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BRIEF REPORT

A Regulatory Variant in *CCR6* Is Associated With Susceptibility to Antitopoisomerase-Positive Systemic Sclerosis

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Objective. **Recognition of the well-known pleiotropism of autoimmune genes supports the concept of a shared pathogenesis across autoimmune diseases such as rheumatoid arthritis (RA) and systemic sclerosis (SSc). Studies have reproducibly demonstrated an association between susceptibility to RA and polymorphisms of the *CCR6* gene, a surface marker for Th17 cells, and the causal variant was recently identified. The present study was thus undertaken to investigate whether *CCR6* polymorphisms could also be associated with susceptibility to SSc.**

Methods. **Twelve tag single-nucleotide polymorphisms (SNPs) of *CCR6*, including the known RA-associated SNP rs3093023, were genotyped in a total of 2,411 SSc patients and 7,084 healthy individuals from 3 European populations (France, Italy, and Germany). Meta-analyses of the data were performed to assess whether an association exists between *CCR6* polymorphisms and susceptibility to SSc or its main subtypes. Direct sequencing of DNA was performed to ascertain whether the functional dinucleotide polymorphism of *CCR6* previously identified in RA (*CCR6DNP*) was also present in SSc.**

Results. **Combined analyses revealed an association between the rs10946216 SNP and SSc susceptibility (odds ratio [OR] 1.13, 95% confidence interval [95% CI] 1.05–1.21, adjusted *P* [P_{adj}] = 0.026). The rs3093023 A allele and rs10946216 T allele were in high linkage disequilibrium, and both were found to confer disease susceptibility in the antitopoisomerase-positive**

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subset of SSc patients (OR 1.27, 95% CI 1.13–1.42, $P_{adj} = 1.5 \times 10^{-3}$ and OR 1.32, 95% CI 1.17–1.48, $P_{adj} = 9.0 \times 10^{-5}$, respectively, relative to healthy controls). Direct sequencing of the DNA of 78 individuals supported the hypothesis that the regulatory dinucleotide *CCR6DNP* could be the causal variant in SSc.

Conclusion. **The results of this study establish *CCR6* as a new susceptibility factor for antitopoisomerase-positive SSc, as demonstrated in 3 European Caucasian populations, confirming the notion that SSc and RA could conceivably share autoimmune risk alleles. The results also suggest a potential role of the interleukin-17 pathway in SSc.**

Systemic sclerosis (SSc) is a chronic systemic disease with a complex pathogenesis. The disease is characterized by early vascular alterations and activation of the immune system, with autoimmune features preceding the deposition of extracellular matrix, leading to systemic fibrosis. Although it is frequently characterized as an autoimmune disease, the mechanisms underlying the early inflammatory phase of SSc, involving both T cells and B cells, remain poorly understood.

In recent years, numerous genetic factors underlying the susceptibility to SSc have been identified, mainly through association studies using candidate gene approaches and a few genome-wide association studies (GWAS) (1,2). The majority of these susceptibility loci belong to pathways that lead to autoimmune responses or inflammation. In these pathways, the loci may be involved in antigen processing (*MHC*), innate immunity (*IRF5*), T cell differentiation and/or activation (*STAT4*, *TNFSF4, CD226*), and signaling (*TNFAIP3*, *TNIP1*, *PTPN22*, *BANK1*, *BLK*, *CD247*) (1). Most of these known susceptibility loci have also been identified in other autoimmune diseases, thus highlighting the existence of a genetic overlap between SSc and other autoimmune diseases and the concept of shared autoimmunity. In particular, several loci, including *PTPN22*, *STAT4*, and *TNFAIP3*, have been reported to be associated with rheumatoid arthritis (RA) (3).

