Tesis Doctoral

BASES MOLECULARES DEL LUPUS ERITEMATOSO SISTÉMICO: IDENTIFICACIÓN DE MARCADORES GENÉTICOS

Memoria presentada por la licenciada Elena Sánchez Rodríguez para optar al grado de Doctor Europeo por la Universidad de Granada.

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Que el trabajo titulado "Bases moleculares del lupus eritematoso sistémico: identificación de marcadores genéticos" ha sido realizado bajo su dirección en el Departamento de Biología Celular e Inmunología del Instituto de Parasitología y Biomedicina "López-Neyra", del Consejo Superior de Investigaciones Científicas en Granada por Dña. Elena Sánchez Rodríguez, Licenciada en Biología por la Universidad de Granada, para optar al GRADO DE DOCTOR EUROPEO por esta misma Universidad.

Granada, 08 de Octubre de 2008

Fdo. Dr. Javier Martín Ibañez

A mis padres y hermano.

A Roge.

ABREVIATURAS

AAF	Anticuerpo anti-fosfolípido
ACG	Arteritis de células gigantes
aCL	Anticuerpo anti-cardiolipina
ACR	Colegio americano de reumatología
ADN	Ácido desoxirribonucleico
ADNcs	ADN de cadena sencilla
ADNdc	ADN de doble cadena
AIJ	Artritis idiopática juvenil
AINES	Antiinflamatorios no esteroideos
AL	Anticuerpo anticoagulante lúpico
ANA	Anticuerpos anti-nucleares
AR	Artritis reumatoide
ARN	Ácido ribonucleico
ARNcd	ARN de cadena doble
ARNcs	ARN de cadena sencilla
ARNm	ARN mensajero
BANK1	Proteína adaptadora de células B con repeticiones de ankirina (B-cell scaffold protein with ankyrin repeat)
BCR	Receptor de la célula B
BTNL2	Butyrophilin-like 2
CD	Célula dendrítica
CIA	Artritis inducida por colágeno
CNV	Variación del número de copias (Copy number variation)
COX2	Ciclooxigenasa 2
CPA	Célula presentadora del antígeno
CTLA-4	Antígeno de linfocitos T citotóxicos 4

CU	Colitis ulcerosa
DT1	Diabetes tipo I
EA	Espondilitis anquilosante
EAE	Encefalomielitis autoinmune experimental
EAI	Enfermedad autoinmune
EBV	Virus de Epstein Barr
EC	Enfermedad de Crohn
EII	Enfermedad inflamatoria intestinal
EM	Esclerosis múltiple
EMSA	Ensayo de mobilidad electroforética
ES	Esclerodermia
FcR	Receptores Fc
FCRL	Fc receptor-like
GWAS	Estudios de asociación del genoma completo (Genome wide association studies)
HLA	Antígeno leucocitario humano (Human leukocyte antigen)
HSA	Antígeno estable al calor (Heat stable antigen)
ICs	Inmunocomplejos
IFN	Interferón
Ig	Inmunoglobulina
IL	Interleuquina
iNOS	Óxido nítrico sintasa inducible
IP3R	Receptor de inositol 1 4 5-trifosfato (Inositol 1 4 5-Triphosphate Receptor)
IRF5	Factor reguador de IFN 5 (IFN regulatory factor)
LAI	Índice de actividad lúpica

LD	Desequilibrio de ligamiento (Linkage disequilibrium)
LES	Lupus eritematoso sistémico
LOD	Logaritmo del cociente de verosimilitud de ligamiento
LPS	Lipopolisacáridos
LRR	Repeticiones de leucinas
LYP	Proteín tirosín fosfatasa linfoide
MAF	Frecuencia del alelo menor (Mminor allele frequency)
MHC	Complejo mayor de histocompatibilidad (Major histocompatibility complex)
MIF	Factor inhibidor de la migración de macrófagos
MMPs	Metaloproteinasas
MyD88	Myeloid differentiation primary-response protein 88
NFκB	Factor nuclear κB
NK	Células asesinas naturales
NL	Nefritis lúpica
OR	Odds ratio
PAMPS	Patrones moleculares asociados a patógenos
PBMC	Células polimorfonucleares de sangre periférica
PDCD1	Muerte celular programada 1 (Programmed cell death 1)
PRR	Receptores de reconocimiento de patrones
PTKs	Proteín tirosín kinasas
PTPN22	Proteín tirosín fosfatasa no receptor 22
PTPs	Proteín tirosín fosfatasas
SLAM	Medida de la actividad del LES
SLEDAI	Índice de actividad de la enfermedad del LES
SNP	Polimorfismo de un solo nucleótido (Single nucleotide

	polymorphism)
STAT4	Transductor de señal y activador de la transcripción 4 (Signal transducer and activator of transcription 4)
Tc	Célula T citotóxica
TCR	Receptor de la célula T
TDT	Test de desequilibrio de transmisión
TGF	Factor de crecimiento transformante (Transforming growth factor)
Th	Células T colaboradoras
TLR	Receptores tipo toll (Toll like receptors)
TNF	Factor de necrosis tumoral
TRAF1	Receptor de TNF asociado al factor 1 (TNF receptor associated factor 1)
T_{reg}	Células T reguladoras
UV	Ultravioleta
WGS	Rastreo sistemático del genoma (Whole genome scan)

1. SUMMARY (Resumen)

Systemic lupus erythematosus (SLE) is the prototype of systemic autoimmune disease, with a complex pathogenesis involving multiple genetic and environmental factors. It is characterized by a diverse array of clinical symptoms, indicative of widespread immune-mediated damage. It is also a heterogenous disease, presenting differently from patient to patient and with no single clinical or immunological feature required to make a formal diagnosis. The pathogenesis behind the disease remains unclear. The main immunological feature is uncorntrolled formation of autoantibodies, leading to excess formation of immune complexes which deposit in different tissues, causing inflammation and tissue damage. The disease primarily affects women in their reproductive years and the estimated prevalence varies between 12 and 64 cases per 100.000 inhabitants in European-derived populations, with a higher prevalence, in general, in non-European-derived populations. For at least 30 years we have known that there is a strong genetic component to SLE: a disease concordante of 2-5% in dizygotic compared with 30-50% in monozygotic twins and a sibling risk ratio (λ_s) of 20-29.

Progress in identifying these genetic factors was initially slow. Most of the genetic factors proposed to date to be involved in SLE have been analyzed by association studies in unrelated patients and controls. Until recently, the investigations of genetic effects in SLE have been mostly association studies of *HLA* genes. The alleles *HLA-DRB1*0301*, *HLA-DRB1*1501* y *HLA-B8* in the MHC have been consistently associated in SLE. The publication of the human genome sequence in 2001 have stimulated renewed interest in genetic. In the last years, genome-wide linkage studies have shown several loci out of the MHC region with association to SLE, and this has accelerated over the last 12 months with the publication of several high-density genome wide association

studies (GWAS) and the identification of several novel candidate genes through fine mapping studies.

The main aim in this doctoral thesis has been to try to identify new genetic markers of susceptibility to SLE using case-control association studies of candidate genes, thus helping to elucidate the genetic basis of this complex disease. The strategies used to select the candidates genes to study have been mainly two: *positional*, based on genes that have previously been found in regions of susceptibility to the disease through linkage studies or genes that are found in areas associated with susceptibility in animal models of SLE; and *functional*, which is to select genes that may have an implication in the pathophysiological mechanisms of the disease as well as genes involved in inflammatory or autoimmune related diseases. To perform these studies we have used several cohorts of SLE patients and healthy controls from different Spanish regions (Granada, Málaga, Sevilla, Lugo and more recently Sabadell, Valencia y Oviedo) and on time worked in collaboration with various international groups from Germany, Italy, Sweden, Argentina and Mexico.

Some of the genes studied in this thesis were selected by the strategy of candidate genes. Among these we studied genes involved in the inflammatory response, because this is a fundamental mechanism in the initiation and perpetuation of the disease. In this group were selected *TLR2*, *TLR4*, *TLR5* and *TLR7* genes, which are surface receptors that play an essential role in the activation and regulation of the innate and adaptative immune response leading to activation of a large number of inflammatory mediators through the activation of the transcription factor NFκB. Taking into account the important role of the transcription factor NFκB in the activation of the inflammatory response, we decided also study the *NFKB1* gene, that encodes the p50 subunit of the molecule NFκB, with susceptibility to SLE.

FcγRs genes are essential mediators of inflammatory effects, cytotoxic antibodies and immune complexes and relate the innate immune system and acquired one another. Within this group we studied the *FCRL3* gene, which belongs to this family of genes (FcγR) and they are believed to have a vital role in increasing the presence of self-reactive B cells.

In addition, within these genes involved in inflammatory response we selected a number of cytokines that promote a Th1 response and thus lead to a situation of inflammation, such as genes of the family of the interleukin 12 (IL-12) : *IL12B*, *IL12RB1*, *IL23A* and *IL23R* genes and two genes that encodes two pro-inflammatory cytokines: *IL18* and *MIF* genes.

Regard the MHC region, where the classical *HLA* genes are, there are also a large numbers of genes very close together characterized of being extended haplotypes that may influence in the susceptibility to disease by increasing the presentation of the immunogenic peptide epitopes in the periphery, resulting in increased activation of T cells, or the presentation of ineffective autoantigen in the thymus, giving rise to more aggresive T cells or less number of regulatory T cells (Treg). The genes studied in this region were the *BTNL2* and *MICA* genes. Other group of selected genes was genes implicated in the regulation of T and B cells. We have selected in this group the *PTPN22* gene, which encodes a lymphoid-specific phosphatasa (Lyp). PTPN22 is a key molecule regulating TCR signalling in T lymphocytes and in recent times has been considered as an important gene in autoimmunity. Other selected gene is *CD24*, which belong to a family of proteins involved in signal transduction by members of the protein tyrosine kinases (PTKs) and is involved in the activation and differentiation of T and B cells.

Finally, we selected a series of genes that have been identified through the new GWAS as genes involved in SLE susceptibility and are also implicated

in the pathogenesis of the disease. Under this heading, the studied genes were: *BANK1*, a gene involved in signalling through the BCR; *STAT4*, a gene that encodes for a transcription factor that mediates the expression of genes in important disease pathways; *TRAF1*, which is suggested to be a negative regulator of TNF-Receptor signalling and *C5*, a central component of the complement pathway.

Our results show that genes which encodes the pro-inflammatory cytokines MIF and IL-18 are associated with susceptibility to SLE. We have found that the -173C allele and the -173C-CAAT$_7$ haplotype of the *MIF* gene are associated with high risk to development SLE. In addition, these variations lead to an increased production of MIF which leads to a series of inflammatory processes that trigger the onset of the autoimmune disease. On the other hand, the *IL18* -1297C allele was also associated with susceptibility to SLE. Besides, additional functional studies conducted revealed the importance of this allele in the expression of IL-18.

Regard to genes involved in T and B cells regulation, we note that the *PTPN22* R620W gene polymorphism, which alters the regulation of T cells and has been proposed as a common susceptibility marker to several autoimmune diseases, is strongly associated with susceptibility to SLE in our population. In addition, we found that the *CD24* A57V polymorphism is also associated with risk to SLE, and this association has been replicated in an independent cohort from Germany.

Within the genes selected by GWAS, we found that the *BANK1* and *STAT4* genes (works done in collaboration with Dr. Marta Alarcón-Riquelme group, Uppsala University, Sweden), which are implicated in T and B cells regulation, are associated with SLE. Our analysis suggests that three genetic variants of the *BANK1* gene, either individually or as haplotypes, confer

susceptibility for SLE and functional studies have demonstrated the functional relevance of two of them. With regard to *STAT4*, we found five polymorphisms associated with SLE in the Spanish population and four of them have been replicated in five independent populations. In addition, we do find a correlation with expression levels of *STAT4* in peripheral blood cells and the three most associated SNPs.

Finally, in the *TRAF1/C5* locus we have found a polymorphism (rs10818488) associated with susceptibility to SLE and this association was replicated in an independent cohort from Crete. This latest work was conducted in collaboration with Dr. Rene Toes group, Leiden University, The Netherlands.

Regarding to the rest of genetic variants tested, we did not find evidences of association with the candidate genes *TLR2*, *TLR4*, *TLR5*, *TLR7*, *NFκB*, *FCRL3*, *IL12B*, *IL12RB1*, *IL23*, *IL23R*, *MICA* and *BTNL2* and SLE. Therefore, we suggest that these genes do not play a critical role in genetic predisposition to SLE in our population.

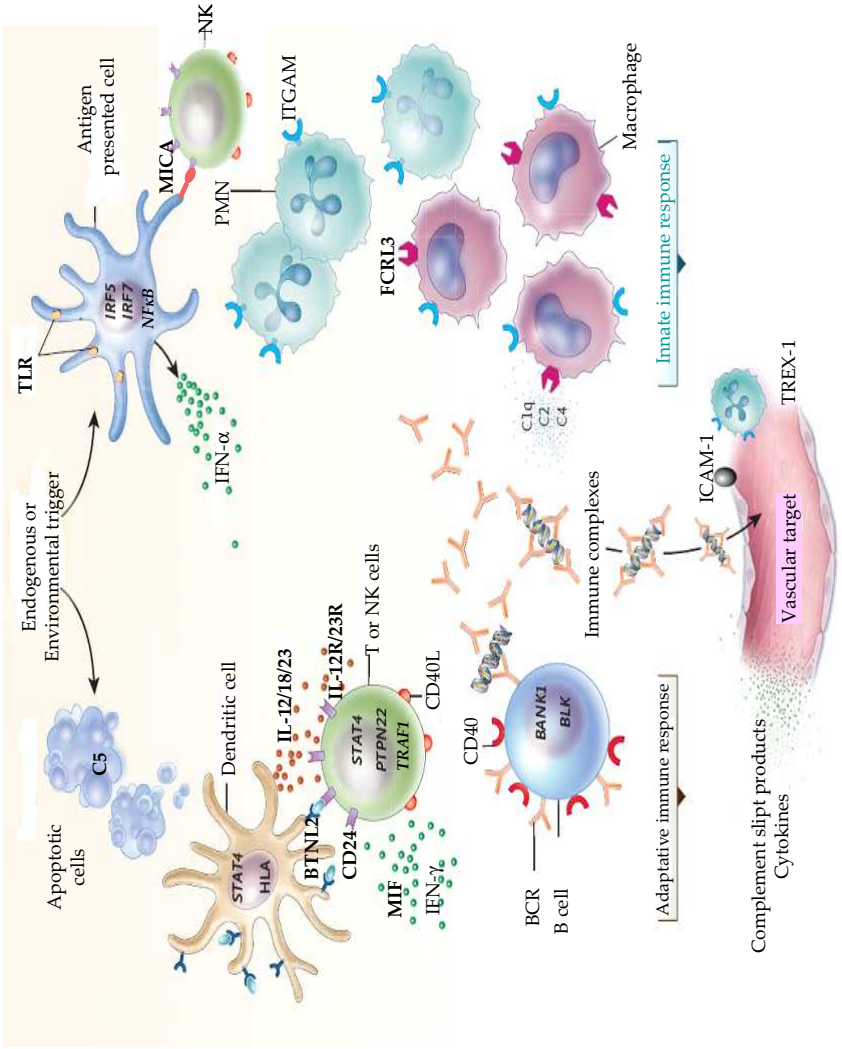

Figure 1.1. Outline of the genes studied in this work and other know genes implicated in susceptiblity to SLE.

2. INTRODUCCIÓN

El primer conocimiento que aparece en un contexto médico de la palabra "lupus" es en la biografía de San Martín en el siglo X (1), mientras que el primer caso conocido de lupus eritematoso sistémico (LES) fue probablemente una momia peruana del sigo VIII, en la cual los investigadores fueron capaces de revelar la presencia de alopecia, hematuria, pleuritis y pericarditis (2). Rogerius Frugardi, también conocido como Roger de Salerno, un cirujano del siglo XII, introdujo el término "noli me tangere" (*no me toques*) para referirse a la ulcera facial. Ferdinand von Hebra describe el *rash* en forma de alas de mariposa en 1845. En 1851, el francés Pierre L. Cazénave introdujo el termino "Lupus érythémateaux" por primera vez. A principios del siglo XIX, Kaposi entre otros, introdujo el termino "discoide" para denominar a las lesiones cutáneas y describe como "sistémico" al lupus cuyas características son fiebre, sinovitis, anemia, afectación pulmonar y coma. En 1880, William Osler estableció firmemente la existencia de una forma de lupus diseminada y otra sistémica. En 1948, Hargraves describió las células LE, lo que representó uno de los mayores avances en la comprensión de la enfermedad. Este fenómeno consiste en la visualización de leucocitos que fagocitan otros leucocitos y en donde los núcleos de aquellos que son fagocitados permanecen intactos. Al principio se conocía muy poco sobre los mecanismos implicados en el desarrollo del fenómeno LE. Sin embargo, este hallazgo fue la base para el entendimiento inicial de la enfermedad como un proceso autoinmune.

