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ANCA patients have T cells responsive to complementary PR-3 antigen

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Abstract

Some patients with proteinase 3 specific anti-neutrophil cytoplasmic autoantibodies (PR3-ANCA) also have antibodies that react to complementary-PR3 (cPR3), a protein encoded by the antisense RNA of the PR3 gene. To study whether patients with anti-cPR3 antibodies have cPR3-responsive memory T cells we selected conditions that allowed cultivation of memory cells but not naïve cells. About half of the patients were found to have CD4+TH1 memory cells responsive to the cPR3$^{138\text{-}169}$-peptide; while only a third of the patients had HI-PR3 protein responsive T cells. A significant number of T cells from patients responded to cPR3$^{138\text{-}169}$ peptide and to HI-PR3 protein by proliferation and/or secretion of IFN-γ, compared to healthy controls while there was no response to scrambled peptide. Cells responsive to cPR3$^{138\text{-}169}$-peptide were not detected in MPO-ANCA patients suggesting that this response is specific. The HLADRB1* 15 allele was significantly overrepresented in our patient group and is predicted to bind cPR3$^{138\text{-}169}$ peptide with high affinity. Regression analysis showed a significant likelihood that anti-cPR3 antibodies and cPR3-specific T cells coexist in individuals, consistent with an immunological history of encounter with a PR3-complementary protein. We suggest that the presence of cells reacting to potential complementary protein pairs might provide an alternative mechanism for auto-immune diseases.

Keywords

ANCA; PR-3; complementary PR-3; autoantigen complementarity; memory T-cells; autoimmune disease

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DISCLOSURE
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Patients with antibodies reactive with our recombinant complementary proteinase 3 (cPR3$^{105–201}$) protein must have experienced an immunological encounter with a cPR3-like antigen. The following studies are based on the proposal that T cells involved in this immunological response remain in a memory cell pool and are identifiable.

Our investigations into complementary proteins in PR3-anti-neutrophil cytoplasmic autoantibody (ANCA) vasculitis disease began after some unexpected results during experiments designed to determine sites on PR3 protein recognized by PR3 autoantibodies. To generate bacterial clones expressing different fragments of human PR3 protein, a blunt-ended ligation strategy was used to clone randomly generated DNA fragments. Consequently, the DNA fragments could insert into the vector in either a sense or anti-sense orientation, and thus some bacterial clones were expressing a protein fragment from anti-sense RNA. Patients' sera were found to react to some of these cPR3-producing clones. Subsequently, a recombinant complementary PR3 protein was produced in a human cell line, termed cPR3$^{105–201}$, which is the complement of the middle third of the sense PR3 protein (105–201 aa (amino acids)). Using this reagent, we proved that anti-cPR3$^{105–201}$-specific antibodies were unique and that they bound PR3-ANCA to form an idiotypic pair.[1] Furthermore, cPR3$^{105–201}$ protein and native PR3 physically interacted characteristic of a complementary protein pair.

Complementary proteins were first defined in the late 1960s by L.B. Mekler,[2,3] who proposed that information embedded within the genetic code could identify proteins that would pair in nature, that is, proteins from sense codons bind proteins from their anti-sense codons. Many researchers have discovered protein partners by using complementary sequences coded by anti-sense codons (reviewed by Heal *et al.*[4] and Tropsha *et al.*[5]).

Earlier studies focused on complementary proteins and their antibody-producing B cells. The present studies focus on T cells. To date, no one has purposefully searched in humans for T cells that respond to complementary protein counterparts of known autoantigens. What has been done in PR3-ANCA disease is a search for PR3-specific reactive T cells. Although a proliferative response was observed, the results failed to show a disease-specific association.[6–12] These data raise the question of why on the one hand we can identify disease-associated PR3 autoantibodies,[13] but on the other hand have difficulty identifying disease-associated T cells.[14] As a new approach, investigations into the potential contributions of complementary proteins could prove informative. In support, a report published in the year 2000 proposed that difficulties identifying disease-associated PR3-specific T cells could be explained if a protein complexed to PR3, and not PR3 itself, was what actually induced T cell help for PR3-specific B cells.[10] Could the protein suggested to be in complex with PR3 be a 'complementary protein'? It has been proposed that complementary proteins when complexed would have a unique structure that appears foreign.[15,16] However, immunological responses incited by a complementary protein not complexed to its sense counterpart must be equally considered at this point.

If patients with PR3-ANCA have experienced an immunological encounter with a cPR3-like antigen, then the T cells involved in that encounter may still be present and identifiable. Herein we establish the first human correlate between T-cell responses and complementary proteins.

RESULTS

Patient study group

Patients with biopsy-proven PR3-ANCA vasculitis (*n*=30) were identified through the Glomerular Disease Collaborative Network between January 2004 and June 2006 (Table 1).

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All patients donated a blood sample were included in the study. However, if a patient's cells from a particular sample did not respond to recall antigens (a mixture of tetanus toxoid, diphtheria toxin, and *Candida*) was assumed anergic and was not included in that particular analysis. Thus, patient enrollment was generally limited to those in remission, slightly active disease on maintenance drugs, or newly diagnosed patients before aggressive treatment. Because of limitations on the amount of blood obtainable per donation, inclusion in the different analyses required that patients donate more than once during the study period (Table 1).

Proliferative response of cPR3$^{138–169}$ peptide-reactive T cells

To test for T cells specific for cPR3, a peptide was synthesized (cPR3$^{138–169}$) containing 32 amino acids of the original recombinant protein (cPR3$^{105–201}$). This sequence was identified in epitope mapping studies as reactive with patients' antibodies,[1] and later confirmed by mass spectrometry (manuscript in review). Control peptides included scrambled amino acids of cPR3$^{138–169}$ and the sense counterpart of PR3 (sense PR3$^{138–169}$). A higher number of CD3$^+$ T cells from patients with PR3-ANCA (Wilcoxon ranked sum test, P=0.0014), but not patients with myeloperoxidase (MPO)-ANCA, proliferated upon encounter with cPR3$^{138–169}$ peptide compared with healthy controls (Figure 1b). In parallel, patients were tested for T cells that proliferated in response to heat-inactivated full-length PR3 protein. An increase in proliferating cells was detected, although the majority of these were from four patients (P=0.01) (Figure 1c). Cells from patients and healthy controls responded similarly to recall antigen stimulants (P=0.79). The percentage of background proliferation (% of total CD3$^+$ cells that were carboxyfluorescein diacetate succinimidyl ester (CFSE)^{dim}) was similar comparing patients (3.0±4.2%) and healthy controls (2.0±2.02%).

Proliferative response to cPR3$^{138–169}$ peptide occurred without additional co-stimulation with cytokines, suggesting that these T cells had a memory cell phenotype.[17] In support, KLH peptide, as a stimulant that requires naive cell proliferation, failed to produce a response using cells from 10 PR3-ANCA patients and 7 MPO-ANCA patients (mean CDI: recall antigens=6.55±8.9; KLH=1.10±0.49) (representative of data—Figure 1d).

T cells produce interferon-γ in response to cPR3$^{138–169}$ peptide

A higher number of T cells from patients secreted interferon-γ (IFN-γ) when stimulated with cPR3$^{138–169}$ peptide, as compared with healthy controls (Wilcoxon ranked sum test, P<0.0001) (Figure 2a). Specificity is supported by a dose-dependent response seen using cells from three patients versus healthy controls (Figure 2b). A significant increase in IFN-γ-secreting cells was also seen with exposure to sense PR3$^{138–169}$ peptide (P=0.008) and to heat-inactivated proteinase 3 (HI-PR3) protein (P=0.03) in contrast to scrambled cPR3$^{138–169}$ peptide (P=0.35) (Figure 2a). There was a tendency of patients' T cells to be less responsive to the recall antigen proteins but not significantly different from healthy controls (Figure 2c). To determine whether CD4$^+$ or CD8$^+$ cell was responding to cPR3$^{138–169}$ peptide, IFN-γ secretion was pharmacologically blocked and cells were analyzed by flow cytometry. A significant increase in CD4$^+$-IFN-γ-positive cells was found versus positive CD8$^+$ cells (Wilcoxon ranked sum test, P=0.005) (Figure 2d).