A large meta-analysis of GWAS studies of RA cohorts, all involving patients with RA of European descent who were positive for RA-typical autoantibodies, confirmed associations with 4 loci that have been implicated in other autoimmune diseases, including the single-nucleotide polymorphism (SNP) rs3093023, located in the *CCR6* gene region (4). A concurrent GWAS study also identified a strong association between the *CCR6* locus and RA in a Japanese population (5). The association was replicated in 2 independent Japanese replication cohorts (5). In addition, a functional triallelic dinucleotide polymorphism of *CCR6*, *CCR6DNP*, was recently discovered (5). Indeed, this *CCR6DNP* polymorphism has been found to correlate with the levels of *CCR6* messenger RNA, been associated with the detection of interleukin-17 (IL-17) in the sera of RA patients, and been shown to exhibit effects on gene transcription, suggesting that this dinucleotide polymorphism is a causal variant (5). The same *CCR6DNP* variant was also associated with susceptibility to several other autoimmune diseases, including Graves' disease and Crohn's disease (5). Very recently, another meta-analysis of GWAS studies strongly confirmed the association of RA with the *CCR6* locus in the Japanese population, and provided convincing evidence to support the existence of some shared genetic risk factors for RA between populations of European and Japanese ancestry (6).

The *CCR6* gene encodes C-C motif chemokine receptor 6, i.e., the receptor for CCL20 and a surface marker for IL-17–producing Th17 cells (7). It is believed that CCL20/CCR6 signaling plays a role in the recruitment of not only immature dendritic cells and their precursors, but also Th17 cells, into sites of potential antigen entry (7). Various disease models for systemic lupus erythematosus and RA have been linked to infiltration of Th17 cells, most of them reporting CCR6 as a key factor for Th17 cell infiltration into the target tissues (8). With regard to this process in patients with SSc, published data are scarce, but studies in an animal model of SSc have suggested that IL-17 might play a role in the development of skin fibrosis (9).

Given the evidence of shared, common autoimmune genes between RA and SSc, together with the increasing data showing that Th17 cells may be a critical factor in autoimmune diseases, the current study sought to investigate whether there are any associations between *CCR6* polymorphisms and susceptibility to SSc.

PATIENTS AND METHODS

Study population. In total, 2,411 SSc patients and 7,084 unrelated, ethnically matched healthy individuals from 3 European populations were included (France, n = 1,082 SSc cases and n = 3,084 controls; Italy, n = 680 SSc cases and n = 1,895 controls; Germany, n = 649 SSc cases and n = 2,105 controls). Detailed phenotypic assessment was carried out in all SSc patients, as previously described (2,10). In addition, in all patients, we determined the cutaneous subtype of SSc, according to the criteria of LeRoy et al (11), and carried out a phenotypic assessment by subtype. All patients were tested for antinuclear antibodies and their putative specificity. Anticentromere antibodies were determined on the basis of their

Table 1. Pooled analysis of associations with the *CCR6* rs3093023 and rs10946216 single-nucleotide polymorphisms (SNPs) in the combined Caucasian (French, Italian, and German) populations in an additive recessive model*

SNP minor/major allele, group	No. of subjects	Genotype			MAF, %	*P*	Adjusted *P*†	OR (95% CI)
		1/1	1/2	2/2				
rs3093023 A/G								
SSc	2,366	511 (21.6)	1,176 (49.7)	679 (28.7)	46.5	0.07		1.06 (0.99–1.14)
lcSSc	1,471	311 (21.1)	730 (49.6)	430 (29.2)	46.0	0.36		1.04 (0.96–1.13)
SSc ACA+	870	182 (20.9)	422 (48.5)	266 (30.6)	45.2	0.89		1.01 (0.91–1.11)
dcSSc	709	165 (23.3)	349 (49.2)	195 (27.5)	47.9	0.034		1.13 (1.01–1.26)
SSc ATA+	648	161 (24.9)	337 (52.0)	150 (23.2)	50.9	6.6×10^{-5}‡	1.5×10^{-3}‡	1.27 (1.13–1.42)
SSc with FA	810	183 (22.6)	407 (50.3)	220 (27.2)	47.7	0.04		1.12 (1.01–1.24)
SSc plus other AID	275	70 (25.5)	135 (49.1)	70 (25.5)	50.0	0.02		1.23 (1.03–1.46)
Healthy controls	6,912	1,360 (19.7)	3,495 (50.6)	2,057 (29.8)	45.0	NA	NA	NA
rs10946216 T/C								
SSc	2,351	528 (22.5)	1,186 (50.5)	637 (27.1)	47.5	0.0012	0.026	1.13 (1.05–1.21)
lcSSc	1,457	318 (21.8)	738 (50.7)	401 (27.5)	47.2	0.035		1.10 (1.01–1.19)
SSc ACA+	860	186 (21.6)	431 (50.1)	243 (28.3)	46.7	0.21		1.07 (0.96–1.19)
dcSSc	710	169 (23.8)	354 (49.9)	187 (26.3)	48.7	0.006	0.13	1.17 (1.05–1.31)
SSc ATA+	649	166 (25.6)	341 (52.5)	142 (21.9)	51.9	4.1×10^{-6}‡	9.0×10^{-5}‡	1.32 (1.17–1.48)
SSc with FA	805	189 (23.5)	405 (50.3)	211 (26.2)	48.6	0.005	0.11	1.17 (1.05–1.30)
SSc plus other AID	268	69 (25.8)	136 (50.8)	63 (23.5)	51.1	0.0049	0.11	1.29 (1.08–1.54)
Healthy controls	5,017	1,015 (20.2)	2,491 (49.7)	1,511 (30.1)	45.1	NA	NA	NA

