Polymorphisms in the Interleukin 4, Interleukin 13, and Corresponding Receptor Genes Are Not Associated with Systemic Sclerosis and Do Not Influence Gene Expression

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ABSTRACT. ***Objective.*** Polymorphisms in the genes encoding interleukin 4 (*IL4*), interleukin 13 (*IL13*), and their corresponding receptors have been associated with multiple immune-mediated diseases. Our aim was to validate these previous observations in patients with systemic sclerosis (SSc) and scrutinize the effect of the polymorphisms on gene expression in various populations of peripheral blood leukocytes.

Methods. We genotyped a cohort of 2488 patients with SSc and 2246 healthy controls from The Netherlands, Spain, United Kingdom, Italy, Germany, and France. Taqman assays were used to genotype single-nucleotide polymorphisms (SNP) in the following genes: (1) *IL4* (–590C>T/rs2243250); (2) *IL4* receptor alpha (*IL4RA*) (Q576R/rs1801275); (3) *IL13* (R130Q/rs20541 and –1112C>T/rs1800925); and (4) *IL13RA1* (43163G>A/rs6646259). The effect of these polymorphisms on expression of the corresponding genes was assessed using quantitative RT-PCR on RNA derived from peripheral blood B cells, T cells, plasmacytoid dendritic cells, monocytes, and myeloid dendritic cells. We investigated whether these polymorphisms influenced development of pulmonary complications over 15 years in patients with SSc.

Results. None of the investigated polymorphisms was associated with SSc or any SSc clinical subtype. We did not observe any effect on transcript levels in the cell subtypes or on development of pulmonary complications.

Conclusion. Our data showed that polymorphisms in *IL4*, *IL13*, and their receptors do not play a role in SSc and do not influence the expression of their corresponding transcript in peripheral blood cells. (J Rheumatol First Release Nov 1 2011; doi:10.3899/jrheum.110235)

Key Indexing Terms:
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Systemic sclerosis (SSc) is a debilitating autoimmune disease featuring immune activation, vasculopathy, and autoantibody production. These processes eventually lead to fibrosis of skin and internal organs[1]. The exact etiology remains to be unraveled, although it is generally accepted that multiple common genetic variants contribute to the risk of developing SSc[2]. Traditionally, SSc has been regarded as a disease propelled by a Th-2 response[3]. This view was mainly based on the increased expression of several signature molecules that are associated with Th-2 response, such as interleukin 4 (IL-4), IL-13, and IL-5 in SSc serum, skin, and bronchoalveolar lavage[4,5]. However, evidence is accumulating that other T cell subsets, including Th-1, Th-17, and T regulatory cells, may also drive the pathology observed in SSc[6,7]. In parallel with the important role of IL-4 and IL-13 in Th-2 responses, both cytokines seem to exhibit a key role in tuning Th-17 responses. For instance, both IL-13 and IL-4 are able to attenuate Th-17 cytokine production[8,9]. Adding to the complexity, cells of the innate immune system may also produce pathologically relevant quantities of IL-13, especially in the context of fibrosing diseases[10,11].

In addition to being involved in the same biologic processes, IL-4 and IL-13 display similar features in structure and signaling. Although they share only 25% homology at the amino acid level, their core structure is very similar. The genes are situated close to each other on chromosome 5q31.1 and are often coregulated. Both cytokines mediate their effects by interacting with the same receptor complex composed of 2 transmembrane proteins, IL-4RA1 and IL-13RA1[12,13]. A second IL-13 receptor with a short cytoplasmic tail, IL-13RA2, binds IL-13 with high affinity and acts as a decoy receptor, although a recent study suggests that it may mediate transforming growth factor ß-induced fibrosis[14].

Polymorphisms of both IL-4 and IL-13 and their receptors have been implicated in susceptibility to asthma[15] and atopic dermatitis and are believed to play a role in psoriasis as well[16,17]. In addition, IL-13 variants have recently been implicated in susceptibility to psoriatic arthritis[18].

