

# Relative Importance of CCR5 and Antineutrophil Cytoplasmic Antibodies in Patients with Wegener's Granulomatosis

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**ABSTRACT. Objective.** Wegener's granulomatosis (WG) is an idiopathic inflammatory condition characterized by upper and lower airway involvement and often renal dysfunction. Sites of tissue injury include pleomorphic cell populations, and classically mononuclear cell infiltrates that may form granulomas. Vascular inflammation (i.e., vasculitis) is often but not always present. Because CCR5 and its ligands influence mononuclear cell trafficking, we sought to identify their expression in pulmonary lesions and to determine whether genetic variations in genes for CCR5 and its ligands influence susceptibility to WG.

**Methods.** Lung biopsies from 4 patients that had classical features of WG were examined for protein expression of CCR5, RANTES, MIP-1 $\alpha$  and MIP-1 $\beta$  using immunohistochemistry. One hundred eighteen Caucasian patients with WG and 127 ethnically matched healthy controls were included in the genetic analysis. Genomic DNAsamples were amplified by PCR. CCR5  $\Delta$ 32 and RANTES -28 and -401 polymorphisms were determined by either specific primers or direct sequencing.

**Results.** CCR5<sup>+</sup> cells were enriched in lung lesions from patients with WG. Enhanced protein concentrations of RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  were present in WG lung lesions, indicating redundancy of ligands for CCR5 in affected tissue. Genetic analyses revealed 3 subsets of patients with WG: (1) circulating antineutrophil cytoplasmic antibody (ANCA) positive and CCR5<sup>+/+</sup> (58%); (2) CCR5<sup>+/+</sup> and ANCA negative (22%); and (3) CCR5  $\Delta$ 32 and ANCA positive (20%). Among patients in whom ANCA were repeatedly absent, none was found to carry the CCR5  $\Delta$ 32 allele. Conversely, patients who possessed the CCR5  $\Delta$ 32 allele were always ANCApositive.

**Conclusion.** CCR5 and its ligands are abundantly present in pulmonary lesions in WG. The absence of a genetic deletion for CCR5 (CCR5  $\Delta$ 32) in WG patients lacking ANCA suggests that CCR5 may exert a particularly important pathogenetic role in those patients. Another subset of patients (~20%) with WG possessed a genetic deletion for CCR5. That each of these patients was ANCA positive implies that an alternative pathway to CCR5 may exist, for which ANCA may be especially important. (J Rheumatol 2003;30:1541-7)

*Key Indexing Terms:*

WEGENER'S GRANULOMATOSIS  
POLYMORPHISMS

CHEMOKINES

RECEPTORS/CHEMOKINES  
CCR5

Wegener's granulomatosis (WG) is a systemic granulomatous disease that is often associated with vasculitis<sup>1-4</sup>. The cause of WG is unknown. When the disease is fully expressed and untreated, death may occur within weeks of

onset. Inflammation and tissue destruction is most common in the nose, sinuses, lungs, and kidneys. Other critical organs may also be involved. Characteristic histologic features of WG are granuloma formation and, when present, vasculitis of small to medium size vessels. Granulomatous inflammation typically includes activated macrophages that may become syncytia ("giant cells"), T cells, neutrophils and occasionally eosinophils. Macrophage/monocyte activation has been well documented in WG<sup>5-7</sup>. Activated T cells with a Th1 phenotype are enriched in sites of inflammatory reactions<sup>8-12</sup>.

The presence of circulating antibodies against granule constituents of myeloid cells (antineutrophil cytoplasmic antibodies, ANCA) is a serological marker for WG that is present in most patients<sup>13-15</sup>. However, a minority of patients with WG do not have detectable circulating ANCA. In addition, the correlation between ANCA titers and disease activity is too crude to recommend ANCA titers as the prin-

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cial guide to therapeutic decision-making<sup>14-16</sup>. These observations have raised questions about whether ANCA is an essential component of disease pathogenesis.