* MAF = minor allele frequency; OR = odds ratio; 95% CI = 95% confidence interval; SSc = systemic sclerosis; lcSSc = limited cutaneous subtype of systemic sclerosis; ACA+ = anticentromere antibody positive; dcSSc = diffuse cutaneous subtype of systemic sclerosis; ATA+ = anti–topoisomerase I antibody positive; FA = fibrosing alveolitis; AID = autoimmune disease (other than rheumatoid arthritis); NA = not applicable.
† *P* values were adjusted using Bonferroni correction for multiple comparisons.
‡ Significant association (at $P < 0.05$).

distinctive immunofluorescence pattern. Anti–topoisomerase I antibodies were determined by counterimmunoelectrophoresis.

The study was approved by our local institutional review boards, and written informed consent was obtained from all subjects. Patients with SSc in whom RA was also diagnosed were excluded prior to the current analysis, in order to avoid bias due to a possible excess of the risk alleles attributable to these patients, as previously described (10).

SNP selection and genotyping. DNA samples from SSc patients and controls were genotyped for 12 tag SNPs of *CCR6* (6q27): rs11575083, rs10946216, rs3093010, rs2071171, rs3093007, rs3093012, rs1855025, rs3093009, rs3798315, rs3093006, rs9459883, and rs3093023. We also included available genome-wide data from 1,984 French controls, 291 Italian controls, and 993 German controls from the Three Cities cohort, HYPERGENES study (http://www.hypergenes.eu), and KORA F4 study, respectively, since some of the *CCR6* tag SNPs were included in the chips used for these projects (2). The rs3093023 SNP is known to be associated with RA (4). In addition, in European Caucasian populations, this SNP appears to be in strong linkage disequilibrium (LD) ($r^2 > 0.80$) with 2 other RA-associated SNPs, rs3093024 and rs963334 (part of the *CCR6DNP*), both of which were found to be associated with RA in a study by Kochi et al (5). Genotyping was performed using a competitive allele-specific polymerase chain reaction (PCR) system (KASPar Genotyping; Kbioscience), as previously described (10).

Sequencing of the *CCR6DNP* polymorphism. To further investigate the potential causal variant, we sought to determine whether rs3093023 could tag the recently identified functional *CCR6DNP*. We randomly selected 78 individuals from the French cohort (42 SSc patients and 36 controls) on the basis of their rs3093023 genotype (A/A, G/A, or G/G), yielding 17 subjects with the A/A genotype, 36 with the G/A genotype, and 25 with the G/G genotype. Genomic DNA was extracted from blood samples and was amplified for direct sequencing by PCR (Macherey-Nagel), using the following forward and reverse sequences: 5′-CAACCACCTTTGAAAGAGCAG-3′ and 5′-CCCTTGTTCATCCCAACCT-3′. DNA products (207 bp in length) were purified using a Multiscreen PCR filter plate (Millipore) and then directly sequenced using a BigDye Terminator Cycle Sequencing kit (version 3.1; Applied Biosystems).

