The Fine Specificity and Cytokine Profile of T-Helper Cells Responsive to the α3 Chain of Type IV Collagen in Goodpasture’s Disease

LINDSAY S. CAIRNS,* RICHARD G. PHELPS,† LAURA BOWIE,* ANDREW M. HALL,* WALAA W.M. SAWEIRS,† ANDREW J. REES,* and ROBERT N. BARKER*

*Department of Medicine and Therapeutics, University of Aberdeen, Aberdeen, United Kingdom; and
†Department of Clinical and Surgical Sciences (Internal Medicine), University of Edinburgh, Edinburgh, United Kingdom

Abstract. Goodpasture’s disease is a severe nephritis characterized by autoantibodies to the α3 chain of type IV collagen, α3(IV)NC1, in the glomerular basement membrane. The disease is very strongly associated with HLA-DR15, the affinities of α3(IV)NC1 peptides for DR15 are known, and elution experiments have identified major naturally processed sequences. Here, the fine specificity and cytokine profile of α3(IV)NC1-reactive T cells from patients with Goodpasture’s disease is defined. Peripheral blood mononuclear cells from patients at diagnosis proliferated in response to significantly more peptides (χ² = 8.6, P = 0.004) from a panel spanning the sequence of α3(IV)NC1 than did those from control DR15-positive donors and were highly focused (P = 0.0002, binomial distribution) on two peptides, α331-90 and α3131-150. Some peptides induced interferon-γ, but none induced IL-4.

Resolution of disease was accompanied by a striking deviation of the responses from proliferation to secretion of the T-regulatory cytokine IL-10, and addition of neutralizing antibody confirmed that such IL-10 production was suppressive. The affinity of the peptides for DR15 molecules was positively correlated (χ² = 14.6, P = 0.00067) with the ability to elicit proliferation. However, unlike foreign antigens, this hierarchy is not due to responses against the major naturally processed peptides, which rarely stimulated proliferation and which have only intermediate affinity for DR15 molecules. It is inferred that the helper response to α3(IV)NC1 in Goodpasture’s disease is dominated by epitopes that are normally inefficiently presented because of processing constraints.

Autoimmunity is thought to be important in the pathogenesis of most forms of glomerulonephritis, which together account for 25% of patients with end-stage renal failure. Current treatments for such autoimmune diseases are unsatisfactory, but the rational design of more effective approaches depends on understanding the mechanisms by which self-tolerance to autoantigens can be lost and restored. Although rare, Goodpasture’s disease provides a unique opportunity for these studies because it is the only glomerulonephritis in which the target of the autoimmune attack is known. Indeed, the Goodpasture antigen is one of the best defined autoantigens of pathologic relevance in any human autoimmune disease.

Goodpasture’s disease is characterized by pathogenic anti-glomerular basement membrane autoantibodies that are invariably specific for the COOH-terminal noncollagenous domain of the α3 chain of type IV collagen, α3(IV)NC1 (1–5). The pathogenesis of many antibody-mediated, organ-specific autoimmune conditions is dependent on T-helper (Th) cells, and Goodpasture’s disease seems to be no exception (6). Patients’ Th cells proliferate specifically with limited T-cell receptor Vβ gene usage when incubated with α3(IV)NC1 (7,8), indicating that this antigen also carries helper epitopes. The importance of Th cells is also suggested by the very strong association with HLA class II alleles: 85% of Caucasoid patients carry HLA-DRB1*1501, and there is a significant dominant-negative association with HLA-DRB1*07 (9). Evidence from experimentally induced Goodpasture’s disease demonstrates that Th cells can be important both for providing help to α3(IV)NC1-specific B cells and in modulating glomerular injury (10–12).

It is important to determine how pathogenic, autoreactive human Th cells escape the processes of deletion and anergy that contribute to self-tolerance. In animal models, autoggressive Th cells can survive such censorship if they recognize self-epitopes that are inefficiently presented as a result of low affinity for the restricting class II molecule (13) or processing constraints (14), but it is unclear whether similar mechanisms are important in human disease. Our previous work that analyzed the processing and presentation of α3(IV)NC1 (15–17) now enables us to address these issues in Goodpasture’s disease. The major naturally processed α3(IV)NC1 peptides that
are displayed bound to DR15 molecules have been identified directly after elution from antigen-pulsed, Epstein-Barr Virus-transformed human B cells (15,16). They comprise three nested sets of peptides each centered on a common core. Furthermore, the affinity of a complete panel of overlapping α3(IV)NC1 peptides for DR15 has been established (16,17). Together, these studies reveal that the selection of the three nested sets for presentation is determined by dominant processing constraints, not solely by affinity for class II molecules (16,17). For the first time, this makes it possible to determine the relationship between the fine specificity of Th cells from patients with an autoimmune disease and the hierarchy with which peptides from the target autoantigen are presented.

The pathogenicity of autoreactive Th cells is related not only to their specificity but also to the patterns of cytokines that they secrete (18). Many models of autoimmune disease are driven by helper responses that are strongly dominated by the Th1 subset and characterized by IFN-γ secretion, whereas inducing a corresponding Th2 bias associated with IL-4 production can prevent or ameliorate the pathology (6,14,19). Furthermore, regulatory Th cells producing IL-10 and/or TGF-β can protect rodents against a wide range of immune-mediated diseases (20) and mediate some forms of mucosal tolerance (21). Despite their relevance to therapy (6,20), virtually nothing is known about the contribution of different Th subsets to human autoimmune diseases such as glomerulonephritis. In Goodpasture’s disease, the pathogenic inflammatory response wanes after several months (22), even in the absence of immunosuppressive therapy, enabling autoantigen-specific Th cells to be studied during both the acute phase of autoimmune destruction and resolution.