To elucidate the role of polymorphisms in the *IL4*, *IL13*, *IL4R*, and *IL13RA1* genes, we aimed to validate previous associations with immune-mediated diseases, including SSc, in a large multinational SSc cohort[19,20,21]. As well, we investigated the possible effect of these polymorphisms on IL-13, IL-13RA1, and IL-4RA expression in B cells, T cells, myeloid dendritic cells (MyDC), plasmacytoid dendritic cells, and monocytes from patients with SSc.

MATERIALS AND METHODS

Patients and controls. The study population consisted of 2488 patients with SSc and 2246 healthy controls matched by geographical region and age. Six case-control sets were of European ancestry (a Spanish cohort: 231 patients with SSc and 250 controls; a Dutch cohort: 143 patients with SSc and 274 controls; a German cohort: 422 patients with SSc and 266 controls; a British cohort: 234 patients with SSc and 98 controls; an Italian cohort: 444 patients with SSc and 362 controls; and a French cohort: 1014 patients with SSc and 996 controls). All patients fulfilled the 1980 American College of Rheumatology classification criteria for SSc[22]. The local ethical committee from each center approved our study. Patients and controls were included in our study after providing written informed consent. All patients were classified as having limited cutaneous (lcSSc) or diffuse cutaneous SSc (dcSSc) using the criteria of LeRoy, *et al*[23]. Patients with SSc changes limited to the skin distal to elbows and/or knees, regardless of facial involvement, fulfill the definition for lcSSc. Patients with more proximal scleroderma skin changes were classified as having dcSSc. The presence of pulmonary fibrosis was investigated by high-resolution computed tomography scan. Restrictive syndrome and diffusion capacity of the lungs was defined as a forced vital capacity (FVC) < 70% of predicted value and a diffusion lung capacity for carbon monoxide (DLCO) < 70% of predicted. Pulmonary artery hypertension (PAH) was diagnosed by right heart catheterization and was considered confirmed if the mean pulmonary artery pressure was > 25 mm Hg at rest with a normal left atrial wedge pressure. We used followup data on decline of FVC and development of PAH from an inception cohort including 358 Dutch and Italian patients, starting inclusion after the onset of the first non-Raynaud symptom and ending at 15 years of followup. The patients were evaluated at least yearly for these complications (Table 1).

Genotyping. Peripheral blood samples (10 ml) collected in EDTA tubes were obtained from each patient and stored at –80°C prior to DNA isolation. Genomic DNA was extracted from leukocytes in peripheral venous blood according to standard protocols. DNA was transferred to 96-well plates. Each plate contained 3 negative controls (H_2O) and 5 duplicate samples (3 within the plate and 2 between the plates). We chose single-nucleotide polymorphisms (SNP) previously shown to influence

Table 1. Basic and clinical characteristics of the 6 cohorts of patients with systemic sclerosis (SSc) in this study.

Characteristic	Netherlands	Spain	Germany	France	Italy	United Kingdom
No.	143	231	422	1014	444	234
Age, yrs (SD)	58 (13)	58 (13)	57 (12)	56 (13)	55 (13)	54 (12)
Disease duration, mo (SD)	131 (82)	144 (90)	113 (109)	128 (98)	140 (138)	155 (92)
Female, % (controls, %)	81 (84)	82 (78)	76 (76)	86 (26)	92 (73)	85 (81)
Limited phenotype, %	69	68	51	63	52	79
Positive anti-topo, %	23	23	26	24	33	15
Positive ACA %	58	39	46	37	32	71
Pulmonary fibrosis on CT scan	32.3	30.7	37.2	39.0	32.1	43.2
Low FVC (< 70% predicted), %	26.1	29.1	18.5	16.2	15.3	30.1
Low DLCO (< 70% predicted), %	33	45.1	50.2	NA	67.2	11.5

ACA: anticentromere antibodies; anti-topo: anti-topoisomerase antibodies; FVC: forced vital capacity; DLCO: diffusion capacity of lung for carbon monoxide; CT: computed tomography.