Chemokines, 8–10 kDa proteins with 4 conserved cysteines, are cell-type-selective chemoattractants that have been divided into 4 subfamilies based on the relative position of their cysteine residues<sup>17-19</sup>. The CXC (or  $\alpha$ ) chemokines have the first 2 cysteine residues separated by one amino acid, while the CC (or  $\beta$ ) chemokines have the first 2 cysteine residues adjacent to each other. Lymphotactin and fractalkine are the only members thus far included in the C and CX3C chemokine subfamilies, respectively. Chemokines function through their G-protein-coupled cell surface receptors expressed on target cells. About 50 chemokines and 18 chemokine receptors have been identified to date. CC chemokine receptor 5 (CCR5), the receptor for  $\beta$ -chemokines RANTES (regulated upon activation normal T-cell expressed and secreted)<sup>20</sup>, macrophage inflammatory protein (MIP)-1 $\alpha$ , and MIP-1 $\beta$ , has been detected mainly on macrophages, T lymphocytes, and dendritic cells<sup>19,21</sup>. Several groups have described increased concentrations of CCR5 ligands and enrichment of CCR5-bearing cells in affected organs from patients with inflammatory/autoimmune diseases<sup>22-25</sup>, suggesting a critical role for the CCR5 signaling pathway in recruiting these cells into sites of inflammatory reactions. Further, CCR5 is expressed mainly on Th1 rather than Th2 T cells<sup>26-28</sup>, and CCR5<sup>+</sup> dendritic cells are capable of promoting Th1 T cell responses via interleukin 12 (IL-12) dependent<sup>29</sup> or independent pathways<sup>30</sup>. Mice lacking CCR5 have defects in macrophage recruitment during host defense against *Cryptococcus neoformans*<sup>31</sup> and have poor formation of granulomas following infection with *Leishmania donovani*. Experimental production of granulomas was not affected by deficiencies of CCR2 or MIP-1 $\alpha$ <sup>32</sup>.

In humans, the CCR5 gene may have a 32 base pair deletion ( $\Delta 32$ ) that creates a truncated, nonfunctional CCR5 protein. The CCR5  $\Delta 32$  allele, a loss-of-function mutation, has been demonstrated by several groups to confer resistance to human immunodeficiency virus (HIV) infection because of the requirement of HIV to utilize CCR5 as a coreceptor for membrane binding and cell penetration<sup>33-38</sup>. Levels of cell surface CCR5 expression in individuals heterozygous for CCR5  $\Delta 32$  (CCR5<sup>+/32</sup>) are reduced to 20–30% of that in CCR5 wild-type (CCR5<sup>+/+</sup>) individuals<sup>39</sup>, suggesting that CCR5  $\Delta 32$  is a dominant negative mutation. CCR5  $\Delta 32$  has been reported to be associated with milder disease in rheumatoid arthritis<sup>40</sup>, reduced risk of asthma<sup>41</sup>, and later onset and fewer relapses of multiple sclerosis<sup>42,43</sup>. Individuals homozygous for CCR5  $\Delta 32$  do not develop rheumatoid arthritis<sup>44</sup>.

The aims of this study were (1) to explore the role of CCR5 in WG by direct examination of this receptor and its

ligands in patient lung biopsies, and (2) to test the possibility of there being a negative association between CCR5  $\Delta 32$  and susceptibility to WG.

## MATERIALS AND METHODS

**Patients and healthy controls.** One hundred eighteen American Caucasian patients and 127 ethnically matched healthy controls were studied. All patients were recruited from the Center for Vasculitis Care and Research, Department of Rheumatic and Immunologic Diseases, The Cleveland Clinic Foundation, from 1996 to 2000. Age and sex matched healthy controls were from the same geographic area as patients. Samples were collected from unrelated friends or spouses of patients. All patients fulfilled the American College of Rheumatology criteria for WG<sup>45</sup>. Fifty-seven patients were male and 61 female. Forty-five patients had renal involvement and 73 did not. One hundred fourteen patients' sera were examined for ANCA. The study was approved by the Institutional Review Board of The Cleveland Clinic Foundation.