2.1. Aspectos clínicos y epidemiológicos del lupus eritematoso sistémico

2.1.1. Características clínicas del LES

El LES es el prototipo de enfermedad autoinmune (EAI) sistémica, caracterizada por ser un desorden crónico, inflamatorio causado por la producción de un amplio espectro de autoanticuerpos. Debido al carácter sistémico de la enfermedad, en pacientes con LES pueden verse afectados multitud de órganos y sistemas (Figura 2.1).

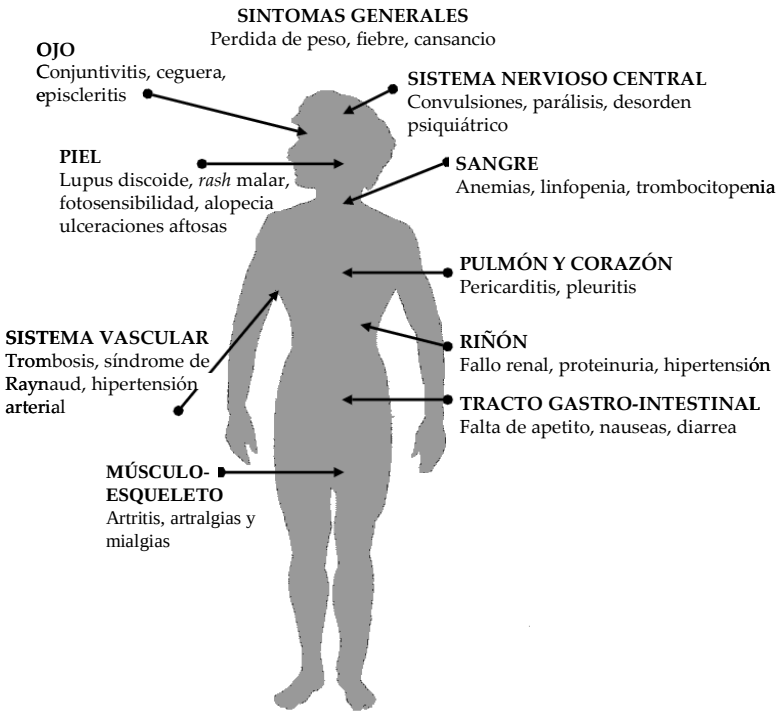

Figura 2.1. Esquema de los diversos órganos y sistemas afectados en pacientes con LES.

Los síntomas generales más habituales son cansancio, fiebre, anorexia y pérdida de peso; estos síntomas casi siempre acompañan a otras manifestaciones más específicas cuando aparecen los brotes de la enfermedad;

siendo frecuente la presencia de linfadenopatías, más o menos generalizadas y esplenomegalia. Entre las afectaciones clínicas más comunes se encuentran:

2.1.1.1. Manifestaciones cutáneas

Las manifestaciones cutáneas en el LES son muy frecuentes. Cuatro de estas manifestaciones cutáneas, *rash* malar, lupus discoide, fotosensibilidad y ulceras orales, están incluidas dentro de los criterios de clasificación del LES. Estas manifestaciones van a variar dependiendo de la raza, ya que el lupus discoide y la alopecia son más comunes en población afroamericana; mientras que el *rash* malar y las ulceras orales son más frecuentes en población caucásica.

Aproximadamente el 60% de los pacientes con LES presentan *rash* malar (característico "en alas de mariposa") que es eritematoso, fotosensible, plano o elevado; se puede localizar en mejillas, puente de la nariz, mentón y pabellones auriculares (Figura 2.2). Estas lesiones cutáneas agudas no dejan cicatriz, aunque pueden aparecer telangiectasias. En las áreas expuestas al sol, es frecuente la aparición de un *rash* maculopapular más generalizado que puede estar relacionado con los brotes de la enfermedad. Con bastante frecuencia existe alopecia, generalmente parcial y de forma irregular, aunque a veces puede ser completa. Lesiones de lupus cutáneo crónico (también conocido como lupus discoide) hay en aproximadamente un 20% de pacientes con LES; cursan con atrofia central y dejan cicatriz, con pérdida permanente de apéndices. Estas lesiones se sitúan generalmente sobre el cuero cabelludo, orejas, cara, zonas de los brazos expuestas al sol y tórax. De importancia pronóstica es que menos del 5% de estos pacientes con lupus discoide desarrollan LES.

Figura 2.2. *Rash* malar en forma de "alas de mariposa".

2.1.1.2. Manifestaciones músculoesqueléticas

La mayoría de los pacientes con LES presentan artralgias y/o mialgias, más que crisis de artritis. Cuando hay artritis, generalmente no erosiva, se suelen afectar las articulaciones interfalángicas proximales, metacarpofalángicas, carpos y rodillas (Figura 2.3). Durante los períodos de actividad de la enfermedad puede haber miositis/miopatía, debido al cuadro de inflamación general del proceso o, a veces, como consecuencias de los tratamientos.

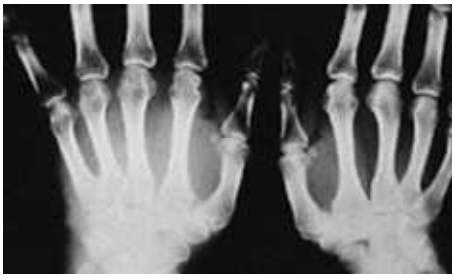

Figura 2.3. Radiografía mostrando las manos con artritis no erosiva en un pacientes de LES.

2.1.1.3. Manifestaciones hematológicas

Las más frecuentes son la anemia de tipo normocítico-normocrómico; leucopenia, generalmente con linfopenia; anemia hemolítica y trombocitopenia moderada.

2.1.1.4. Manifestaciones cardiopulmoranes

La pleuritis y derrame pleural son frecuentes en los enfermos con LES y, en ocasiones, pueden ser la forma de debut de la enfermedad o una complicación durante los brotes. Menos frecuentes son la neumonitis lúpica, la fibrosis pulmonar, hipertensión pulmonar, síndrome de distrés respiratorio y hemorragia intraalveolar masiva.

La pericarditis es la manifestación cardiaca más frecuente en el LES, cursando generalmente con escaso derrame pericárdico, aunque en alguna ocasión se puede llegar al taponamiento cardiaco. Los pacientes con LES tienen también un elevado riesgo de sufrir ateroesclerosis (3, 4).

2.1.1.5. Manifestaciones gastrointestinales

Los síntomas más comunes a nivel del aparato digestivo son náuseas, diarrea y malestar inespecífico. En bastantes ocasiones se deben a efectos secundarios de los tratamientos instaurados. La complicación más grave es la vasculitis intestinal.

2.1.1.6. Manifestaciones renales

La mayoría de los pacientes con LES tienen depósitos de inmunocomplejos (ICs) en los glomérulos, aunque sólo un 25-50% de ellos presentan datos clínicos de nefritis con edemas, proteinuria, hematuria y cilindruria.

2.1.1.7. Manifestaciones del sistema nervioso

La afectación del sistema nervioso central aparece habitualmente en enfermos de LES activo con síntomas a nivel de otros órganos. Se pueden afectar distintas zonas del cerebro, meninges, médula y nervios craneales y

periféricos. La manifestación más frecuente es la disfunción cognoscitiva, junto con alteraciones del ánimo del tipo de ansiedad y depresión; también es común la jaqueca, y algunos pacientes pueden tener convulsiones.

2.1.1.8. Manifestaciones vasculares

La complicación más importante a nivel vascular es la trombosis. Esta manifestación se relaciona con los anticuerpos antifosfolipídicos (AAF), ya sea anticoagulante lúpico (AL) o anticuerpos anticardiolipina (aCL). Otras manifestaciones vasculares son: el síndrome de Raynaud, hipertensión arterial secundaria y la coagulación arterial diseminada.

2.1.1.9. Manifestaciones oculares

Puede ser muy diversa la participación ocular en los enfermos de LES y así no es infrecuente que en el curso evolutivo de la enfermedad puedan presentar conjuntivitis, episcleritis, síndrome de Sjögren, uveítis y neuritis óptica. La complicación ocular más grave es la vasculitis retiniana, que puede producir ceguera.

Debido al amplio espectro de manifestaciones clínicas y a la alternancia entre períodos de exacerbación y remisión, el diagnóstico de LES puede no ser evidente. Además, existen otras enfermedades que se asemejan al LES. Desde 1971 se han establecido criterios de clasificación de la enfermedad con el fin de establecer parámetros de inclusión de pacientes en estudios clínicos (Tabla 2.1). Una primera modificación de estos criterios fue realizada en 1982 (5) y la última fue hecha en 1997 (6). La presencia de cuatro o más criterios, en forma sucesiva o simultánea durante cualquier período de observación, permite clasificar a un paciente con LES (6).

1. Eritema facial	Eritema fijo, plano o elevado sobre eminencias malares
2. Lupus discoide	Lesiones cutáneas eritematosas, con cambios en la pigmentación y cicatrices residuales
3. Fotosensibilidad	*Rash* malar causado por exposición a luz UV.
4. Úlceras orales	En la cavidad oral o nasofaríngea, usualmente con poco dolor, observadas por un facultativo
5. Artritis	No erosiva, que afecte a dos o más articulaciones periféricas con dolor, inflamación o derrame articular
6. Serositis	Pleuritis o pericarditis, roce o evidencia de derrame pericárdico
7. Alteración renal	Proteinuria > 0.5 g/dl, > 3+ o cilindros celulares o hemáticos
8. Alteración neurológica	Convulsiones o psicosis, sin otra causa
9. Alteración hematológica	Anemia hemolítica; leucopenia (< 4000/mm^3) o linfopenia (<1500/mm^3) en dos o más ocasiones o trombocitopena (<10000/mm^3), en ausencia de fármacos que las produzcan
10. Alteración inmunológica	Anticuerpos anti-ADN (anticuerpos contra el ADN nativo), anti-Sm (anticuerpos frente al antígeno nuclear Sm) y/o anticuerpos antifosfolípidos
11. Anticuerpos antinucleares	Titulo elevado de ANAs (anticuerpos anti nucleares) detectados por inmunofluorescencia o ensayo equivalente, en algún momento de la evolución, en ausencia de fármacos que los induzcan

Tabla 2.1. Criterios de clasificación del LES según el Colegio Americano de Reumatología (ACR), revisados en 1997.

Aunque estos criterios de clasificación del LES han sido establecidos para facilitar la identificación de pacientes, esta claro que el LES encierra numerosos fenotipos diferentes. Estos fenotipos varían desde un LES medio-leve, caracterizado por *rash* malar y dolor en la articulaciones que apenas requiere medicación, a un LES severo y multisistémico. Debido a esta variabilidad fenotípica en los pacientes con LES, se han establecido diversos índices para medir la actividad de la enfermedad: índice de actividad de la enfermedad del LES (SLEDAI), medida de la actividad del lupus sistémico (SLAM) y el índice de la actividad lúpica (LAI). Estos diversos índices indican el número de órganos y sistemas afectados, así como la inclusión/exclusión de datos serológicos.

2.1.2. Tratamiento del LES

Su objetivo es el control del proceso autoinmune. Debe realizarse de acuerdo al compromiso predominante. El tratamiento se suele dividir en manejo no-farmacológico y farmacológico. Dentro del primero se resalta evitar la exposición a la luz solar y el uso de protector solar en pacientes con manifestaciones cutáneas. Así, el uso de píldoras anticonceptivas en altas dosis o el uso de terapia de reemplazo hormonal están contraindicados en estos pacientes. Otro aspecto no-farmacológico importante es la disminución de riesgos cardiovasculares como llevar una alimentación sana y dejar de fumar, dada la alta incidencia de muerte prematura de pacientes con LES debido a una ateroesclerosis acelerada (7).

El tratamiento farmacológico de los pacientes con LES puede dividirse en cuatro grupos: los anti-inflamatorios no esteroideos (AINES), esteroides, antimaláricos y agentes citotóxicos (8) (Tabla 2.2).

Tratamiento	Eficacia (en casos medios/moderados)	Eficacia (en casos severos)	Toxicidad
Corticoesteroides	Usualmente efectivo (dosis bajas)	Efectivo algunas veces (dosis elevadas)	Alta
AINES	Efectivos algunas veces	No efectivo	Moderada
Antimaláricos	Efectivo algunas veces/usado durante un largo periodo de tiempo	No efectivo	Baja
Azatioprina	Usualmente efectivo	Efectivos algunas veces	Moderada
Metotrexato	Usualmente efectivo	Desconocido	Moderada
Leflunomida	Desconocido	Desconocido	Moderada
Ciclosporina	No usado	Efectivos algunas veces	Alta
Ciclofosfamida	No usado	Efectivos algunas veces	Alta

Tabla 2.2. Tratamientos habituales en el LES.

Durante los últimos años se han probado multitud de nuevos fármacos inmunomoduladores, aunque ninguno de ellos ha sido aprobado como tratamiento global en el LES. Algunas de estas nuevas terapias usadas en grupos seleccionados de pacientes son plasmaféresis, gammaglobulinas intravenosas, ciclosporina, micofenolato mofetil, depleción de linfocitos B con anticuerpos monoclonales (ejemplo. Rituximab) y transplante de células madre hematopoyéticas (9, 10).

2.1.3. Epidemiología del LES

Una de las características del LES es que tiene una predominancia femenina, siendo la proporción mujer:hombre de 9 a 1 (Figura 2.4). Aunque el LES puede aparecer a cualquier edad, es más frecuente en mujeres en edad reproductiva (entre 15-50 años) (11). Cuando la enfermedad se desarrolla durante la infancia o en edad adulta (después de la menopausia), difiere de la forma clásica, con menos predominancia femenina y diferente presentación clínica (12, 13).

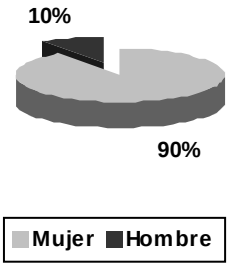

Figura 2.4. Relación mujer:hombre a desarrollar LES.

La incidencia del LES se ha triplicado desde los años setenta, de 1.51 por cada 100.000 habitantes (1950-1979) a 5.56 por cada 100.000 habitantes (1980-1992) (14). Este incremento parece ser debido a un más temprano diagnóstico de la enfermedad y a la inclusión de casos leves. Su incidencia y prevalencia

varían según las poblaciones estudiadas, siendo la enfermedad más común en poblaciones afroamericanas (200 por cada 100.000 habitantes) y en poblaciones asiáticas que en poblaciones caucásicas (aproximadamente 40 casos por cada 100.000 habitantes) (15, 16).

La prevalencia del LES se incrementa desde África hacia Europa, un fenómeno conocido como la hipótesis de "gradiente de prevalencia" (17, 18). Los iniciadores potenciales de este gradiente incluyen tanto factores genéticos y ambientales como diferencias nutricionales o exposición a patógenos.

El curso clínico de la enfermedad también varía entre las poblaciones. En el caso de la nefritis lúpica (NL) se ha demostrado que es más frecuente y produce un daño orgánico más rápido en población hispana y afroamericana que en caucásicos (19-21). También se ha demostrado que en pacientes de LES latinoamericanos y afroamericanos hay una mayor presencia de enfermedad renal, pericarditis y lesiones discoides que en los pacientes de raza blanca (22).

2.2. Bases moleculares e inmunológicas del LES

Aunque los eventos exactos por los cuales se rompe la tolerancia inmunológica en el LES se desconocen, multitud de estudios se están realizando con el fin de ayudarnos a comprender el desorden inmunológico que se produce en esta enfermedad. Se sabe que la apoptosis quizás explique como el sistema inmune reconoce predominantemente antígenos intracelulares. En el LES han sido descritos fallos en la eliminación de células apoptóticas y esos defectos pueden dar lugar a una elevada producción de macrófagos, los cuales van a presentar los antígenos intracelulares a las células T y B, dando lugar al proceso autoimmune (23). Los diferentes patrones de citoquinas expresados en pacientes de LES también son importantes en la patogénesis de la enfermedad. Las últimas investigaciones han centrado su

atención en la sobreexpresión de interferones (IFN) tipo I y su ruta de señalización (también llamado "firma del interferón") (24). Además el LES está caracterizado por una respuesta exagerada de las células B que dan lugar a la producción de un amplio espectro de autoanticuerpos y una elevada activación de células T (Figura 2.5).