susceptibility to immune-mediated diseases and that are protein-altering or associated with altered expression levels[19,20,21]. In addition, the *IL13RA1* SNP (rs6646259) covers the largest part of the *IL13RA1* gene because it is situated in a large haplotype block. Taqman assays were used for genotyping SNP within *IL4* (–590C>T/rs2243250/assay ID: C__16176216_10) and *IL4R* (Q576R/rs1801275/assay ID: C___2351160_20). In the *IL13* gene 2 SNP were genotyped, R130Q (rs20541/assay ID: C___2259921_20) and –1112C>T (rs1800925/assay ID: C___8932056_10). In the *IL13RA1* gene, the 43163G>A variant (rs6646259/assay ID: C__11770516_10) was genotyped. Taqman assays were performed according to the manufacturer's protocol using the 7500 Fast Real-Time polymerase chain reaction (PCR) system (Applied Biosystems, Foster City, CA, USA). Results were analyzed using Sequence Detection Software version 1.4. A competitive allele-specific PCR system (Kaspar Genotyping, KBioscience, Hoddesdon, UK) was used to genotype these SNP in the French sample as reported[24].

Isolation of cell subsets and expression analysis. We isolated peripheral blood mononuclear cells (PBMC) from heparinized venous blood by density-gradient centrifugation, using samples from 25 patients with SSc and 9 controls provided by Boston University Medical Center (Boston, MA, USA). Subsequently, BDCA4+ (plasmacytoid dendritic cells), CD3+ (T cell), CD19+ (B cell), CD1C+ (MyDC), and CD14+ (monocyte) cells were isolated by magnetic cell separation techniques according to the manufacturer's protocol as described[25]. RNA was purified by AllPrep DNA/RNA columns (Qiagen, Valencia, CA, USA) and cDNA was synthesized by I-script (Bio-Rad, Hercules, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed on an Mx3005P QPCR System (Stratagene). Each primer set yielded a product with a dissociation curve composed of a single peak. Ct values for duplicate samples were averaged and the amount of cDNA relative to a housekeeping gene (*GAPDH*) was calculated with the ΔCt method. Primers (Table 2) were derived from the Harvard PrimerBank or were created with Primer3 software[26,27].

Data analysis. Significance levels were calculated with 2x2 contingency tables and Fisher's exact test using SPSS 16.0. To account for multiple testing, the Bonferroni adjustment was applied (significance threshold p = 0.002). Homogeneity of OR among cohorts was calculated using Breslow-Day and Woolf Q methods and calculation of pooled OR was performed under a fixed-effects model (Mantel-Haenszel metaanalysis). Power calculations using the pooled sample size (2488 patients with SSc, 2246 controls) showed that we achieved a power of detecting a relative risk of 1.2 of 97% (both rs1801275 and rs20541), 95% (rs1800925), and 92% (rs2243250). Since *IL13RA1* is located on the X chromosome, we performed an analysis in the female population only (SSc n = 2036, controls n = 1035); doing this we still reached a power of 84% to detect a relative risk of 1.2 (rs6646259). Survival analysis was performed using Kaplan-Meier curves and significance levels were calculated with log-rank (Mantel-Cox) statistics. Cox proportional hazards survival regression was used to determine relative risks.

RESULTS

After genotyping, no divergence in Hardy-Weinberg equilibrium was observed. The minor allele frequencies (MAF) of the SNP tested were all in accord with frequencies reported by the HapMap project (www.hapmap.org). The 6 SSc populations showed very little variation in MAF. According to the Breslow-Day statistics, no significant heterogeneity affecting the 6 European populations was detected, justifying a metaanalysis. We observed no significant deviations in genotype and allele frequencies in any of the polymorphisms tested in patients with SSc compared to controls. Initially, we observed a divergence in allele distribution of the rs1800925 polymorphism in the French dcSSc (p = 0.02) and antitopoisomerase-positive (p = 0.03) SSc samples compared to controls (supplementary data available from the authors upon request). However, after correction for

Table 2. Primer sequences used for RT-PCR.

