A candidate gene study identifies a haplotype of CD2 as novel susceptibility factor for systemic sclerosis

E. Koumakis[1,2], M. Bouaziz[3], P. Dieudé[4,5], A. Cauvet[1], B. Ruiz[1], P. Airò[6], D. Cusi[7], M. Matucci-Cerinic[8], E. Salvi[9], G. Cuomo[10], E. Hachulla[11], E. Diot[12], P. Caramaschi[13], V. Riccieri[14], J. Avouac[1,2], Y. Allanore[1,2]

Eugénie Koumakis, MD
Matthieu Bouaziz, PhD
Philippe Dieudé, MD
Anne Cauvet
Barbara Ruiz
Paolo Airò, MD, PhD
Daniele Cusi, MD
Marco Matucci-Cerinic, MD, PhD
Erika Salvi, MD
Giovanna Cuomo, MD
Eric Hachulla, MD, PhD
Elisabeth Diot, MD
Paola Caramaschi, MD
Valeria Riccieri, MD
Jérôme Avouac, MD, PhD
Yannick Allanore, MD, PhD

The authors' affiliations are listed on page S-47.

Please address correspondence to:
Prof. Yannick Allanore,
Hôpital Cochin,
Service de Rhumatologie A,
27 rue du Faubourg Saint Jacques,
75014 Paris, France.
E-mail: yannick.allanore@cch.aphp.fr

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ABSTRACT

Objective. *Systemic sclerosis (SSc) is a rare autoimmune disease (AID) with a complex genetic aetiology. Evidence for a shared pathogenesis across AIDs is given by the well-known pleiotropism of autoimmune genes. Recently, several unbiased approaches have identified an association between polymorphisms of the CD2 gene, and rheumatoid arthritis (RA) susceptibility. The objective of this study was to investigate whether CD2 polymorphisms are associated with SSc.*

Methods. *Two SNPs of CD2, rs624988 and rs798036, were genotyped in a total of 1,786 SSc patients and 2,360 healthy individuals from two European populations (France and Italy). Meta-analyses were performed to assess whether an association exists between CD2 polymorphisms or haplotypes and SSc or its main subtypes.*

Results. *The combined analyses revealed an association between the rs624988 A allele and SSc susceptibility: p_{adj}=0.023, OR=1.14 (95%CI 1.04-1.25). Single marker analysis did not reveal any association between rs798036 and SSc. Haplotype analysis identified that the A-T haplotype, previously described in RA, was associated with higher susceptibility for SSc (p_{adj}=0.029, OR=1.14, 95%CI 1.04-1.25) and with the positive anticentromere antibody sub-group of SSc patients (p_{adj}=0.009, OR=1.19 95%CI 1.07-1.32). Genotype-mRNA expression correlations revealed that the CD2 risk haplotype was associated with decreased CD2 mRNA expression in SSc patients.*

Conclusion. *Our study establishes CD2 as a new susceptibility factor for SSc, in a European Caucasian population, confirming the sharing of autoimmune risk factors by SSc and RA.*

Introduction

Systemic sclerosis (SSc) is a chronic systemic disease with a complex pathogenesis characterised by early vascular alterations and activation of the immune system with autoimmune features preceding the deposition of extracellular matrix leading to systemic fibrosis (1). Although it is frequently characterised as an autoimmune disease (AID), the mechanisms underlying the early inflammatory phase, involving both T and B cells, remain poorly understood.

In recent years, numerous genetic factors underlying disease susceptibility to SSc have been identified, mainly through association studies using candidate gene approaches and a few genome-wide association studies (GWAS) (2, 3). The vast majority of these susceptibility loci belong to pathways leading to auto-immune responses or inflammation and are involved in antigen processing (*MHC*), innate immunity (*IRF5*), T-cell differentiation and/or activation (*STAT4*, *TNFSF4, CD226*), and signalling (*TNFAIP3*, *TNIP1*, *PTPN22*, *BANK1*, *BLK*, *CD247*) (2). Most of these susceptibility loci have also been identified in other AIDs, in particular with SLE and RA, highlighting the existence of a genetic overlap between SSc and other AIDs and the concept of shared autoimmunity (4).