We asked if the complex of peptides, cPR3$^{138–169}$ with sense PR3$^{138–169}$, would have a differential effect on stimulating capacity. Eight patients' T-cell samples were examined for INF-γ secretion in response to complexed peptides versus individual peptides. KLH peptide was included as a negative control. Rather than enhancing the INF-γ response, mixing abrogated the cPR3$^{138–169}$ peptide effect (Figure 3).

Compiling the data from the proliferation and cytokine studies, a statistical analysis of people who respond to the various stimulants versus those who did not respond was determined based

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on the mean plus 2 s.d. of healthy controls setting $P \leq 0.05$ as significant (Table 2). The analysis of the proliferation data indicated that a significant percentage of patients (54%) responded to $cPR3^{138–169}$ in contrast to the percentage of HI-PR3 protein responders (31%), which was not statistically different from healthy controls. Analysis of the INF-γ data indicated that a significant number of patients responded to both $cPR3^{138–169}$ and HI-PR3 protein.

Specificity of responses to fragments of $cPR3^{138–169}$ peptide

$cPR3^{138–169}$ peptide bears some homologies to a number of bacterial proteins.[1] It was questioned whether this 32 aa peptide had characteristics similar to a pathogen-derived superagonist. Three 16 aa overlapping peptide fragments were tested for stimulatory characteristics with the supposition that superantigen-like sequences would bias reactivity toward one fragment. IFN-γ responses were not restricted to only one sequence within a particular fragment (Table 3), that is, no superantigen sequences were apparent, thus supporting the specificity of responses to $cPR3^{138–169}$.

Human leukocyte antigen allele frequencies in the patient group

Differences in the immunogenetic backgrounds of patients, in particularly human leukocyte antigen (HLA) class II molecules, may account for observed immune responses to $cPR3^{138–169}$ peptide. Of 48 HLA-DR alleles from 24 patients, 11 were DRB1*15 at a frequency of 0.229. Statistical comparisons of observed frequencies to the expected frequencies of 0.093 in the general population[18] indicated an overrepresentation of this allele in the patient group (Fisher's exact test, $P=0.006$) (Table 4). There was no statistical differences for the alleles DQB1*6 ($P=0.18$), DQB1*5 ($P=0.99$), DQB1*3 ($P=0.57$), and DQB1*2 ($P=0.26$).

To address whether the DRB1*15 protein is a binder of $cPR3^{138–169}$ peptide, we performed an analysis using the Immune Epitope and Analysis Resource database.[19] HLA II molecules bind 8-aa fragments, and therefore the various alleles might preferentially bind different regions of the 32 aa $cPR3^{138–169}$ peptide. DRB1*01 and DRB1*15 bind with high affinity ($IC_{50}<50$) (Table 5). DRB1*03, DRB1*07, DRB1*11, and DRB1*13 are predicted to bind with intermediate affinity, whereas DRB1*04 is the least likely to bind with a low affinity for only one region of the fragment.

Individual variability among longitudinal samples

The substantiation of the validity-specific T-cell responses in the patient group lies in the employment of multiple methodologies using repetitive patient samplings. A graphical representation of each patient's T-cell responsiveness overtime provides a look at the potential for variable outcomes (Figure 4a). Patient A was positive in all three assays with the proliferation studies (2004), fluorescence-activated cell sorter (FACS) (2005), and enzyme-linked immunospot assay (ELISPOT) (2006). However, patient K was negative in 2004 for proliferation and positive in 2005 for ELISPOT assay. Explanations for variability are not obvious, as both patients were in remission and drug regimens remained consistent. We asked if disease status could explain, in part, the variability. Statistical comparisons of patients in remission (BVAS=0) with those not in remission (BVAS score >0) indicated an equal distribution within the responsive T-cell group and the non-responder group (Fisher's exact test $P=0.99$) (Figure 4b). Other potential explanations such as type of medication (Table 1) or environmental-related factors were not identifiable. Variability is inevitable and appears to be a common occurrence in studies of human subjects.[10]

Coexistence of cPR3-specific T cells with anti-cPR3-specific antibodies

A critical question concerning the functional consequences of $cPR3^{138–169}$ peptide-reactive $CD4^+$ TH1 cells is whether they were involved in cPR3-specific B-cell maturation and antibody

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expression. To determine whether the patients enrolled in the T-cell study were positive anti-cPR3-specific antibodies, at any point during the course of their disease, all available-banked sera samples were screened. If a positive serum sample was identified for an individual, that individual was considered to have experienced an immunological response, which could be correlated with the production of a memory T-cell pool. The mean value for patients was 27.96 ±21.20, compared with healthy controls at 15.59±5.51 (Wilcoxon ranked sum analysis, P=0.05). All patients with detectible antibodies were positive for reactive T cells (Figure 5a). Because availability of sera samples was limited for some patients, a positive antibody episode may have been missed, so it is uncertain whether the remaining patients with positive for T cells ever had the antibodies. A ranked linear regression analysis indicated a likelihood of P-value of 0.0086 that, if patients had cPR3$^{138–169}$ peptide-reactive T cells, they would also have the reactive antibodies. The data support the conclusion that a complementary PR3 protein (or its mimic) was presented as a helper T-cell epitope stimulating B-cell maturation and antibody production.

DISCUSSION

This is the first report of disease-related T-cell responsiveness to a protein complementary to a known autoantigen. The data reveal cPR3$^{138–169}$-responsive T cells of the CD4^{+}-TH1 class, a T-cell subtype that is capable of delivering signals for B-cell maturation.[20] There was a significant correlation between the presence of anti-cPR3 antibodies and responsive T cells on an individual basis. These data are consistent with a complementary protein-specific component in immunological events of PR3-ANCA vasculitis autoimmune disease. Proliferation-based analyses of HI-PR3 protein responsiveness were significant when considering cell number, although the major contribution of positive cells was from only four patients and 4 of 13 responders did not reach significance, similar to published results.[10] However, HI-PR3 protein-responsive T cells were found to have a positive correlation with disease, compared with healthy controls, as assessed using the IFN-γ secretion assay. Nonetheless, the magnitude of the response was far below that seen in response to cPR3$^{138–169}$. One explanation for the discordance of data between assays is that the ELISPOT assay can detect responses of fewer cells and thus is more sensitive.

The process that led to cPR3$^{138–169}$-specific T cells, B cells, and antibodies required degradation of a complementary PR3 protein followed by the display of peptides by HLA class II molecules on the B-cell surface to T-helper cells. Genotyping HLA alleles of patients revealed that DRB1*15 is overrepresented in this group, and furthermore, DRB1*15 molecules are predicted to bind cPR3$^{138–169}$ peptide with high affinity (HLA Ligand/Motif Database). The HLA-DR2 (which includes DRB1*1501, DRB1*1502, DRB1*1601, and DRB1*1602) allele group has been associated with ANCA vasculitides in the past.[21–24] Moreover, patients with DR2 were more likely to have persistently positive ANCA. HLA-DR2 has been associated also with multiple sclerosis,[25] anti-GBM disease,[26] and other chronic granulomatous diseases such as sarcoidosis[27] and leprosy.[28] An interesting case study reported a young boy homozygous for HLA-DR2 who developed anti-GBM disease coincident with ulcerative colitis.[29] It was proposed that the patient's homozygosity at HLA-DR2 was a predisposing factor for developing both diseases. We identified three patients who typed as DRB1*15 homozygosis and all three tested positive for cPR3$^{138–169}$ peptide-specific T cells. The HLA-DR4 (DRB1*0401 and DRB1*0404) allele group has also been reported to have some associations with Wegener's vasculitis.[22,30] What is particularly interesting about DR4 in our studies was that three of five patients who were negative for responses to cPR3$^{138–169}$ peptide were the DRB1*04 allele group and two of these have sinus-limited disease (the only two in the study). A number of other positive and negative associations with HLA genes have been reported in systemic vasculitis but what is clear is that there is yet to be a consensus of a consistent allelic association.[31–33]

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A limitation when studying human T cells is that the only available sample is peripheral blood cells, unlike animal studies where spleens and lymph nodes are available. Using circulating cells, we found that the number of spots in the ELISPOT assay were less than published animal studies using spleens. Others report similarly low numbers of spots from human peripheral cells and propose this is expected for low-frequency reactive cells.[34] These are not unexpected as memory cells are thought to primarily reside in the spleen and peripheral lymphoid tissue, with low numbers of cells found in the circulation. Our efforts to expand the T cells in culture to increase the number of spots were unsuccessful. What is comforting is the degree of concordance between the proliferation studies and the ELISPOT assay with repetitive patient samplings. Even with these limitations, we successfully demonstrated a strong statistically significant response in patients compared with healthy controls.