Statistical analysis. Statistical analyses were conducted using the *Arthritis & Rheumatism* recommendations for genetic association studies (12), and included power calculations and haplotype analyses. Tests for conformity with Hardy-Weinberg equilibrium (HWE) were performed using a standard chi-square test (1 degree of freedom). All odds ratios (ORs) are provided with the 95% confidence intervals (95% CIs). Individual association analyses to assess possible associations of *CCR6* SNPs with SSc were performed by comparing the allelic distribution between cases and controls using the Fisher's exact test. The same procedure was applied to subgroups stratified according to SSc phenotype. We applied a conservative Bonferroni correction for multiple testing, that took into account both the number of phenotypic subsets and the number of tag SNPs tested (n = 22). An adjusted *P* value of less than 0.05 was considered statistically significant.

Meta-analysis of *CCR6* rs3093023 and rs10946216 SNPs. The Breslow-Day method was applied to calculate the homogeneity of ORs between the 3 cohorts. The combined data for the 3 populations were subsequently analyzed by

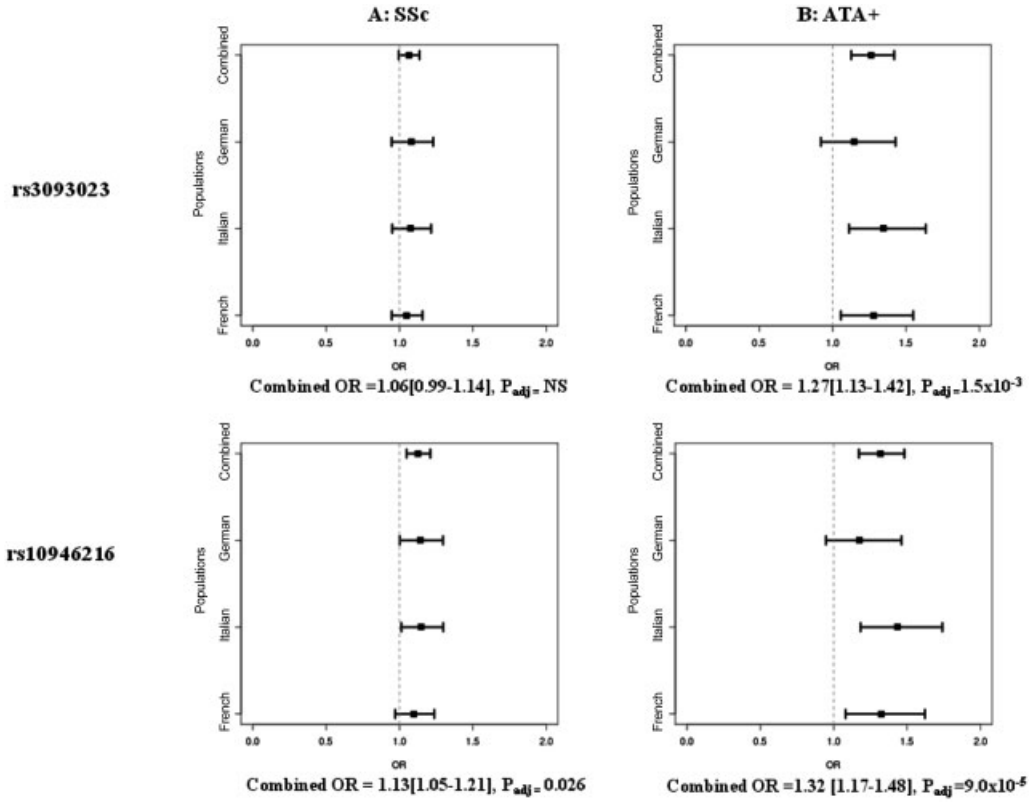

Figure 1. Association of *CCR6* single-nucleotide polymorphisms rs3093023 and rs10946216 with systemic sclerosis (SSc), as determined in a meta-analysis of the combined data from 3 European Caucasian populations. Forest plots show the results of the meta-analysis of the pooled data (combined) and the data from each cohort (German, Italian, and French) for the likelihood of the presence of *CCR6* rs3093023 (top) and *CCR6* rs10946216 (bottom) in patients with SSc (**A**) and in the subset of SSc patients with anti–topoisomerase I antibodies (ATA+) (**B**). Bars show the odds ratio (OR) with 95% confidence intervals, relative to healthy controls (set at 1.0), analyzed using the Cochran-Mantel-Haenszel test. NS = adjusted *P* value (P_{adj}) not significant.

