. Briefly, cells were grown to subconfluence in Dulbecco's modified Eagle's medium (Gibco-BRL) with 5% fetal calf serum (MBM, Bourne End, UK) and penicillin/streptomycin (Gibco-BRL). Twenty micrograms of  $\alpha 3(IV)NC1$  plasmid DNA in phosphate-buffered saline (PBS) and 5% DEAE-dextran (Pharmacia, Uppsala, Sweden) were added to the cells for 30 min. Whole medium containing 80  $\mu\text{M}$  chloroquine (Sigma) was then added for an addi-

tional 2.5 h. After this, the supernatant was washed off and medium containing 10% DMSO was added for 2.5 min, aspirated, and replaced with fresh medium. Medium was collected every 72 h for a total of 10 d. The supernatant material was passed over a column containing anti-FLAG antibody, eluted using glycine-HCl (pH 3.5), neutralized, and dialyzed with PBS.

Collagenase-solubilized GBM (CS-GBM) was produced as described previously, by sieving cadaveric human kidneys obtained at autopsy and isolating glomeruli (26). These were disrupted by sonication and digested with collagenase I (Sigma). Tetanus toxoid was purchased from Evans Medical (Leatherhead, UK).

### *Limiting Dilution Analyses*

**Patients and Control Subjects.** Patients presenting with histologically and serologically confirmed Goodpasture's disease underwent venesection as soon as the diagnosis was confirmed and before treatment, if possible. However, only one patient (patient 3) had not received any immunosuppressive therapy at the time of venesection. All other acutely presenting patients had begun their treatment regimens within the previous 2 wk. The study received local research ethics committee approval.

Presentation of these patients was with acute renal failure in all cases and with pulmonary hemorrhage in two cases. All except two of the patients survived and renal recovery occurred for six patients, although three experienced residual renal impairment. Treatment varied according to the clinical situation but usually consisted of daily oral administration of prednisolone (starting at 60 mg and decreasing by 10 to 15 mg/wk until a dose of 20 mg was reached, after which decrements were of 2.5 mg/fortnight), daily oral administration of cyclophosphamide (2 mg/kg), and daily or near-daily plasma exchange (4-L exchanges for 14 d). For one patient (patient 1), additional pulsed methylprednisolone therapy (three daily pulses of 0.5 g) had been administered at the referring hospital. One patient (patient 3) who was dialysis-dependent at the time of presentation was not initially undergoing immunosuppressive therapy but subsequently developed pulmonary hemorrhage and received conventional treatment as described above. Immunosuppressive therapy was withdrawn during a period of 6 mo after presentation. Cyclophosphamide was withdrawn at 2 mo, and oral steroid treatment was discontinued by 6 mo for all except three patients. These three patients continued to receive low doses of steroids as follows: at 12 mo, to treat continued nephrotic-range proteinuria (patient 1, 15 mg of prednisolone); after delayed pulmonary hemorrhage (patient 3, 15 mg of prednisolone); and at 10 yr, to treat concurrent myasthenia gravis (patient 6, 5 mg of prednisolone). The patient characteristics, treatments, and outcomes are summarized in Table 1.

PBMC were isolated (see below) and stored in liquid nitrogen until required. Patients underwent tissue-typing using conventional DNA methods. Cells were also obtained from a number of patients after the acute disease, in some cases as long as 10 yr after the initial presentation. Control samples were from healthy volunteers.

**Cell Isolation and Enrichment.** PBMC were obtained by separating whole blood using Lymphoprep (Nycomed, Oslo, Norway). CD4<sup>+</sup> T cells and antigen-presenting cells (APC) were purified by immunomagnetic depletion using Dynabeads (DynaL, Oslo, Norway). PBMC were incubated with primary antibody for 45 min at  $4^{\circ}\text{C}$  on a roller and then washed twice in PBS. Anti-mouse Ig-coupled Dynabeads were washed in medium [RPMI 1640 medium (ICN Pharmaceuticals, Thame, UK) with 10% human AB serum] and added, according to the recommendations of the manufacturer, for two 45-min periods at  $4^{\circ}\text{C}$ , on a roller. Bound cells were removed after