The primary purpose of this study was to exploit the detailed knowledge of the presentation of α3(IV)NC1 (15–17) to determine whether T cells specific for the autoantigen of pathologic relevance in a human autoimmune disease recognize the most abundant naturally processed epitopes or those that are less efficiently presented. We therefore mapped the fine specificity of α3(IV)NC1-responsive Th cells from patients with Goodpasture’s disease. To determine the breadth of the responses and to avoid selection pressures inherent in cloning, we took advantage of techniques successfully developed for studying polyclonal T cells specific for other antigens (23–26). The Th subset of the responding cells was also identified at different stages of nephritis, by measuring the cytokines secreted. The results not only represent a first step in the development of specific therapy for glomerulonephritis but also elucidate the mechanisms underlying the loss of self-tolerance in human autoimmune disease.

**Materials and Methods**

**Patients and Donors**

Eight patients who had presented with rapidly progressive glomerulonephritis as a result of Goodpasture’s disease were studied (Table 1). Diagnosis was based on the detection of circulating antibodies specific for α3(IV)NC1 by ELISA and/or Western blotting and the use of indirect immunofluorescence to demonstrate a linear deposition of IgG along the glomerular basement membrane of frozen renal biopsy sections. Renal biopsies from each patient showed that all sampled glomeruli contained crescents, indicating that extensive capillary damage had occurred. All six patients who could be typed at DRB1 carried HLA DRB1*1501 alleles that are associated with susceptibility to Goodpasture’s disease (9). Initial blood samples were drawn from patients 1 to 6 at clinical presentation with acute disease; patients 2 to 5 had received no immunosuppressive therapy, and patient 1 had been given a short course of immunosuppressive treatment 4 mo before the first sample was obtained. Follow-up samples were taken from patients 1, 2, 4, and 6 at the time of dialysis, and blood was also drawn late in the course of disease from patients 7 and 8, who became available to the study 24 and 6 mo, respectively, after clinical presentation. Control blood samples were obtained by venipuncture from five HLA-DR15–positive, healthy donors. All blood was collected into preservative-free lithium heparin tubes and stored at room temperature before processing, which was completed within 24 h.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age at presentation and gender</th>
<th>DRB1 allele</th>
<th>Time (months) at which samples drawn relative to presentation</th>
<th>Duration of immunosuppressive treatment at first samplea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>First</td>
<td>Second</td>
</tr>
<tr>
<td>1</td>
<td>56 F</td>
<td>15, 14</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>55 M</td>
<td>15, 4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>73 M</td>
<td>ND</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>62 M</td>
<td>15, 15</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>54 F</td>
<td>15, 4</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>70 F</td>
<td>15, 11</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>7</td>
<td>82 M</td>
<td>ND</td>
<td>24</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>54 F</td>
<td>15, 7</td>
<td>6</td>
<td>–</td>
</tr>
</tbody>
</table>

a Cyclophosphamide, prednisolone, and plasma exchange.

b Abridged treatment because of toxicity and poor renal outlook.

c No immunosuppressive treatment given at any time.
Antigens and Mitogens

A panel of 22 peptides was synthesized to span the sequence of α3(IV)NC1 (1–5), based on a series of 20-mers with 10 amino acid overlaps (Table 2). The composition of the peptides was confirmed by mass spectrometry (16). For stimulation assays, the peptides were added to cultures at 20 μg/ml, as in other studies (23, 24). Reombinant α3(IV)NC1 was prepared as described previously (5) and used to stimulate cultures at 10 μg/ml.

The control antigen Mycobacterium tuberculosis purified protein derivative (PPD; Statens Seruminstitut, Denmark) was dialyzed extensively against PBS (pH 7.4) and filter-sterilized before addition to cultures at 10 μg/ml. PPD readily provokes recall responses in vitro, because most UK citizens have been immunized with Bacillus Calmette-Guérin (25). Concanavalin A (Con A) was obtained from Sigma (Poole, Dorset, UK) and used to stimulate cultures at 10 μg/ml.

Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) were separated from fresh blood samples by density gradient centrifugation (Lymphoprep; Nycomed, Denmark). In one case (patient 3), PBMC were stored under liquid nitrogen before culturing. The viability of PBMC used in all experiments was >90%, as judged by trypan blue exclusion.