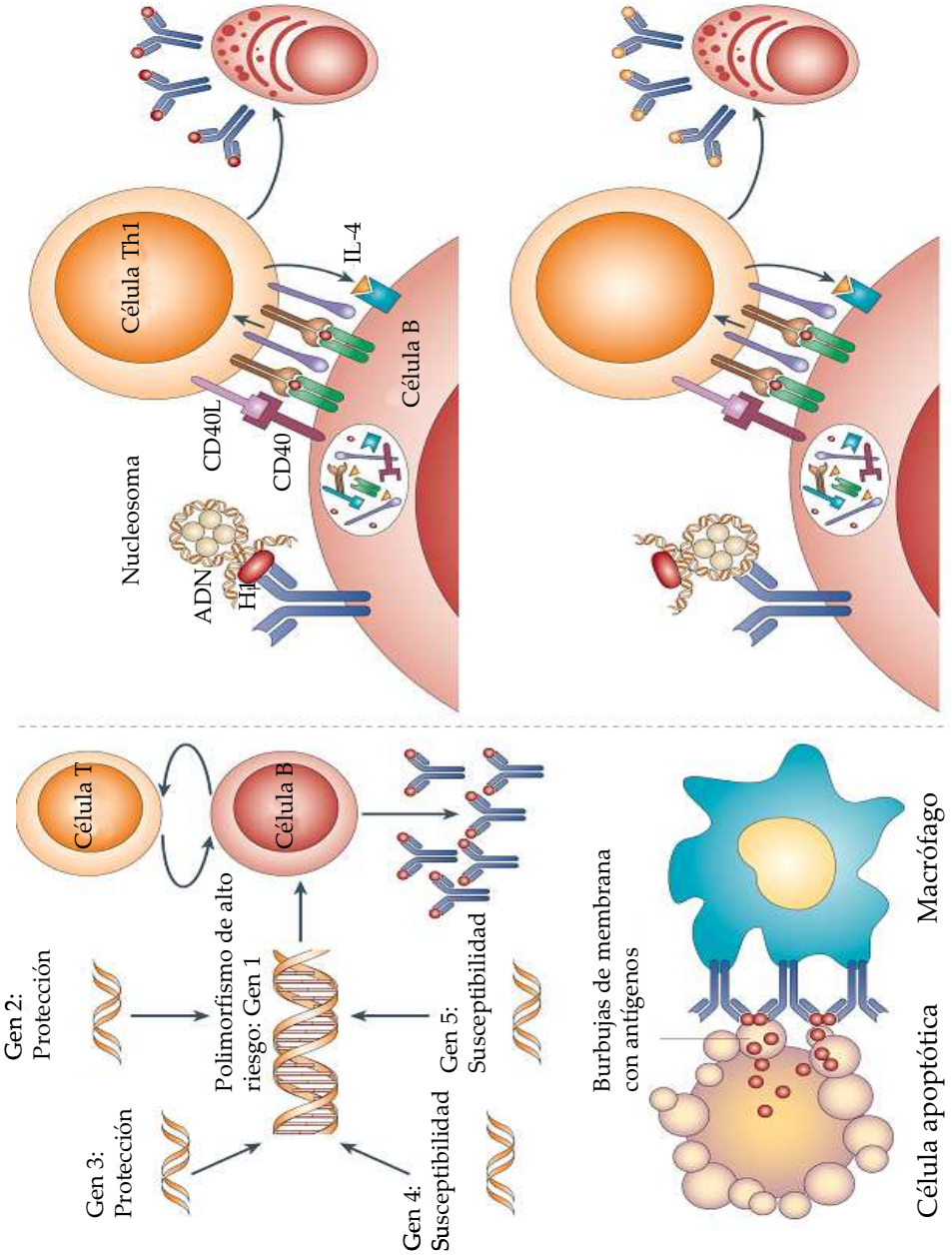

Figura 2. 5. Mecanismos inmunológicos en el LES.

2.2.1. Autoanticuerpos en el LES

La presencia de autoanticuerpos es una característica esencial del LES, y dos de los criterios de clasificación de la enfermedad refieren a la detección de algunos de estos anticuerpos (6). Aunque un amplio rango de diferentes autoanticuerpos se han descrito en la sangre de pacientes con LES, solo unos pocos de ellos aparecen en más del 10% de pacientes con la enfermedad (13).

Los anticuerpos anti-nucleares (ANA) son unos de los más importantes en el LES (25), ya que más del 95% de los pacientes presentan elevados títulos de ANA durante el curso de la enfermedad. Sin embargo, el test para ANA no es específico del LES y resultados positivos para este test pueden darse en otro tipo de desordenes de tejido conectivo. En 1967, en los riñones de un paciente que padecía NL se observó que contenían anticuerpos unidos a ADN de doble cadena (ADNdc) (26). Esos ANA eran autoanticuerpos que se unen a constituyentes normales – en este caso ADNdc– de las células y tejidos de los pacientes. La gran importancia de estos anticuerpos anti-ADNdc ha sido recientemente confirmada (10). Estos anticuerpos son altamente específicos en el LES, ya que están presentes en el 70% de los enfermos (10), frente al 0.5% presente en individuos sanos o pacientes con otras enfermedades autoinmunes como la artritis reumatoide (AR) (10). Los niveles de anticuerpos anti-ADNdc presentes en el suero de los pacientes refleja la actividad de la enfermedad (27), aunque no en todos los pacientes. Entre los pacientes que presentan niveles elevados de estos autoanticuerpos y están en una fase inactiva de la enfermedad, el 80% van a comenzar a presentar una actividad clínica en los 5 años posteriores a la detección de esos niveles elevados de anticuerpos (28). Estos anticuerpos anti-ADNdc se han visto también altamente asociados con NL (29). Sin embargo, no todos los anticuerpos anti-ADNdc son patogénicos.

Algunos pacientes que presentan estos anticuerpos en sangre no muestran evidencia de actividad de la enfermedad (30). Además, en estudios realizados en animales, se ha visto que no todos los anticuerpos anti-ADNdc causan efectos patológicos. Afortunadamente, algunos isotipos y sus propiedades de unión facilitan la caracterización de los anticuerpos anti-ADNdc patogénicos de los no patogénicos. Diversos estudios muestran que los niveles de anticuerpos de inmunoglobulina G (IgG) que se unen al ADNdc están más relacionados con la actividad de la enfermedad y el daño tisular que los anticuerpos IgM o anti-ADN de cadena sencilla (ADNcs) (10, 31).

Otros autoanticuerpos encontrados en biopsias renales de pacientes con LES han sido: anti-Ro (un complejo ribonucleoprotéico), anti-La (una proteína de unión al ARN) y anti-Sm o anti-Smith (partículas nucleares que contienen diferentes polipéptidos) (32). Aunque no se ha probado que estos autoanticuerpos tengan un papel clave en el desarrollo de la NL. Las evidencias más claras en cuanto a su acción en NL apuntan a los anticuerpos anti-ADNdc, anti-nucleosomas y anti-α actinina (27, 33, 34). La presencia de anticuerpos anti-Ro y anti-La, confiere en embarazadas riesgo de bloqueo aurículo-ventricular fetal. Por otro lado los anticuerpos frente el receptor de N-metil-D-aspartato se han relacionado con el sistema nervioso central en el LES (35).

2.2.2. Daño tisular originado por los autoanticuerpos en el LES

La mayoría de los estudios de daño tisular mediado por autoanticuerpos realizados en pacientes con LES se han centrado en el papel de los anticuerpos anti-ADNdc en pacientes con NL. Se han desarrollado dos principales teorías a este respecto; ambas coinciden en que la unión de los anticuerpos al ADNdc no es probablemente el mayor determinante crítico del

daño tisular. El ADNdc extracelular se origina principalmente en forma de nucleosomas, que son fragmentos de cromatina que las células liberan cuando entran en apoptosis. Se ha propuesto que los anticuerpos anti-ADNdc patogénicos en los pacientes con LES se unen a nucleosomas que han entrado en el torrente circulatorio, dando lugar a que esos complejos anticuerpo-nucleosoma se establezcan en la membrana basal de los glomérulos renales (36). Esos ICs tienen la capacidad de activar al complemento, desencadenándose así la glomerulonefrítis. Todos estos eventos han sido demostrados en modelos animales (37, 38).

El segundo modelo propone que los anticuerpos anti-ADNdc, anti-nucleosomas, o ambos pueden tener una reacción cruzada con proteínas del riñón, teniendo así un efecto patogénico directo sobre las células renales. Entre la multitud de antígenos presentes en el riñón, la mayoría de los estudios apuntan hacia la α-actinina como el principal responsable (39, 40). Aunque los anticuerpos anti-α-actinina no son específicos del LES, esos anticuerpos, cuando están presentes en el suero de pacientes con LES, pueden servir como marcador de compromiso renal.

2.2.3. Células T en el LES

Las células T juegan un papel importante en la regulación del sistema inmune. El LES está caracterizado por un disbalance entre linfocitos T citotóxicos (Tc) y linfocitos T colaboradores (Th). Las células T en pacientes con LES reconocen a través de sus receptores de células T (TCR) autoantígenos como partículas de ribonucleoproteínas, aumentando la afinidad de las células B autoreactivas, dando lugar a la producción de autoanticuerpos (Figura 2.6). Estos antígenos son presentados a través de una molécula del complejo mayor de histocompatibilidad principal (MHC) que se encuentra en la superficie de

las células presentadoras de antígeno (CPA). La presentación de este complejo antígeno-MHC no es suficiente para estimular a la célula T. Está unión debe ir acompañada de una segunda interacción de coestimulación con el linfocito T. Existen varias parejas de coestimuladores moleculares, incluyendo la pareja CD40-CD40 Ligando y CD28-B7, las cuales son capaces de generar la segunda señal requerida para la activación de la célula T (41). Otra posible interacción puede ocurrir entre la molécula B7 y CTLA-4 (antígeno de linfocitos T citotóxicos 4), la cual es inhibitoria. Si se produce la señal positiva mediante la interacción de CD28-B7, la célula T es activada, se produce la liberación de citoquinas y se promueve la situación de inflamación. Por el contrario, si domina la señal negativa mediada por la interacción CTLA-4-B7, la activación de la célula T se suprime (Figura 2.7).

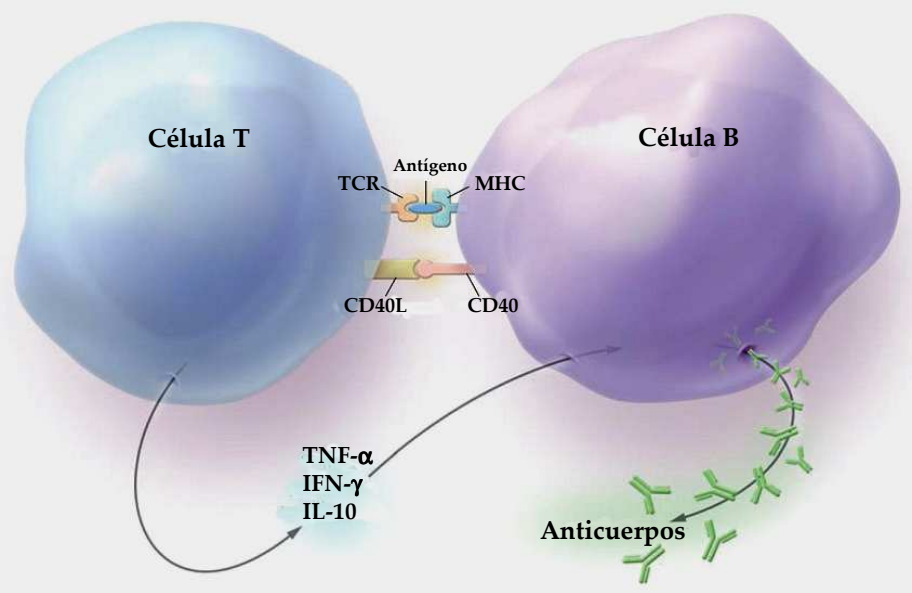

Figura 2.6. Interacción célula T-célula B.

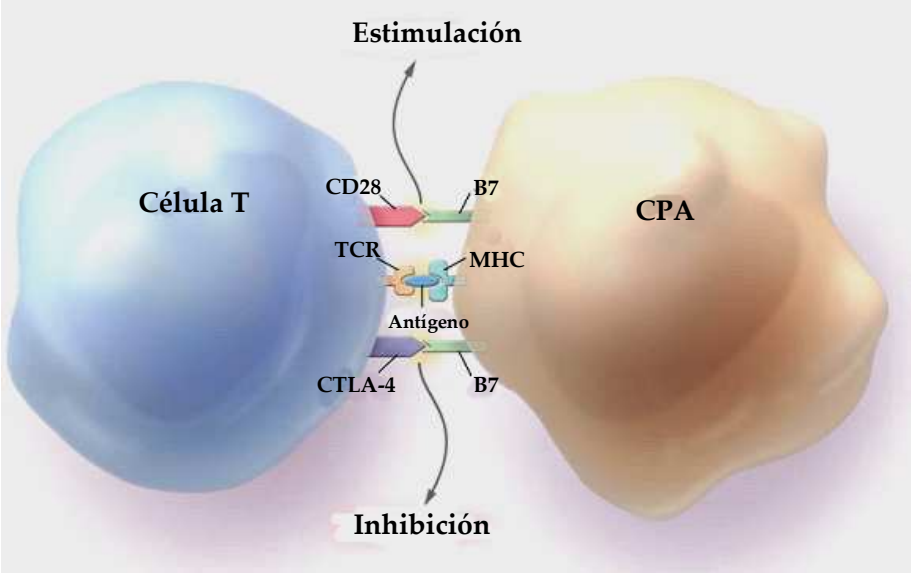

Figura 2.7. Interacción entre la célula T y la célula presentadora de antígeno

2.2.4. Papel de las células B en el LES

Aunque diversas hipótesis apuntan a que las anormalidades de las células T juegan un papel esencial en la evolución de la autoinmunidad y el daño tisular ocurrido en el LES. Datos recientes indican que la capacidad de respuesta excesiva de las células B frente a una estimulación autoinmune quizás sea una característica esencial de la enfermedad (42). Un dato que apoya la hipótesis de las células B es la estrecha relación entre el LES y la producción de patrones característicos de autoanticuerpos, algunos de los cuales como ya se ha descrito con anterioridad, están claramente involucrados en el daño tisular. Descubrir el papel central de los autoanticuerpos en el LES ha sido un desafío, ya que estudios realizados con ratones modificados genéticamente predispuestos a padecer LES (MRL *lpr*/*lpr*) desarrollan nefritis y vasculitis a pesar de ser incapaces de secretar autoanticuerpos. Ratones MRL *lpr*/*lpr* deficientes en células B no desarrollan glomerulonefrítis ni vasculitis, lo cual implica que las células B y no los autoanticuerpos, tienen un papel

esencial en la enfermedad en este modelo animal de LES (43). En humanos, hay evidencias claras de que al menos la afectación de algunos tejidos está mediada por autoanticuerpos, aunque la anormal activación y función de las células B que se produce en estos pacientes pueda estar también contribuyendo al desarrollo de la enfermedad. Es sabido que en pacientes de LES se produce una sobreactivación de las células B. Esto incluye anormalidades en la activación, señalización y migración de las células B;; en el aumento constitutivo de la expresión de moléculas coestimuladoras; el aumento en la producción de diversas citoquinas que fomentan a su vez la estimulación de las células B; un marcado aumento de la actividad mutacional; y anormalidades en la selección positiva y negativa de células B que pueden directa o indirectamente contribuir al desarrollo del LES (42).

Recientemente se ha evaluado el papel de las células B en el sistema inmune, indicando que funcionan más que como simples precursoras de células secretoras de anticuerpos. En una respuesta inmune normal, las células B son las precursoras de células secretoras de anticuerpos, funcionan como CPA y también regulan las funciones de un gran número de células implicadas en la respuesta inmune, como por ejemplo células T o células dendríticas (CD) (Figura 2.8).