**Immunohistochemistry.** Open lung biopsies from 4 patients with WG were fixed using 4% paraformaldehyde. Tissue specimens were cut to 8  $\mu$ m thickness. Sections were pretreated by steaming for 30 min in 10 mM citrate buffer, followed by incubation with protein block and avidin and biotin blocking solutions (Dako, Carpinteria, CA, USA) at room temperature for 10 min. Slides were incubated with primary antibodies at 4°C overnight. Appropriate biotinylated secondary antibodies were added and incubated at room temperature after washing with phosphate buffered saline (PBS). After incubation with the Avidin/Biotin peroxidase system (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions, a DAB peroxidase substrate kit (Vector) was used for color development after washing with PBS. Mouse anti-human CCR5, goat anti-human MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES were obtained from R&D Systems (Minneapolis, MN, USA). The working dilutions were 1:200, 1:100, 1:400 and 1:200, respectively. Biotinylated secondary anti-mouse and goat antibodies (Vector) were used at dilutions of 1:2000 and 1:1000, respectively.

**DNA extraction.** DNA was prepared from EDTA-preserved peripheral blood leukocytes using a standard proteinase K digestion and phenol/chloroform method.

**CCR5  $\Delta 32$  genotyping.** Primers 32f: 5'-CAT CAT CCT CCT GAC AAT CGA-3', and 32r: 5'-CCAGCC CCAAGATGACTA TC-3' were synthesized commercially (Invitrogen, Carlsbad, CA, USA). Polymerase chain reactions (PCR) were performed in a thermal cycler (Perkin Elmer 9700) with PCR parameters as described<sup>40</sup>. A preincubation for 10 min at 95°C was introduced since the AmpliTaq<sup>®</sup> Gold DNA polymerase (Perkin Elmer) was employed to ensure accuracy of the amplifications. PCR products were analyzed on 3% NuSieve<sup>®</sup> GTG agarose gels and visualized by 0.1% ethidium bromide staining.

**RANTES -401 and -28 promoter polymorphisms.** Two single nucleotide polymorphisms (SNP) located in the promoter region of the gene encoding for RANTES (*Rantes*) with functional importance have recently been identified<sup>46,47</sup>. Allele-specific primers for the -28 G to C substitution polymorphism were designed as follows: 28G: 5'-TCC CCT TAG GGG ATG CCC CTG-3', and RR: 5'-GCG CAG AGG GCAGTA GCA ATG-3' amplified G allele at position -28, while 28C: 5'-TCC CCT TAG GGG ATG CCC CTC-3' and RR amplified C allele, both yielding PCR products of 172 bp in length. PCR were performed with parameters as follows: 95°C for 10 min; 94°C 30 s, 60°C 30 s, 72°C 90 s for 30 cycles; 72°C for 5 min; and then stored at 4°C. PCR products were examined using 2% Nusieve<sup>®</sup> GTG agarose. Accuracy of the genotyping using allele-specific primers was verified by direct sequencing using an ABI 377 sequencer (Perkin Elmer).

Primers 401F: 5'-GGACCC TCC TCA ATAAAAC-3', and 401R: 5'-TGG CAG TTA GGA CAG GAT C-3' were used to amplify a fragment containing the polymorphism located at -401 in the promoter of *Rantes*. A G to A nucleotide substitution results in an additional consensus-binding

site of the GATA transcription factor family<sup>47</sup>. The location of this polymorphism was identified at -403 previously<sup>48</sup>. PCR were performed as above with annealing temperature of 55°C. PCR products were purified and subjected to sequence analysis on an ABI 377 sequencer (Perkin Elmer).

**ANCA measurement.** Serum samples were routinely examined for the levels of ANCA using MPO, PR3-specific ELISA (Scimedx Co., Denville, NJ, USA) and by standard indirect immunofluorescence on ethanol fixed neutrophils. Patients with detectable concentrations of circulating ANCA on any occasion during the disease course were classified as ANCA positive, while patients with undetectable ANCA on at least 2 occasions were regarded as ANCA negative.