Table 2. Sequences of α3(IV)NC1 peptides

<table>
<thead>
<tr>
<th>α3&lt;sub&gt;a,b&lt;/sub&gt;</th>
<th>Sequence</th>
<th>Presentation by DRA/DRB1*&lt;sup&gt;15&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–20</td>
<td>SPATWTRGFVFTFRHQSQA</td>
<td>IA</td>
</tr>
<tr>
<td>11–31</td>
<td>VPTRHSQTTAIPSCPETGVP</td>
<td>LA</td>
</tr>
<tr>
<td>21–40</td>
<td>IPSCPETGVPLYSGFSLV</td>
<td>IA/NP</td>
</tr>
<tr>
<td>31–50</td>
<td>LYSGFSLVQGHQRAHGQD</td>
<td>IA</td>
</tr>
<tr>
<td>51–70</td>
<td>LGTLGSCLRFTTMPFLFCN</td>
<td>IA</td>
</tr>
<tr>
<td>61–80</td>
<td>FTTMPFLFCNVNDVCNFA</td>
<td>IA/NP</td>
</tr>
<tr>
<td>71–91</td>
<td>VNDVCNFAQNDYSWLSVPA</td>
<td>IA</td>
</tr>
<tr>
<td>81–100</td>
<td>NDYSYWLSIPALRPMNAPI</td>
<td>IA</td>
</tr>
<tr>
<td>91–110</td>
<td>ALMPMNMAPITGRALEYPS</td>
<td>IA</td>
</tr>
<tr>
<td>101–120</td>
<td>TGRALEYPSRTCVCEGPA</td>
<td>HA</td>
</tr>
<tr>
<td>111–130</td>
<td>RCTVCEGPAIAAVHSQTTD</td>
<td>IA</td>
</tr>
<tr>
<td>121–140</td>
<td>IAIAVHSQTTDIPPCPHGIS</td>
<td>LA</td>
</tr>
<tr>
<td>131–150</td>
<td>IPPCPHGWISLWKGSFIMF</td>
<td>HA</td>
</tr>
<tr>
<td>141–160</td>
<td>LWKGSFIMFTSAGSEGTQ</td>
<td>HA</td>
</tr>
<tr>
<td>151–170</td>
<td>TSGASEGTQGALASPSCLE</td>
<td>IA/NP</td>
</tr>
<tr>
<td>161–180</td>
<td>ALASPSCLEEFRAFSPLEC</td>
<td>IA/NP</td>
</tr>
<tr>
<td>171–190</td>
<td>EFRAFSPFLECHGRTMCNYS</td>
<td>IA</td>
</tr>
<tr>
<td>181–200</td>
<td>HGRGTCNYYNSYSFWSLAL</td>
<td>HA</td>
</tr>
<tr>
<td>191–210</td>
<td>NSYSFWSLALNPERMFRKPI</td>
<td>IA</td>
</tr>
<tr>
<td>201–220</td>
<td>NPERMFRKIPSTVKAEGLE</td>
<td>IA</td>
</tr>
<tr>
<td>211–230</td>
<td>PSTVKAEGLEKISSRCQVCM</td>
<td>IA</td>
</tr>
<tr>
<td>221–234</td>
<td>KISSRCQVCMKKKRH</td>
<td>LA</td>
</tr>
</tbody>
</table>

a Peptides are named by position in the sequences of α3(IV)NC1 numbered relative to the sequence SPAT... marking the beginning of the NC1 domain.
b The classification is based on biochemical identification of major sets of naturally processed and presented peptides from α3(IV)NC1 (15, 16) and on DR15/peptide binding data (16, 17). Peptides that include the core of the sets of naturally processed sequences are marked NP. Peptides have high affinity (HA), intermediate affinity (IA), or low affinity (LA) for DR15.

Proliferation Assays

As previously described (23–26), PBMC were cultured at a concentration of 1.25 × 10<sup>6</sup> cells/ml in the Alpha modification of Eagle’s medium (Life Technologies, Paisley, UK) supplemented with 5% autologous serum, 4 mM L-glutamine (Sigma), and 20 mM HEPES (pH 7.2; Sigma). All plates were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. The cell proliferation in culture was estimated from the incorporation of 3<sup>H</sup>-thymidine in triplicate microtiter wells 5 d after stimulation with antigen. Proliferation results are presented either as the mean CPM ± SD of the triplicate sample or as a stimulation index (SI), expressing the ratio of mean CPM in stimulated versus unstimulated control cultures. The mean coefficient of variation was <10%, and an SI >2.5 is interpreted as representing a significant positive response. All positive responses recorded were also >1000 CPM, except in the case of patient 3, whose PBMC were judged to have been suppressed by previous frozen storage.

Blocking HLA Class II Restricted Proliferation

Blocking mAb specific for HLA-DR, -DP, or -DQ (supplied by Pharmingen/Becton Dickinson, Oxford, UK) were dialyzed thoroughly against PBS before addition to cultures at the previously determined optimum concentration of 2.5 μg/ml (23–25).

Measurement of Cytokine Production

The production of IFN-γ, IL-4, IL-10, and active TGF-β<sub>1</sub> in cultures was measured by a highly sensitive cellular ELISA, using a method reported previously (26). Briefly, flat-bottomed microtiter plates (Nunc, Roskilde, Denmark) were coated with 50 μl per well of monoclonal anti-cytokine capture antibody (Pharmingen) diluted in 0.05 M carbonate coating buffer (pH 9.6). Five days after stimulation with peptide, duplicate 100-μl aliquots of PBMC cultures were transferred into wells coated with antibody. After incubation of PBMC with capture antibody for 24 h at 37°C, 100 μl of the appropriate biotinylated monoclonal detection antibody (Pharmingen) in 0.2% BSA/PBS was added to the wells, and incubated at room temperature for 45 min. Aliquots of 100 μl ExtraAvidin-alkaline phosphatase conjugate (Sigma) were added to each of the wells and incubated at room temperature for 30 min. The plates were developed by incubating 100 μl well p-nitrophenyl phosphate (Sigma) 1.0 mg/ml in 0.05 M carbonate buffer (pH 9.6). The absorbance at 405 nm was measured using a multiscan plate reader (Labsystems, Basingstoke, UK). Cytokine secretion was calculated by interpolation from a standard curve generated by incubating duplicate wells with doubling dilutions of recombinant human IFN-γ, IL-4, IL-10, and TGF-β<sub>1</sub> (Pharmingen). Results are presented either as the mean concentration of duplicate wells or as the ratio of mean concentration in stimulated versus unstimulated control cultures (SI). The mean coefficient of variation for assays was <10%, and an SI >2.0 is interpreted as representing a significant positive response.

Blocking Antibody

For inhibiting the effects of IL-10, a neutralizing antibody specific for this cytokine (Pharmingen) was added to newly established cultures at 1 ng/ml (23).