calculating the pooled ORs, using a Cochran-Mantel-Haenszel test for stratified analysis.

Power calculation. The power of the data from the meta-analysis was assessed using the R program (version 2.15.2). Taking into account the expected frequency of the *CCR6* rs3093023 minor allele (40.6%) in the general population, the combined set of 2,411 SSc cases and 7,084 controls provided a power of 98.0% to detect an association between SSc and these variants, with an OR of 1.3 at the 5% significance level.

RESULTS

Association of *CCR6* polymorphisms with SSc and its main subtypes. Twelve *CCR6* polymorphisms were genotyped in 3 European populations (French, Italian, and German). The average genotyping call rate was higher than 98% in all 3 cohorts. Of note, the number of controls from each cohort differed between the various tested SNPs according to the availability of the gene chips provided by collaborators. Because of our large experience and previous studies using these samples, which were obtained from subjects of different geographic origins but of the same ethnicity, we were confident that the homogeneity of the 3 cohorts could be validated. Therefore, we directly performed combined analyses of the data in order to achieve the largest statistical power. Homogeneity of the cohorts was indeed confirmed by the Breslow-Day test, the results of which showed no evidence of interpopulation heterogeneity.

We thus conducted a meta-analysis of the pooled data from the 3 cohorts, using a Cochran-Mantel-Haenszel test under fixed effects. All SNPs studied in the *CCR6* gene region were in HWE in Caucasian controls. We found an association between the *CCR6* rs10946216 polymorphism and susceptibility to SSc in the pooled analysis (Table 1).

To investigate the possible association of the *CCR6* polymorphisms with specific clinical features of SSc, we stratified the patients according to the main