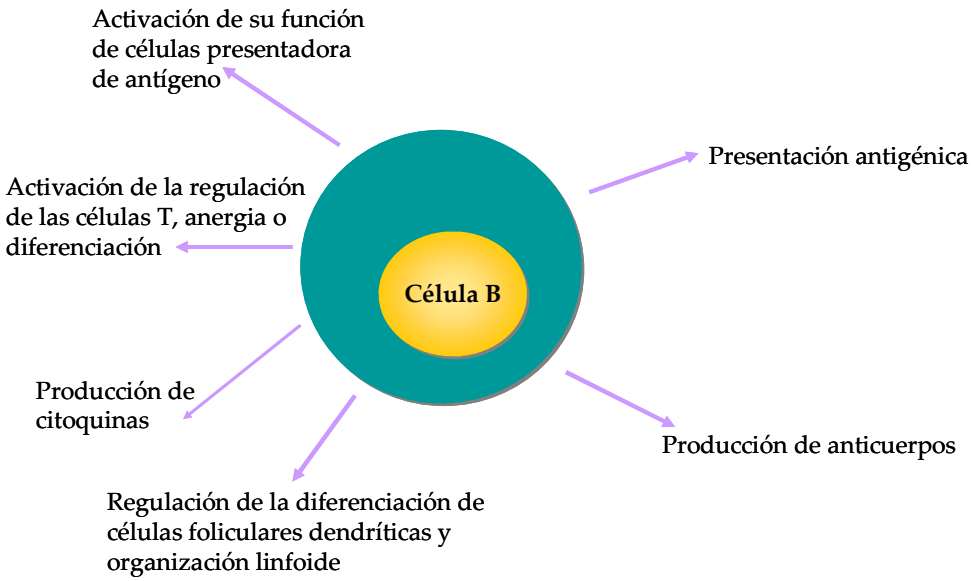

Figura 2.8. Función de las células B en la respuesta inmune normal.

Por el contrario, en el caso del LES diversos factores genéticos pueden incrementar la reactividad de las células B, lo cual puede dar lugar directamente a la producción de autoanticuerpos y finalmente generar el daño orgánico. Este proceso también puede aumentar la capacidad de las células B de aumentar la función de otros tipos celulares implicados en la respuesta de las células B. Además, los distintos factores genéticos pueden directamente alterar la función de las células T y de las CPA, así como la producción de citoquinas o la disponibilidad de antígenos endógenos, lo que puede contribuir probablemente a un aumento de susceptibilidad de las células B, y dar lugar a la autoinmunidad y finalmente al daño orgánico (Figura 2.9).

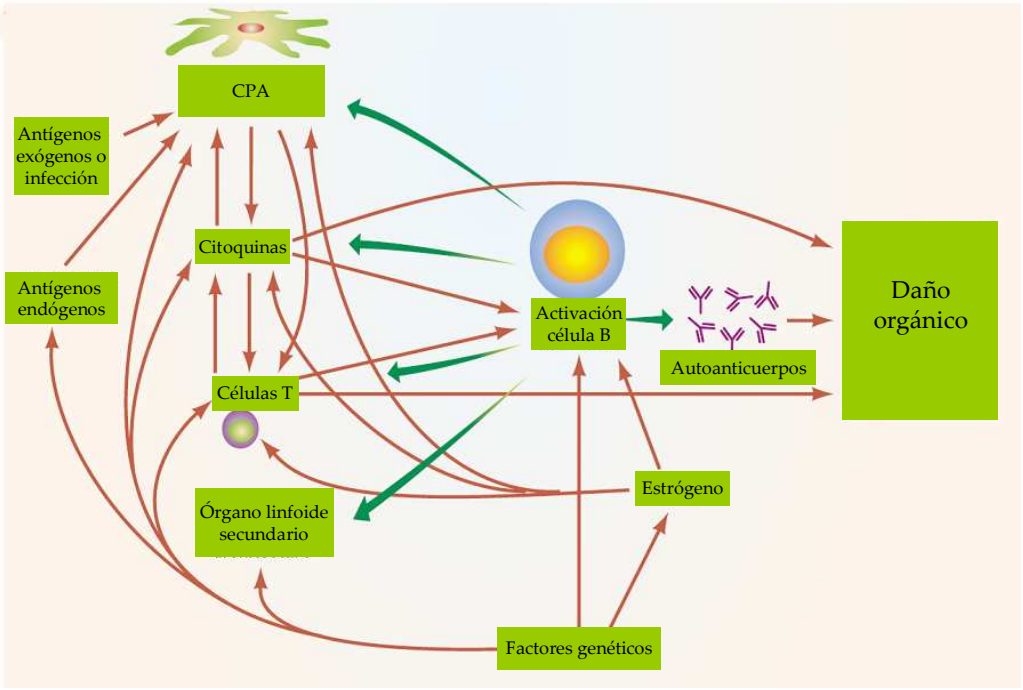

Figura 2.9. Regulación alterada de la célula B en el LES.

2.2.5. Fuentes de autoanticuerpos en el LES

Una de las fuentes principales de anticuerpos son los cuerpos celulares liberados como resultado de la apoptosis. Durante la apoptosis se forman burbujas de material celular en la superficie de la célula moribunda. Los antígenos que están normalmente ocultos dentro de la célula van a quedar expuestos en la superficie de esas burbujas (Figura 2.10). Esos antígenos expuestos son principalmente nucleosomas, Ro, La y aniones fosfolipídicos (44). En pacientes con LES son frecuentes anticuerpos frente a estos antígenos.

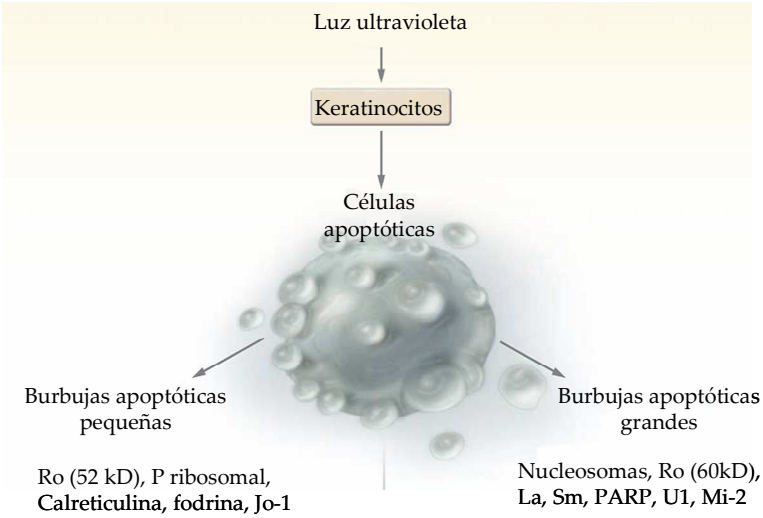

Figura 2.10. Inducción de burbujas en la superficie celular durante la apoptosis.

Así mismo, los pacientes con LES presentan una anormal eliminación de los cuerpos apoptóticos (23). C1q juega un papel determinante en la fagocitosis de esos cuerpos apoptóticos mediante su unión a estos, los cuales pueden ser engullidos por macrófagos que presenten receptores C1q en su superficie. De manera que deficiencias en el complemento quizás sea un punto clave en la baja eliminación de estos residuos que ocurre en el LES. Deficiencias homocigóticas de C1q, C2 y C4 son desordenes raros, pero la presencia de alguna de estas condiciones genéticas es un fuerte factor de predisposición al LES (45-47).

2.2.6. Citoquinas en el LES

La producción de citoquinas en pacientes con LES difiere de la que ocurre en individuos sanos o pacientes con otras EAI como la AR, incluso esta producción difiere entre los diversos fenotipos de la enfermedad (48). Por ejemplo, los niveles de interleuquina 6 (IL-6) están aumentados en el líquido cerebro espinal de pacientes con LES que presentan síntomas neurológicos, sin embargo este incremento no ocurre en pacientes que no presentan estos

síntomas (49). Parece ser que en esta enfermedad el balance de citoquinas es más importante en determinar el fenotipo o severidad de la enfermedad que en determinar susceptibilidad a la misma (Figura 2.11).

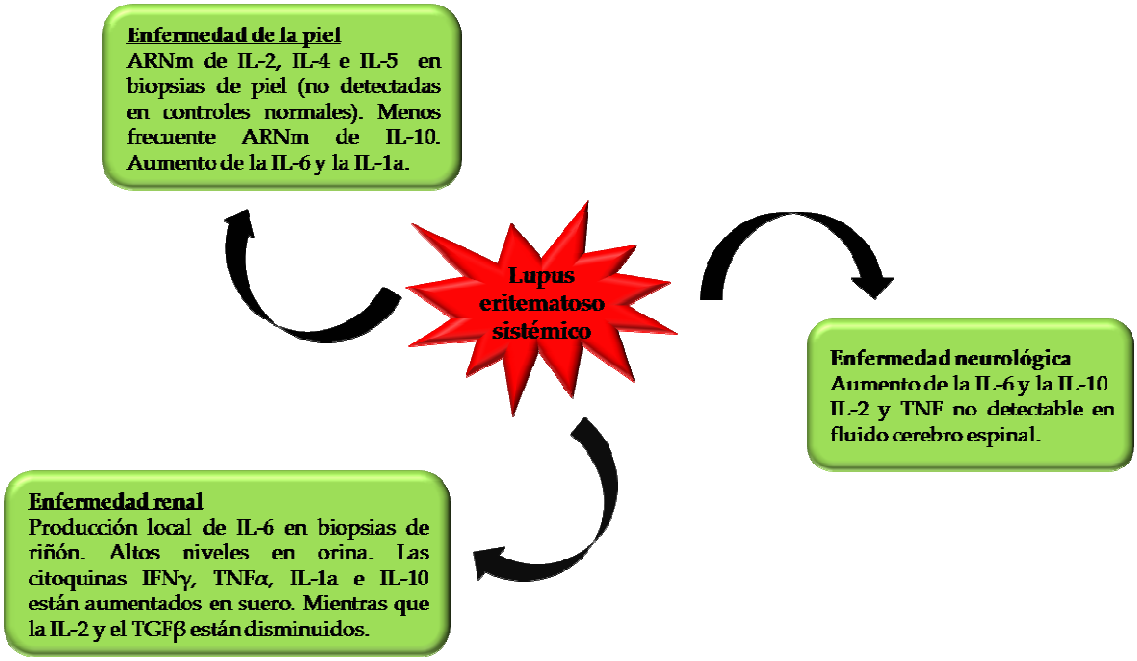

Figura 2.11. Resumen del perfil anormal de citoquinas en piel, riñón y sistema neurológico en el LES.

Las células Th CD4+ pueden dividirse en dos subpoblaciones principales basándose en las citoquinas producidas por estas células. Las células Th1 que producen principalmente IFN-γ e IL-2 (citoquinas pro-inflamatorias) y que promueven la inmunidad mediada por células, y las células Th2 que secretan principalmente IL-4, IL-5 e IL-10 (citoquinas anti-inflamatorias) están asociadas con la respuesta inmune humoral, lo cual induce la producción de autoanticuerpos (50). En muchos desordenes autoinmunes, como es el caso del LES, se ha demostrado que el disbalance entre la producción de citoquinas Th1 y Th2 juega un papel clave en la inducción y el desarrollo de la enfermedad (51). En pacientes con LES, los niveles en suero de citoquinas anti-inflamatorias como IL-4, IL-6 e IL-10 están elevados (52-54), mientras que se produce un disminución en los niveles de

citoquinas pro-inflamatorias como IL-2 (55). Debido a esto, el LES ha sido considerado históricamente como una enfermedad tipo Th2. Sin embargo, esta hipótesis se esta poniendo en entredicho en los últimos tiempos (51), ya que se ha demostrado que los niveles de IFN-γ en el suero de pacientes con LES están aumentados significativamente (56) y que un disbalance hacia una predominancia de la respuesta tipo Th1 está asociada con una aceleración del síndrome lupus-like en ratones (57).

2.2.7. Otros trastornos en la regulación inmune del LES

La depuración de los ICs por las células fagocíticas es un mecanismo que se encuentra alterado en los pacientes con LES (58). La fagocitosis de IgG2 e IgG3 disminuida puede explicarse por alteraciones en los receptores para inmunoglobulinas (FcγR), cuyo polimorfismo genético ha sido asociado a la enfermedad (58, 59). También se ha determinado que la fagocitosis no-inflamatoria de células apoptóticas está disminuida (60). La persistencia de estos cuerpos apoptóticos podría servir como inmnógenos para la inducción de linfocitos autoreactivos y como antígenos para la formación de complejos inmunes. Por otra parte, la supresión de la actividad de las células B por parte de los linfocitos T CD8+ supresores y las células asesinas naturales (NK) está alterada (61, 62). Esta supresión disminuida puede ser un factor que facilite la perpetuación de la enfermedad.

2.2.7.1. Apoptosis

Defectos en la apoptosis pueden llevar a una mayor supervivencia de los linfocitos patogénicos. Esta hipótesis ha sido demostrada en modelos murinos de LES. El ratón MRL/*lpr*, portador del gen *lpr*, asociado con un defecto de los receptores Fas, desarrolla una EAI similar al LES humano. Esta

molécula Fas cuando interactúa con su ligando (FasL) traduce señales de apoptosis (63). Dado que esta interacción se encuentra defectuosa, los ratones presentan una linfoproliferación importante que compromete, entre otros órganos, los riñones (glomerulonefrítis) (64). Otro tipo de modelo, el ratón *Gld*/*gld*, caracterizado por presentar una molécula FasL no funcional, presenta también linfoproliferación, hipergammaglobulinemia y depósito de ICs en el riñón (65). Ambos modelos, aunque utilizados para el estudio de la enfermedad, difieren del modelo humano, en el que se observa linfopenia en vez de linfoproliferación y la expresión de Fas-FasL es normal (66, 67).

El papel de la apoptosis en el LES parece deberse más a alteraciones en la depuración de células apoptóticas por parte de los macrófagos, la cual se encuentra disminuida (60). Las razones que explican esta alteración no son claras, pero pueden estar asociadas a defectos cualitativos y cuantitativos de proteínas del complemento como C2, C4 y C1q. Los pacientes con deficiencias homocigóticas de estos componentes padecen un lupus severo a una edad temprana (68).

2.2.7.2. Alteraciones en el sistema del complemento

Como ya se ha expuesto anteriormente, individuos con alteraciones en la vía clásica del complemento (C4, C1q) tienen mayor riesgo de presentar LES. En la práctica clínica, niveles bajos del complemento indican actividad de la enfermedad, en particular en el riñón (69). Dos hipótesis se han propuesto para explicar el desarrollo de LES en personas con deficiencias del complemento. La primera de ellas, ya señalada, es la "hipótesis de depuración" que plantea que una inadecuada depuración de cuerpos apoptóticos genera una inapropiada activación de células T y B autoreactivas. La segunda hipótesis, llamada "hipótesis de tolerancia", plantea que los

componentes de la inmunidad innata son importantes en la selección negativa de los linfocitos autorreactivos, principalmente de las células B específicas para autoantígenos. Los componentes C1q y C4 aumentan la presentación de antígenos a las células B inmaduras en la médula ósea. Cuando estas células B inmaduras encuentran un antígeno en estas circunstancias son seleccionadas negativamente. Así se disminuye el proceso de maduración y activación de células B autoreactivas (70).

2.3. Etiología del LES

La causa exacta por la que se produce el LES es desconocida, pero se sabe que el LES es una enfermedad multifactorial compleja que surge como resultado de la combinación de factores genéticos, factores ambientales, factores hormonales y eventos estocásticos ocurridos al azar, los cuales provocan una alteración de la respuesta inmune (71) (Figura 2.12).

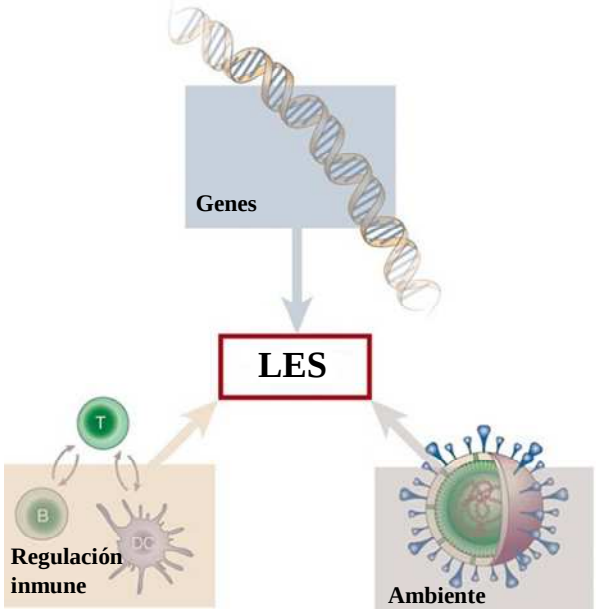

Figura 2.12. Factores que contribuyen al desencadenamiento del LES.