Results

Proliferative Responses of PBMC to the Panel of Peptides Spanning the Goodpasture Antigen

Blood was obtained from six patients with Goodpasture’s disease (Table 1). A panel of 22 synthetic peptides, spanning the sequence of α3(IV)NC1, was screened for the ability to
stimulate the proliferation of PBMC from each of the five patients with newly diagnosed, untreated Goodpasture’s disease and from patient 1, who had been partially treated (Figure 1). PBMC from all patients exhibited proliferative responses against two or more Goodpasture antigen peptides, with a striking predilection ($P = 0.0002$, binomial distribution) for peptides $\alpha_3^{71-90}$ and $\alpha_3^{131-150}$, which were stimulatory in every case. Three other peptides, $\alpha_3^{1-20}$, $\alpha_3^{31-40}$, and $\alpha_3^{141-160}$, each provoked responses by PBMC from four of the patients.

In comparison with the results obtained in the patients, PBMC from healthy HLA-DR15–positive donors (Figure 2) mounted significantly fewer proliferative responses to the $\alpha3(IV)NC1$ peptides ($8 \text{ versus } 31.3\%$ of 110 peptide/donor or 132 peptide/patient combinations; $\chi^2 = 8.6, P = 0.004$). In particular, peptides $\alpha_3^{71-90}$ and $\alpha_3^{131-150}$, which were stimulatory in all of the patients with Goodpasture’s disease, respectively elicited proliferative responses by PBMC from none or only one of the control donors. It should be noted that failure of control volunteers’ PBMC to respond is specific to the

![Figure 1](image1.png)

**Figure 1.** Peripheral blood mononuclear cells (PBMC) from all patients with Goodpasture’s disease proliferate in response to peptides from the sequence of $\alpha3(IV)NC1$. Peptides are numbered by position of their first amino acid in the sequence of $\alpha3(IV)NC1$. $\text{-----}$, level of proliferation taken as representing a positive response (stimulation index [SI] = 2.5).

![Figure 2](image2.png)

**Figure 2.** Limited responsiveness of PBMC from healthy control HLA-DR15–positive donors when stimulated to proliferate with peptides from the $\alpha3(IV)NC1$ sequence. Peptides are numbered by position of their first amino acid in the sequence of $\alpha3(IV)NC1$. $\text{-----}$, level of proliferation taken as representing a positive response (SI = 2.5). The HLA-DR types of the donors were DR1/DR15 (1), DR15/DR15 (2), DR13/DR15 (3), DR15/DR15 (4) and DR4/DR15 (5).
α3(IV)NC1 peptides, because they all proliferated when stimulated with the T-cell mitogen Con A or the recall foreign antigen PPD (results not shown). The greater responsiveness of patients’ versus control individuals’ PBMC to α3(IV)NC1 peptides is still more striking in view of the weaker proliferation elicited in the patient group by Con A (mean 49% of control donor responses) and PPD (mean 14% of control donor responses), probably caused by the immunosuppressive effects of renal failure at the time of sampling (27–29).

To determine whether the responses to α3(IV)NC1 peptides were HLA class II restricted, we tested blocking antibodies specific for HLA-DR, -DQ, and -DP (23–25) for the ability to inhibit α3(IV)NC1 peptide-induced proliferation when sufficient PBMC could be obtained. In patient 2, anti-DR, -DP, or -DQ was added to PBMC cultures stimulated with peptides α321–40 or α3131–150. Anti-DR effectively blocked proliferation against the α3(IV)NC1 peptides (in three replicate experiments, mean inhibition 77% for peptide α321–40; 77% for peptide α3131–150). The addition of anti-DP resulted in a smaller inhibition (in three replicate experiments, mean inhibition 59% for peptide α321–40; 59% for peptide α3131–150), whereas anti-DQ had little effect (in three replicate experiments, mean inhibition 9% for peptide α321–40; 22% for peptide α3131–150). Experiments of this type were also performed using PBMC from patient 4, again showing that anti-DR blocking antibodies had the greatest inhibitory effects. Thus, in three replicate experiments, mean inhibition by anti-DR was 44% for peptide α331–50; 59% for peptide α331–50, and 41% for peptide α3131–150. Blocking DP or DQ molecules was less effective (in three replicate experiments, mean inhibition 59% for peptide α321–40; 59% for peptide α3131–150). The sample was taken within 1 mo of presentation. Peptides are numbered by position of their first amino acid in the sequence of α3(IV)NC1. No IL-4 or TGF-β was detected in any of the cultures. *** level taken as representing a positive response (SI = 2.5 for proliferation, 2.0 for cytokine production).

The type of cytokine responses mounted by α3(IV)NC1 peptide-reactive Th cells from patients with Goodpasture’s disease was examined. Cultures of PBMC stimulated with α3(IV)NC1 peptides were assayed for production of the respective Th1 and Th2 cytokines IFN-γ and IL-4 and the potentially inhibitory cytokines IL-10 and active TGF-β1. The results obtained with a second sample of PBMC taken from patient 2 within 1 mo of presentation are illustrated in Figure 3, and data from all five patients with active disease are summarized in Figure 4. Although cytokine secretion was less frequent than proliferation after stimulation with α3(IV)NC1 peptides, IFN-γ responses were not uncommon (12% of 110 peptide/patient combinations) and associated with proliferation ($\chi^2 P = 0.04$). In contrast, no peptides elicited IL-4 production by PBMC from any of the patients, despite the use of a well-validated, highly sensitive assay capable of measuring even weak IL-4 responses (26). IL-10 was produced by the initial PBMC samples in response to some peptides (16.5% of peptide/patient combinations), but active TGF-β1 was not detected in any cultures stimulated with the peptide panel.