Gene	Forward Primers (5'–3')	Reverse Primers (5'–3')	PrimerBank ID
GAPDH	ATG GGG AAG GTG AAG GTC G	GGG GTC ATT GAT GGC AAC AAT A	7669492a1
IL13	GAA GGC TCC GCT CTG CAA T	TCT GGG TCT TCT CGA TGG CA	26787978a1
IL13RA1	ACT CCT GCT TTA CCT AAA AAG GC	GCA CTA CAG AGT CGG TTT CCT	4504647a1
IL4RA	TCA TGG ATG ACG TGG TCA GT	CAG GTC AGC AGC AGA GTG TC	*

* Primer was newly designed with Primer3 software[27].

multiple testing using the Bonferroni adjustment, no results remained significant. In addition, a metaanalysis taking into account all 6 European SSc populations did not reveal a significant effect for any of the investigated variants on SSc susceptibility or clinical phenotypes (Table 3). When we corrected for sex in our analysis for the non-X chromosome-located SNP, no significant differences were observed. Finally, we performed a recessive and dominant analysis, which yielded similar negative results (data not shown).

The implication of IL-4 and IL-13 in many immune-mediated pulmonary diseases[11,14,28,29,30,31,32] led us to further investigate the role of functional variants in the *IL4* and *IL13* genes with special emphasis on pulmonary involvement. For this purpose we used followup data on FVC decline and PAH development from 358 Dutch and Italian patients, starting at the date of onset of the first non-Raynaud symptom and ending at 15 years. Patients were evaluated at least once a year for these complications. None of the polymorphisms influenced development of these complications significantly in the followup period.

To investigate whether polymorphisms in *IL13*, *IL4RA*, and *IL13RA1* affect gene expression, the levels of transcripts for these genes were determined by qRT-PCR in purified populations of monocytes, plasmacytoid dendritic cells, myeloid dendritic cells, and T cells from the peripheral blood of healthy controls and patients with SSc. Overall expression levels of IL-13 were low, with either undetectable or very high Ct values in most samples, precluding a comparative analysis for this gene. No statistically significant differences in the expression of IL-13RA1 or IL-4RA were found in the cell subsets comparing SSc patients and controls (Figure 1). In addition, neither the polymorphism in IL-13RA1 nor that in IL-4RA influenced expression of their corresponding gene in any cell type tested (Figure 2).

DISCUSSION

We observed that 5 common polymorphisms in the coding regions of *IL4* and *IL13* or their corresponding receptors are not associated with SSc susceptibility in 6 European populations. The role of *IL4* and *IL13* has been addressed in mul-

Table 3. Combined analysis of the 5 polymorphisms investigated, using Mantel-Haenszel analysis in a fixed model for estimation of combined effects.

SNP	Subtype	Total No.	Minor Allele Frequency	p*
IL13 rs1800925	SSc	1832	0.19	0.15
	lcSSc	1125	0.18	0.11
	dcSSc	558	0.20	0.06
	ACA	689	0.18	0.67
	Anti-topo	426	0.20	0.07
	Controls	1869	0.17	
IL13 rs20541	SSc	2474	0.37	0.34
	lcSSc	1520	0.35	0.14
	dcSSc	723	0.41	0.97
	ACA	1046	0.29	0.77
	Anti-topo	563	0.41	0.75
	Controls	2246	0.44	
IL13RA1 rs6646259 (females only)	SSc	2037	0.19	0.2
	lcSSc	1211	0.16	0.78
	dcSSc	556	0.23	0.19
	ACA	800	0.13	0.25
	Anti-topo	413	0.22	0.85
	Controls	1035	0.16	
IL4 rs2243250	SSc	1829	0.16	0.46
	lcSSc	1128	0.16	0.7
	dcSSc	553	0.15	0.3
	ACA	692	0.16	0.81
	Anti-topo	422	0.14	0.26
	Controls	1869	0.15	
IL4R rs1801275	SSc	1819	0.20	0.11
	lcSSc	1125	0.19	0.64
	dcSSc	547	0.20	0.16
	ACA	685	0.19	0.4
	Anti-topo	419	0.20	0.19
	Controls	1879	0.19	

* Mantel-Haenszel p value. SSc: systemic sclerosis; lcSSc: limited cutaneous SSc; dcSSc: diffuse cutaneous SSc; ACA: anticentromere antibodies; anti-topo: antitopoisomerase antibodies.