Table 1. Characteristics of patients and their immunosuppressive treatment at the time of T cell isolation<sup>a</sup>

Patient No.	Clinical Features	Antibody Titer (%) <sup>b</sup>	Creatinine Concentration ( $\mu$ M)	Cells	Treatment when Cells Were Obtained	Duration of Treatment (d)	Outcome	Survival (mo)
Acute								
1	ARF, PH	18	390	NF	MP, PEX, CYC, OP	7	R	A (28)
2	ARF	45	DD	F	PEX, OP, CYC	10	DD	D (135)
3	ARF	80	DD	NF	None	7	DD	A (21)
4	ARF	60	DD	F	PEX, OP, CYC	2	DD	D (18)
Convalescence								
1	None	0	150	NF	OP	360	CRF	A (28)
3	PH	0	DD	NF	OP	230	DD	A (21)
5	None	0	174	NF	None		CRF	A (24)
6	None	0	400	NF	OP	(10 yr)	CRF	A (160)
7	None	0	132	NF	None		R	A (125)
8	None	0	125	NF	None		R	A (120)

<sup>a</sup> ARF, acute renal failure; PH, pulmonary hemorrhage; DD, dialysis-dependent; MP, pulsed methylprednisolone; OP, orally administered prednisolone; CYC, orally administered cyclophosphamide; PEX, plasma exchange; R, recovery; CRF, chronic renal failure; F, frozen; NF, not frozen; A, alive; D, died.

<sup>b</sup> Normal range, <15%.

passage over a magnet, and the remaining cells were washed in whole medium. APC were obtained by depleting PBMC of T cells with anti-CD4 and anti-CD8 mAb (Serotec, Oxford, UK). CD4<sup>+</sup> T cells were obtained by first performing a PBMC-adherence step at 37°C and then incubating the nonadherent cells with anti-CD33, -CD19, -CD16, -CD14, -CD56, and -CD8 mAb (all from Serotec), to deplete B cells, monocytes, macrophages, dendritic cells, natural killer cells, and CD8<sup>+</sup> T cells, respectively. Cell purity was confirmed by staining with anti-CD4/CD8 and anti-CD3/DR mAb (Becton Dickinson, Mountain View, CA), and flow cytometry was performed with an EPICS XL flow cytometer (Coulter Electronics, Luton, UK).

**T-Cell Proliferation.** APC were pulsed overnight at 37°C with medium alone, recombinant  $\alpha$ 3(IV)NC1 at 5  $\mu$ g/ml, CS-GBM at 20  $\mu$ g/ml, or tetanus toxoid (Evans Medical) at 1:1000 dilution. After being washed in PBS, the cells were plated in complete medium [RPMI 1640 medium containing 2 mM L-glutamine, 50  $\mu$ g/ml penicillin, and 50  $\mu$ g/ml streptomycin (Gibco-BRL), with 10% human AB serum] in 96-well, round-bottomed plates (Nunc, Roskilde, Denmark). CD4<sup>+</sup> T cells were maintained at 4°C overnight, counted, and plated in doubling dilutions into the 96-well plates; 24 replicates were plated for each T-cell dilution. The final 24 wells contained only APC and served as negative controls. After 6 d, the wells were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine for the last 16 h of incubation. Plates were collected and counted in a  $\beta$ -counter.

**Calculation of Frequencies and Antigen/Autologous Mixed Lymphocyte Reaction Ratios.** The frequencies of CD4<sup>+</sup> T cells reacting to each APC preparation were calculated using the modified score function of the maximal likelihood method, as described previously (27). Calculations were performed using a newtonian iterative method with Microsoft Excel version 5 (Microsoft, Redmond, WA). Only results with *P* values of >0.05 ( $\chi^2$  test) were used for further analysis. Because incubation of CD4<sup>+</sup> T cells with APC in the absence of antigen produces a proliferative response, known as the autologous mixed lymphocyte reaction (AMLR), all frequencies were then expressed as the ratio of the antigen-specific response to the AMLR; this ratio was termed the frequency index. Derivation of the

frequency index is indicated by the following equation: frequency index = frequency of CD4<sup>+</sup> cells reacting to antigen-pulsed APC/frequency of CD4<sup>+</sup> cells reacting to unpulsed APC. Assays were highly reproducible within subjects with respect to T-cell frequencies, whether fresh or frozen cells were used.