In Goodpasture’s disease, the autoantibody response spontaneously remits over time (22). To determine whether this apparent reestablishment of self-tolerance is associated with changes in the Th-cell response against α3(IV)NC1 peptides, we obtained repeat blood samples from three patients 4, 9, or 12 mo after presentation. Figure 5 shows that for all patients, the responses elicited by the peptide panel, with few exceptions, deviated significantly from proliferation to IL-10 production over time. The deviation is striking even when only responses that are above the threshold for significant SI are compared: for patient 1, the number of peptides that elicit proliferation drops from five to one, whereas those that stimulate IL-10 rise from one to 10. Similarly, for patients 4 and 6, the respective number of peptides that elicit proliferation drops from five to zero and from nine to four, whereas those that stimulate IL-10 rise from zero to five and from zero to six. These shifts cannot be attributed to the effects of immunosuppressive treatment, which had been withdrawn from patient 1 before either sample was taken, whereas patients 4 and 6 had received no such therapy. Moreover, the deviation was specific to the α3(IV)NC1 peptides because there was an opposing trend for responses to Con A or control antigen (mean change in response to control stimuli over time, +150% for proliferation, −16% for IL-10 production).
Although the main aim was to characterize T-cell epitopes on \( \alpha3(IV)\)NC1, the ability of PBMC from two patients with acute disease to respond against recombinant \( \alpha3(IV)\)NC1 was also tested and found to reflect the type of response elicited by \( \alpha3(IV)\)NC1 peptides. Figure 6 demonstrates that recombinant \( \alpha3(IV)\)NC1 stimulation of a sample taken at presentation from patient 1 induced strong proliferation and IFN-\( \gamma \) production, with little IL-10, and no detectable IL-4 or TGF-\( \beta \). The recombinant autoantigen also elicited a similar pattern of response by PBMC taken within 1 mo of presentation from patient 2 with active disease. Furthermore, testing of PBMC obtained from patient 2 5 mo later revealed that the response to recombinant \( \alpha3(IV)\)NC1, again like the peptide panel, deviates from proliferation toward IL-10 production over time.

To confirm that the T-cell response to epitopes on \( \alpha3(IV)\)NC1 is dominated by IL-10 in late disease, we also samples tested from patients 7 and 8, who respectively had presented 24 and 6 mo previously. The results obtained using cells from patient 7 are illustrated in Figure 7, and it can be seen that, as in the other late samples, IL-10 production is the major response to recombinant \( \alpha3(IV)\)NC1 and to the \( \alpha3(IV)\)NC1 peptide panel. The question of whether such IL-10 responses were suppressive was addressed by setting up duplicate cultures, with or without a blocking antibody specific for this cytokine. Figure 7 also demonstrates that neutralization of IL-10 allowed the development of Th proliferative responses to \( \alpha3(IV)\)NC1 peptides, which were accompanied by IFN-\( \gamma \) production (not shown). The pattern of results in patient 8 was similar, with predominant IL-10 secretion being replaced by significant proliferation in response to multiple peptides in cultures to which neutralizing antibody had been added. In each patient, there is a significant correlation (\( R_s = 0.6, P = 0.003 \)) between IL-10 levels in \( \alpha3(IV)\)NC1 peptide-stimulated cultures and the increase in proliferative response when IL-10 is neutralized.

Proliferative Responses of PBMC to \( \alpha3(IV)\)NC1 Peptides with High, Intermediate, or Low Affinity for DR15 and to Peptides that Contain Naturally Processed \( \alpha3(IV)\)NC1 Sequences

Immune responses to foreign antigens are usually focused on peptides with highest affinity for restricting MHC molecules (30), but some studies have suggested that this relationship may be absent or reversed for autoantigens (6,13,14). To examine this issue in Goodpasture’s disease, we determined the frequency of proliferative responses to the \( \alpha3(IV)\)NC1 peptides categorized by their previously determined affinity for DR15 molecules (see Table 2) (15–17), according to the ranges proposed by Buus et al. (30). The results, summarized in

**Figure 4.** Summary of IFN-\( \gamma \) and IL-10 responses by PBMC from the patients with acute Goodpasture’s disease after stimulation with the 22 peptides from the panel spanning the sequence of \( \alpha3(IV)\)NC1. Peptides are numbered by position of their first amino acid in the sequence of \( \alpha3(IV)\)NC1. For each peptide, dots (·) show the size of response by PBMC from each of the five patients tested. --- level taken as representing a positive response (SI = 2.0). Bars show the number of patients in whom positive responses were recorded.

**Figure 5.** Th cells from patients 1, 4, and 6 with Goodpasture’s disease deviate from proliferative to IL-10 responses against peptides from the sequence of \( \alpha3(IV)\)NC1. Responses of PBMC samples obtained 5, 4, or 12 mo apart from the respective patients are compared after stimulation with the 22 peptides spanning the sequence of \( \alpha3(IV)\)NC1. Peptides are classified (Table 2) according to their presentation by DR15 molecules (\( \triangle \), high affinity; \( \Box \), intermediate affinity; \( \bigtriangleup \), naturally processed; \( \bullet \), low affinity). For each patient, the loss of proliferative responses in the second sample compared with the first is significant (Wilcoxon signed rank test, \( P < 0.001 \)) and the gain in IL-10 responses is also significant (patient 1, \( P < 0.001 \); patient 4, \( P < 0.05 \); patient 6, \( P < 0.001 \)).
Figure 6. Th cytokine production and proliferation by PBMC from patients with Goodpasture’s disease after stimulation with recombinant α3(IV)NC1. Proliferative IFN-γ and IL-10 responses are shown for the initial PBMC sample taken from patient 1 at presentation (A) and for the samples taken 1 mo (B) and 6 mo (C) after presentation from patient 2. Responses in antigen-stimulated (■) and -unstimulated (□) control cultures are illustrated. No IL-4 or TGF-β was detected in any of the cultures. The late sample of PBMC from patient 2 tested in C did not proliferate (SI < 2) in response to any from a selection of the peptides (α321–40, α351–70, α371–91, α391–106, α3101–120, α3131–150, and α3141–160) that had stimulated proliferation earlier, during active disease (see Figures 1 and 3).