A topic for discussion is why T cells specific for the PR3 autoantigen have been difficult to find at significant levels. Clayton and Savage designed a system to determine whether PR3-specific B cells require T cell help to produce antibodies using peripheral blood lymphocytes culture system. Their conclusions were that B cells from patients produce PR3 antibodies through a T-cell-independent pathway or through some nonspecific B-cell stimulation.[35] Nonetheless, PR3-specific T cells are identifiable. Consider for a moment that anti-idiotypic antibody processes are involved in the generation of PR3-specific antibodies. This possibility was supported when mice immunized with $cPR3^{105–201}$ peptide developed not only anti-$cPR3^{105–201}$ antibodies but also antibodies that reacted with human-native PR3. These mouse anti-human PR3 antibodies produced a cytoplasmic-staining pattern on human neutrophils identical to that produced by patients' PR3-ANCA. Thus, in these mice, the derivation of the anti-human PR3-reactive antibody must have occurred through an anti-idiotypic response incited by human-specific complementary protein.[1] The anti-idiotypic process is initiated with a T-cell and B-cell response against a PR3 complementary protein. Antibodies can regulate each other by suppressing or augmenting the immune reaction in a manner that would perpetuate autoimmune disease.[36–38] An antibody is immunogenic by virtue of its non-germline-encoded antigen-binding site. B cells are known to spontaneously display endogenous V region peptides on their HLA class II molecules and activate $CD4^+$ T cells.[39,40] Display of immunoglobulin-derived peptides (idiotopes) on APC HLA II molecules can occur by several routes. Monocytes and dendritic cells phagocytize antigen–antibody complexes bound to surface F_c receptors, and they directly phagocytize soluble antibodies through routine environmental sampling. Host antibodies are then degraded and loaded onto HLA II molecules and displayed on the APC surface in a manner similar to foreign antigens.[41] Alternatively, B cells endocytose antigens that ligate to surface immunoglobulin (the B-cell receptor) and process these proteins for display on HLA II molecules.[42–44] How can this information be incorporated into understanding PR3-ANCA generation? Experimental evidence indicates that animals immunized with human complementary PR3 protein not only develop antibodies reactive with the immunogen, but also the development of human-specific PR3 antibodies. That has been observed in mice,[1] rabbits, and chickens (unpublished data). Similarly, a research group that studies La/sodium chloride-sodium citratein buffer (SSB)-specific autoantibodies associated with Sjogren's syndrome and systemic lupus erythematosus found that mice immunized with the autoantigen's complementary peptide counterpart elicited antibodies against the immunogen and anti-idiotypic antibodies that reacted with the sense autoantigen.[45] It has been demonstrated in multiple autoimmunity animal models that anti-idiotypes, raised against autoantibodies, induced anti-anti-idiotypes that possessed characteristics of the initial autoantibodies and caused disease after immunization.[46,47]

A crucial question is the source of the actual complementary PR3 proteins that triggered the immunological responses described here. Ongoing studies are addressing this by probing for proteins from patient material that react with our antibodies from rabbits immunized with complementary peptides. The possibilities remain that it could be carried in by a microbe with

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proteins homologous to the complementary protein[48,49] or that patients aberrantly transcribe and translate it.[1] Somewhat encouraging, we have detected anti-sense transcripts in patients using an anti-sense-specific primer for the reverse transcriptase reaction and PCR.[1] Whether these transcripts are—or even can be—translated is unclear, although there are reports of translated anti-sense transcripts.[50]

A recent review proposed that 'complementary proteins, which occur naturally, or result from cellular dysfunction, might be more common than recognized currently. This implies that the role of complementary proteins in autoimmunity merits increasing investigation.'[51] Understanding when and how complementary proteins initiate autoimmune disease will depend on discovering where these proteins come from. Nevertheless, there is enough evidence to warrant a closer look.

MATERIALS AND METHODS

Patients

All subjects gave written informed consent and participated in the study according to the guidelines of the UNC Institutional Review Board (IRB no. 97-MED-44). Patients enrolled in the study with PR3-ANCA disease (n=30) included 11 females and 19 males; mean age of 50.3 years (26–79 years); 3 blacks, 25 Caucasians, and 2 others (Table 1). Mean of PR3-ANCA titers across samples was 53.9 (range: 3.2–170.0). Healthy controls were recruited on site for blood donations (n=34). The disease control group of seven MPO-ANCA patients included five females and two males, mean age of 45 years (21–65 years), 1 black, 5 Caucasians, and 1 Asian. Limits on the amount of blood obtainable per donation required that the different methodologies in this study be done in tandem. Thirteen of 30 patients donated blood more than once during the study's 2-year period. Studies for anti-cPR3 antibody reactivity required the use of banked sera samples. Healthy control sera were from approved kidney transplant donors (n=12).

Proteins and synthetic peptides

Recombinant $cPR3^{105–201}$ protein was produced as previously described:[1] sequence: DAGLAARDESANVMWPAEEGDHGDIELLQDLGWGVVGTHAAPAHGQALGAVGH WLVLLWQLDCGDGGTEVGWAAQLDEENVVQFVLRVVVVQKHLSHREVLLGGLL RPHVVGSEHHVHQALGYVPQAVRGRQHEAG.

Synthetic peptides from Alpha Diagnostic (San Antonio, TX, USA) were included as follows:

$cPR3^{138–169}$: N-DLGWGVVGTHAAPAHGQALGAVGHWLVLLWQL-C (32 aa)

Fragment 1—$cPR3^{138–153}$: N-DLGWGVVGTHAAPAHG-C (16 aa)

Fragment 2—$cPR3^{146–161}$: N-THAAPAHGQALGAVGH-C (16 aa)

Fragment 3—$cPR3^{154–169}$: N-QALGAVGHWLVLLWQL-C (16 aa)

Sense-$PR3^{138–169}$: N-QLPQQDQPVPHGTQCLAMGWGRVGAHDPPAQV-C (32 aa)

Scrambled peptide: N-LWAGDWVALGLGAWLAGLHVHAQTPHVQVGGL-C (32 aa)

Native PR3 (Wieslab AB, Lund, Sweden) was passed over an Extracti-Gel AffinityPak detergent-removing column (Pierce, Rockford, IL, USA) and heat-inactivated (100°C/10 min) [12] to linearize the protein.[9]

Recall antigen mixture contained tetanus toxoid (2 μg/ml) and diphtheria toxin (2 μg/ml) (List Labs, Campbell, CA, USA), plus 15 μg/ml of *Candida* (Allermed Lab, San Diego, CA, USA).

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Other agents are concavalin A (Con A) (1 μg/ml), phorbol 12-myristate 13-acetate (PMA) (25 ng/ml), and ionomycin (1 μg/ml) (Sigma, St Louis, MO, USA).

Cell stimulations

Blood was collected into sodium heparin CPT Cell Preparation tubes (BD Vacutainer, Franklin Lakes, NJ, USA) and peripheral blood mononuclear cells were isolated per instructions. Stimulants included peptides (2–25 μg/ml), HI-PR3 (2–10 μg/ml), recall antigen mixture, and either ConA or PMA plus ionomycin. Peptide-solvent dimethyl sulfoxide was added to controls.