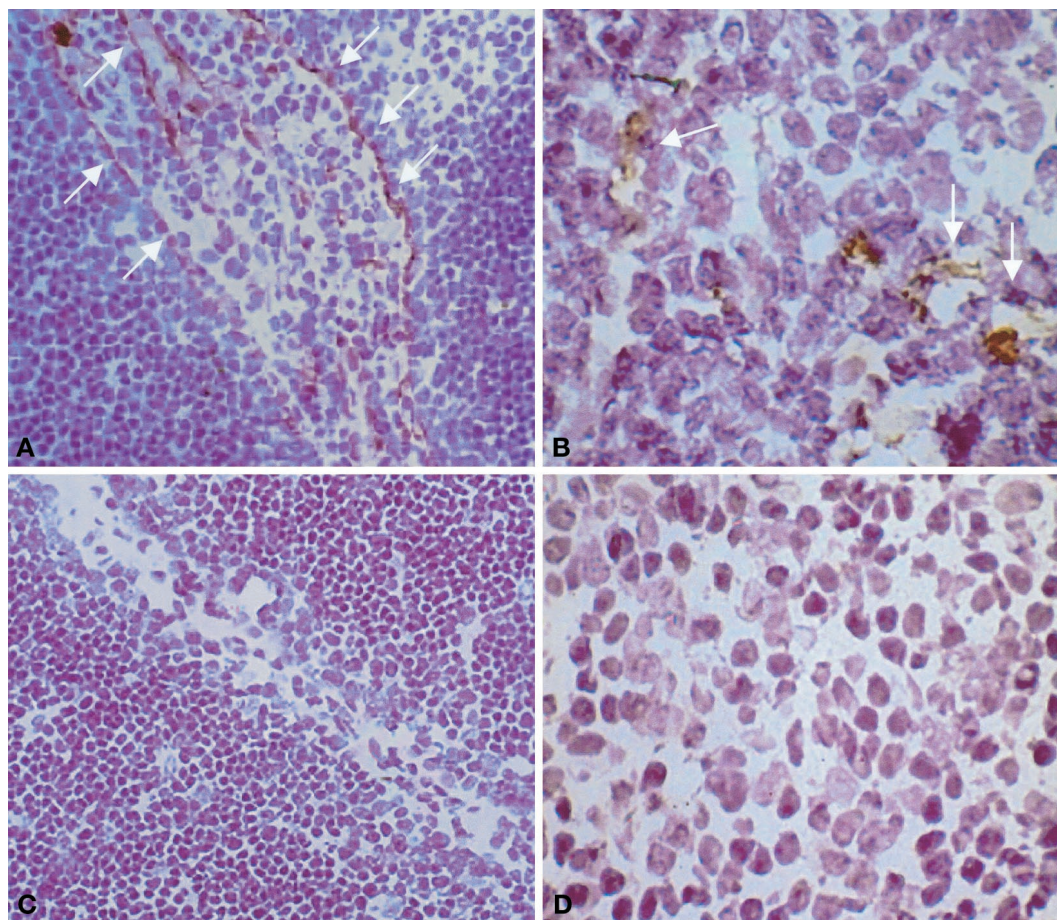
## Results

### *The GA Is Expressed in Normal Human Thymus*

Samples of human thymus were obtained from children undergoing cardiothoracic surgery and were analyzed for the presence of the GA by immunohistochemical analyses and RT-PCR. Thymic sections stained with mAb P1 exhibited positive staining, confirming that the GA is expressed in the thymus (Figure 1, A and B). Intense staining was observed in the perivascular space throughout the thymus, delineating vascular structures. Furthermore, numerous discrete areas of staining, which appeared to represent isolated thymic cells, were observed throughout the medulla (Figure 1B). No cortical staining was observed in any section. Thymic sections stained with isotype-matched (IgG1) control Ig exhibited no staining in the cortex or medulla (Figure 1, C and D). Sections of renal tissue from patients with Goodpasture's disease demonstrated classic linear staining along the GBM (data not shown), as described previously (3).