Among the RA susceptibility loci identified to date, *CD2* has recently emerged through different unbiased approaches (5, 6). In a strategy focusing on variants within protein-coding regions (*e.g.* missense, nonsense and synonymous variants), Diogo *et al.* identified that a missense variant rs688738 (c.798C>A [p.His266Gln]) and a non-coding variant, rs624988, within the *CD2* locus, independently contributed to the risk of RA (5). A previous study using a computational method that applies statisti-

cal text mining to PubMed abstracts, Gene Relationships Across Implicated Loci (GRAIL), had already identified and convincingly replicated an association between the rs11586238 *CD2* SNP (p=1×10^{-6} replication) and RA (6).

CD2 is a surface antigen of the human T-lymphocyte lineage expressed on peripheral blood T cells. CD2 mediates adhesion between T cells and antigen presenting cells expressing the CD2 ligand, CD58. In addition to its role in promoting adhesion, CD2, by itself or in concert with TCR stimulation, is able to transduce signals which lead to T cell activation. In addition, antigen-responsive memory T cells express higher levels of CD2 than naive T cells which are largely antigen unresponsive.

Given the evidence of shared common autoimmune genes between RA and SSc, the current study sought to investigate the association of *CD2* polymorphisms with SSc.

Table I. Characteristics of SSc patients included in the study.

	French cohort (n = 1027)	Italian cohort (n = 808)	Combined cohort (n = 1835)
Age (mean ± SD years)	57.5 ± 13.9	57.7 ± 13.6	57.6 ± 13.8
Male/female (%)	154/873 (15.0)	88/720 (10.9)	242/1593 (13.2)
Disease duration (mean ±SD years)	10.7 ± 7.6	14.0 ± 12.5	12.3 ± 10.4
lcSSc (%)	67.4	69.8	68.5
dcSSc (%)	32.6	30.2	31.5
Fibrosing alveolitis on CT (%)	39.0	39.4	39.2
FVC (mean ±SD, %)	95.8 ± 23.4	94.9 ± 23.9	95.3 ± 23.6
FVC < 75% (%)	19.7	24.0	21.8
Digital Ulcers (%) (ever occured)	40.3	45.7	42.7
ACA + (%)	42.9	38.9	41.0
ATA+ (%)	27.2	36.5	31.6
Associated AID (%)	17.6	15.0	16.6

*SSc: systemic sclerosis; lcSSc: limited cutaneous SSc; dcSSc: diffuse cutaneous SSc; FA: fibrosing alveolitis (defined by typical ground-glass or honeycombing patterns on HRCT scan); FVC: forced vital capacity; ACA: anticentromere antibody; ATA: antitopoisomerase antibody; associated AID: at least one associated autoimmune disease.

Patients and methods

Study population

We performed a case-control association study, using a European Caucasian cohort consisting of 1,835 SSc patients and 2,401 healthy unrelated ethnically matched individuals from two European populations (France: SSc cases 1,027; controls 1,048; Italy: SSc cases 808; controls 1,353). Only individuals with European ancestry were included in the study (defined as all 4 grandparents being of European Caucasian ancestry). This cohort has already been used in several previous genetic studies and high homogeneity between the various group samples has been demonstrated (7). Detailed phenotypic assessment was carried out for all SSc patients, as previously described (3, 8). The characteristics of the SSc patients are shown in Table I. For all patients with SSc, we determined LeRoy's cutaneous subtype and carried out a phenotypic assessment. All patients were tested for antinuclear antibodies and their putative specificity. Anticentromere antibodies were determined on the basis of their distinctive immunofluorescence pattern. Anti-topoisomerase I antibodies were determined by counterimmunoelectrophoresis. A fibrosing alveolitis was defined as the presence of typical features on high resolution computerised tomography (HRCT) of the chest of any typical ground-glass, reticulation or honeycombing patterns. The study was approved by local institutional review boards, and written informed consent was obtained from all subjects. SSc patients known as having associated RA were excluded prior to analysis in order to avoid bias due to a possible excess of the risk alleles attributable to these patients, as previously described (7).