**Statistical analysis.** A chi-square test with Yates' correction was used to compare the allelic and genotypic frequencies between patients and controls and subgroups of patients. Odds ratios (OR) and 95% confidence intervals (CI) for relative risks were calculated after use of Fisher's exact test if necessary. Alleles we examined were previously shown to be associated with certain inflammatory/autoimmune disorders and to influence the gene expression. Under such circumstances, it has been proposed that correction for multiple statistical significance tests is not necessary<sup>49</sup>. To interpret our data in a cautious manner, probability (p) values were further corrected (pc, Bonferroni correction) by multiplying the number of comparisons made at each locus, including number of subgroups of patients and number of alleles or genotypes. All p values were 2 sided. A pc value less than 0.05 was considered significant.

## RESULTS

**CCR5 and its ligands in lung biopsies from patients with WG.** High levels of RANTES expression were found in open lung biopsies from patients with WG (Figure 1), which is consistent with a recent report<sup>50</sup>. An abundance of MIP-1 and MIP-1β was found in inflammatory lesions, but not in the adjacent tissue that appeared normal. These chemokines were produced by "giant cells" as well (Figure 1). Compared to the adjacent normal lung tissues (Figure 2A), CCR5<sup>+</sup> cells were significantly enriched in inflamed lung tissues from patients with WG (Figures 2B, 2C, 2D). Numerous CCR5<sup>+</sup> leukocytes were found tethering to pulmonary vascular endothelial cells (Figure 2E). These findings do not occur in normal tissues (Figure 2A). In contrast, predominantly CCR5<sup>-</sup> cells were found free in the center of the vessel lumen, indicating that CCR5 is a key molecule mediating leukocyte transmigration from the microcirculation to areas of injury<sup>17</sup>. These results suggest that RANTES/MIP-1/MIP-1β and CCR5 ligand receptor pairs play an important role in the recruitment of CCR5-bearing cells in granulomatous inflammation.

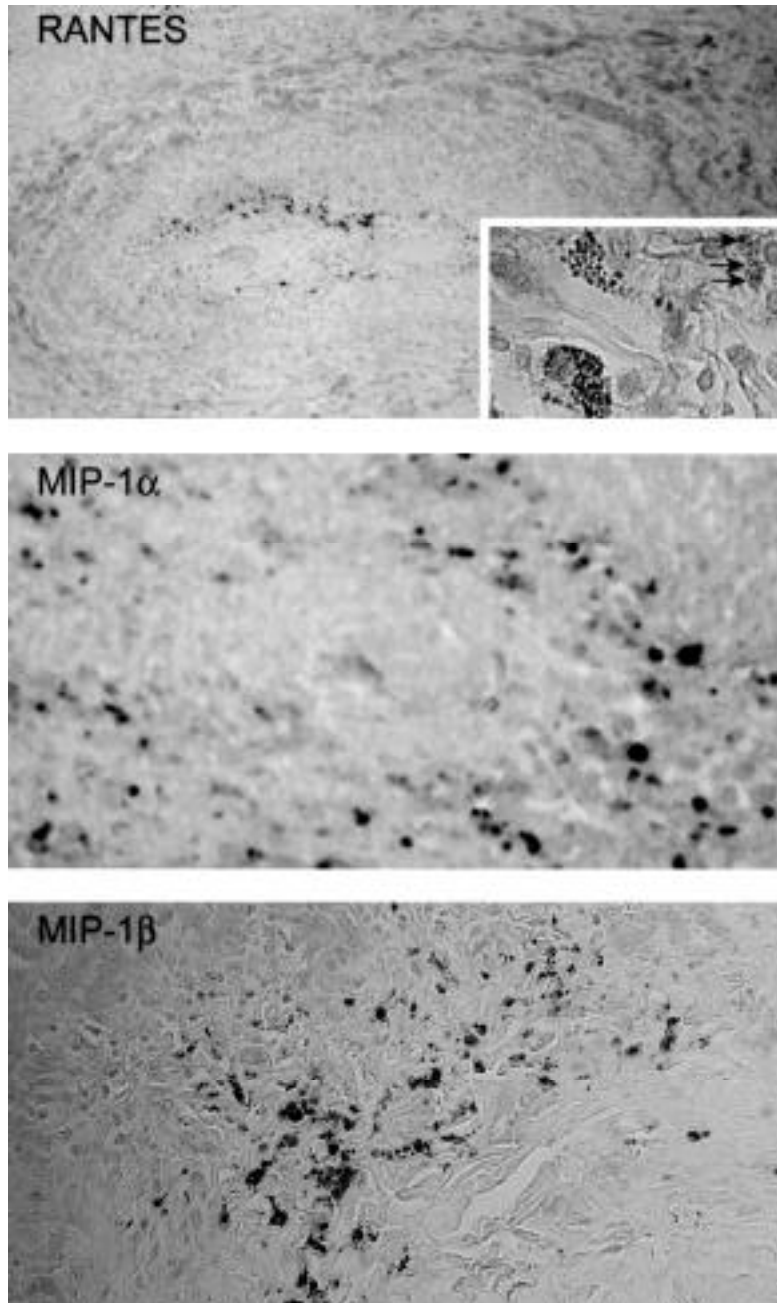
**CCR5 pathway in the absence of ANCA.** Among 114 patients tested for ANCA, 89 were positive and 25 were repeatedly negative. Among the 25 patients with undetectable ANCA, 19 had WG without renal involvement. The genotypic and allelic distributions of CCR5 32 showed no significant difference between patients and healthy controls and were similar to those reported previously in American Caucasian populations<sup>35,38</sup>. There was no significant difference between the observed (Table 1) and the expected frequencies calculated by Hardy-Weinberg equilibrium. However, differences were noted among patients in subset