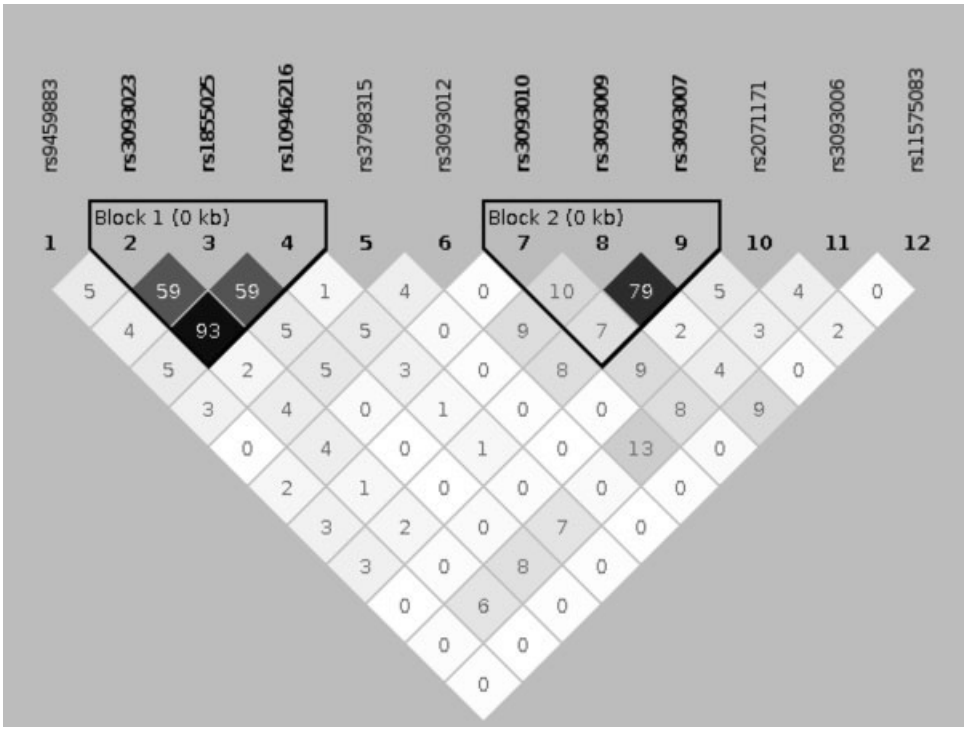

Figure 2. Linkage disequilibrium (LD) of polymorphisms and haplotype block structure of the *CCR6* gene within the combined healthy control population. Blocks connecting pairs of single-nucleotide polymorphisms (SNPs) are shaded according to the strength of the LD between the SNPs, ranging from 0.0 (solid white) to 1.0 (solid black), as measured by r^2 values, which are given as numeric values within each box.

SSc subtypes. We observed a significant increase in the frequency of the *CCR6* rs3093023 A allele and rs10946216 T allele in the antitopoisomerase-positive subset of SSc patients compared with controls (for rs3093023, OR 1.27, 95% CI 1.13–1.42, adjusted P [P_{adj}] $= 1.5 \times 10^{-3}$; for rs10946216, OR 1.32, 95% CI 1.17–1.48, $P_{adj} = 9.0 \times 10^{-5}$) (Table 1 and Figure 1).

In addition, intracohort analyses found that the frequencies of the *CCR6* rs3093023 A allele and rs10946216 T allele were significantly increased in antitopoisomerase-positive SSc patients compared with SSc patients who were negative for antitopoisomerase antibodies (for rs3093023, 50.9% versus 45.3% [OR 1.25, 95% CI 1.09–1.42, $P = 0.0009$]; for rs10946216, 51.9% versus 46.5% [OR 1.24, 95% CI 1.08–1.41, $P = 0.0014$]). No association was observed between antitopoisomerase-negative SSc and the presence of either rs3093023 (OR 1.01, 95% CI 0.94–1.10, $P = 0.74$) or rs10946216 (OR 1.07, 95% CI 0.98–1.16, $P = 0.13$). Details on the allelic distribution of the 2 SNPs in each of the cohorts is available from the corresponding author upon request. No association was found between the SSc subtypes and any of the other *CCR6* SNPs investigated (rs11575083, rs3093010, rs2071171, rs3093007, rs3093012, rs1855025, rs3093009, rs3798315, rs3093006, or rs9459883).

***CCR6* haplotype analysis and LD relationship in healthy controls.** The 2 SNPs found to be associated with susceptibility to SSc, rs3093023 and rs10946216, were in strong LD ($r^2 \geq 0.80$) in the combined European Caucasian control population (Figure 2). Two of the haplotypes formed of these 2 SNPs had a frequency of >5% in controls. The AT haplotype, containing the minor alleles of the rs3093023 and rs10946216 SNPs, was more frequent among SSc patients than among controls (46% versus 44%; $P = 0.0096$) in the combined cohort. Moreover, the AT haplotype was also found to be significantly associated with anti–topoisomerase I–positive SSc (haplotype frequency of 50% in antitopoisomerase-positive SSc patients versus 44% in controls; $P = 6.9 \times 10^{-6}$) (results available from the corresponding author upon request).

Sequencing of the *CCR6DNP* polymorphism. Direct sequencing of the functional dinucleotide polymorphism of *CCR6*, *CCR6DNP*, in DNA samples from 78 individuals for whom the rs3093023 genotype was

known revealed the presence of 5 genotypes: the TG/TG genotype (23.1%), the heterozygous genotypes TG/CG (46.2%), TG/CA (1.3%), and CG/CA (3.8%), and the homozygous CG/CG genotype (25.6%) (results available from the corresponding author upon request). Presence of the *CCR6DNP* TG/TG genotype was correlated with presence of the A/A genotype of rs3093023 in 94.1% of SSc patients. These results suggest that the rs3093023 A allele could tag the *CCR6DNP* TG allele in the entire genotyped cohort.