SNP selection and genotyping

DNA samples from SSc patients and controls were genotyped for the *CD2* SNPs rs624988 and rs798036 consisting of the two independent SNPs driving the best signal of association with RA at the *CD2* locus in the study by Diogo *et al*. We also included available genome-wide data from 720 Italian controls from the HYPERGENES (www.hypergenes.eu) cohort, since the *CD2* rs624988 and rs798036 SNPs were included in the chips used for this project (2). Genotyping was performed using a competitive allele-specific polymerase chain reaction system (KASPar Genotyping, Kbioscience, Hoddeston, UK), as previously described (8).

Quantification of gene expression by peripheral blood mononuclear cells (PBMC)

In order to assess a possible genotype-phenotype correlation, *CD2* gene expression by peripheral blood mononuclear cells was measured by TaqMan quantitative real-time PCR.

The total RNA was obtained using a RNA extraction kit (RNeasy Mini Kit, Qiagen) followed by a c-DNA reversion transcription step (SuperScript II Reverse Transcriptase, Invitrogen). Gene expression was performed using quantitative Real-Time Polymerase Reaction (Universal Master Mix II, Applied Biosystems). All primers were obtained by a predesigned gene expression assay (Applied Biosystems).

Statistical analyses

Statistical analyses were conducted using the recommendations published in Arthritis and Rheumatism (8), and included power calculation and haplotype analysis. Tests for conformity with Hardy-Weinberg equilibrium (HWE) were performed using a standard chi-square test (1 degree of freedom). Homogeneity between the 3 cohorts was confirmed by the performance of Breslow-Day method and therefore the combined data were subsequently analysed by calculating the pooled ORs using a Cochran-Mantel-Haenszel test under fixed effects. Individual association analyses of *CD2* SNPs were performed by comparing cases and controls with Fisher's exact test on allelic distribution. The same procedure was applied to subgroups stratified according to SSc

Table II. Pooled analysis of the CD2 rs624988 and rs798036 SNPs in the combined Caucasian populations (French and Italian) in an additive recessive model.

SNP, phenotype	n	MAF(%) §	Genotype Distribution			P	P_{adj}†	OR (95% CI)
rs624988		**A**	**AA (%)**	**AG (%)**	**GG (%)**			
SSc	1781	41.0	296 (16.6)	860 (48.3)	625 (35.1)	0.0038	**0.023**	1.14 [1.04-1.25]
LcSSc	1155	41.0	184 (15.9)	573 (50.0)	398 (34.4)	0.016	NS	1.13 [1.02-1.26]
SSc ACA+	671	42.0	103 (15.4)	352 (52.4)	216 (32.2)	0.0095	**0.057**	1.18 [1.04-1.38]
DcSSc	534	41.0	96 (18.0)	250 (46.8)	188 (35.2)	0.021	NS	1.17 [1.02-1.34]
SSc ATA+	521	40.0	82 (15.7)	254 (48.8)	185 (35.5)	0.16	NS	1.10 [0.96-1.26]
SSc-FA	647	42.0	110 (17.0)	318 (49.2)	219 (33.8)	0.0089	**0.053**	1.18 [1.04-1.34]
SSc plus other AID	239	40.0	34 (14.2)	122 (51.0)	83 (34.7)	0.34	NS	1.10 [0.90-1.33]
Controls	2344	38.0	344 (14.7)	1083 (46.2)	917 (39.1)	NA		
rs798036		**A**	**AA (%)**	**AT (%)**	**TT (%)**			
SSc	1786	9.0	20 (1.1)	295 (16.5)	1471 (82.4)	0.58	NS	0.96 [0.83-1.11]
LcSSc	1161	10.0	12 (1.0)	203 (17.5)	946 (81.5)	0.94	NS	1.01 [0.85-1.19]
SSc ACA+	677	11.0	9 (1.3)	127 (18.8)	541 (79.9)	0.29	NS	1.11 [0.96-1.35]
DcSSc	534	8.0	7 (1.3)	75 (14.0)	452 (84.6)	0.15	NS	0.84 [0.66-1.06]
SSc ATA+	520	8.0	5 (1.0)	77 (14.8)	438 (84.2)	0.15	NS	0.84 [0.66-1.06]
SSc-FA	647	9.0	8 (1.2)	106 (16.4)	533 (82.3)	0.73	NS	0.97 [0.79-1.19]
SSc plus other AID	237	9.0	6 (2.5)	32 (13.5)	199 (84.0)	0.86	NS	0.97 [0.71-1.34]
Controls	2365	10.0	31 (1.3)	405 (17.1)	1929 (81.6)	NA		