analyses based on CCR5 32 genotypes and ANCA status (positive vs negative). CCR5 32 was significantly under-represented in patients lacking ANCA (0/25, 21.9% of WG cohort) compared to those positive for ANCA and compared to controls. Conversely, patients who possessed the CCR5 32 (23/89) allele were always ANCA positive. No significant associations between CCR5 32 and sex of patients, renal involvement, and the age of disease onset were found. **Polymorphisms in the promoter region of Rantes.** Two polymorphisms that have been shown to influence the expression of Rantes were examined. No differences in genotypic and allelic distributions between patients and controls were found (Table 2). Preliminary analyses of the 2 polymorphisms with clinical phenotypes of WG showed no differences between subgroups of patients (data not shown). In our current dataset, the frequency of G allele in the polymorphism at position -28 of Rantes was akin to those reported previously in Caucasians<sup>51</sup>, but was significantly lower than that in the Japanese data<sup>46</sup>, which may be related to differences in ethnicity of populations in each study.

## DISCUSSION

We observed high levels of RANTES, MIP-1, and MIP-1β in lung biopsies from patients with WG. Significantly elevated numbers of CCR5<sup>+</sup> cells were evident in granulomatous lung lesions. A minority of patients with WG may have a genetic deletion for CCR5, suggesting the presence of important alternative receptors mediating leukocyte migration into sites of inflammation, and of alternative disease pathways. Remarkably, among patients who lacked ANCA, there were no instances of individuals having the CCR5 32 null allele. The absence of CCR5 32 in WG patients lacking ANCA suggests that CCR5 may exert a particularly important role in pathogenesis in those patients. In addition, the converse observation that all patients in the WG subset who possessed the CCR5 32 allele were ANCA positive suggests that an ANCA associated pathway may be more critical in those individuals. These data have led us to postulate that when CCR5 pathway is markedly reduced or absent, the additional absence of ANCA dramatically diminishes the likelihood of WG.