Debido a que el 90% de los pacientes con LES son mujeres, parece probable que las hormonas femeninas jueguen un papel importante en la enfermedad, aunque un papel protectivo de las hormonas masculinas en hombres o un efecto genético ligado al cromosoma X también es posible. A pesar de que numerosos estudios con tratamientos hormonales se han realizado en pacientes con LES (72, 73), aún no está claro como esas hormonas sexuales podrían promover la enfermedad. En el LES el embarazo, es por definición, un evento de alto riesgo, tanto por la elevada frecuencia de resultados fetales adversos, como por el riesgo potencial para la madre. Ya que la enfermedad renal activa y la hipertensión son importantes predictores de pérdidas fetales y nacimiento prematuros, respectivamente. La mayoría de las perdidas fetales ocurren en asociación con los AAF, los cuales son frecuentes en pacientes de LES, y que están asociados con pérdidas en mujeres sanas. Los anticuerpos anti-Ro/SSa también presentes en el LES, están asociados a bloqueo cardíaco congénito. Los estudios de los efectos del embarazo sobre la actividad lúpica han proporcionado resultados heterogéneos. Lockshin y Tincani (74), no encuentran un empeoramiento de la actividad de la enfermedad, mientras que Petri y col. sí refieren una frecuencia incrementada de las exacerbaciones durante el embarazo, cuando se compara con pacientes no embarazadas y después del parto (75).

Diferentes fármacos son capaces de desencadenar una variante de LES conocido como lupus inducido por fármacos. Los fármacos más conocidos que producen este efecto son la procainamida, hidrazalina y la quinidina (76, 77). Otro factor externo que parece influir en la aparición del LES es tener antecedentes de alguna enfermedad viral. Identificar qué virus producen este efecto ha sido un gran reto. Actualmente se cree que el virus Epstein-Barr

(EBV) es importante, ya que se ha encontrado una asociación entre el comienzo del LES y haber presentado una infección por EBV (78).

La radiación ultravioleta (UV) es uno de los factores ambientales más evidentes asociados al LES, ya que produce una exacerbación tanto del lupus cutáneo como del sistémico (79-81).

2.4. Contribución de factores genéticos en el LES.

El LES muestra una importante agregación familiar, con una frecuencia más alta de la enfermedad en los familiares de primer grado de los pacientes que el resto de la población. Además, en las familias de pacientes con LES pueden observarse en algunos casos, otras EAI como la anemia hemolítica, la tiroiditis autoinmune y la AR, entre otras (82). La agregación familiar se cuantifica mediante el coeficiente λs, que se define como el cociente de dividir la prevalencia de la enfermedad en hermanos de individuos afectados de LES entre la prevalencia de la población general. El coeficiente λs en el LES es de 20 (83). Los factores genéticos son importantes en la predisposición a la enfermedad pero no indispensables, en efecto, la concordancia de LES en gemelos monocigotos (30-50%) es muy superior a la observada en gemelos dicigotos (2-5%) (84, 85). Sin embargo, la mayoría de los casos de LES son esporádicos, lo que apoya la idea de una etiología multifactorial, en la que factores ambientales también participan.

Desde el punto de vista genético esta es una enfermedad compleja, en la cual múltiples genes contribuyen a la susceptibilidad de la misma. Solo en una pequeña proporción de pacientes (menos del 5%) un único gen pudiera ser el responsable del LES. Tal es el caso de los pacientes con deficiencias homocigóticas de factores del complemento (68). Los genes más estudiados

con respecto a su implicación en el LES son hasta la fecha aquellos ubicados en el MHC (83, 84, 86, 87).

2.4.1. El complejo mayor de histocompatibilidad (MHC)

El MHC es un complejo de genes ubicados en el brazo corto del cromosoma 6, en la región 6p21.3. Se caracteriza por ser altamente polimórfico y poligénico. Sus componentes principales son los genes del antígeno leucocitario humano (*HLA*) de clase I y clase II, cuyas proteínas participan respectivamente en la presentación de péptidos tanto intracelulares como extracelulares. De manera que presentan un papel clave en el mantenimiento de la tolerancia inmunológica (88, 89). Las moléculas MHC se expresan en la superficie de las CPA y están formadas por una porción extracelular que contiene a la cavidad de unión de péptidos, seguida de una par de dominios de tipo inmunoglobulina anclados a la membrana celular y un dominio intracitoplasmático. La molécula HLA de clase I completamente ensamblada, es un heterodímero, formado por una cadena α, una cadena β2 microglobulina y el péptido que va a ser presentado al linfocito T CD8+. En esta región se encuentran los genes que codifican para los genes *HLA-A*, *B* y *C* entre otros. Las moléculas HLA de clase II están formadas por dos cadenas unidas no covalentemente: cadena α y cadena β, que se encargan de presentar los péptidos a los linfocitos T CD4+, estas moléculas codifican principalmente a los genes *HLA-DR*, *DQ* y *DP* (Figura 2.13).

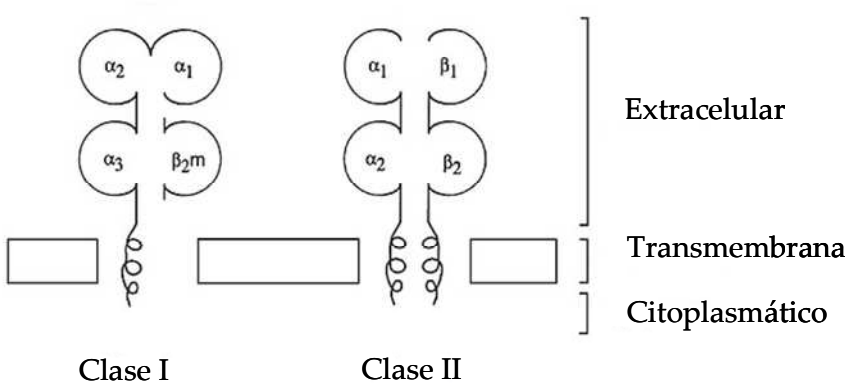

Figura 2.13. Estructura de las moléculas MHC.

La región de clase III se sitúa entre la I y la II, y contiene genes importantes en el sistema inmune innato, incluyendo el factor de necrosis tumoral α (TNF-α) y algunos componentes del complemento como C2 y C4 (Figura 2.14).

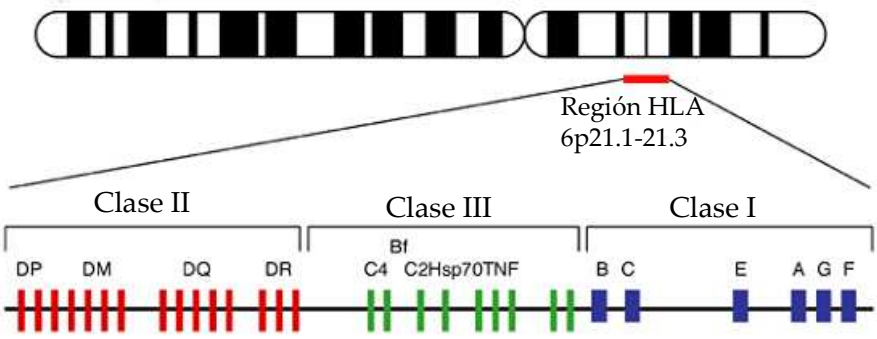

Figura 2.14. Esquema genético de la distribución de los genes de las regiones de clase I, clase II y clase III del MHC; ubicados en la posición 6p21.

La primera asociación genética entre el LES y los genes *HLA* descrita fue en 1971, con el gen *HLA* de clase I *B8* (87). Posteriores estudios han demostrado que la asociación más contundente en el LES en población blanca, es con los genes *HLA* de clase II *DR2* (*DRB1*1501*) y *DR3* (*DRB1*0301*), este último se encuentra en desequilibrio de unión con el *B8* (90-92). Desde que se

describiera la primera asociación entre los genes *HLA* y el LES numerosos estudios se han realizado al respecto, los cuales han sido ampliamente revisados (1, 93-95). Varios estudios han sugerido que la asociación presente entre estos genes *HLA* de clase II y el LES es predominantemente a nivel de la producción de autoanticuerpos específicos más que con la enfermedad en si (95-97).

A pesar de la relevancia de estos alelos *HLA* en la predisposición genética al LES, se estima que estos solo forman una parte de la contribución genética total a la enfermedad (98). Por lo tanto, deben existir numerosos genes que influyen en el LES fuera de la región HLA.

Hasta hace solo unos años se utilizaban principalmente dos aproximaciones para identificar estos genes no-MHC, como causantes de enfermedades complejas como el LES: los estudios de ligamiento y los estudios de asociación de genes candidatos.

2.4.2. Estudios de ligamiento

Los estudios de ligamiento se usan para identificar regiones cromosómicas dentro del genoma que contengan genes de predisposición a la enfermedad (99). Estos estudios se realizan en familias que tengan al menos dos o más miembros afectados (100) y tienen la capacidad de localizar genes de susceptibilidad sin tener un previo conocimiento de la etiología de esta.

El primer paso para detectar ligamiento entre una región (también llamada locus o loci en plural) y un fenotipo, es generar un mapa cromosómico utilizando marcadores genéticos en las familias estudiadas. Para que el análisis sea robusto y con alto poder estadístico hay que usar una serie de marcadores altamente informativos separados uniformemente a lo largo de los cromosomas, usualmente cada 20 centimorgans (cM), en todos los sujetos de

las familias a estudiar. Se considera que un marcador es altamente informativo cuando es muy polimórfico y es poco probable encontrar individuos con los dos alelos iguales (homocigotos). Si los dos alelos son distintos (heterocigotos) en la mayoría de sujetos de una familia, se puede seguir con facilidad la herencia de cada uno de ellos a lo largo de generaciones sucesivas. Como consecuencia, se puede determinar cuántos alelos idénticos comparten dos individuos concretos de la familia. Los marcadores genéticos más utilizados en la búsqueda de ligamiento son los microsatélites que son repeticiones cortas de 2 a 6 nucleótidos que se ubican uno tras otro, en lugar de las mutaciones de un solo nucleótido ó SNPs ya que estos son menos informativos.Otra limitación de estos estudios en enfermedades complejas, es su limitado poder para detectar genes con un efecto modesto o pequeño, de manera que se necesitan un gran número de familias. Debido al gran número de marcadores genéticos analizados en estos rastreamientos del genoma, se incrementa la probabilidad de que ocurran falsos positivos (errores tipo I). Para evitar estos errores se utiliza el método de máxima verosimilitud (101). Para obtener un valor de significancia estadística, se transforma logarítmicamente el cociente de verosimilitud en lo que se denomina *LOD score* (logaritmo del cociente de verosimilitud de ligamiento). Estos valores de significancia estadística oscilan entre 3.3 y 3.6 cuando el ligamiento es significativo ($P<0.05$) y de 1.9 a 2.2 cuando se sugiere un posible ligamiento. Así mismo para validar estos estudios se debe confirmar el ligamiento con un valor de P de 0.01 en un grupo independiente del inicial.

En el caso del LES se han llevado a cabo varios estudios de rastreo sistemático del genoma (conocidos como whole genome scans, WGSs) en poblaciones de diferente origen étnico (Figura 2.15).

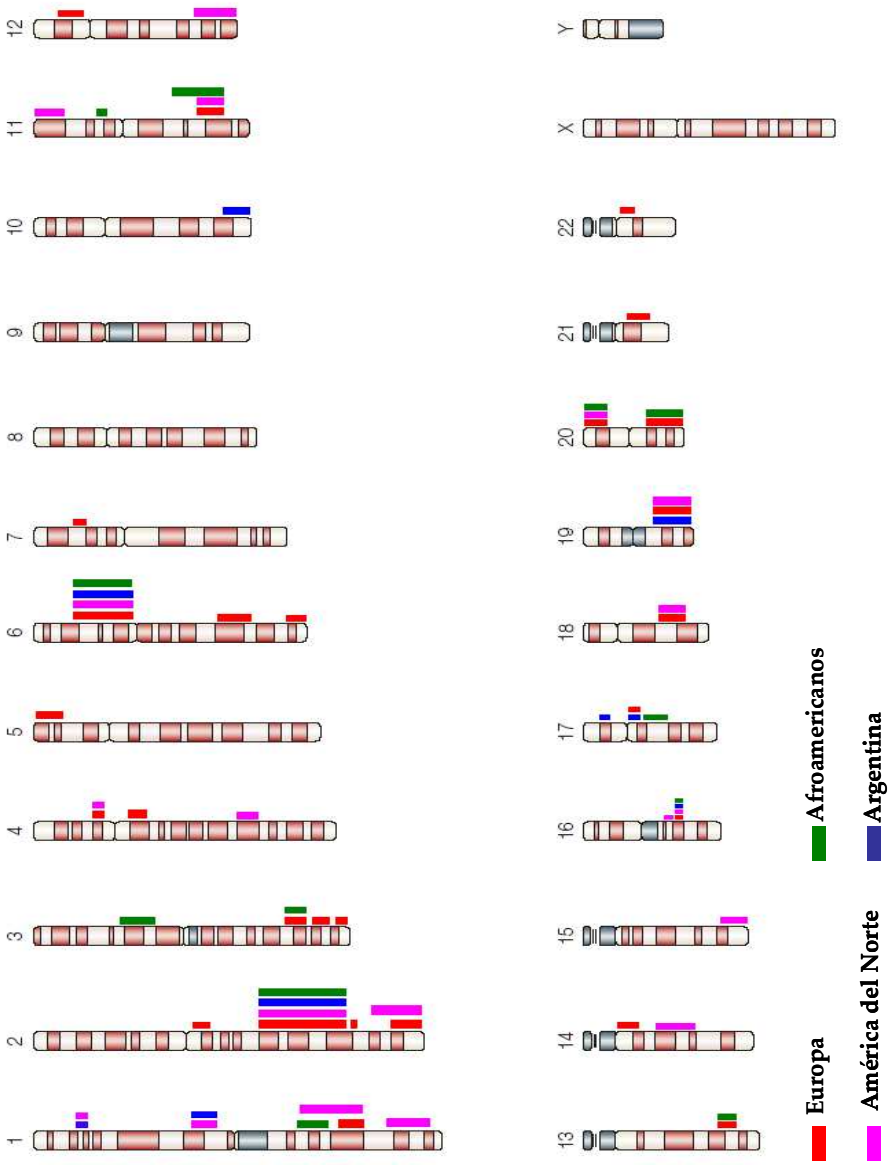

Figura 2.15. Localización cromosómica de las regiones de ligamiento a LES por los estudios de WGS.

En el LES seis regiones se han demostrado como fuertemente asociadas a la enfermedad con un valor de *LOD score* por encima de 3.3 (84) (Tabla 2.3).

Locus	*LOD score*	Genes candidatos	Modelo de ratón
1q22-24	3.45	*FcγRIIa, CRP, FCγRIIb, FCγRIIIa, PBX1*	Sle1a, Sle1b
1q41-42	3.50	*PARP*	Sle1d, Bxs3
2q35-37	4.25	*PDCD1*	Bxs1
4p15-16	3.90	*CD38, BST1, ZNF36*	Sle6
6p11-22	4.20	*HLA Clase II, C4a, TNFα*	Sles1
16q12-13	3.85	*NOD2*	Lbqw1

Tabla 2.3. Regiones cromosómicas confirmadas de asociación al LES

2.4.3. Estudios de asociación de genes candidatos

La segunda aproximación usada para la búsqueda de posibles genes asociados con las enfermedades complejas son los estudios de asociación de genes candidatos (102). Estos estudios buscan detectar una asociación entre uno o más polimorfismos genéticos y un rasgo característico. Los estudios de asociación difieren de los de ligamiento en que el mismo alelo (ó alelos) está asociado con ese rasgo de manera similar en toda la población, mientras que los estudios de ligamiento permiten que diferentes alelos estén asociados con ese rasgo en familias diferentes. Estos estudios de asociación pueden hacerse entre pacientes no relacionados (casos) y controles sanos para identificar marcadores que difieran significativamente entre los dos grupos o bien en grupos familiares formados normalmente por un individuo enfermo y sus progenitores (102). Este tipo de estudios normalmente tienen mayor poder para detectar asociaciones pequeñas o débiles que los de ligamiento.

Numerosos estudios genéticos de asociación de genes candidatos se han llevado a cabo en el LES hasta la fecha, los cuales han producido resultados alentadores pero aparentemente inconsistentes (ref). La mayoría de las asociaciones publicadas no han sido replicadas en poblaciones diferentes a las asociadas inicialmente. Estas discrepancias podrían deberse, al menos en parte, a la heterogeneidad genética. Diferentes alelos, e incluso diferentes genes, pueden producir susceptibilidad a la enfermedad en distintos grupos étnicos. Otra explicación podría ser el insuficiente poder estadístico de los estudios caso-control realizados hasta la fecha para detectar odds ratios (OR) modestas.