CFSE assay

Cytoplasmic proteins were fluorescently labeled with CFSE (0.1 μm) for 15 min (Molecular Probes, Eugene, OR, USA). Subsequent proliferation in the absence of CFSE results in decreased fluorescence intensity by one-half with each cell division. Cells were cultured at 1×10^6 cells per ml for 6 days with proteins (10 μg/ml) or peptides (25 μg/ml). $CD3^+$ cells were labeled with phycoerythrin (PE) mouse anti-human CD3 monoclonal antibody (BD PharMingen, San Diego, CA, USA) and analyzed by FACScan linked to a CELLQuest software system (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). The cell division index (CDI) is based on 5000 $CFSE^{bright}$ $CD3^+$ cells as previously described.[52]

$$\mathrm{CDI}=\frac{\left(\frac{\mathrm{CFSE}^{\mathrm{dim}}}{\mathrm{CFSE}^{\mathrm{bright}}/5000}\right)^{\mathrm{treated}}}{\left(\frac{\mathrm{CFSE}^{\mathrm{dim}}}{\mathrm{CFSE}^{\mathrm{bright}}/5000}\right)^{\mathrm{untreated}}}$$

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Enzyme-linked immunospot assay

Cells were plated at 100 μl per 2×10^6 cells per milliliter in triplicate on MultiScreen 96-Well Filtration Plates (Millipore, Bedford, MA, USA)—coated with anti-human IFN-γ monoclonal antibody (Pierce). Treatments included peptides (5 μg/ml), HI-PR3 (2 μg/ml), recall antigens, and Con A. IFN-γ-releasing cells were detected with biotinylated mouse anti-human IFN-γ antibody (Pierce) (2 μg/ml) and streptavidin (Southern Biotech, Birmingham, AL, USA) and AEC solution, containing 3-amino-9-ethylcarbazole tablet, *N*,*N*-dimethylformamide and hydrogen peroxide (Sigma). Data were analyzed using ImmunoSpot reader (ImmunoSpot 3 software, version 3.2; Cellular Technology Ltd, Cleveland, OH, USA).

Intracellular cytokine production of $CD4^+$ and $CD8^+$ cells

Cells (0.8×10^6/ml per well) were cultured with HI-PR3 (10 μg/ml) or peptides (5 μg/ml) for 4 days. PMA + ionomycin (6 h) was a positive control. Brefeldin A (Sigma) was added (10 μg/ml) (4 h). Cells were fixed using FACS Lysing Solution and FACS Permeabilizing Solution 2 (Becton Dickinson Immunocytometry Systems) and were labeled with FastImmune anti-human-IFN-γ-FITC antibody (BD PharMingen) and anti-human CD4- or CD8-PerCP-labeled antibodies (Becton Dickinson Immunocytometry Systems) and analyzed by FACScan.

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HLA typing

Low-resolution HLA-DRB1 and HLA-DQB1 typing was performed by PCR with sequence-specific primers (PCR-SSP) using the Pel-Freez DRDQ 3T SSP UniTray system (Dynal Biotech, Brown Deer, WI, USA) as per instructions. Allele frequencies of the general population were from the Allele Frequency Database (http://www.allelefrequencies.net/test).[18] The Immune Epitope Database and Analysis Resource (IEDB) (http://www.immuneepitope.org/home.do) were used for predictions of HLA II alleles that bind cPR3 peptides.

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Detection anti-cPR3 antibodies in sera by ELISA

High-binding plates (Coster, Cambridge, MA, USA) were coated overnight at 4 °C with recombinant cPR3$^{105–201}$ protein (5 μg/ml). Sera were added (1:100) and reactive antibodies were detected with alkaline phosphatase-goat anti-human antibody (Chemicon, Temecula, CA, USA) plus alkaline phosphatase substrate (Bio-Rad, Hercules, CA, USA) and read on a VersaMax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Values are percentage of positive control (rabbit anti-his-tag antibody) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) compared with the mean plus 2 s.d. of healthy controls.

Statistical analysis

The Wilcoxon rank-sum test was used as a nonparametric alternative to the two-sample *t*-test for analysis of T-cell responses rank-sum to compare patients and healthy controls. The Fisher's exact test was used for analysis of HLA allelic frequencies among patients as compared with a US population database of Caucasians from Bethesda (n=307). An individual homozygous for an allele was counted as contributing two alleles to that group. A Bonferroni correction was used since there were multiple comparisons, with P-values of <0.005 required for statistical significance. Ranked linear regression analysis was used to determine associations between complementary protein responsive T cells and reactive antibodies.

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REFERENCES

1. Pendergraft WF III, Preston GA, Shah RR, et al. Autoimmunity is triggered by cPR-3(105–201), a protein complementary to human autoantigen proteinase-3. Nat Med 2004;10:72–79. [PubMed: 14661018]
2. Mekler LB. Specific selective interaction between amino acid groups of polypeptide chains. Biofizika 1969;14:581–584. [PubMed: 5396298]
3. Mekler LB. On the specific mutual interaction of amino acid residues of polypeptide chains and amino acid residues with codons. Oncology 1973;27:286–288. [PubMed: 4704740]
4. Heal JR, Roberts GW, Raynes JG, et al. Specific interactions between sense and complementary peptides: the basis for the proteomic code. Chembiochem 2002;3:136–151. [PubMed: 11921391]
5. Tropsha A, Kizer JS, Chaiken IM. Making sense from antisense: a review of experimental data and developing ideas on sense–antisense peptide recognition. J Mol Recognit 1992;5:43–54. [PubMed: 1472380]
6. Brouwer E, Stegeman CA, Huitema MG, et al. T cell reactivity to proteinase 3 and myeloperoxidase in patients with Wegener's granulomatosis (WG). Clin Exp Immunol 1994;98:448–453. [PubMed: 7994909]
7. Ballieux BE, van der Burg SH, Hagen EC, et al. Cell-mediated autoimmunity in patients with Wegener's granulomatosis (WG). Clin Exp Immunol 1995;100:186–193. [PubMed: 7743653]
8. Griffith ME, Coulthart A, Pusey CD. T cell responses to myeloperoxidase (MPO) and proteinase 3 (PR3) in patients with systemic vasculitis. Clin Exp Immunol 1996;103:253–258. [PubMed: 8565308]
9. King WJ, Brooks CJ, Holder R, et al. T lymphocyte responses to anti-neutrophil cytoplasmic autoantibody (ANCA) antigens are present in patients with ANCA-associated systemic vasculitis and persist during disease remission. Clin Exp Immunol 1998;112:539–546. [PubMed: 9649227]
10. van der Geld YM, Huitema MG, Franssen CF, et al. *In vitro* T lymphocyte responses to proteinase 3 (PR3) and linear peptides of PR3 in patients with Wegener's granulomatosis (WG). Clin Exp Immunol 2000;122:504–513. [PubMed: 11122262]