ACA: anti-centromere antibodies; AID: autoimmune disease (other than RA); ATA: anti-topoisomerase I antibodies; dcSSc: diffuse cutaneous SSc; FA: fibrosing alveolitis; lcSSc: limited cutaneous SSc; MAF §: minor allele frequency (according to Cochran-Mantel-Haenszel test for the combined European Caucasian populations); (n) refers to the number of pooled populations analysis; NS: non significant; OR: odds ratio; SNP: single nucleotide polymorphisms; SSc: systemic sclerosis; † after Bonferroni correction; 95% CI - 95% confidence interval.

phenotype. All odds ratios (ORs) are provided with their 95% confidence intervals. We applied a Bonferroni correction for multiple testing, that took into account the number of phenotypic subsets (n=6). An adjusted *p*-value <0.05 was considered as statistically significant.

Results

Single marker analysis

Two SNPs were genotyped in two European populations (French and Italian). Because of our large experience and previous studies using these samples, we were confident with the homogeneity of the 3 cohorts of different geographical origin but of same ethnicity. Homogeneity of the cohorts was confirmed by the Breslow–Day test showing no evidence of interpopulation heterogeneity. Therefore, we directly performed combined analyses in order the get the largest power, as previously done (Table II) (10), using the Mantel-Haenszel test under fixed effects. All SNPs studied in the *CD2* gene region were in HWE in Caucasian controls. Among the 2 SNPs of interest, the expected minor allele frequencies (MAF) range from 6 to 42%; therefore we provide a power calculation for these 2 MAF. For a 6% MAF our power to detect an association is >92% for an OR of 1.5 and of 57% to detect an OR of 1.3. For a 42% MAF our power is >98% to detect an OR of 1.3 or higher. Genotype frequencies were in HWE in the control population for all of the SNPs investigated.

We found an association between the *CD2* rs624988 SNP and susceptibility to SSc in the combined cohort (Table II): OR=1.14 [95% CI 1.04-1.25] p_{adj}=0.023 (Table II). No association was observed between the rs798036 SNP and SSc or any of the clinical subtypes investigated.

CD2 haplotype analysis and LD relationship in healthy controls (Table III)

In the combined European Caucasian control population, the rs624988 SNP was independent of the rs798036 SNP (r^2 =0.01). Among the four *CD2* haplotypes formed by these two variants, only the A-T haplotype was associated with higher susceptibility for SSc (OR=1.14, 95%CI 1.04-1.25) (Fig. 1). When stratifying for disease subtype, the A-T haplotype was mainly associated with the subgroup of patients with ACA+ (OR=1.19).