Because negative selection is thought to occur as a result of antigen presentation to developing thymocytes by thymic epithelial cells and thymic dendritic cells (28), we sought to define whether these cells were also capable of synthesizing the GA. RNA was extracted from whole thymus and from isolated thymic epithelial cells grown as thymic explants. The latter were confirmed to be totally pure epithelial cell cultures by positive staining with cytokeratin (100% of cells positive for cytokeratin). In RT-PCR with  $\alpha$ 3(IV)NC1-specific primers, a





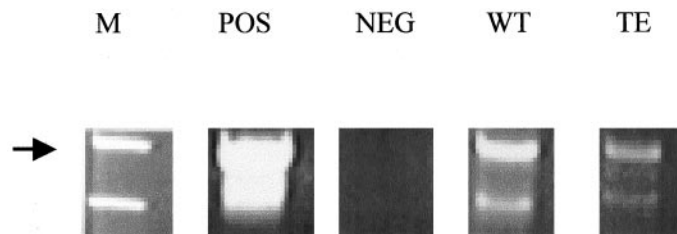
**Figure 1.** (A) Immunohistochemical analysis of human thymic tissue stained with monoclonal antibody (mAb) P1, showing staining in the perivascular spaces throughout the thymus, delineating vascular structures (white arrows). (B) High-power view of the thymic medulla stained with mAb P1, revealing numerous discrete areas of staining throughout the medulla, suggesting isolated thymic epithelial cells (white arrows). (C) Control section of thymus stained with an isotype-matched (IgG1) control antibody, revealing no positive staining. (D) High-power view of the thymic medulla stained with an isotype-matched (IgG1) control antibody, revealing no positive staining. Magnifications:  $\times 200$  in A and C;  $\times 400$  in B and D.

clear band was amplified from both whole-thymus RNA and thymic epithelial cell RNA (Figure 2). The positive control sample was  $\alpha 3(\text{IV})\text{NC1}$  cDNA. The DNA products were confirmed to be  $\alpha 3(\text{IV})\text{NC1}$  by their exhibiting a pattern of bands identical to that of control  $\alpha 3(\text{IV})\text{NC1}$  cDNA after digestion with the appropriate restriction enzymes.

#### *Frequencies of GA-Specific T Cells Are Elevated in Acute Disease and Decrease with Time*

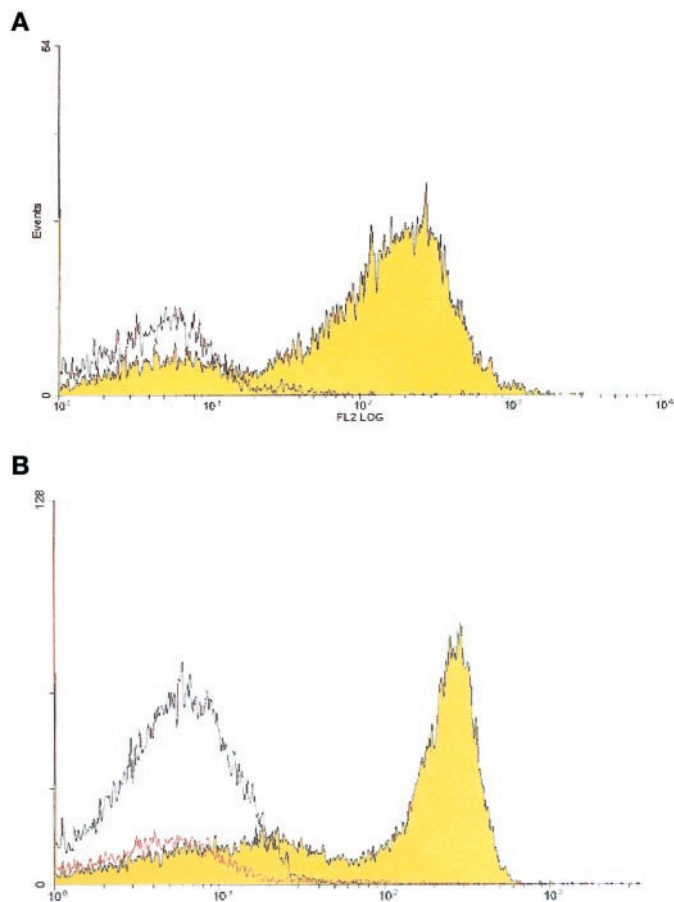
All patients and one-half of the healthy control subjects expressed DR15 and/or DR4 HLA class II alleles, with which the disease is strongly associated (17). Three patients were prospectively monitored for up to 1 yr. Cell isolation and enrichment from blood samples or frozen PBMC were successfully performed in all cases. After cell purification, flow cytometry demonstrated that CD4<sup>+</sup> cells represented 88 to 95% of all CD3<sup>+</sup> cells. APC were enriched such that the majority of the cells were positive for DR antigens, with <10% of cells staining for CD3 (Figure 3).