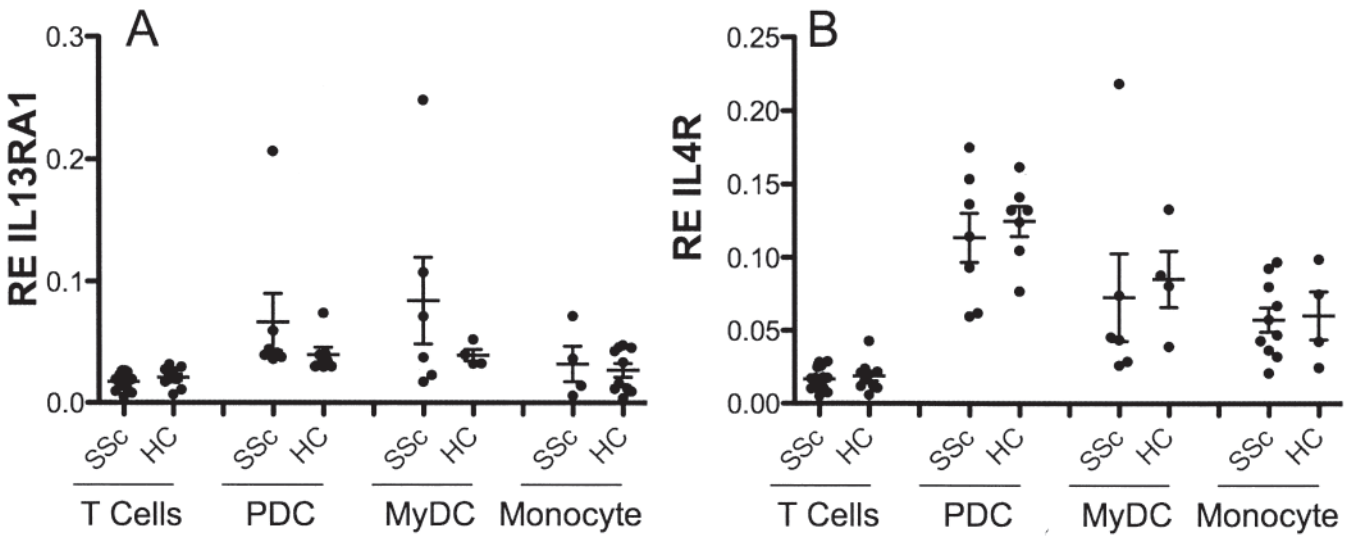

Figure 1. Messenger RNA levels of (A) IL-13RA1 and (B) IL-4RA in T cells, plasmacytoid dendritic cells (PDC), myeloid dendritic cells (MyDC), and monocytes from healthy controls (HC) and patients with SSc. No significant differences were observed between controls and patients for either transcript in any cell type. RE: relative expression compared to GAPDH.