Figure 7. Neutralization of IL-10 releases suppressed Th proliferative responses to epitopes on α3(IV)NC1. PBMC were obtained 24 mo after presentation from patient 7 and stimulated with recombinant α3(IV)NC1 or the peptides spanning the sequence of α3(IV)NC1. IL-10 production is shown, together with proliferation in the presence or absence of a neutralizing antibody specific for IL-10. There is a significant correlation (Rr = 0.6, P = 0.003) between IL-10 levels elicited by peptide and the increase in proliferative response when IL-10 is neutralized. Peptides are numbered by position of their first amino acid in the sequence of α3(IV)NC1. Level taken as representing a positive response (SI = 2.5 for proliferation, 2.0 for cytokine production).

The hierarchy of proliferative responses to α3(IV)NC1 peptides, categorized by affinity for DR15, is reminiscent of that for particular foreign proteins and their restricting elements (30), where the relationship is thought to reflect the ability of MHC molecules to select peptides for display to T cells. However, the same explanation may not be true for all antigens, because MHC affinity is only one determinant of efficient presentation, and different criteria may be relevant to the selection of foreign and self-peptides for display. We therefore considered our data in relation to previous biochemical studies, which have identified the major peptides that are naturally processed from α3(IV)NC1 and presented by DR15 (15,16). Peptides designated NP in Table 2 contain the entire common immunized mice (31). In striking contrast, Figure 8 shows that the α3(IV)NC1 NP peptides α321–40, α361–80, and α3161–170 respectively stimulated proliferation by PBMC from only two, one, or none of the six patients. Therefore, the dominant autoreactive T-cell responses to α3(IV)NC1 in Goodpasture’s disease are not specific for the major naturally processed peptides from this autoantigen but target other peptides with...
higher affinity for DR15 that are less efficiently presented, presumably because of processing constraints (15,16).

Although there was no predilection for proliferative responses against NP peptides containing the cores of the nested sets, two of the most commonly targeted peptides overlap with naturally processed sequences. Thus, peptide α3(IV)NC1, one of the peptides that was stimulatory in all patients, contains a DR15 binding motif (VCNASRNDY) that overlaps that of the second of the naturally processed nested sets (LFCNVNDVNF; in peptide α3(IV)NC1 and is fully enclosed by the proposed naturally processed α3(IV)NC1 segment RFTTMPFLFCNVNDVNFASRN (15–17). Similarly, peptide α3(IV)NC1, which was stimulatory in four patients, contains the DR15 motif LFVQGNQR and is entirely within the proposed naturally processed segment PSCPEGTVPLYGSFSFLFVQGNQRAGH (15–17). However, the other peptides that elicited proliferation by PBMC from most or all patients, peptides α3(IV)NC1 and α3(IV)NC1, share no overlap with any previously identified naturally processed sequences.

IL-10 Responses of PBMC to α3(IV)NC1 Peptides with High, Intermediate, or Low Affinity for DR15 and to Peptides That Contain Naturally Processed α3(IV)NC1 Sequences

The question that arises is whether the T cells that produce IL-10 in response to the peptide panel, which may have a regulatory role, are selected on the same criteria and share the same specificities as those that proliferate and are associated with active disease. There is a significant positive correlation between the number of proliferative and IL-10 responses elicited by each α3(IV)NC1 peptide in the patient group as a whole (\(R = 0.5, P = 0.019\)) and between the magnitude of the two types of response in one of the two patients that exhibited the deviation from proliferation to IL-10 production (patient 4; \(R = 0.47, P = 0.029\)). Furthermore, there is a significant relationship between the categorization of peptides by affinity for DR15 and the frequency of IL-10 responses (\(\chi^2 = 4.4, P = 0.036\), reminiscent of the association seen for proliferative responses. Thus, peptides with low affinity for DR15 rarely stimulated IL-10 production (IL-10 responses in 12% of patient/low-affinity combinations), and high-affinity peptides were overrepresented (IL-10 responses in 46% of patient/high-affinity peptide combinations). Together, these results indicate a considerable overlap in the α3(IV)NC1 peptides that elicit proliferative and IL-10 responses, but the two sets of stimulatory sequences are not identical. In contrast to the proliferation data, each of the three peptides that contain the cores of the naturally processed sets, α321–40, α361–80, and α361–170, were capable of stimulating IL-10 production, and these responses were relatively common (IL-10 responses in 33% of patient/naturally processed peptide combinations).

Discussion

This report addresses a key question in understanding the breakdown of self-tolerance in human autoimmune kidney disease, namely whether self-peptides recognized by the Th cells that drive the pathogenic response are normally either efficiently or inefficiently processed and presented (14). The results show that the Th cell proliferative response to α3(IV)NC1 in patients with Goodpasture’s disease is dominated by peptides that do not correspond to naturally processed sequences but that have high affinity for the disease-associated MHC class II molecule and are deduced to be cryptic because of processing constraints (15,16). The second main finding is that the response to the peptides deviates toward IL-10 production as the disease resolves spontaneously, consistent with the view that regulatory T cells can restore self-tolerance.

The observation of proliferative responses to peptides α31–90 and α3131–150 by T cells from all patients with Goodpasture’s disease is striking and reveals a consistency comparable to that documented for the HLA association and autoantibody response. The HLA association is remarkable both for its simplicity, with 85% of affected individuals, including those tested here, inheriting DRB1*1501, and for its strength across the entire patient population (odds ratio, 8.5) (9). However, the pathogenic response is also known to be highly focused and consistent between patients, with pathogenic autoantibody primarily recognizing a conformational epitope encompassing α3(IV)NC1 residues in the region spanned by peptides α321–40 and α361–170 (3–5). This remarkable uniformity affords unusual advantages for investigation in human disease and here yields interpretable results from a relatively small study.