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11. Winek J, Mueller A, Csernok E, et al. Frequency of proteinase 3 (PR3)-specific autoreactive T cells determined by cytokine flow cytometry in Wegener's granulomatosis. J Autoimmun 2004;22:79–85. [PubMed: 14709416]
12. Popa ER, Franssen CF, Limburg PC, et al. *In vitro* cytokine production and proliferation of T cells from patients with anti-proteinase 3- and antimyeloperoxidase-associated vasculitis, in response to proteinase 3 and myeloperoxidase. Arthritis Rheum 2002;46:1894–1904. [PubMed: 12124874]
13. Williams RC Jr, Staud R, Malone CC, et al. Epitopes on proteinase-3 recognized by antibodies from patients with Wegener's granulomatosis. J Immunol 1994;152:4722–4737. [PubMed: 8157982]
14. Van Der Geld YM, Simpelaar A, Van Der Zee R, et al. Antineutrophil cytoplasmic antibodies to proteinase 3 in Wegener's granulomatosis: epitope analysis using synthetic peptides. Kidney Int 2001;59:147–159. [PubMed: 11135067]
15. Blalock JE, Bost KL. Ligand receptor characteristics of peptides encoded by complementary nucleic acids: implications for a molecular recognition code. Recent Prog Horm Res 1988;44:199–222. [PubMed: 2464186]
16. Blalock JE, Smith EM. Hydropathic anti-complementarity of amino acids based on the genetic code. Biochem Biophys Res Commun 1984;121:203–207. [PubMed: 6547339]
17. Lovett-Racke AE, Trotter JL, Lauber J, et al. Decreased dependence of myelin basic protein-reactive T cells on CD28-mediated costimulation in multiple sclerosis patients. A marker of activated/memory T cells. J Clin Invest 1998;101:725–730. [PubMed: 9466965]
18. Middleton, D.; Menchaca, L.; Rood, H., et al. Tissue Antigens. 2003. p. 403-407.New allele frequency database: http://www.allelefrequencies.net
19. Sathiamurthy M, Hickman HD, Cavett JW, et al. Population of the HLA ligand database. Tissue Antigens 2003;61:12–19. [PubMed: 12622773]
20. Campbell DJ, Kim CH, Butcher EC. Separable effector T cell populations specialized for B cell help or tissue inflammation. Nat Immunol 2001;2:876–881. [PubMed: 11526405]
21. Elkon KB, Sutherland DC, Rees AJ, et al. HLA antigen frequencies in systemic vasculitis: increase in HLA-DR2 in Wegener's granulomatosis. Arthritis Rheum 1983;26:102–105. [PubMed: 6130772]
22. Spencer SJ, Burns A, Gaskin G, et al. HLA class II specificities in vasculitis with antibodies to neutrophil cytoplasmic antigens. Kidney Int 1992;41:1059–1063. [PubMed: 1381003]
23. Peen E, Williams RC Jr. What you should know about PR3-ANCA. Structural aspects of antibodies to proteinase 3 (PR3). Arthritis Res 2000;2:255–259. [PubMed: 11094437]
24. Gencik M, Borgmann S, Zahn R, et al. Immunogenetic risk factors for anti-neutrophil cytoplasmic antibody (ANCA)-associated systemic vasculitis. Clin Exp Immunol 1999;117:412–417. [PubMed: 10444278]
25. Gregersen JW, Kranc KR, Ke X, et al. Functional epistasis on a common MHC haplotype associated with multiple sclerosis. Nature 2006;443:574–577. [PubMed: 17006452]
26. Robertson J, Wu J, Arends J, et al. Characterization of the T-cell epitope that causes anti-GBM glomerulonephritis. Kidney Int 2005;68:1061–1070. [PubMed: 16105036]
27. Morais A, Pires N, Alves H, et al. HLA-class I and II genotyping in sarcoidosis patients. Rev Port Pneumol 2005;11:32–33.
28. Hegazy AA, Abdel-Hamid IA, Ahmed el SF, et al. Leprosy in a high-prevalence Egyptian village: epidemiology and risk factors. Int J Dermatol 2002;41:681–686. [PubMed: 12390192]
29. Hibbs AM, Bznik-Cizman B, Guttenberg M, et al. Ulcerative colitis in a renal transplant patient with previous Goodpasture disease. Pediatr Nephrol 2001;16:543–546. [PubMed: 11465800]
30. Jagiello P, Gencik M, Arning L, et al. New genomic region for Wegener's granulomatosis as revealed by an extended association screen with 202 apoptosis-related genes. Hum Genet 2004;114:468–477. [PubMed: 14968360]
31. Griffith ME, Pusey CD. HLA genes in ANCA-associated vasculitides. Exp Clin Immunogenet 1997;14:196–205. [PubMed: 9493788]
32. Papiha SS, Murty GE, Ad'Hia A, et al. Association of Wegener's granulomatosis with HLA antigens and other genetic markers. Ann Rheum Dis 1992;51:246–248. [PubMed: 1550412]
33. Hagen EC, Stegeman CA, D'Amaro J, et al. Decreased frequency of HLA-DR13DR6 in Wegener's granulomatosis. Kidney Int 1995;48:801–805. [PubMed: 7474667]

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34. Schloot NC, Meierhoff G, Karlsson Faresjo M, et al. Comparison of cytokine ELISpot assay formats for the detection of islet antigen autoreactive T cells. Report of the Third Immunology of Diabetes Society T-Cell Workshop. J Autoimmun 2003;21:365–376. [PubMed: 14624759]
35. Clayton AR, Savage CO. Production of antineutrophil cytoplasm antibodies derived from circulating B cells in patients with systemic vasculitis. Clin Exp Immunol 2003;132:174–179. [PubMed: 12653854]
36. Erlanger BF, Cleveland WL, Wassermann NH, et al. Auto-anti-idiotype: a basis for autoimmunity and a strategy for anti-receptor antibodies. Immunol Rev 1986;94:23–37. [PubMed: 3492425]
37. Hill BL, Erlanger BF. Monoclonal antibodies to the thyrotropin receptor raised by an autoantiidiotypic protocol and their relationship to monoclonal autoantibodies from Graves' patients. Endocrinology 1988;122:2840–2850. [PubMed: 2453350]
38. Erlanger BF. Auto-anti-idiotypy, autoimmunity and some thoughts on the structure of internal images. Int Rev Immunol 1989;5:131–137. [PubMed: 8691045]
39. Munthe LA, Kyte JA, Bogen B. Resting small B cells present endogenous immunoglobulin variable-region determinants to idiotope-specific CD4(+) T cells *in vivo*. Eur J Immunol 1999;29:4043–4052. [PubMed: 10602015]
40. Munthe LA, Os A, Zangani M, et al. MHC-restricted Ig V region-driven T-B lymphocyte collaboration: B cell receptor ligation facilitates switch to IgG production. J Immunol 2004;172:7476–7484. [PubMed: 15187126]
41. Dembic Z, Schenck K, Bogen B. Dendritic cells purified from myeloma are primed with tumor-specific antigen (idiotype) and activate CD4+ T cells. Proc Natl Acad Sci USA 2000;97:2697–2702. [PubMed: 10706628]
42. Bogen B, Malissen B, Haas W. Idiotope-specific T cell clones that recognize syngeneic immunoglobulin fragments in the context of class II molecules. Eur J Immunol 1986;16:1373–1378. [PubMed: 3096740]
43. Weiss S, Bogen B. B-lymphoma cells process and present their endogenous immunoglobulin to major histocompatibility complex-restricted T cells. Proc Natl Acad Sci USA 1989;86:282–286. [PubMed: 2492101]
44. Weiss S, Bogen B. MHC class II-restricted presentation of intracellular antigen. Cell 1991;64:767–776. [PubMed: 1847667]
45. Papamattheou MG, Routsias JG, Karagouni EE, et al. T cell help is required to induce idiotypic–anti-idiotypic autoantibody network after immunization with complementary epitope 289–308aa of La/SSB autoantigen in non-autoimmune mice. Clin Exp Immunol 2004;135:416–426. [PubMed: 15008973]
46. Shoenfeld Y. The idiotypic network in autoimmunity: antibodies that bind antibodies that bind antibodies. Nat Med 2004;10:17–18. [PubMed: 14702622]
47. Shoenfeld Y. Idiotypic induction of autoimmunity: a new aspect of the idiotypic network. FASEB J 1994;8:1296–1301. [PubMed: 8001742]
48. Preston GA, Pendergraft WF III, Falk RJ. New insights that link microbes with the generation of antineutrophil cytoplasmic autoantibodies: the theory of autoantigen complementarity. Curr Opin Nephrol Hypertens 2005;14:217–222. [PubMed: 15821413]
49. Pendergraft WF III, Pressler BM, Jennette JC, et al. Autoantigen complementarity: a new theory implicating complementary proteins as initiators of autoimmune disease. J Mol Med 2005;83:12–25. [PubMed: 15592920]
50. Huelseweh B, Kohl B, Hentschel H, et al. Translated anti-sense product of the Na/phosphate co-transporter (NaPi-II). Biochem J 1998;332(Pt 2):483–489. [PubMed: 9601078]
51. McGuire KL, Holmes DS. Role of complementary proteins in autoimmunity: an old idea re-emerges with new twists. Trends Immunol 2005;26:367–372. [PubMed: 15927527]
52. Mannering SI, Morris JS, Jensen KP, et al. A sensitive method for detecting proliferation of rare autoantigen-specific human T cells. J Immunol Methods 2003;283:173–183. [PubMed: 14659909]