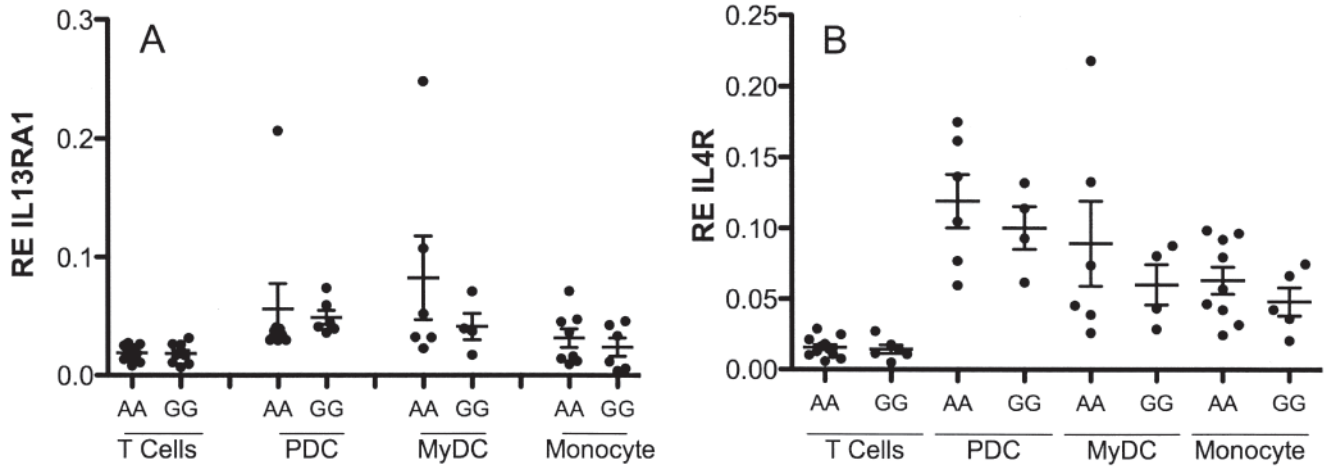

Figure 2. Messenger RNA levels of (A) IL-13RA1 and (B) IL-4RA in T cells, plasmacytoid dendritic cells (PDC), and myeloid dendritic cells (MyDC), segregated by genotype for rs6646259 and rs1801275, respectively. Neither polymorphism significantly influenced gene expression in patients with SSc or controls.

tiple autoimmune diseases including SSc[5,10,14,15,16,17,18,29,30,31,32,33,34,35,36]. The functional genetic variants included in our study have been previously associated with immune-mediated diseases; however, they do not seem to contribute to the pathogenesis of SSc. Further, genetic variation at the *IL13RA1* and *IL4RA* loci did not influence gene expression across a variety of immune cells. However, differences in expression at the protein level or within immune cells of lesional tissue cannot be excluded. On the other hand, a recent genome-wide association study that covered these genes fully also did not reveal a statistically significant association[37]. To fully exclude a role for these genes in the pathogenesis of SSc, resequencing would be necessary to cover rare variants as well. It must be noted that our study did not address a variant in the *IL13RA2* gene previously associated with SSc in a small French cohort (n = 107)[38]. This variant was left out because a possible overlap between the French cohort in our study and the cohort in the previous report could not be excluded. Considering the size and power of our study to detect significant deviations of all the 5 variants between cases and controls, the lack of association is unlikely to be caused by a type 2 error. The patients in our study are well described and were evaluated following conventional guidelines[22,23], making disease classification bias unlikely. The patients in the followup cohort were included upon the first evaluation of a non-Raynaud SSc symptom; following from this it may be that patients with extremely progressive disease and with high mortality were not included in this analysis. On the other hand, the period of patient evaluation covered 15 years and therefore reflects the great majority of patients who develop pulmonary complications later in the disease course, making these results

relevant. In SSc mouse models, fibrosis after bleomycin administration is dependent on IL-13[10]. In addition, both cytokines are able to drive collagen production by fibroblasts directly[39]. However, T cells do not seem to be the key producers of these cytokines, and other cell types are likely to be the main producers in SSc[40]. For instance, alveolar macrophages have been found to produce this profibrotic cytokine in pulmonary fibrosis[32]. We did not observe statistically significant differences in immune cell subtypes of SSc and the healthy controls. However, it was recently reported that patients with SSc display heterogeneity at the gene expression level that may not be reflected in the clinical phenotype[41]. We cannot exclude the possibility that an analysis of RNA expression similar to ours across molecular subtypes of SSc might reveal an association particularly in patients that show an immune cell activation signature. In addition, genetic variation at the *IL13RA1* and *IL4RA* loci did not influence gene expression across a variety of immune cells. Differences in expression at the protein level or within immune cells of lesional tissue cannot be excluded. Finally, expression levels of resting cells were measured, whereas differences may become more apparent when examined in a proinflammatory environment.

Our results make it likely that the higher levels of IL-4 and IL-13 observed in patients with SSc are not caused by common genetic variations, but result from upstream immune activation mechanisms that promote Th-2 maturation or the differentiation of innate immune cells that can also produce these cytokines[3,4]. For instance, aberrations in expression of genes upstream of *IL4* and *IL13* have recently been identified in SSc; these aberrations, instead of polymorphisms in *IL4* and *IL13*[42], may influence expression of these genes.

We could not replicate the previous association of the rs1800925 polymorphism in the *IL13* gene with SSc, which is most likely due to the small population of patients with SSc involved in the initial study (n = 107)[43]. In addition, we did not observe an association between genetic variants of *IL4*, *IL4RA*, or *IL13RA1* and SSc susceptibility and/or phenotype. Further studies are needed to investigate the mechanisms involved in the upregulation of and response to *IL4* and *IL13* observed in SSc.

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