DISCUSSION

To our knowledge, the present study, which analyzed data from a very large population of European Caucasian subjects, is the first to demonstrate an association between *CCR6* polymorphisms and susceptibility to SSc. Moreover, the most remarkable association was observed with the antitopoisomerase antibody–positive subset of SSc, for both the rs3093023 SNP (OR 1.27) and the rs10946216 SNP (OR 1.32). Results of the intracohort analyses further supported this preferential association with the antitopoisomerase-positive subtype, suggesting that the risk variant could contribute to a disease-specific phenotype and, furthermore, might be considered to be a specific marker of severe disease, since antitopoisomerase antibody positivity is known to be associated with more severe disease, both in terms of skin diffusion and according to the extent of organ involvement (13). An association between *CCR6* risk alleles and disease-specific autoantibody production has also been demonstrated in patients with RA. Indeed, different patterns of allelic associations between anti–citrullinated protein antibody (ACPA)–positive RA and ACPA-negative disease have been described at several loci, including *CCR6* (14).

It is noteworthy that this association with *CCR6* has not been previously found in GWAS studies in patients with SSc. This might be attributed to a possible underestimation of the effect sizes in previous studies, which were low-to-moderate and may not have reached genome-wide significance, but had true effects. Furthermore, phenotypic and genotypic heterogeneity may also explain some of the false-negative results in prior GWAS studies. Although the splitting of subjects into distinct phenotypic subtypes may decrease the power to identify potential associated loci, we believe that it is a major step in the genetic analysis of complex traits.

It is also important to point out that the comparison of patients with SSc of a particular disease subset to patients without this trait (case–case analysis) is more relevant for biomarker identification than is a comparison of patients with the disease manifestation to unaffected controls (case–control analysis). However, in studies of rare diseases, this may be a limitation, because collection of large sample sizes that would allow analysis of relevant disease subsets could be difficult. Results of the studies in the 3 independent cohorts included herein were mainly suggestive of an association between *CCR6* polymorphisms and the antitopoisomerase-positive SSc subset (results available from the corresponding author upon request), but the current meta-analysis was able to reveal a convincing and significant association.

The strong LD observed between the *CCR6* SNPs rs10946216 and rs3093023 in our control population raised the question as to what might be the causal variant in SSc. Both of these SNPs are located in noncoding regions. To further address this question, we sought to determine whether the functional triallelic dinucleotide polymorphism *CCR6DNP*, which was previously identified as being associated with RA in a study by Kochi et al (5), was present in our SSc cohort, and whether this SNP was in LD with the rs3093023 SNP, for which an association with SSc was identified in the present study. The very strong correlation observed between the *CCR6DNP* TG allele and the *CCR6* rs3093023 A allele supports the hypothesis that this functional variant could be the causal variant responsible for the association with the anti–topoisomerase I antibody–positive subset of SSc.

In patients with SSc, the exact mechanisms underlying the early inflammatory stage of disease, leading to the ultimate stage of fibrosis, remain largely unknown. Evidence for a T cell–driven autoimmune response is supported by histologic findings in the skin of SSc patients during the inflammatory phase, which is marked by the presence of mononuclear cell infiltrates containing T cells, preceding the development of fibrosis. Several lines of evidence point to a role of Th17 cells and of Th17-derived cytokines in the early stage of disease pathogenesis (9,15). Indeed, increased frequencies of circulating Th17 cells have been detected in patients with SSc (9). Elevated serum levels of IL-17A and increased IL-17A expression in peripheral blood lymphocytes and skin lesions have been found in SSc patients (15). Moreover, IL-17 has been found to enhance the proliferation of human fibroblasts in vitro, and the profibrotic effects of IL-17 have been demonstrated in a murine bleomycin-induced skin fibrosis model (9). However, it has been suggested that although IL-17+ cells directly promote inflammation, they may have inhibitory effects on human myofibroblasts (15). Taken

together, these data suggest that IL-17–blocking agents could represent interesting therapeutic approaches in SSc, as supported by recent findings in other rheumatic diseases (16).