Para evitar estos errores hay que seleccionar un tamaño muestral adecuado y garantizar la homogeneidad de la población. Para ello es imprescindible que el grupo de controles sea lo más parecido posible al de pacientes en edad, sexo y origen étnico (103). Este tipo de sesgo se evita en los estudios familiares basados en el test de desequilibrio de transmisión (TDT), mediante el cual la transmisión preferencial del alelo (o haplotipo) analizado desde padres heterocigotos a los descendientes afectados prueba la asociación del alelo (o haplotipo) analizado con susceptibilidad a la enfermedad.

A pesar de las limitaciones de los estudios de asociación de genes candidatos, gracias a estos ha sido posible en parte la identificación de las asociaciones con el LES más fuertes aparte de los genes *HLA* hasta la fecha. Estas asociaciones vienen dadas por los genes *FCGR*, *PDCD1*, *PTPN22* e *IRF5*.

2.4.3.1. Desequilibrio de ligamiento

La asociación que no ocurre al azar entre dos alelos que se encuentran en diferente posición en una población se conoce como desequilibrio de ligamiento (LD). Esto ocurre porque los dos alelos se heredan juntos más

frecuentemente que lo que cabría esperar basándose en su frecuencia alélica en la población. Este LD es dependiente de la historia demográfica de la población y de la tasa de recombinación entre los loci (104). Eventos como la recombinación pueden acabar con el LD entre alelos, y los patrones de LD que observamos en diferentes poblaciones son el resultado de recombinación, mutación, migración, deriva aleatoria, selección, apareamiento no al azar y tamaño poblacional. El desequilibrio de ligamiento (LD) puede medirse entre dos loci, A y B con dos alelos cada uno, usando la siguiente fórmula: $D= f(A_1B_1) - f(A_1)f(B_1)$, donde $f(A_1B_1)$ representa la frecuencia del haplotipo observado del alelo 1 de ambos loci A y B, y la frecuencia esperada del haplotipo $f(A_1)f(B_1)$. Normalmente el LD es medido mediante la D' unidireccional y la r^2 bidireccional en estudios genéticos. Cuando el LD es completo $D'= 1$ y $r^2= 1$ (105).

2.4.4. Selección de genes candidatos

El primer paso a la hora de diseñar un estudio de asociación es la elección del gen candidato a investigar. La elección de genes candidatos se puede hacer siguiendo dos estrategias. Una de estas estrategias es la *posicional*, basada en genes que con anterioridad hayan sido encontrados en regiones de susceptibilidad a la enfermedad mediante estudios de ligamiento, o bien genes que se encuentren en regiones sinténicas asociadas a susceptibilidad en modelos animales de LES. La segunda estrategia es la *funcional*, la cual consiste en seleccionar genes que puedan tener una implicación en los mecanismos fisiopatológicos de la enfermedad, así como genes implicados en enfermedades inflamatorias o autoinmunes relacionadas. Estas dos estrategias no son excluyentes sino complementarias.

2.4.5. Selección de marcadores genéticos

Hay varios tipos de variaciones genéticas naturales, como inserciones/deleciones de un bajo número de nucleótidos, duplicaciones de largos segmentos del genoma, variaciones del número de copias de algunos genes (CNVs), todos ellos estudiados en susceptibilidad a enfermedades. Sin embargo, las variaciones genéticas más comunes y usadas en estudios de ligamiento y de asociación son los llamados microsatélites y los SNPs (Figura 2.16).

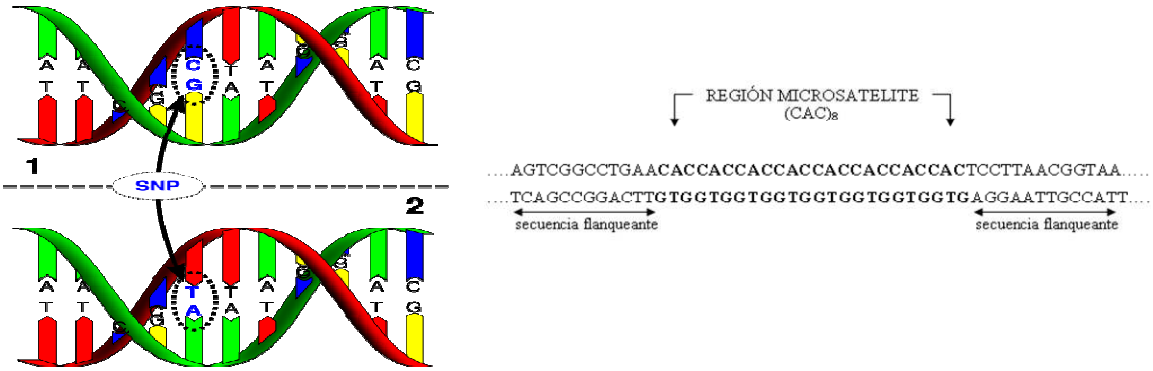

Figura 2.16. Representación esquemática de un SNP y un microsatélite.

Los *microsatélites*, son repeticiones cortas repetidas de 2 a 6 nucleótidos que se ubican uno tras otro. Generalmente se encuentran en regiones no codificantes del ADN. Son neutros, co-dominantes y poseen una alta tasa de mutación, lo que los hace muy polimórficos, condición que permite usarlos como marcadores genéticos en WGS.

Los *SNPs*, son mutaciones de un solo nucleótido cuya frecuencia del alelo minoritario debe ser mayor al 1% en la población (106). Los SNPs están teniendo cada vez más importancia en estudios genéticos. Aunque el hecho de que los SNPs sean principalmente bi-alélicos hace que sean menos

informativos en estudios genéticos que los microsatélites (107). Sin embargo, los SNPs son mucho más comunes en el genoma, con cerca de 12 millones de SNPs que están disponibles en las bases de datos, como la Database of Single Nucleotide Poymorphisms (dbSNP) from Nacional Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/SNP). Por otro lado los avances en la tecnología de genotipado de estos SNPs, hacen que cada vez sean más fáciles, baratos y rápidos de genotipar que los microsatélites. Además los SNPs ofrecen la posibilidad de realizar un mapeo fino del genoma debido a la cercana posición de uno respecto a otro y su baja tasa de mutación (en el rango de 10^{-8}) (108, 109), lo que hace que cada vez sean más preferidos y usados en estudios de ligamiento y búsqueda de asociación a lo largo del genoma.

Dentro de todos estos SNPs localizados a lo largo del genoma, los SNPs que alteran la función, tales como aquellos que se encuentran en las regiones codificantes del gen y dan lugar a cambios de aminoácidos en la proteína, los que modulan la transcripción o afectan a procesos de splicing, unión de factores de transcripción o modifican la secuencia del ARN no codificante, son mejores candidatos a la hora de plantear un estudio de asociación (Figura 2.17).

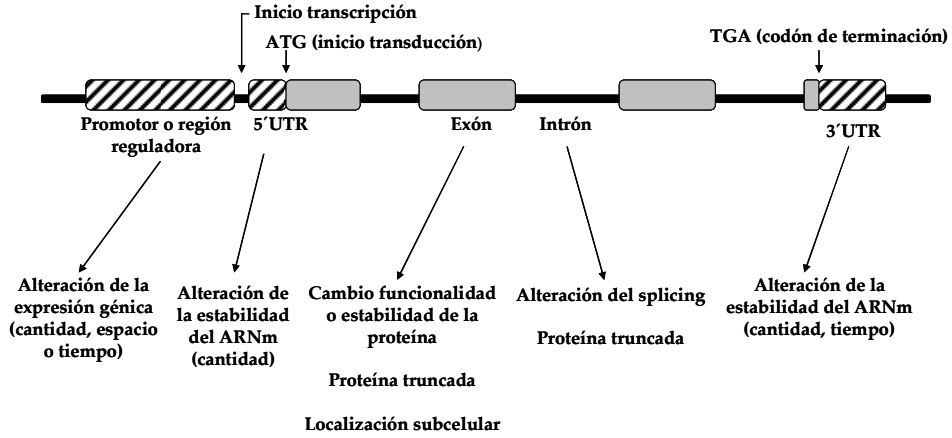

Figura 2.17. Implicaciones funcionales de los SNPs dependiendo de su localización dentro de un gen.

2.5. Estudios de asociación del genoma completo

En los últimos años gracias a los nuevos avances ha sido posible el desarrollo de los estudios de asociación del genoma completo (GWAS). Estos avances han consistido principalmente en un mayor conocimiento de los patrones de variación génica humana con el proyecto internacional HapMap (www.hapmap.org) (110), la disponibilidad de nuevas técnicas de genotipado, con densa cobertura del genoma y costes más baratos y por último la recolección de más amplias y mejor caracterizadas cohortes de pacientes y controles.

Hasta la fecha numerosos GWAS han aparecido en diversas enfermedades, incluyendo EAI, como la AR, enfermedad inflamatoria intestinal (EII), diabetes tipo 1 (DT1) y LES. Estos estudios en el LES han mostrado asociación con regiones ya conocidas de susceptibilidad a la enfermedad como los genes *HLA* en 6p21 y *PTPN22* (111). Además, se han identificado nuevas e interesantes regiones de susceptibilidad al LES que necesitan ser confirmadas en estudios replicativos para poder esclarecer el papel de esas nuevas en regiones en la susceptibilidad a la enfermedad.

En la realización de esta tesis doctoral se han utilizado dos tipos de estrategias fundamentales para la búsqueda de posibles genes de susceptibilidad al LES: estrategia de genes candidatos y genes seleccionados mediante GWAS. Los genes seleccionados en cada caso son los que se detallan a continuación.

2.6. Genes seleccionados mediante la estrategia de genes candidatos

Teniendo en cuenta los dos criterios principales de selección de genes candidatos, funcional y posicional, hemos elegido una serie de genes para investigar su papel en el LES mediante estudios de asociación caso-control. Estos genes han sido clasificados en base a su implicación en los distintos procesos fisiopatológicos de la enfermedad.

2.6.1. Genes implicados en la respuesta inflamatoria

2.6.1.1. Receptores tipo Toll (TLRs)

El sistema inmune innato ofrece un mecanismo de defensa rápido y eficaz contra patógenos microbianos. La respuesta inmune frente a microorganismos invasores es iniciada mediante el reconocimiento de patrones moleculares asociados a patógenos (PAMPS) a través de receptores de reconocimiento de patrones (PRR). Fundamentalmente, el reconocimiento de PAMPS se realiza a través de los receptores tipo Toll (TLRs) (112, 113). Los TLRs se expresan constitutivamente en numerosas células del sistema inmune y están diseñados para detectar y eliminar agentes patógenos mediante la activación de la respuesta inmune innata y adaptativa, y particularmente para la inducción de la respuesta Th1 (112). Los TLRs pertenecen a la familia de receptores transmembrana tipo I, la cual se caracteriza por tener un dominio rico en repeticiones de leucinas (LRR) y un dominio intracelular Toll/IL-1R (TIR) (114-116). Esta familia de receptores incluye 11 miembros, cada uno de los cuales reconoce distintos componentes de bacterias, hongos, virus tales como ARN de cadena doble (ARNcd) y ARN de cadena sencilla (ARNcs) (113,

117) aunque también pueden unirse a antígenos propios como proteínas del choque térmico entre otras (118). La capacidad de los TLRs de reconocer distintos ligandos y su distinta localización celular, hacen que estos receptores difieran también en las vías de señalización que ellos activan. Después del reconocimiento de sus ligandos, los TLRs activan las vías de señalización intracelular que desencadenan la activación de NF-κB y otras vías dependientes de MAP kinasas lo cual conduce a la expresión de citoquinas y moléculas coestimuladoras (119). Hasta la fecha se han descrito dos vías de activación a través de TLRs, una dependiente de la proteína adaptadora MyD88 (myeloid differentiation primary-response protein 88) y otra independiente (119) (Figura 2.18). Mientras que algunos TLR, como TLR4 pueden señalizar a través de ambas vías (120).

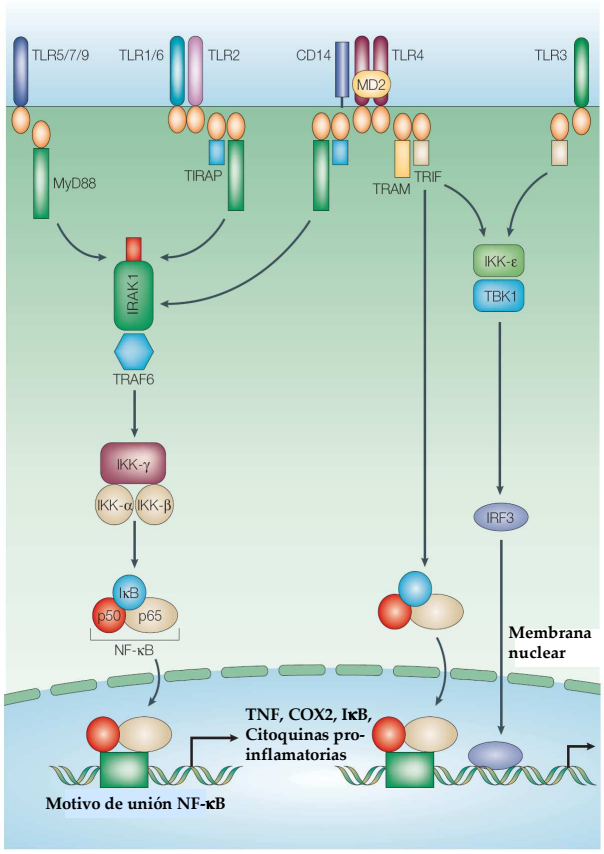

Figura 2.18. Diferentes vías de señalización a través de los TLRs.

La señalización a través de TLRs conduce a la sobreexpresión de moléculas coestimuladoras por parte de la CPA, facilitando la subsiguiente activación del sistema inmune adaptativo. Por tanto, el sistema inmune innato controla respuestas inmunes adaptativas a través de la provisión de una segunda señal a la estimulación de las células T. Debido a éste importante papel del sistema inmune innato, se ha postulado que una alteración en la regulación del reconocimiento de patógenos por parte de este sistema podría estar asociada con autoinmunidad. Por otra parte cada día parece estar más claro el papel de los TLRs en la activación de células B autoreactivas (121).

Debido al importante papel que parecen tener estos genes *TLR* en el proceso inflamatorio y la respuesta inmunológica que se produce en pacientes con LES, decidimos estudiar el papel de diversos polimorfismos de los genes *TLR2*, *TLR4*, *TLR5* y *TLR7* en el LES.

2.6.1.2. Factor nuclear κB (*NFKB*)

El factor nuclear κB (NFκB), es un factor de transcripción de gran importancia en procesos inflamatorios e inmunes, ya que regula la transcripción de un gran número de genes implicados en la respuesta inmune, moléculas de adhesión, diferenciación, proliferación, angiogénesis y apoptosis (122). Se ha observado que anormalidades en la regulación de NFκB están implicadas en diversas patologías humanas como por ejemplo enfermedades inflamatorias, deficiencias inmunes, diabetes, ateroesclerosis y también en tumores. Debido a que NFκB es responsable de la regulación de muchos otros genes involucrados en la progresión de la enfermedad, variantes en los genes que codifican para la proteína NFκB podrían estar potencialmente involucrados en el desarrollo de la enfermedad (123).

Las moléculas de la familia NFκB/REL están compuestas por heterodímeros codificados por los genes *RELA* (p65), *NFKB1* (p50; p105), *NFKB2* (p52; p100), *c-REL* y *REL* (124). La forma activa de NFκB más abundante es un heterodímero compuesto por la subunidad p65 asociada con p50 ó 52 (125). La actividad de NFκB es estimulada por medio de diversas vías de señalización como la unión de patógenos a receptores de superficie (LPS-TLRs), la inducción de citoquinas (TNF-α e IL-1) o tras la activación a través del TCR (Figura 2.19). Tras estos estímulos, se produce una cascada de señalización que produce la fosforilación, ubiquitinación y degradación del inhibidor de NFκB (IκB), lo cual facilita su degradación por el proteosoma. De esta forma NFκB se libera y esto posibilita su traslocación al núcleo, donde da lugar a la activación de numerosas moléculas implicadas en procesos inflamatorios y autoinmunes tales como citoquinas pro-inflamatorias, quimioquinas, moléculas de adhesión, metaloproteinasas (MMPs), ciclooxigenasa 2 (COX2) y óxido nítrico sintasa inducible (iNOS).