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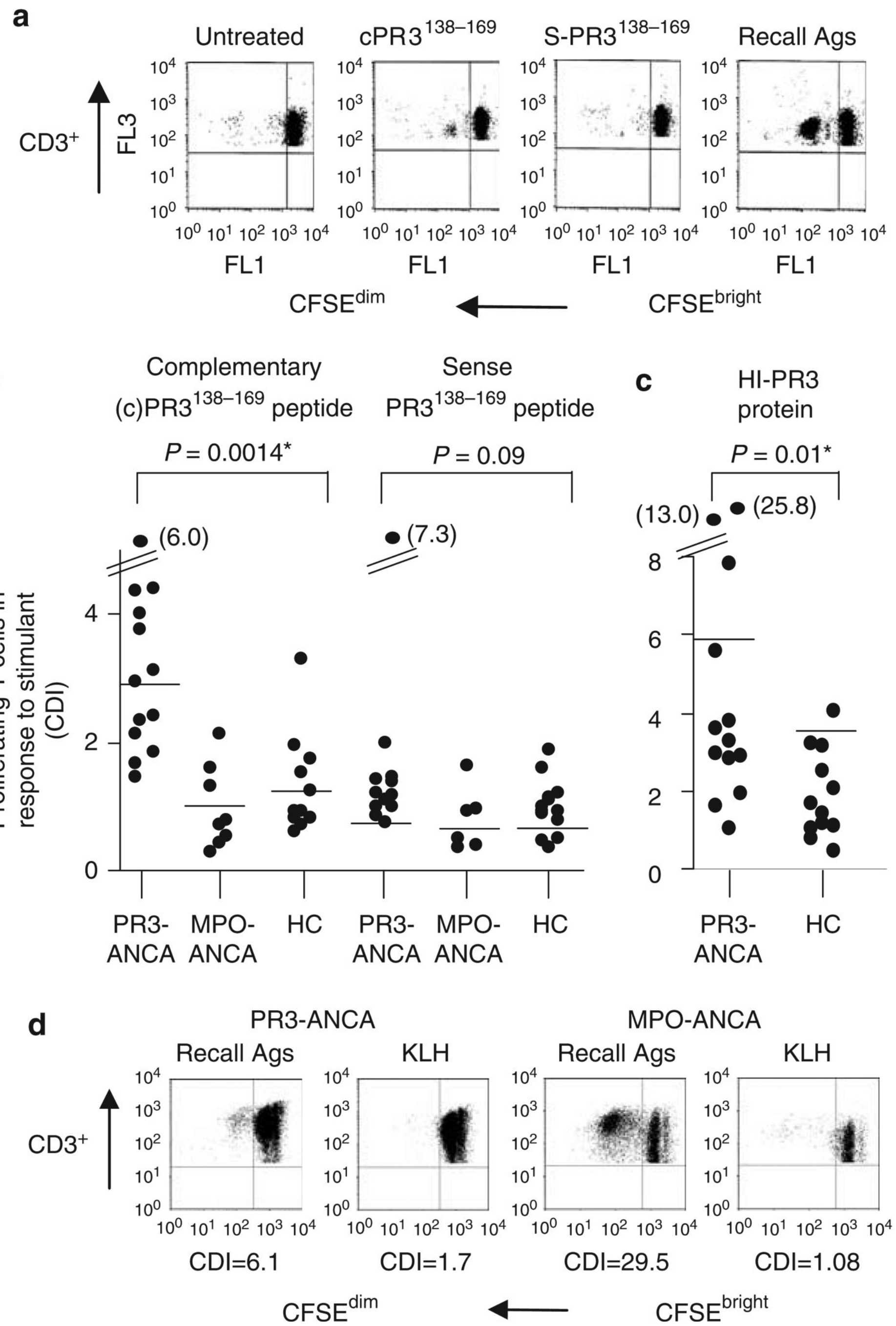

Figure 1. Patients with PR3-ANCA vasculitis harbor T cells that proliferate, when stimulated with complementary (c)PR3$^{138–169}$ peptide

(**a**) Representation flow cytometric data; CD3^{+} T cells were gated and analyzed for the presence of a CFSE^{dim} subset (upper left quadrant). (**b** and **c**) Proliferative response of CD3^{+} T cells of vasculitis patients with PR3-ANCA, MPO-ANCA, and healthy controls (HC) after antigen stimulation. Solid horizontal lines indicate mean values. Comparisons between groups were done using the Wilcoxon ranked sum test.(**d**) Positive T-cell response to recall antigen coincident with non-responsiveness to KLH indicates responding cells are memory cells versus naive cells.

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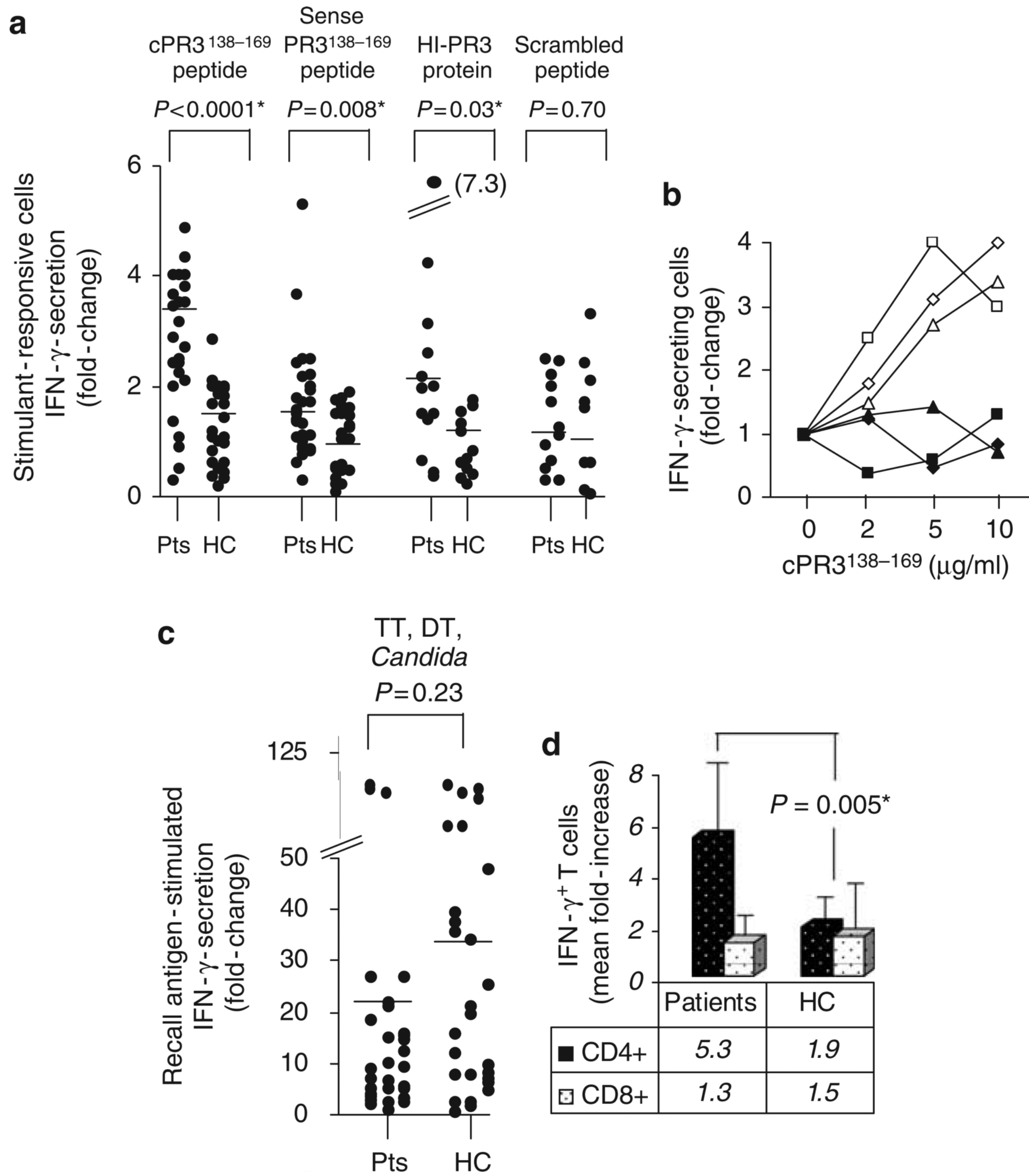

Figure 2. cPR3$^{138–169}$ peptide stimulates CD4^{+} T cells to release IFN-γ

(**a**) ANCA vasculitis patients (Pts) and healthy controls (HC) were analyzed for cytokine secreting T cells by ELISPOT (data indicate fold change of stimulated cells versus unstimulated). (**b**) Response to cPR3$^{138–169}$ peptide was dose-dependent. Three patients (open symbols) and three healthy controls (closed symbols) were analyzed for IFN-γ release after stimulation with varying concentrations of peptide. (**c**) Responses to recall antigens (mixture containing tetanus toxoid, diphteria toxin and candida) were similar between groups. (**d**) FACS analysis intracellular IFN-γ levels identified cPR3$^{138–169}$ peptide-responsive cells as CD4^{+}. Comparisons between groups were done using the Wilcoxon ranked sum test.