The frequencies of T cells proliferating in response to CS-



**Figure 2.** Reverse transcription-PCR analyses of human thymic epithelial cells (TE) and whole thymus (WT), with negative control samples (NEG) and positive control samples (POS) consisting of  $\alpha 3$  chain of type IV collagen [ $\alpha 3(\text{IV})\text{NC1}$ ] cDNA, after 35 cycles of amplification. M, DNA ladder markers. Arrow, 721-bp band.

GBM, recombinant  $\alpha 3(\text{IV})\text{NC1}$ , tetanus toxoid, and autologous APC alone were calculated (as summarized in Table 2). The frequencies for CS-GBM varied from 1:10,000 to 1:123,000 and those for recombinant  $\alpha 3(\text{IV})\text{NC1}$  varied from 1:6289 to 1:65,359 during acute disease. However, the AMLR also varied considerably in both control subjects and patients



**Figure 3.** (a) Flow cytometric histogram of antigen-presenting cells, showing negative control cells (solid line) and HLA-DR-positive cells (filled profile). (b) Flow cytometric histogram of responder T cells, showing negative control cells (solid line, upper profile), CD8<sup>+</sup> cells (solid line, lower profile), and CD4<sup>+</sup> cells (filled profile).

(from 1:8475 to 1:79,000 and from 1:9346 to 1:159,000, respectively). Therefore, to enable more meaningful comparisons between assays and among individuals, an antigen-specific frequency/AMLR frequency ratio was calculated and termed the frequency index (Figure 4). All samples tested exhibited a measurable AMLR, and there was no significant difference in the AMLR between patients and control subjects. Not all patients exhibited a measurable frequency for tetanus toxoid, whereas all control subjects did. For patients, the CS-GBM frequency index varied from 1.26 to 3.96. There was a similar CS-GBM frequency index range for control subjects. There was, however, a significant difference between patients during their acute disease period and control subjects with respect to the frequency index for  $\alpha 3(\text{IV})\text{NC1}$ -specific CD4<sup>+</sup> T cells ( $P < 0.05$ , Mann-Whitney  $U$  test). All patients tested with  $\alpha 3(\text{IV})\text{NC1}$  exhibited a frequency index of  $>1$ , whereas the control subjects did not.

For the patients who were tested during the acute disease phase and during convalescence, up to 12 mo later (patients 1 and 3), there were decreases in the frequency indices for both CS-GBM and  $\alpha 3(\text{IV})\text{NC1}$ . The frequency index for tetanus toxoid for

patient 1 increased during the same period. For one of the convalescent patients (patient 5), the frequency for CS-GBM was low but the frequency for  $\alpha 3(\text{IV})\text{NC1}$  was elevated, despite the fact that the patient was disease-free and without circulating anti-GBM antibodies. Patients who had experienced disease  $>10$  yr earlier (patients 6, 7, and 8) exhibited no detectable frequencies for CS-GBM or  $\alpha 3(\text{IV})\text{NC1}$ . Comparison of  $\alpha 3(\text{IV})\text{NC1}$ -specific frequency indices at different disease time points demonstrated a trend for reduction in  $\alpha 3(\text{IV})\text{NC1}$ -specific T-cell frequencies with time (Figure 5). These differences did not reach statistical significance, however, because of the small numbers of patients evaluated at each time point.