The results of the present study demonstrate an association of the most severe SSc subset, antitopoisomerase antibody–positive SSc, with a regulatory variant of the *CCR6* gene. Thus, *CCR6* appears to be a newly identified susceptibility gene for SSc and for this specific subtype of SSc. The findings also demonstrate a potential role of Th17 cells in SSc, thus opening possibilities for the development of new treatment strategies.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Allanore had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES

1. Mayes MD. The genetics of scleroderma: looking into the post-genomic era. Curr Opin Rheumatol 2012;24:677–84.
2. Allanore Y, Saad M, Dieude P, Avouac J, Distler JH, Amouyel P, et al. Genome-wide scan identifies TNIP1, PSORS1C1, and RHOB as novel risk loci for systemic sclerosis. PLoS Genet 2011;7:e1002091.
3. Zhernakova A, van Diemen CC, Wijmenga C. Detecting shared pathogenesis from the shared genetics of immune-related diseases. Nat Rev Genet 2009;10:43–55.
4. Stahl EA, Raychaudhuri S, Remmers EF, Xie G, Eyre S, Thomson BP, et al. Genome-wide association study meta-analysis identifies seven new rheumatoid arthritis risk loci. Nat Genet 2010;42:508–14.
5. Kochi Y, Okada Y, Suzuki A, Ikari K, Terao C, Takahashi A, et al. A regulatory variant in CCR6 is associated with rheumatoid arthritis. Nat Genet 2010;42:515–9.
6. Okada Y, Terao C, Ikari K, Kochi Y, Ohmura K, Suzuki A, et al. Meta-analysis identifies nine new loci associated with rheumatoid arthritis in the Japanese population. Nat Genet 2012;44:511–6.
7. Schutyser E, Struyf S, Van Damme J. The CC chemokine CCL20 and its receptor CCR6. Cytokine Growth Factor Rev 2003;14:409–26.
8. Hirota K, Yoshitomi H, Hashimoto M, Maeda S, Teradaira S, Sugimoto N, et al. Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. J Exp Med 2007;204:2803–12.
9. Okamoto Y, Hasegawa M, Matsushita T, Hamaguchi Y, Huu DL, Iwakura Y, et al. Potential roles of interleukin-17A in the development of skin fibrosis in mice. Arthritis Rheum 2012;64:3726–35.
10. Dieude P, Guedj M, Wipff J, Avouac J, Hachulla E, Diot E, et al. The PTPN22 620W allele confers susceptibility to systemic sclerosis: findings of a large case–control study of European Caucasians and a meta-analysis. Arthritis Rheum 2008;58:2183–8.
11. LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA Jr, et al. Scleroderma (systemic sclerosis): classification, subsets, and pathogenesis. J Rheumatol 1988;15:202–5.
12. Plenge RM, Bridges SL Jr, Huizinga TW, Criswell LA, Gregersen PK. Recommendations for publication of genetic association studies in Arthritis & Rheumatism. Arthritis Rheum 2011;63:2839–47.
13. Perera A, Fertig N, Lucas M, Rodriguez-Reyna TS, Hu P, Steen VD, et al. Clinical subsets, skin thickness progression rate, and serum antibody levels in systemic sclerosis patients with antitopoisomerase I antibody. Arthritis Rheum 2007;56:2740–6.
14. Eyre S, Bowes J, Diogo D, Lee A, Barton A, Martin P, et al. High-density genetic mapping identifies new susceptibility loci for rheumatoid arthritis. Nat Genet 2012;44:1336–40.
15. Truchetet ME, Brembilla NC, Montanari E, Lonati P, Raschi E, Zeni S, et al. Interleukin-17A+ cell counts are increased in systemic sclerosis skin and their number is inversely correlated with the extent of skin involvement. Arthritis Rheum 2013;65:1347–56.
16. Hueber W, Patel DD, Dryja T, Wright AM, Koroleva I, Bruin G, et al. Effects of AIN457, a fully human antibody to interleukin-17A, on psoriasis. Sci Transl Med 2010;2:52ra72.