Functional consequences of the CD2 A-T haplotype with regard to CD2 mRNA expression

We then investigated the potential influence of the CD2 risk haplotype on *CD2* mRNA expression by PBMCs from 55 SSc patients and 45 control subjects. There was no difference in *CD2* mRNA expression levels between individuals carrying the *CD2* homozygous *A-T* haplotype than in those who did not. Among SSc patients however, *CD2* mRNA expression levels were significantly lower in those patients carrying the *CD2* homozygous *A-T* haplotype (*p*=0.007) (Fig. 2). CD2 mRNA expression was also significantly lower in SSc patients than in control subjects (*p*=0.009).

Discussion

To our knowledge, the present study, using a large European Caucasian population, is the first to identify *CD2* as a new SSc susceptibility gene. Single-marker analyses found a statistically significant association, although of weak effect size, between rs624988, a common non-coding single-nucleotide variant, and SSc susceptibility, whereas no association was found with rs798036. The rs798036 SNP has been

Table III. Haplotype association analysis for the CD2 SNPs rs624988 and rs798036.

Rs624988	Rs798036	Phenotype	Frequency in cases	Frequency in controls	*p*-value	P_{adj} †	OR(95%CI)*
A	T	SSc	0.39	0.36	0.0048	**0.029**	1.14 (1.04-1.25)
		LcSSc+	0.40		0.015	NS	1.19 (1.03-1.37)
		ACA+	0.39		0.0016	**0.009**	1.19 (1.07-1.32)
		DcSSc	0.38		0.021	NS	1.13 (1.02-1.26)
		ATA+	0.38		0.26	NS	1.09 (0.94-1.25)
		SSc-FA	0.39		0.012	NS	1.18 (1.04-1.38)
		SSc plus other AID	0.37		0.50	NS	1.07 (0.87-1.32)

ACA: anti-centromere antibodies; AID: autoimmune disease (other than RA); ATA: anti-topoisomerase I antibodies; dcSSc: diffuse cutaneous SSc; FA: fibrosing alveolitis; lcSSc: limited cutaneous SSc; NS: non significant; *OR: odds ratio of comparison with the more common haplotype in the population; SSc: systemic sclerosis; †after Bonferroni correction; 95% CI - 95% confidence interval.

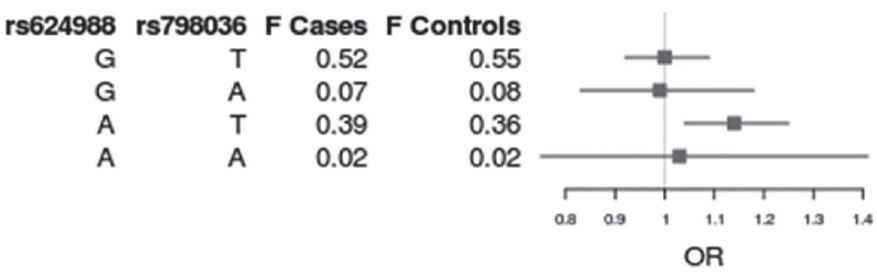

Fig. 1. Results from the haplotype analysis using the rs624988 and rs798036 SNPs.

reported to be a proxy of rs699738 (r2=1), a missense variant inducing a substitution of a histidine for a glutamine at position 266 of CD2 (5).

Although this CD2 association has not been found in GWAS in SSc, CD2 was an interesting candidate for SSc susceptibility given its important role in the activation of the immune system, the high number of SSc susceptibility genes involved in immune pathways, and the concept of shared autoimmune genes. CD2 has been convincingly found to be associated with susceptibility to RA (5,6). Diogo *et al*. first described the association between the A-T haplotype formed by the risk alleles of rs624988 and rs798036, and RA susceptibility. In the present study, although no association was found between SSc and the rs798036 A allele, a significant association was found with the A-T risk haplotype, shared with RA. The association with SSc was found only with the positive anti-centromere antibody sub-group of SSc patients. The absence of association in single marker analysis may be attributable to power limitation for rs798036 given the MAF of 10% in the control population.