## Discussion

Three novel observations are described here. First, significantly elevated frequencies of CD4<sup>+</sup> T cells specific for  $\alpha 3(\text{IV})\text{NC1}$  were detected in patients at the time of acute Goodpasture's disease. Second, the frequencies of autoreactive T cells steadily decreased with time. Third, expression of  $\alpha 3(\text{IV})\text{NC1}$  was clearly detectable, by immunocytochemical and PCR analyses, in the normal human thymus.

The autoantigen specificity of B cells in this disease is well established and is highly conserved among patients (4,23). In contrast, definition of the antigen specificity of autoreactive T cells has been elusive. This has led to uncertainty regarding whether the determinants recognized by T cells are derived from the  $\alpha 3(\text{IV})\text{NC1}$  domain or whether they are located on another protein that is internalized with  $\alpha 3(\text{IV})\text{NC1}$  by antigen-specific B cells. The results described here suggest that this molecule is a relevant autoantigen in Goodpasture's disease. Clearly, these findings do not exclude the possibility of there being T-cell epitopes in other elements of the GBM. However, the demonstration of increased frequencies of T cells that are reactive with this molecule justifies further investigation of how the antigen is processed and presented, particularly by the DR alleles that are associated with disease susceptibility.

Although significantly increased frequencies for  $\alpha 3(\text{IV})\text{NC1}$  were detected during acute disease, in comparison with control values, the absolute frequencies were low. Similar frequencies have been reported for autoreactive T cells in other autoimmune diseases (29). These frequencies can be contrasted with the frequencies measured after immunization against typical exogenous antigens, such as tetanus toxoid, which usually range from 1:1000 to 10,000 (30). There are several possible explanations for the small numbers of T cells detectable in peripheral blood. One possibility is that most of the autoreactive T cells are sequestered in the diseased organs and the draining lymph nodes. This possibility is difficult to address in patients. However, in mouse models of allograft rejection, it seems that reactivity in circulating T cells reflects events in the graft itself (31,32).

A second possibility, which is favored by our data, is that most autoantigen-specific T cells are deleted in the thymus. It was recently demonstrated that a number of autoantigens are expressed in the thymus, and it is becoming clear that the thymus may present a vast array of self-proteins, including those that are sequestered or have limited tissue distribution

**Table 2.** Frequencies of CD4<sup>+</sup> T cells reacting in the AMLR and in response to CS-GBM,  $\alpha 3(\text{IV})\text{NC1}$ , and tetanus toxoid and calculated frequency indices for CS-GBM-,  $\alpha 3(\text{IV})\text{NC1}$ -, and tetanus toxoid-specific T cells in patients and control subjects<sup>a</sup>

Patient No.	1/Frequency For AMLR	1/Frequency For CS-GBM	CS-GBM-FI	1/Frequency For $\alpha 3(\text{IV})\text{NC1}$	$\alpha 3(\text{IV})\text{NC1}$ -FI	1/Frequency For TT	TT-FI
<b>Acute</b>							
1	39,370	9,950	3.96	ND	ND	17,492	2.25
2	159,489	93,897	1.70	65,359	2.44	70,423	2.26
3	156,006	123,625	1.26	58,997	2.64	ND	ND
4	9,346	4,785	1.95	6,289	1.49	9,217	1.01
<b>Convalescent, &lt;1 yr</b>							
1	31,847	12,837	2.48	16,340	1.95	1,942	16.4
3	6,640	ND	ND	5,782	1.15	ND	ND
5	10,972	8,703	1.26	5,298	2.07	ND	ND
<b>Convalescent, 10 yr</b>							
6	12,531	24,096	0.52	15,873	0.79	14,535	0.86
7	26,316	22,124	1.19	23,202	1.13	21,277	1.24
8	62,993	105,794	0.59	ND	ND	ND	ND
<b>Control</b>							
1	82,497	58,384	1.41	123,780	0.67	7,601	10.9
2	12,272	6,602	1.85	14,714	0.83	2,724	4.5
3	36,653	35,146	1.04	36,653	1.0	4,587	8.0
4	56,180	23,697	2.37	37,594	1.49	ND	ND
5	21,298	ND	ND	16,495	1.29	2,651	8.03
6	8,479	2,686	3.16	6,934	1.22	2,438	3.48
7	42,478	31,249	1.35	ND	ND	6,622	6.4
8	68,475	37,115	1.84	ND	ND	11,120	6.16
9	29,700	77,038	0.38	ND	ND	9,284	3.05