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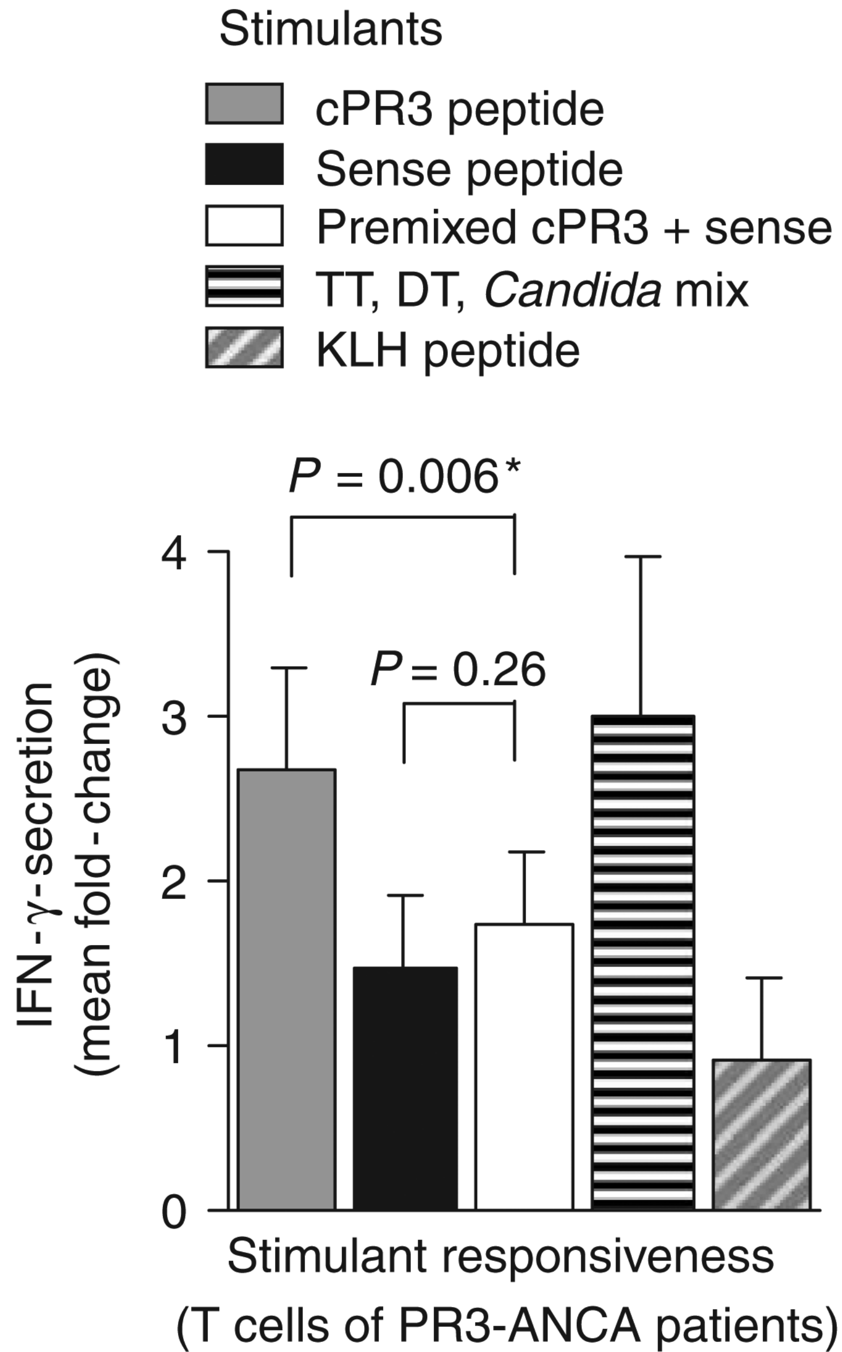

Figure 3. Complex of cPR3 peptide with sense peptide did not enhance T-cell responsiveness

Responsiveness was measured by ELISPOT analysis of IFN-γ-secreting cells. Results are shown as mean fold change as compared with mock control. Positive T-cell response to recall antigens (mixture containing tetanus toxoid (TT), diphtheria toxin (DT), and *Candida*) and non-responsiveness to KLH indicates responding cells are memory cells versus naive cells.

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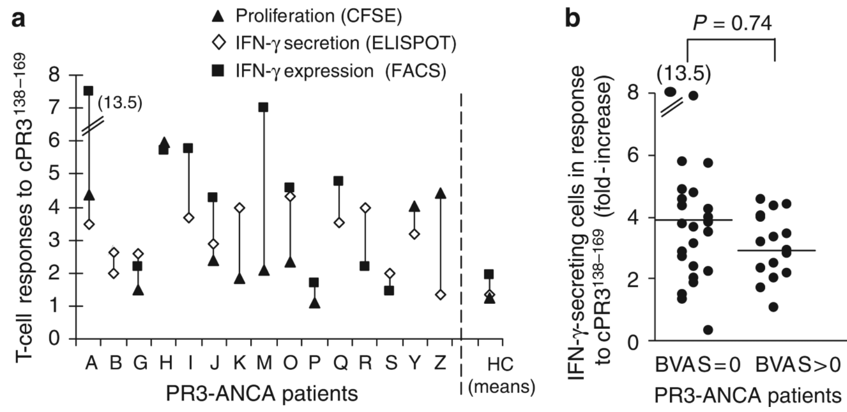

Figure 4. Patients' T cell responses vary with time

(**a**) Comparison of results from each patient that donated blood more than once during the 2-year interval of the study. Mean value of healthy controls (HC) for each assay shown for comparison. (**b**) *y* axis is the ELISPOT value (fold change over untreated) per patient sample plotted against the BVAS activity of the patient on the day the sample was donated on the *x* axis.

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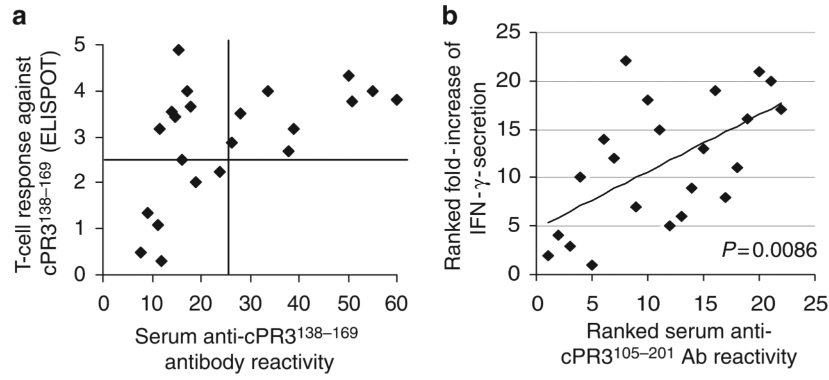

Figure 5. Positive T-cell responses to cPR3$^{138–169}$ peptide were seen in patients who previously had anti-cPR3$^{138–169}$ antibodies

(**a**) The cross bars on the plot indicate the mean plus 2 s.d. (ELISPOT=2.65; ELISA=26.6) of the normal subjects included in the studies. Nine of 22 patients had positive T-cell reactivity and serum antibody reactivity. (**b**) Values were ranked from highest to lowest ELISPOT values. Ranked linear regression indicated a correlation coefficient of 0.545 (P=0.0086).