The frequency of proliferative responses by PBMC from
patients was highest for the group of α3(IV)NC1 peptides that bind DR15 molecules most strongly. The association does not reflect generic peptide binding preferences of class II molecules because no such relationship was found with peptide affinity for DR1 or DR7 and is much stronger than that for DR51. This evidence that many of the proliferative responses were restricted by DR15, together with the effectiveness of anti-DR antibodies in blocking proliferation, supports the view that DR15 molecules are important in presenting α3(IV)NC1-derived sequences to pathogenic Th cells in Goodpasture’s disease (9,15–17). In particular, because all of the patients’ PBMC responded to peptides α371–90 and α3131–150 and DR15 was expressed in every typed patient, it is likely that both of these dominant peptides are presented to α3(IV)NC1-specific T cells on DR15 molecules. A structural analysis also concluded that DR15 was the predominant restricting locus in Goodpasture’s disease and that this role accounts for the association with DRB1*150x (9). However, it was also shown here that the response to peptide α3131–150 could be partially blocked by anti-DP antibodies, and it remains possible that the minor stimulatory α3(IV)NC1 peptides, which vary between patients, are not restricted by DR15 but the other expressed MHC class II alleles. Thus, despite the predominance of DR15-restricted peptides, there is also evidence for diversification of the Th-cell response against α3(IV)NC1, comparable to data from other human autoimmune diseases (23,32).

Self-peptides that are available for presentation in the thymus or periphery can delete or anergize the corresponding autoreactive Th cells (13,14). Even α3(IV)NC1, which has a fairly restricted distribution (33) in the basement membranes of the glomerulus and alveolus, would be expected to induce these forms of tolerance, because it is also detectable in the human thymus (34) and even in normal blood and urine (35). One explanation for the development of autoimmune disease is that a defect in patients allows potentially autoreactive Th cells specific for naturally processed self-peptides, which are normally censored from the repertoire, to escape and become activated. Our characterization of responses to α3(IV)NC1 enabled this possibility to be addressed for the first time in a human autoimmune disease. We took advantage of the biochemical identification of the major peptides that are naturally processed and presented from α3(IV)NC1 by DR15 bearing antigen presenting cells (APC), which include three nested sets centered on core sequences contained in peptides α321–40, α361–80, and α3161–180 (15,16). We show here that Th cells from patients with Goodpasture’s disease rarely proliferated in response to these peptides and conclude that they are not important targets for the pathogenic response.

Alternatively, tolerance may be broken by self-peptides that do not normally induce deletion or anergy because of inefficient presentation but that are presented at higher levels in autoimmune disease and can then activate the corresponding T cells (14). The current study supports this hypothesis by demonstrating that the Th-cell response in Goodpasture’s disease is skewed toward α3(IV)NC1 peptides that seem normally to be inefficiently presented as a result of processing constraints (15,16). The relationship between high affinity for DR15 and targeting of peptides by the patients’ T-cell responses mirrors that described for foreign antigens (30,31), but, in contrast to the analysis of antigens such as HEL (31), high affinity of α3(IV)NC1 peptides for DR15 molecules does not correlate with natural processing and presentation (15,16). We reason that the high-affinity peptides that commonly stimulate autoreactive T cells from patients, including peptide α3131–150, are normally inefficiently presented as a result of processing constraints: When naturally processed, their ability to bind DR15 should enable them to out-compete the members of the three nested NP sets and ensure that they were detectable at a higher level. Recent data support the view that destructive processing accounts for the poor presentation of the major T-cell epitopes from α3(IV)NC1, because all three are exquisitely sensitive to endosomal proteases (Phelps et al., manuscript in preparation). Processing events that prevent display of these α3(IV)NC1-derived peptides with high affinity for DR15 would be expected to render such sequences cryptic regardless of class II type.

Cryptic epitopes that are important targets for the helper response in Goodpasture’s disease may become available to stimulate T cells and initiate autoimmune pathology as a result of previous acute nephritis. For example, an inflammatory milieu may promote changes in the peptides presented by cleaving α3(IV)NC1 before internalization into APC, by differential activation of processing pathways with APC, or by simply increasing the availability of free antigen (14). These possibilities are supported by links between the onset of Goodpasture’s disease and inflammatory events such as renal vasculitis, membranous nephropathy, and renal trauma (36). Alternatively, antigen processing of α3(IV)NC1 may be modulated by bound autoantibodies (37) in Goodpasture’s disease.

Failure to present effectively particular self-peptides for censorship of the T-cell repertoire can be due to low affinity for class II molecules rather than processing constraints (13,14), and such peptides can induce autoimmune responses in animal models. For example, the major autoantigenic peptide in one form of experimental allergic encephalomyelitis fails to delete the corresponding T cells because it binds very weakly to its restricting element and therefore is displayed inefficiently (13). However, our data provide no evidence that self-tolerance is broken by Th cells specific for such epitopes in Goodpasture’s disease, because PBMC from the patients exhibited virtually no responsiveness to the α3(IV)NC1 peptides that previously have been shown to have very low affinity for HLA-DR15 molecules (16,17).

As already discussed, α3(IV)NC1 peptides that contain the entire cores of the three naturally processed nested sets rarely stimulated proliferation by patients’ T cells. Although two of the peptides that most commonly elicited proliferative responses by patients’ PBMC, α371–90 and α331–50, do overlap naturally processed core sequences, it is likely that the stimulatory epitopes that they contain are distinct from the cores. Thus, proliferation against peptides α371–90 or α331–50 was rarely accompanied by responses to the adjacent peptides α361–80 or α321–40, which contain the entire cores of the respective naturally processed sets, and both peptides α371–90 and α331–50 include DR15 binding motifs not found within...
these cores. We hypothesize that the repertoire may be purged of T cells that recognize each of the first two segments of naturally processed α3(IV)NC1 bound to DR15 only in the most favorable register (most abundant so identified in elution studies) but not of T cells that recognize the same segments bound in less favorable registers (expanded in disease).