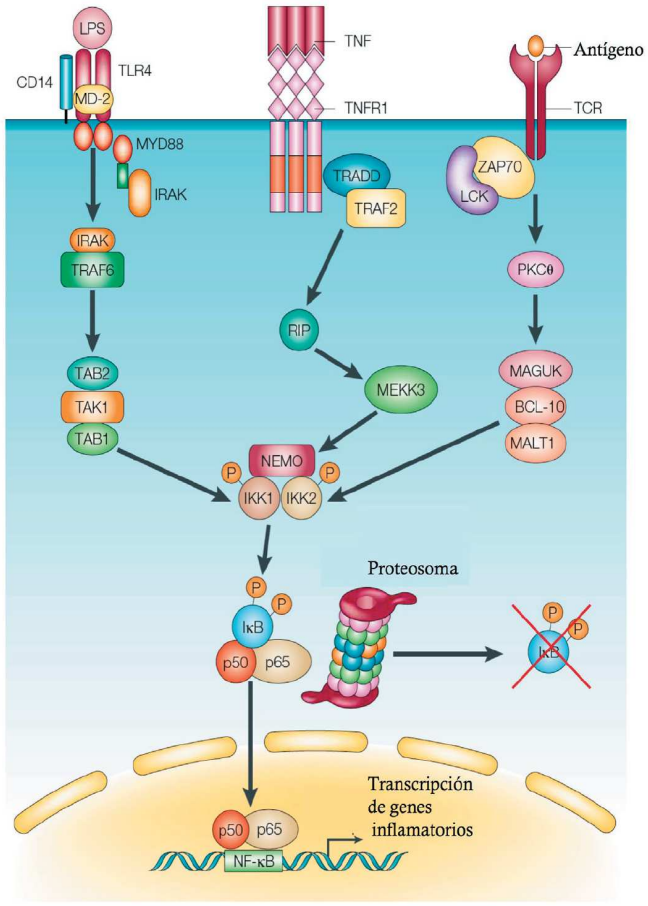

Figura 2.19. Rutas de activación de NFκB

El factor NFκB se encuentra activado en un gran número de EAI, como la AR, enfermedad celíaca, EII o la esclerosis múltiple (EM), sugiriéndose un papel de este factor en el control de la inflamación (126, 127). Sin embargo, la activación de NFκB se ha visto que está significativamente disminuida en pacientes con LES (128, 129). Esto indica que el mecanismo de regulación de NFκB difiere en las distintas EAI, lo cual puede deberse en parte a los factores genéticos.

Debido a su importante papel en la activación de los procesos inflamatorios, el gen *NFκB* se considera un buen gen candidato en el estudio de enfermedades autoinmunes como el LES.

2.6.1.3. Receptores tipo Fc (*FCRL3*)

Los receptores de la parte Fc de la inmunoglobulina G (IgG) (FcγRs) son mediadores esenciales de efecto inflamatorio, de complejos inmunes y anticuerpos citotóxicos (130, 131). Los *FcγRs* son genes candidatos de susceptibilidad a EAI ya que relacionan el sistema inmune innato y el adquirido entre si. Una nueva familia de genes, los Fc receptor-like (*FCRL*, también llamados *FCRH*), tienen una gran homología estructural y secuencial con los genes *FcγRs* (132), y aunque aún no se conocen ni su función ni sus ligandos se piensa que pueden influir en el desarrollo de células B y aumentar la presencia de células autoreactivas en los centros germinales (133).

El gen *FCRL3* (también conocido como *SPAP2* ó *IRTAP3*) se localiza en la región cromosómica 1q21-q22 (134), en el cual colocalizan el cluster de genes *FCRL1-FCRL5* y la familia de genes *FcγR II/III* (Figura 2.20). La región 1q21-23 se considera un locus de susceptibilidad a diversas EAI incluido el LES (83, 135) (136). Además, la región sinténica de ratón de la 1q21 humana se encuentra en el cromosoma 3 y se ha visto que también es un loci de susceptibilidad a diversos modelos de enfermedades autoinmunes (137), como artritis inducida por colágeno (CIA) (138), encefalomielitis autoinmune experimental (EAE) (139), LES (140) ó diabetes insulino dependiente (141). Un gran número de genes candidatos localizados dentro de esta región como son *FcγR II/III* (136), (142, 143), el ligando de Fas (144-146) y la proteína C reactiva (147) muestran asociación con el LES. Sin embargo, debido al gran bloque de LD que forman los genes *FCRL* y *FcγR II/III* es difícil encontrar la verdadera variante genética asociada a la enfermedad en esta región genómica (148). En un reciente estudio Kochi y col encuentran el gen *FCRL3* asociado con varias EAI entre ellas el LES (133).

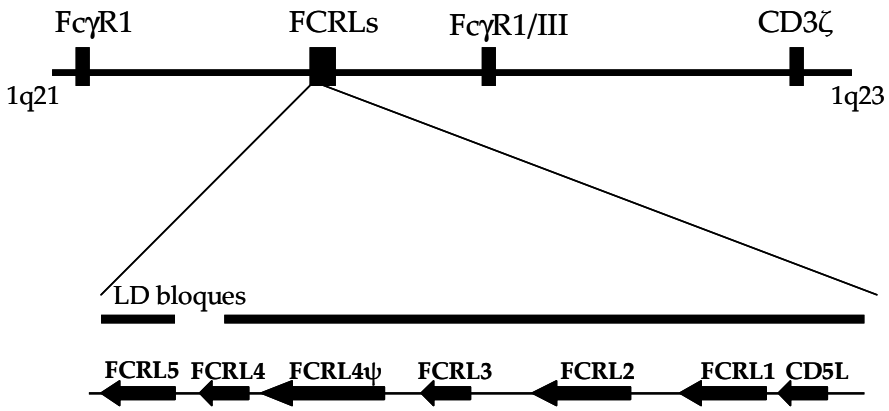

Figura 2.20. Estructura del cluster FCRLγ.

Concretamente el polimorfismo -169C/T (fcrl3_3) de este gen se encontró asociado con la enfermedad en una población japonesa (133). En el mismo estudio demostraron que este SNP altera la expresión de *FCRL3* ya que se ve alterada la unión de NF-κB al promotor de *FCRL3*, regulando de este modo la expresión de *FCRL3*. De manera que decidimos estudiar esta y otras variantes génicas del gen *FCRL3* en relación con el LES en nuestra población.

2.6.1.4. Citoquinas

Las citoquinas tienen un papel muy importante dentro del sistema inmune y la mayoría de ellas se han asociado de una manera u otra con diferentes enfermedades inflamatorias y autoinmunes. De una manera muy general en el estudio de autoinmunidad, se ha postulado que citoquinas producidas por células Th1 promueven síndromes que son órgano-específicos, por ejemplo la DT1, y que, en contraste, citoquinas producidas por células Th2 promueven una enfermedad sistémica como el LES (71). La clasificación de las citoquinas es algo compleja, porque pueden pertenecer a más de una categoría. Las citoquinas pueden clasificarse de varias maneras, según la fase de la

respuesta inmune en la que actúen: citoquinas de las respuesta innata (IL-1, TNF, IL-12, IL-23, IFN tipo 1, IL-10, quimioquinas), o de la respuesta específica (IL-2, IL-4, IL-5, IFNγ, TGFβ); según su función biológica: hematopoyéticas (SCF, IL-3, IL-7,EPO, GM-CSF, M-CSF, G-CSF), de crecimiento y de diferenciación (factor de crecimiento epidérmico, factor de crecimiento derivado de fibroblastos, TGFβ), quimiotácticas (IL-8, MIP-1α, MIP-1β, MCP-1, RANTES), inmunorreguladoras (TGFβ, IFNα, IL-2, IL-4, IL-5, IL-9), pro-inflamatorias (MIF, IL-1α y β, TNFα y β, IL-6, IL-17, IL-18); y anti-inflamatorias (IL1Ra, IL-4, IL-10, IL-13). Como ya se mencionó con anterioridad en esta tesis doctoral, el disbalance de citoquinas que se produce en pacientes de LES, sugiere que estas moléculas y los genes que las codifican pudieran estar implicadas en la susceptibilidad a la enfermedad. De manera que decidimos estudiar varios de estos genes de citoquinas para ver su posible asociación con el LES en nuestra población:

a) Citoquinas de la respuesta innata: *IL12*, *IL12R*, *IL23* e *IL23R*.

b) Citoquinas pro-inflamatorias : *MIF* e *IL18*.

2.6.2. Genes dentro de la región MHC

Dentro de la región MHC se han identificado 224 genes, haciendo de esta región una de las de más densidad génica en el genoma humano. De esos genes, aproximadamente unos 128 son expresados y estas proteínas están involucradas directamente en el sistema inmune (149). Como ya se comentó anteriormente, los genes clásicos HLA de clase I y clase II dentro del MHC (*HLA-A, -B, -C, -DR, -DQ* y *–DP*) son probablemente los más conocidos hasta la fecha, debido a su importante papel en el transporte de péptidos y en la presentación antigénica a las células T (150). Estos genes se caracterizan por ser altamente polimórficos (151, 152), lo cual se manifiesta en la gran variación

existente en las frecuencias alélicas y haplotípicas de estos genes tanto dentro, como entre las distintas poblaciones o grupos étnicos (153). Otra de las características del MHC es la de generar haplotipos extendidos. Debido a la proximidad de los genes, en este complejo se dan asociaciones preferenciales entre los diferentes loci, heredándose en bloque, con una probabilidad mayor que la que tiene cualquier otro grupo de genes, explicada por el azar, lo cual disminuye la probabilidad de recombinación entre loci. Haplotipos del MHC pueden influir en la susceptibilidad a una enfermedad aumentando la presentación de epítopes del péptido inmunogénico en la periferia, dando como resultado la activación aumentada de la célula T, o por la presentación ineficaz de autoantígenos en el timo, originando células T más agresivas o menor número de células T reguladoras (Treg).

Hasta la fecha los genes *HLA* no clásicos más asociados a LES se encuentran en la región HLA de clase III y son el *TNF* y los genes del complemento (154). Debido al importante papel que juegan estos genes y sus posibles haplotipos en susceptibilidad a EAI, decidimos estudiar dos genes dentro del MHC relacionados con la activación de las células T: *MICA* y *BTNL2*.

2.6.2.1. MICA

Dentro de la familia de moléculas HLA de clase I no clásicas se encuentran las moléculas MICA que junto con las MICB forman dicha familia (155). Estas moléculas son ligandos de los receptores NKG2D expresados en la superficie de células T CD8+ y NK (156). El gen *MICA* se encuentra aproximadamente a 40kb de los genes *HLAB*, y se localiza entre los genes *BAT1* y *HLAB* (Figura 2.21.a), son altamente polmórficos en humanos, con más de 50 alelos descritos, pero su relevancia funcional aún es desconocida. La

estructura de MICA está formada por tres dominios externos (α1, α2, y α3), un dominio transmembrana (TM) y un dominio citoplasmático (CY) (Figura 2.21.b) (155), y son expresadas preferencialmente en fibroblastos y células epiteliales. A pesar de la alta homología con los otros genes *HLA* de clase I, a diferencia de ellos las moléculas MICA no unen β-microglobulina y péptidos derivados del procesamiento antigénico (157, 158).

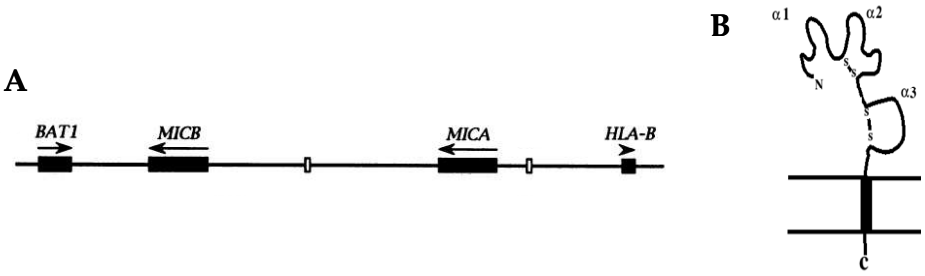

Figura 2.21. a) Localización del gen *MICA*. b) Esquema de la molécula MICA.

El hecho de que la región comprendida entre los genes *HLAB* y *BAT1* haya sido implicada en el desarrollo de varias EAI como miastenia gravis (159), enfermedad de Behçet (160) o DT1 (161) y que las células T γδ que reconocen péptidos unidos a MICA, podrían estar potencialmente involucradas en EAI; parece posible investigar la correlación entre polimorfismos genéticos de *MICA* y el desarrollo del LES.

2.6.2.2. Gen similar a la butirofilina 2 (*BTNL2*)

Los miembros de la familia de genes *BTN* son miembros de la superfamilia de las inmunoglobulinas. Los genes *BTN* codifican un número pequeño y variable de ectodominios consecutivos similares a las inmunoglobulinas, con un péptido señal en el extremo N-terminal y en el C-terminal hay una hélice transmembrana que ancla la proteína a la membrana de la CPA (162, 163). BTNL2 (molécula similar a la butirofilina 2) es un miembro de esta familia de genes caracterizado por adoptar la estructura de

una proteína transmembrana tipo I, con dos dominios extracelulares inmunoglobulina variables (IgV) y un dominio inmunoglobulina constante (IgC) y comparten una alta homología con las moléculas B7 (164, 165) (Figura 2.21). Estos receptores son importantes en la iniciación y terminación de la respuesta inmune celular a través de la interacción de sus ligandos CD28 con la proteína CTLA-4.

Aunque la función exacta de BTNL2 en humanos no está aún definida, se ha observado recientemente que en ratones su función es la de inhibir la activación de las células T de una manera independiente de IL-2 y parece que también interacciona con las células B (166). El gen *BTNL2* está situado aproximadamente a 170kb del gen *HLA-DRB1*, justo en la frontera entre las regiones MHC de clase II y clase III, y presenta un alto grado de LD con este locus (164, 167).

Todos estos datos indican que el gen *BTNL2* tiene un potencial papel inmunológico. Esta función junto con el hecho de encontrarse en una región fuertemente ligada a asociación con LES, sugieren que *BTNL2* es un buen gen candidato desde el punto de vista funcional y posicional para estudios de asociación a LES.

2.6.3. Genes implicados en la regulación de las células T y B

2.6.3.1. Proteín tirosín fosfatasa no receptor 22 (*PTPN22*)

En los últimos años ha surgido un nuevo gen de susceptibilidad a múltiples EAI. Este gen es una proteín tirosín fosfatasa (PTPs) que en un primer estudio se encontró asociado con susceptibilidad a DT1 (168). Desde que Bottini y col. en 2004 describieran la primera asociación entre el polimorfismo R620W (1858C/T ó rs2476601) del gen *PTNP22* (proteín tirosín

fosfatasa no receptor 22) y DT1 (168), esta tirosín fosfatasa intracelular se ha convertido en el mayor factor genético común de riesgo a autoinmunidad en humanos fuera de la región del MHC conocido hasta la fecha.

En gen *PTPN22* codifica a una PTP, las cuales tienen un papel crítico en la transducción de señales en las células T (169), y se ha observado que pequeñas alteraciones en estas PTPs pueden provocar una disfución inmunológica y enfermedades en humanos (170). En concreto, el gen *PTPN22* codifica a una proteína linfoide tirosín fosfatasa (Lyp), la cual está involucrada en el control de la activación de las céulas T. Bajo condiciones normales, esta enzima (Lyp) trabaja como un "regulador negativo" inhibiendo la señalización del TCR junto a la kinasa intracelular CSK. Esta actividad combinada inhibe la activación de la tirosín kinasa LCK que está involucrada en los eventos tempranos de la señalización del TCR (Figura 2.22).

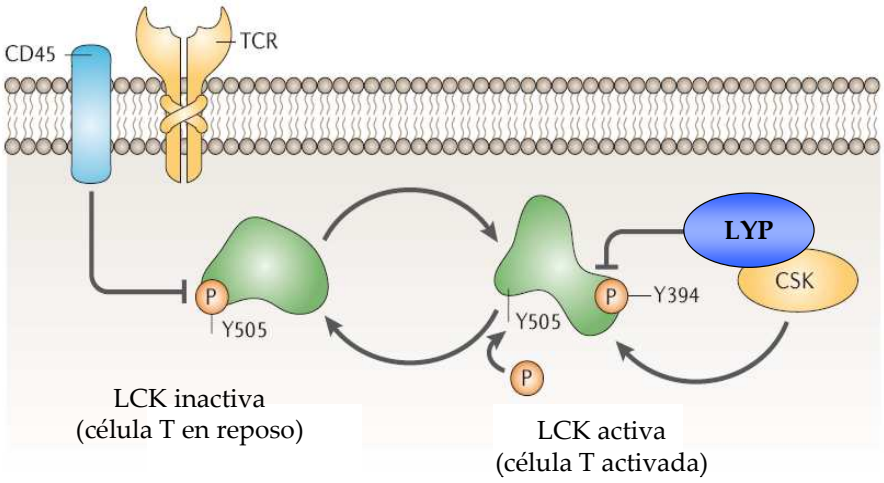

Figura 2.22. Regulación de la actividad de LCK por LYP y CSK. Cuando el TCR es activado la LCK es desfosforilada en su tirosina Y505 por CD45. Posteriormente LCK se autofosforila en Y394, iniciándose así la cascada de señalización que resultará en la activación de la célula T. Sin embargo los reguladores negativos LYP y CSK, son capaces de desfosforilar el residuo Y394 y volver a fosforilar el Y505. De esta manera, LYP inhibe la activación de la célula T (171). El polimorfismo R620W del gen *PTPN22* rompe el sitio de unión de LYP a CSK, que es fundamental para su función.