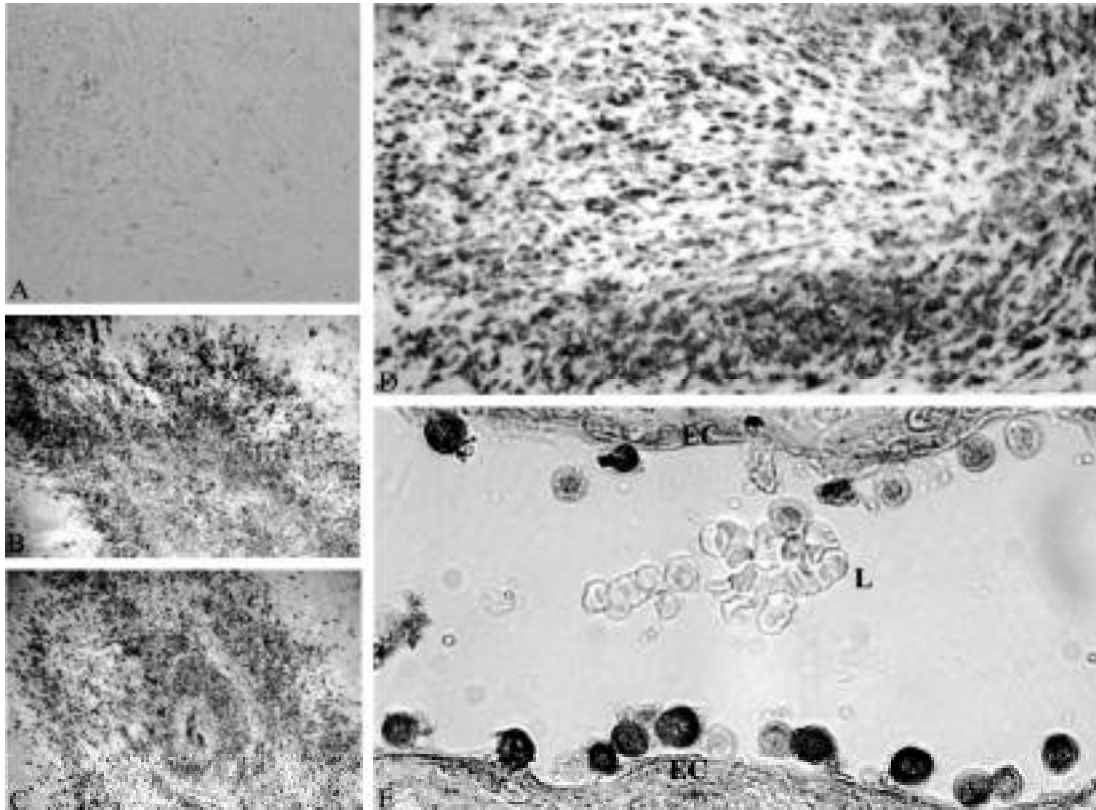
The role of ANCA in the pathogenesis of WG and microscopic polyangiitis has been studied extensively<sup>14</sup>. Several groups have demonstrated that ANCA roughly correlates with disease severity and enhances neutrophil and macrophage activation *in vitro*. However, direct injection of human ANCA into mice, which presumably elicits mouse ANCA through anti-idiotypic network *in vivo*, did not generate granulomas<sup>52</sup>, indicating that ANCA may not be a crucial factor for granuloma formation, a pathologic hallmark of WG. Inconsistent correlations between titers of ANCA and disease activity in some patients and absence of ANCA in others further suggest the existence of other, perhaps more important pathogenetic factor(s), such as CCR5.



*Figure 1.* Expression of CCR5 ligands in lung tissue from patients with WG. Immunohistochemistry revealed numerous cells secreting RANTES, MIP-1, and MIP-1 $\beta$  in areas of granuloma formation (upper and middle panels) and sites with nonspecific patterns of inflammation (lower panel) as well. In addition to cell associated RANTES in the center of the lesion, secreted RANTES was distributed especially around the lesion (upper panel). Compared to the RANTES-expressing leukocytes (arrows), cells with larger cell bodies were also seen to express RANTES (upper panel insert). These are representative findings with similar results from 4 patients.