To date, and to the best of our knowledge, the functional consequences of the risk CD2 haplotypes common to RA and SSc remain undescribed. CD2 and its ligand CD58 are co-stimulatory molecules involved in the early stages of the immune response, playing an important role both in the adhesion of T

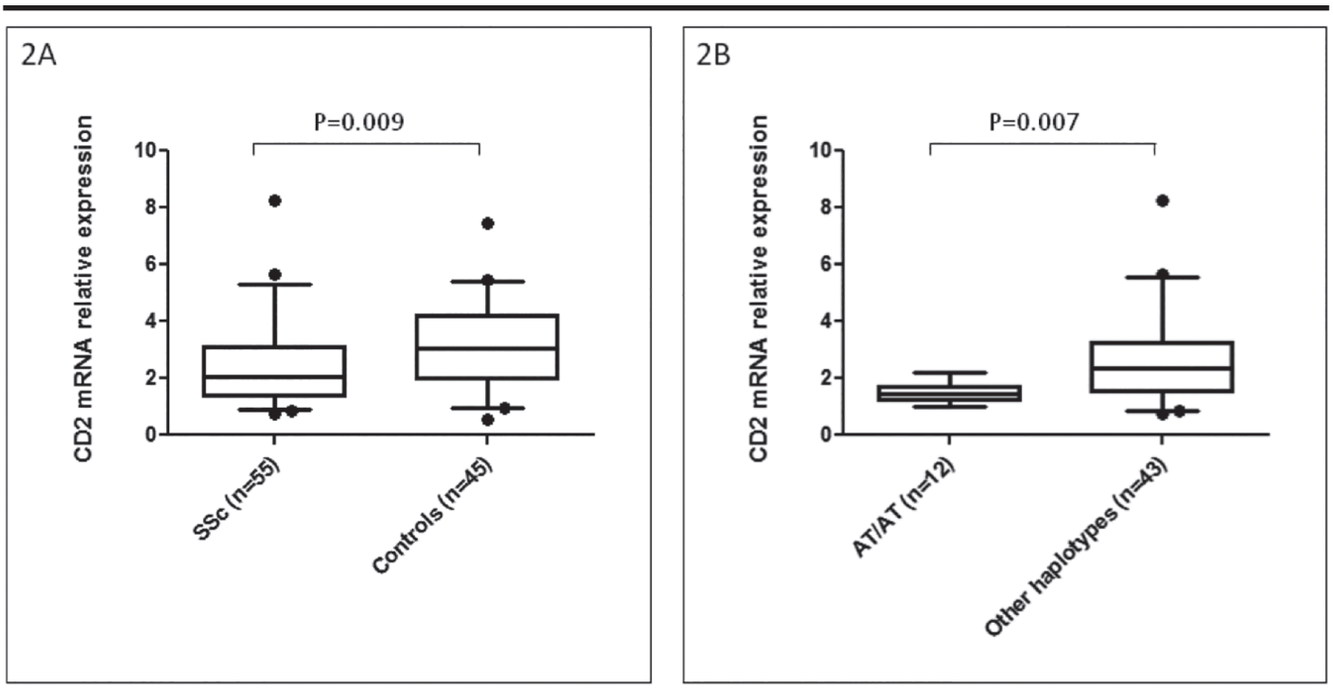

Fig. 2. Influence of the *CD2* A-T haplotype on *CD2* mRNA expression by peripheral blood mononuclear cells, as measured by quantitative TaqMan polymerase chain reaction. 2A. Expression of *CD2* mRNA was significantly reduced in SSc patients as compared with healthy controls. 2B. Among SSc patients, expression of *CD2* mRNA was significantly reduced in carriers of the homozygous CD2 A-T haplotype as compared with carriers of other haplotypes. Results are expressed as median and 25^{th}_75^{th} percentiles.