<sup>a</sup> AMLR, autologous mixed lymphocyte reaction; CS-GBM, collagenase-solubilized glomerular basement membrane;  $\alpha 3(\text{IV})\text{NC1}$ ,  $\alpha 3$  chain of type IV collagen; FI, frequency index; TT, tetanus toxoid; ND, not done.

(21,33–35). Levels of expression of these autoantigens were recently demonstrated to be inversely correlated with a predisposition to develop autoimmune diseases (33,36,37). Therefore, susceptibility to autoimmunity may be partly related to a capacity to centrally delete autoreactive T cells (38,39), and this capacity may be related to the level of thymic autoantigen expression. Despite the process of negative selection, there is evidence that, in certain autoimmune diseases, self-reactive T cells escape thymic deletion through low-avidity interactions with thymic cells expressing the autoantigen (22).

We have demonstrated, at the mRNA and protein levels, that the autoantigen is expressed in the thymus, specifically in thymic epithelial cells. These cells are known to be important in antigen presentation to developing thymocytes during negative selection (28). Therefore, an antigen with such distribution would be expected to be shed or packaged (in exosomes) and taken up by neighboring cells to be processed and presented in association with class II MHC on the cell surface. This process would enable central deletion of high-avidity autoreactive T cells during development of the immune repertoire (40,41). We have not been able to compare the levels of thymic GA expression in patients and control subjects, and there remains the possibility that patients may be deficient in this expression and may thus fail to delete autoreactive T cells.

The T-cell frequencies for CS-GBM, which contains many different potential antigens, were the same for patients and control subjects. This finding is reminiscent of the data from proliferation assays that we reported previously; proliferation in response to affinity-purified GBM preparations was observed in 50% of healthy control subjects (19). It is also in keeping with other reports of autoreactive cells in healthy individuals (42). However, there was a significantly increased frequency of T cells reactive with the specific autoantigen  $\alpha 3(\text{IV})\text{NC1}$  in patients at presentation, compared with control subjects. The reduction in  $\alpha 3(\text{IV})\text{NC1}$ -specific T-cell frequencies among patients with time is not simply attributable to concurrent immunosuppressive treatment, because the highest T-cell frequencies were observed for patients undergoing heavy immunosuppressive regimens and lower frequencies were observed for patients receiving minimal or no immunosuppressive therapy and control subjects. Furthermore, for patient 1, the frequency index for tetanus toxoid was markedly elevated during convalescence, compared with presentation, whereas the frequency index for  $\alpha 3(\text{IV})\text{NC1}$  decreased during the same period. Finally, the T-cell frequencies for recombinant  $\alpha 3(\text{IV})\text{NC1}$  are not simply related to the expression system used to generate the antigen, because others have demonstrated that it is only the specific  $\alpha 3$  (and  $\alpha 4$ ) chains generated