In many examples of experimental autoimmunity, Th1 responses seem to be pathogenic, whereas Th2 responses mediate protection from disease (6,14,19). A similar dichotomy may be relevant to Goodpasture’s disease, because Th cells from patients with newly diagnosed disease secrete IFN-γ but not IL-4 in response to the α3(IV)NC1 peptides. However, in contrast to other pathogenic Th1 responses (6,19), relatively few α3(IV)NC1 peptides elicited IFN-γ, which may be a consequence of the immunosuppressive effects of uremia in patients with Goodpasture’s disease (27–29). Autoantigen-specific regulatory T cells secreting inhibitory cytokines such as IL-10 or TGF-β can prevent a wide range of immune-mediated pathology in animal models, and, in particular, the breaking and restoration of self-tolerance in autoimmune diseases is controlled by their presence or activation (20,21). The current study provides some of the first evidence that such regulatory Th cells also play a role in controlling human autoimmune diseases because changes in IL-10 production in response to α3(IV)NC1 peptides coincided with different stages in pathology. Resolution of glomerular inflammation over time in three patients was associated with a dramatic deviation from proliferative to IL-10 responses. Any IL-10 responses already seen at the time of presentation may reflect the tendency for patients with Goodpasture’s disease to come to diagnosis late in the progression of renal pathology, when the switch away from proliferative responses may have already begun. Experiments to test the reactivity of peripheral blood T cells to recombinant α3(IV)NC1 demonstrated that, as with the peptide panel, initial responsiveness during glomerular inflammation was Th1 in character but deviated toward IL-10 secretion in later disease. In an additional two patients, from whom samples were obtained only in late disease, the predominant response to α3(IV)NC1 epitopes was again found to be IL-10 production. Furthermore, it was confirmed that such IL-10 responses were suppressive because neutralization of the cytokine dramatically revealed proliferative responses to multiple α3(IV)NC1 peptides and to recombinant α3(IV)NC1. The persistent absence of IL-4, despite increases over time in IL-10 production against both peptides and recombinant antigen, indicates a switch toward a regulatory, rather than a Th2, response. Our observations are supported by an earlier report that Th cells from patients who have recovered from Goodpasture’s disease lose the ability to proliferate in response to α3(IV)NC1 (38), but the associated cytokine responses have not previously been examined. Parallel work on another antibody-mediated disease, autoimmune hemolytic anemia (AIHA), also reveals a switch away from proliferative to suppressive IL-10 Th-cell responses associated with remission (23). There is a contrast between the cycles of proliferative and IL-10 responses recorded over time in patients with AIHA and the consistent deviation to IL-10 production in Goodpasture’s disease, which was observed in all six patients with late disease from whom samples were tested against α3(IV)NC1 peptides and/or recombinant antigen. However, this difference in immune reactivity mirrors the natural history of the two diseases, because AIHA is typically chronic and relapsing, whereas Goodpasture’s disease is characterized by one episode of acute glomerular inflammation that resolves but leaves permanent damage. Together, these studies suggest that Th cells from patients with Goodpasture’s disease that proliferate in response to α3(IV)NC1 are pathogenic cells capable of providing help for anti-α3(IV)NC1 autoantibodies and/or driving local inflammation in the glomerulus and that these effector functions are suppressed by IL-10–secreting regulatory cells that develop later in the course of disease.

The results also begin to define the criteria that shape the human regulatory T-cell repertoire. The significant correlation between the α3(IV)NC1 peptides that induce proliferation and IL-10 production demonstrates that autoreactive T cells that mediate the two types of response share a number of specificities. Furthermore, there is a hierarchy in the ability to stimulate IL-10 when the peptides are categorized by affinity for DR15, as was found for proliferative responses. These associations indicate that at least some T cells that are capable of producing IL-10 recognize the immunodominant α3(IV)NC1 epitopes that, despite their high affinity for DR15, seem to be inefficiently presented as a result of processing constraints, as discussed earlier. In addition, however, other Th cells that secreted IL-10 were specific for peptides that contain the cores of the previously identified (15,16) nested sets of naturally processed α3(IV)NC1 sequences. This contrasts with the inability of such sequences to elicit proliferative responses and is consistent with the view that although some T cells that recognize abundantly displayed self-peptides are censored from the repertoire, others can survive and attain a regulatory phenotype (39).

This study is a first step toward specific immunotherapy for glomerulonephritis. Peptides that contain dominant self-epitopes from the Goodpasture antigen could be effective therapeutic tolerogens if given by a mucosal route before irreversible damage is sustained by the glomeruli (6,13,21), and the targeted stimulation of regulatory cells could also restore tolerance (20,21). Either approach could potentially be effective in more common forms of glomerulonephritis if the re-induction of tolerance to α3(IV)NC1 exerted a bystander effect on responses to other renal autoantigens. Furthermore, the demonstration that the autoreactive T-cell response is skewed toward α3(IV)NC1 peptides with high affinity for DR15 but that these are inefficiently processed sequences provides a unique insight into the mechanisms of human autoimmune disease.

Acknowledgments
This work was supported by the National Kidney Research Fund of the UK. We are grateful to the clinicians at Edinburgh (Dr. R. Winney, Prof. A. N. Turner) and Milan (G. Remuzzi), who cooperated in obtaining blood samples from patients with Goodpasture’s disease.
References


Copyright © American Society of Nephrology. Unauthorized reproduction of this article is prohibited.