CD4 memory T cells and monocytes/macrophages are the major cell populations expressing CCR5<sup>21,26</sup>. Injured tissue in WG is enriched with CCR5<sup>+</sup> cells (Figure 2) and CD4<sup>+</sup>CD45RO<sup>+</sup> memory T cells<sup>50</sup>. CCR5<sup>+</sup> mononuclear cells are therefore important in inflammatory reactions in

WG and the formation of granulomas in general. Significantly elevated levels of CCR5 ligands, i.e., RANTES<sup>50</sup>, MIP-1, and MIP-1 $\beta$ , in the inflammatory lesions provide the driving force for the migration of CCR5<sup>+</sup> cells (Figure 1). Our data did not reveal an important role for



**Figure 2.** Significant enrichment of CCR5-bearing cells and adhesion of CCR5<sup>+</sup> leukocytes to endothelium in granulomatous lesions. CCR5<sup>+</sup> cells were abundant in inflammatory lesions and granulomas in lung tissues from patients with WG (B and C, original magnification  $\times 25$ ; D,  $\times 200$ ), while few CCR5<sup>+</sup> cells were found in normal lung tissue (A, original magnification  $\times 100$ ). Note the relative paucity of CCR5<sup>+</sup> cells in the adjacent area of the lesions. Numerous leukocytes were found to adhere to endothelium (EC) in the inflammatory lesions. The majority of cells adhering to EC were CCR5 positive, while those freely floating in the vessel lumen (L) were CCR5 negative (original magnification  $\times 630$ ). These are representative findings with similar results from 4 patients.

**Table 1.** Genotypic and allelic frequencies of CCR5  $\Delta 32$  in patients with WG and healthy controls.

	Patients, n = 118 (%)	Controls, n = 127 (%)	Circulating ANCA	
			Pos, n = 89 (%)	Neg, n = 25 (%)
Genotype				
+/+	95 (80.5)	107 (84.3)	66 (74.2)	25 (100.0)*
+/ $\Delta 32$	22 (18.6)	20 (15.7)	22 (24.7)	0
$\Delta 32/\Delta 32$	1 (0.9)	0	1 (1.1)	0
$\Delta 32$ carrier				
Yes	23 (19.5)	0 (15.7)	23 (25.8)	0
No	95 (80.5)	107 (84.3)	66 (74.2)	25 (100.0) <sup>†</sup>

\*  $p = 0.003$ ,  $pc = 0.018$ , 95% CI 1.2–1.6, compared with patients with circulating ANCA, and  $p = 0.046$  compared with healthy controls. <sup>†</sup>  $p = 0.003$ ,  $pc = 0.012$ , compared to patients with circulating ANCA. ANCA: antineutrophil cytoplasmic antibody; n: number of individuals.

polymorphisms in the promoter region of *Rantes* (mutations at  $-401$  and  $-28$ ) in WG disease expression.

WG occurred in individuals in our study who were either CCR5  $\Delta 32$  carriers and ANCA producers or CCR5-intact regardless of ANCA status. That no WG patient lacking ANCA was positive for CCR5  $\Delta 32$  allele suggests an essen-

tial role for an intact CCR5 pathway in this subset of patients. On the other hand, although CCR5 may well participate in the cascade of host defense in patients with ANCA, the presence of ANCA might diminish the requirement for CCR5. This was indicated by the similar frequency of CCR5  $\Delta 32$  in this subgroup of patients compared with

**Table 2.** Genotypic and allelic frequencies of C/G polymorphism at position -28 and G/A polymorphism at -401 in RANTES gene in patients with WG and healthy controls.

	WG, n = 118 (%)	Controls, n = 127 (%)
<b>-28 Genotype</b>		
C/C	112 (94.9)	122 (96.1)
C/G	6 (5.1)	5 (3.9)
<b>Allele</b>		
C	230/2n (97.5)	249/2n (98.0)
G	6/2n (2.5)	5/2n (2.0)
	WG, n = 103 (%)	Controls, n = 70 (%)
<b>-401 Genotype</b>		
G/G	80 (77.7)	59 (84.3)
G/A	21 (20.4)	10 (14.3)
A/A	2 (1.9)	1 (1.4)
<b>Allele</b>		
G	181/2n (87.9)	128/2n (91.4)
A	25/2n (12.1)	12/2n (8.6)

healthy controls and the occurrence of WG in one CCR5<sup>32/32</sup> patient. These observations suggest that at least one of these immunoinflammatory pathways must be present for WG to occur.

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