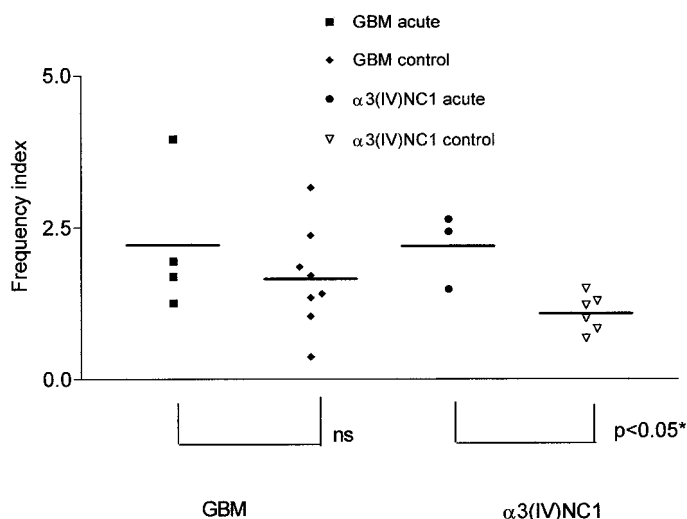


Figure 4. Graph comparing the frequency indices for collagenase-solubilized glomerular basement membrane (GBM) and  $\alpha 3(\text{IV})\text{NC1}$  for patients with acute disease and control subjects. Each point represents one subject tested. Bars show the mean ratio for each group. There was a significant difference between patients and control subjects only with respect to  $\alpha 3(\text{IV})\text{NC1}$  ( $P < 0.05$ , Mann Whitney- $U$  test).

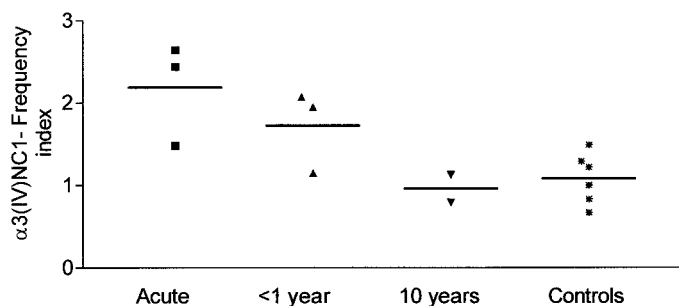


Figure 5. Graph showing the differences in  $\alpha 3(\text{IV})\text{NC1}$  frequency indices for patients at presentation (Acute) and 12 mo and 10 yr after the initial presentation, compared with control subjects. There seems to be a decrease in the frequencies of autoreactive  $\alpha 3(\text{IV})\text{NC1}$ -specific T cells with time after disease presentation.

in such an expression system that are capable of inducing disease in experimental models (43–45). Furthermore, control subjects exhibited no detectable frequencies for recombinant  $\alpha 3(\text{IV})\text{NC1}$ .

When these data are considered together, it seems that, despite the presence of the GA in the thymus, autoreactive T cells do escape thymic deletion in patients and are observed at increased frequency during periods of active disease. With time, these cells decrease in number, possibly as a result of regulation or deletion (39). This may in part explain the lack of disease recurrence and the decrease in antibody levels with time. Interestingly, our patients all received immunosuppressive therapy, which may have been sufficient to regulate T-cell autoreactivity. Disease recurrence has been observed, albeit rarely (5,46). It is tempting to speculate that, for these patients experiencing relapses, there was poor long-term regulation of autoreactive T cells and thus, after

immunosuppressant withdrawal or because of other triggers, disease recurred. One such patient, who had successfully undergone transplantation, experienced relapse only after withdrawal of immunosuppressive therapy (46). Another patient experienced relapse when his immunosuppressive therapy was reduced in association with continued smoking (5). Indeed, for one of our current patients (patient 5), the frequency of  $\alpha 3(\text{IV})\text{NC1}$ -autoreactive T cells has remained high 1 yr after presentation, despite the fact that the patient is free of disease, with undetectable autoantibody levels. Longer follow-up periods will allow us to monitor whether the patient experiences relapse or exhibits a decrease in the frequency of autoreactive T cells. Further investigations into the mechanisms underlying the loss of regulation of  $\alpha 3(\text{IV})\text{NC1}$ -specific autoreactive T cells, their epitope specificity, and the basis of HLA-DR-linked disease susceptibility are required before the pathogenesis of this disease is sufficiently well understood for the design of antigen-specific immunotherapy.

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