lymphocytes to antigen-presenting cells and also in signal transduction (11–13). CD58 is widely distributed in the synovial tissue and there is evidence suggesting an up-regulation of the CD2/CD58 pathway in the synovial tissue of RA patients (14–16). A few years ago, alefacept, a recombinant human CD58-Ig fusion protein that binds to CD2 and prevents its interaction with CD58, was shown to be effective both in human psoriatic arthritis and in a murine model of collagen-induced arthritis (14, 16). However, the role of CD2 in autoimmunity remains unclear as it appears that CD2 signalling may enhance or inhibit T-B cell interactions (18). In particular, signalling through CD2 has been found to induce effector Tregs to exert an immediate suppressor activity (19). CD2 signalling is reported to increase the expression of FoxP3 in human $CD25^{hi}CD127^{lo}$ Tregs (19). Allelic variants of CD58 have been found to be associated with increased risk of developing multiple sclerosis (MS). In a recent study on MS, a protective rs2300747 allele was found to be associated with an increase in CD58 mRNA expression in lymphoblastic cell lines and in peripheral blood mononuclear cells (20). Functional investigations further suggested that the increase in CD58 expression mediated by this protective allele, led to the up-regulation of FoxP3 through engagement of CD2, leading to enhanced function of $CD4^{+}$CD25high regulatory T cells (20). This study has some limitations.We show potential effects of the risk *CD2* haplotype on CD2 mRNA expression. Indeed, *CD2* mRNA expression was reduced in SSc patients carrying the *CD2* homozygous risk *A-T* haplotype, This might lead to lower surface expression of the CD2 protein or with a different activity of the protein, which may in turn confer susceptibility to develop autoimmunity. However, since spliced mRNA or post trancription modification may also influence protein expression, it will be important to confirm in future studies that the decrease in CD2 correlates with reduced CD2 protein expression. This will be crucial to provide practical suggestion relevant for clinical practice. Furthermore, we did not observe a significant difference in CD2 mRNA expression between individuals carrying or not the *CD2* risk haplotype when patients and controls were pooled together. This suggests that other genetic factors may contribute to this difference in SSc patients. This will need to be further investigated.

Altogether, our results identify the *CD2* locus as new susceptibility factor for SSc in a Caucasian population. Functional investigations suggest an effect of the risk haplotype on *CD2* mRNA expression and should open the door to further studies to better understand the role of CD2 and SSc and autoimmunity.

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Authors' affiliations

[1]INSERM, Institut Cochin, INSERM U1016, Sorbonne Paris Cité, and Paris Descartes University, Paris, France;
[2]Paris Descartes University, Rheumatology A Department, Cochin Hospital, APHP, Paris, France;
[3]UMR CNRS-8071/INRA-1152, Université d'Evry Val d'Essonne, Evry, France;
[4]Paris Diderot University, Rheumatology Dept., Hôpital Bichat Claude Bernard, APHP, Paris, France;
[5]Paris Diderot University, INSERM U699, Hôpital Bichat Claude Bernard, Paris, France;
[6]Rheumatology and Clinical Immunology, Spedali Civili, Brescia, Italy;
[7]Institute of Biomedical Technologies, National Centre of Research, Milan, Italy;
[8]Department of Experimental and Clinical Medicine, Division of Rheumatology, AOUC, University of Florence, Italy;
[9]Department of Health Sciences, University of Milan, Italy;
[10]Department of Clinical and Experimental Medicine, Rheumatology Unit, Second University of Naples, Italy;
[11]Dept. of Internal Medicine, Hôpital Claude Huriez, Université de Lille II, France;
[12]INSERM U618, IFR 135, CHU Bretonneau, Tours, France;
[13]Unità di Reumatologia, Azienda Ospedaliera Universitaria Integrata, Verona, Italy;
[14]Rheumatology, Dept. of Internal Medicine and Medical Specialities, "Sapienza" University of Rome, Italy.

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