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Table 1

Information on patients with PR3-ANCA vasculitis

Patients (*n*=30)	Samples (*n*=49)	Sex	Race	Age (years)	HLA II DRB1 alleles	Clinical diagnosis	*BVAS score	Treatment
A	1[a]	M	W	31	03, 10	WG	0	MMF
	2[b]						0	MMF
	3[c]						0	MMF
B	1[c]	M	B	50	04, 15	WG	0	AZA, GC
	2[c]						0	AZA, GC
C	1[c]	M	W	58	15, 15	CSS	0	AZA
D	1[c]	M	W	42	03, 14	MPA	0	MMF
E	1[b]	M	W	72	03, 11	WG	0	CYC, GC
F	1[c]	F	W	47	03, 15	MPA	0	MMF
	2[c]						0	MMF
G	1[a,b]	M	W	69	03, 07	WG	0	CYC, GC
	2[c,b]						0	AZA, GC
H	1[a,b]	F	W	66	04, 07	WG	0	GC
I	1[b]	M	W	26	NA	MPA	0	AZA, GC
	2[c]						0	AZA, GC
J	1[a,b]	M	W	38	01, 14	MPA	0	Off therapy
	2[c]						0	Off therapy
	3[c]						0	Off therapy
K	1[a]	M	W	57	03, 11	MPA	0	AZA
	2[c]						0	AZA
L	1[a]	M	W	64	NA	MPA	0	CYC
M	1[a,b]	M	B	32	04, 15	WG	0	Off therapy
N	1[c]	M	W	26	07, 15	WG	0	Off therapy
O	1[c]	F	B	55	15, 15	WG	3	AZA
	2[b]						3	AZA
	3[c]						0	AZA

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Patients (n=30)	Samples (n=49)	Sex	Race	Age (years)	HLA II DRB1 alleles	Clinical diagnosis	*BVAS score	Treatment
	4^c						3	AZA
P	1^a	M	W	34	01, 04	WG	5	GC
	2^c						1	MMF, GC
Q	$1^{a,c}$	F	W	79	11, 13	MPA	2	MMF
	2^c						0	MMF
	3^c						0	Off therapy
R	1^c	M	W	59	07, 15	WG	3	CYC
	2^c						0	Off therapy
S	$1^{b,c}$	F	W	56	01, 04	WG	7	AZA, GC
T	1^c	F	W	56	11, 13	WG	6	MMF[d]
U	1^c	M	Other	31	03, 07	WG	5	MMF
V	1^c	M	W	47	01, 07	WG	3	MMF, GC
W	1^c	F	W	26	01, 07	WG	3	AZA, CsA
X	1^a	M	W	50	04, 12	MPA	3	MMF, CsA, GC
Y	1^a	F	W	63	03, 04	WG	12^e	CYC, GC
	2^c						3	AZA, GC
Z	1^a	F	W	59	15, 15	MPA	11^e	CYC, GC
	2^c						0	MMF, CsA
	3^c						0	MMF
AA	1^c	M	W	60	NA	MPA	0	MMF
BB	1^c	M	W	37	NA	WG	0	MMF
CC	1^c	F	A	52	NA	WG	25^e	CYC, GC
DD	1^c	F	W	66	NA	WG	16^e	GC

A, Asian; AZA, azathioprine; B, Black; CFSE, carboxyfluorescein diacetate succinimidyl ester; CsA, cyclosporine; CSS, Churg–Strauss syndrome; CYC, cyclophosphamide; ELISPOT, enzyme-linked immunospot assay; F, female; FACS, fluorescence-activated cell sorter; GC, glucocorticoids; HLA, human leukocyte antigen; IFN-γ, interferon-γ; M, male; MMF, mycophenolate mofetil; MPA, microscopic polyangiitis; NA, not applicable; PR3-ANCA, proteinase 3-specific anti-neutrophil cytoplasmic autoantibodies; WG, Wegener's granulomatosis; W, white.

Assays performed per sample are as follows:

[a] Proliferation by CFSE staining

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[b] IFN-γ levels by FACS

[c] IFN-γ secretion by ELISPOT.

[d] Status post-rituximab therapy.

[e] Onset of disease.

[*] BVAS-Birmingham vasculitis activity score.

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Table 2

Analysis of individuals with positive T-cell responses

Analytical measure	Stimulants	Patients % responders	Healthy controls % responders	Statistical significance
Proliferation				
	cPR3$^{138-169}$	54% (7 of 13)	8% (1 of 13)	*P*=0.03
	Sense PR3$^{138-169}$	17% (2 of 12)	8% (1 of 13)	*P*=0.60
	HI-PR3	31% (4 of 13)	8% (1 of 13)	*P*=0.30
IFN-γ secretion				
	cPR3$^{138-169}$	52% (13 of 25)	4% (1 of 24)	*P*=0.0003
	Sense PR3$^{138-169}$	20% (5 of 25)	0% (0 of 23)	*P*=0.051
	HI-PR3	38% (5 of 13)	0% (0 of 12)	*P*=0.040

IFN-γ, interferon-γ.

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Table 3

Analysis of overlapping fragments of cPR3$^{138–169}$ to screen for potential superantigen characteristics

	Spot number cPR3$^{138–153}$ (Fragment 1)[a]	Spot number cPR3$^{146–161}$ (Fragment 2)[a]	Spot number cPR3$^{154–169}$ (Fragment 3)[a]
Patients			
B	1.55	1.15	2.65
T	2.41	3.00	4.41
V	2.56	1.00	3.00
R	1.90	1.90	2.50
A	2.50	2.50	5.10
U	0.90	1.70	2.10
P	0.50	0.43	1.18
O	3.68	3.68	3.08
J	1.67	4.27	2.71
D	3.30	2.30	1.90
Q	6.27	3.91	5.73
C	6.80	1.50	2.25
Controls			
H-A	0.72	0.04	0.74
H-B	0.31	0.92	1.38
H-C	0.65	0.45	0.65
H-D	0.29	0.77	0.51
H-E	1.41	1.14	1.68
H-F	0.68	1.13	0.92
H-G	1.25	0.69	0.13
H-H	0.61	0.96	0.76
H-I	0.12	0.12	0.36
H-J	4.40	0.27	1.60
H-K	1.05	1.26	2.32
H-L	0.50	1.40	1.60

cPR3, complementary proteinase 3; ELISPOT, enzyme-linked immunospot assay.

cPR3$^{138–169}$: DLGWGVVGTHAAPAHGQALGAVGHWLVLLWQL.

Fragment 1: DLGWGVVGTHAAPAHG.

Fragment 2: THAAPAHGQALGAVGH.

Fragment 3: QALGAVGHWLVLLWQL.

[a]The ELISPOT data are expressed as positive spots of treated wells minus spots on untreated wells.

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Table 4

Comparison of HLA-DRB1 allele frequencies of patients with a population frequency database

Locus	Allele group	Alleles observed	Observed frequency	Expected frequency	*P*-value
DRB1	*01	5	0.104	0.128	0.82
	*03	8	0.167	0.110	0.24
	*04	7	0.146	0.159	0.96
	*07	7	0.146	0.145	1.00
	*10	1	0.021	0.014	0.53
	*11	4	0.083	0.103	0.81
	*12	1	0.021	0.010	0.41
	*13	2	0.042	0.121	0.15
	*14	2	0.042	0.045	1.00
	*15	11	0.229	0.093	0.0061*

HLA, human leukocyte antigen.

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Table 5

HLA II alleles predicted to recognize cPR3 peptide amino acid sequences, based on the immune epitope and analysis resource database

Locus	Allele group	Sequence	[a]Predicted affinity IC_{50} (nM)
DRB1	*0101	WGVVGTHAA	7.0
		PAHGQALGA	57.0
		WGTHAAPA	83.1
		THAAPAHGQ	104.6
		GTHAAPAHG	114.6
		GQALGAVGH	142.1
	*0301	VGHWLVLLW	606.7
	*0401	WGVVGTHAA	61,341.5
		GHWLVLWQ	146.5
	*0701	VGTHAAPAH	1.2
		GAVGHWLVL	101.6
		QALGAVGHW	466.5
		LGAVGHWLV	1253.0
	*10	Not available	
	*1101	LGWGVVGTH	461.5
		VGHWLVLLW	2056.4
		Not available	
	*1302	GTHAAPAHG	226.7
		GWGVVGTHA	282.2
		ALGAVGHWL	410.7
		WGVVGTHAA	486.3
	*14	Not available	
	*1501	HGQALGAVG	7.3
		HWLVLLWQL	189.8
		AVGHWLVLL	243.6
		LGWGVVGTH	913.4

cPR3, complementary proteinase 3; HLA, human leukocyte antigen.

$cPR3^{138-169}$: DLGWGVVGTHAAPAHGQALGAVGHWLVLLWQL.

Fragment 1: DLGWGVVGTHAAPAHG.

Fragment 2: THAAPAHGQALGAVGH.

Fragment 3: QALGAVGHWLVLLWQL.

[a]Interpretation of predicted affinities: IC_{50} values<50=high affinity; IC_{50} values<500=intermediate affinity; IC_{50} values<5000=low affinity. No known T-cell epitope has an IC_{50} value.