El gen *PTPN22* mapea en la región de ligamiento a LES 1p13 (172). Además se ha observado que en las células T de pacientes de LES se produce un aumento en los patrones de fosforilación de las PTP (173, 174), y que ratones knockout para *ptpn22* tienen alteraciones en la señalización por TCR y un aumento en la producción de células T memoria y de autoanticuerpos (175).

Debido al importante papel que tiene en la patogénesis del LES la regulación de las células T y que el gen *PTPN22* esta claramente implicado en este y otros procesos inmunológicos, decidimos estudiar la variante R620W de dicho gen en nuestra población de LES.

2.6.3.2. CD24

CD24, antiguamente conocida como heat-stable antigen (HSA), es una proteína glicosilfosfatidilinositol de anclaje a la membrana en la superficie celular, las cuales están involucradas en la transducción de señales por miembros de la familia de las proteín tirosín kinasas (PTKs). Esta proteína es expresada por una amplia variedad de tipos celulares que pueden participar en la patogénesis del LES, incluyendo células T activadas, células B, granulocitos maduros, macrófagos y células dendríticas entre otras (176-179). Aunque la función biológica de CD24 no está clara, se ha demostrado que esta molécula puede actuar como ligando de P-selectinas en células tumorales (180). Esta unión se cree que podría ser importante en la diseminación de las células tumorales y a su vez podría ser un factor clave en el reclutamiento de linfocitos al interior del foco inflamatorio en enfermedades que cursan con un proceso inflamatorio como el LES. Por otra parte CD24 también se ha visto implicada en la activación y diferenciación de las células B (181), y se considera un importante mediador en una vía co-estimuladora independiente de CD28, en la activación de las células T CD4 y CD8 (178), así como un punto clave en

la homeostasis de las células T en EAI (182). Además, CD24 juega un papel importante en la unión de ciertas moléculas de adhesión como VLA-4 y VCAM-1 (183), las cuales son importantes en la co-estimulación linfocitaria en tejidos específicos y en sitios de inflamación en los pacientes de LES (184, 185) (Tabla 2.4).

Tipo celular	Ligandos	Función
Células B activadas	?	Co-estimulación células T
Macrófagos	?	Preparación células T
Astrocitos	?	Co-estimulación células T
Oligodendrocitos	?	Co-estimulación células T
Células T	?	Promover la homeostasis
Células dendríticas	?	Suprimir la homeostasis
Pre células B	?	Apotosis
Pre células B	VLA4-VCAM1, fibronectina	?
Células madre neuronales	?	Suprimir la proliferación
Neuronas	CD171	Suprimir el crecimiento de las neuritas
Neuronas	CD24	?
Cáncer de pulmón	P-selectinas	Metastasis
Células cancerígenas	?	Crecimiento

Tabla 2.4. Funciones diversas mediadas por CD24 y uniones específicas dependiendo del tipo celular que la exprese.

El gen *CD24* (186) se ha identificado como un gen localizado en el cromosoma 6q21-25 (186), región previamente asociada con LES y otras EAI (187, 188). Debido a su implicación funcional en la fisiopatología del LES y su localización en un posible locus de asociación a dicha enfermedad, el gen *CD24* es un buen candidato en estudios de asociación a LES.

2.7. Genes seleccionados mediante GWAS

2.7.1. Proteína adaptadora de células B rica en repeticiones de ankirina (*BANK1*)

En uno de los primeros GWAS realizados en el LES se observó que una variante no sinónima (rs10516487) del gen *BANK1* estaba fuertemente asociada con susceptibilidad a LES (189).

Una de las características principales del LES a nivel inmunológico es la hiperactividad de células B que se produce en el transcurso de la enfermedad. Esta activación de las células B puede estar mediada a través de sus receptores BCR y dicha activación es dependiente de PTKs como Lyn y Syk. Se ha encontrado una proteína adaptadora de células B rica en repeticiones de ankirina llamada BANK1 (190, 191), que es un nuevo sustrato de PTKs. BANK1 se expresa en células B y es fosforilada en residuos de tirosina gracias a la estimulación del BCR, y esta fosforilación es mediada principalmente por Syk (Figura 2.23.).

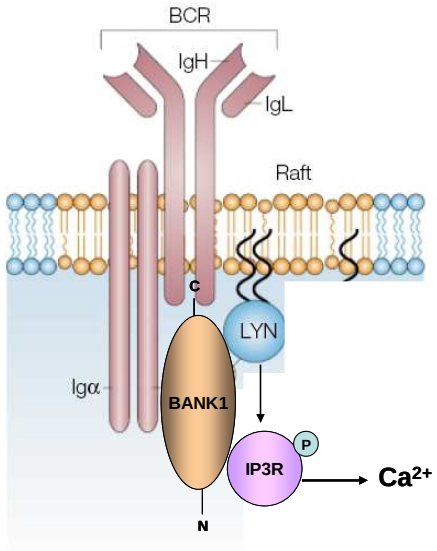

Figura 2.23. Esquema de señalización a través del BCR.

Cuando BANK1 es fosforilado va a promover su asociación con Lyn y el canal de calcio IP3R, facilitándose así la fosforilación y activación del IP3R por Lyn y liberándose Ca^{2+} desde el retículo endoplasmático (192, 193). Todo esto sugiere que un aumento de la unión de BANK1 a proteínas efectoras de señalización, podría dar lugar a un estado de hiperactividad de células B o a una desregulación en la activación de dichas células, lo cual desencadenaría un estado de autoinmunidad. Sin embargo, el papel de BANK1 en células B aún no está claro, ya que hay discrepancias sobre si su función en la activación de las células B es activadora o inhibidora (192, 194).

Debido a que *BANK1* se expresa específicamente en células B, su potencial papel en la regulación de la activación de dichas células y que se encuentra en una región cromosómica de asociación a LES (4q28) (172), se sugiere que es un buen gen candidato en estudios de susceptibilidad genética a LES.

2.7.2. Transductor de señal y activador de la transcripción 4 (*STAT4*)

La región cromosómica 2q se ha encontrado asociada a LES en diversos estudios de rastreamiento del genoma (188, 195), en concreto el locus *CD28-CTLA4-ICOS* (196). Recientemente, se ha realizado un mapeo fino de esta región 2q en pacientes de AR para determinar que variantes son las responsables de tal asociación (197) (Figura 2.24). En este estudio encontraron que el SNP rs7574865 del gen *STAT4* estaba fuertemente asociado con susceptibilidad a AR y LES en varias cohortes independientes.

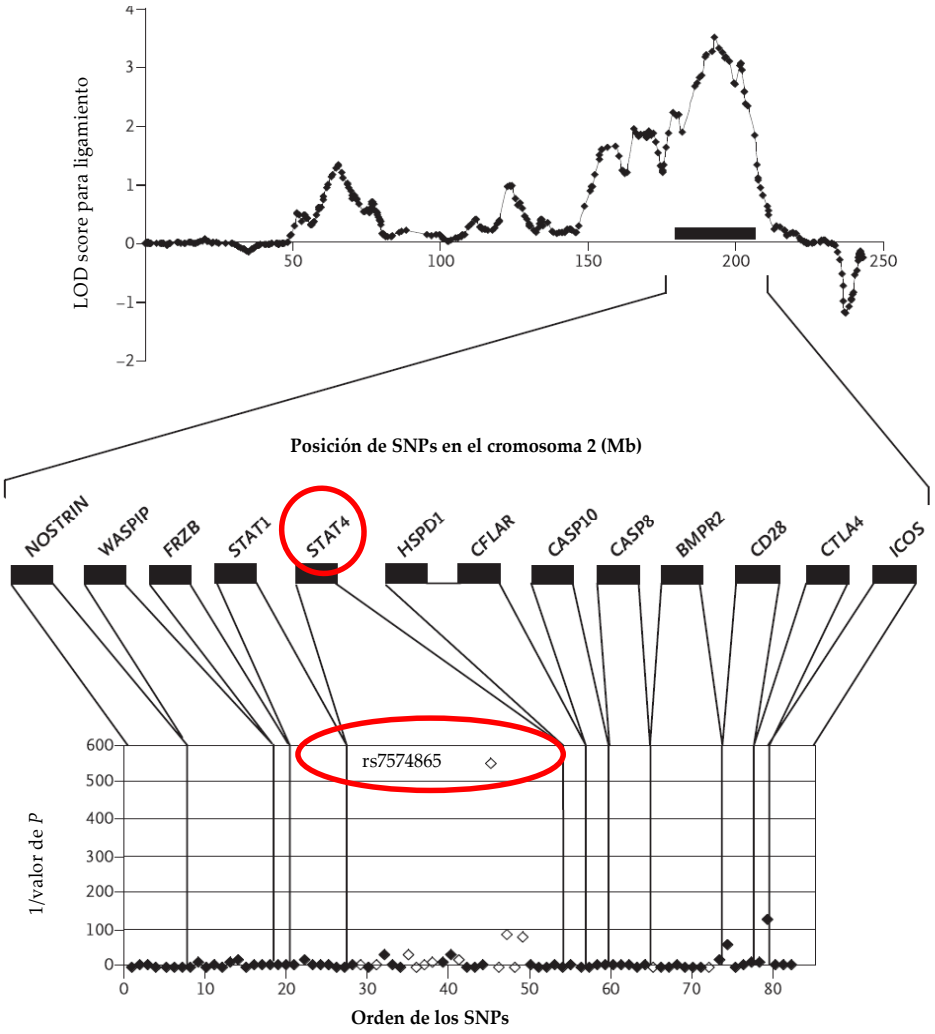

Figura 2.24. Picos de asociación de SNPs en la región 2q.

La regulación de la expresión de citoquinas requiere la cooperación de ciertos factores de transcripción específicos de células T, como STAT4. STAT4 induce la transcripción de IFN-γ en respuesta a la interacción de la IL-12 con el receptor IL-12R (198, 199) (Figura 2.25). Ratones deficientes en STAT4 pierden muchas respuestas estimuladas por IL-12, incluida la inducción de IFN-γ y la diferenciación de las células Th1 (200, 201). Estos ratones generalmente son resistentes a sufrir algunas EAI tales como la artritis inducida por proteoglicanos, EAE y diabetes (202). Sin embargo, en el modelo murino de lupus New Zealand, ratones deficientes en STAT4 muestran una nefritis

acelerada y un incremento de la mortalidad (203, 204). Todos estos hallazgos indican que una deficiencia en la expresión de STAT4 esta directamente asociada con una respuesta Th1 dañada y asociada a enfermedades inmunes.

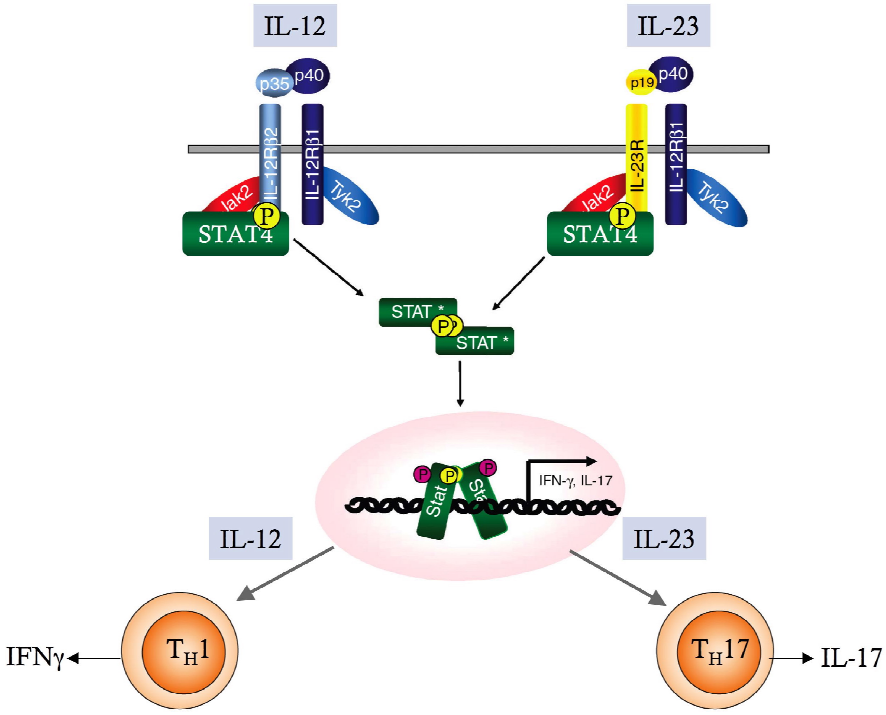

Figura 2.25. Señalización de IL-12 e IL-23 a través de STAT4.

Dada la importancia en la etiopatogénia del LES de las células Th1, y que STAT4 parece estar regulando dichos tipos celulares, este gen podría estar afectando en el LES a través de una señalización alterada en esta ruta. Además, se ha visto que deficiencias de este gen dan lugar a una mayor severidad de la enfermedad en modelos murinos de LES, y que se encuentra en una región cromosómica asociada a LES en diversas poblaciones. Debido a la potencial relevancia que parece tener este gen *STAT4* en la patogénesis del LES, decidimos estudiar dicho gen en nuestra población de pacientes de LES.

2.7.3. Receptor de TNF asociado al factor 1/componente del complemento 5 (*TRAF1/C5*)

Otra de las regiones que se han visto recientemente asociados a distintas EAI como la AR mediante GWAS es la región *TRAF1/C5*, que se encuentra situada en la región cromosómica 9q33.2.

El gen receptor de TNF asociado al factor 1 (*TRAF1*, *TNF receptor-associated factor 1*) codifica a una proteína intracelular que media señales de transducción a través de los receptores de TNFR 1 y 2 y a través de CD40. TNF es una citoquina crítica en la patogénesis del LES (205), y antagonistas de TNF se han propuesto como un tratamiento efectivo para la enfermedad y en particular en la nefritis (206, 207). Uno de los papeles del TNF más importantes en el LES es la diferenciación y proliferación de las células T, jugando un papel pro-inflamatorio en la enfermedad (207). Ratones deficientes en *TRAF1* presentan una proliferación exagerada de células T y de activación en respuesta al TNF o cuando son estimuladas a través del complejo TCR, sugiriéndose por tanto que TRAF1 actúa como un regulador negativo de esas vías de señalización (208). Además TRAF1 se ha visto asociada a un gran número de proteínas citoplasmáticas, la mayoría de ellas implicadas en la activación de NFκB, JNK y apoptosis (209) (Figura 2.26).

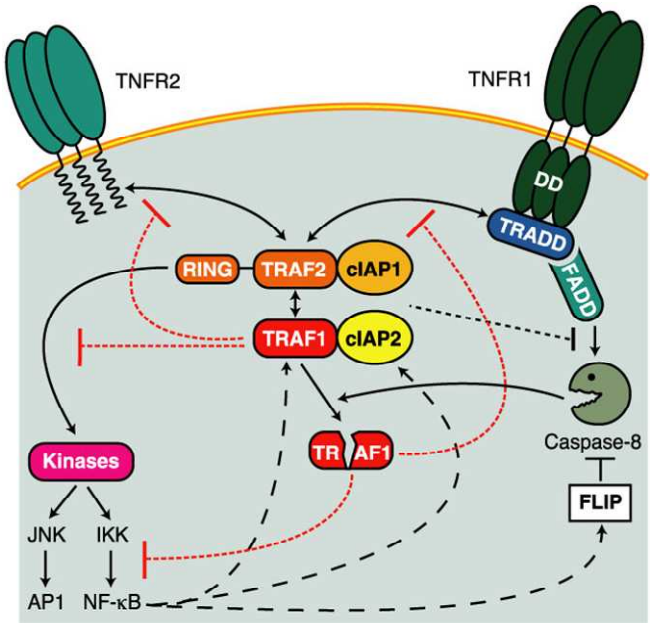

Figura 2.26. Representación de la actividad de TRAF1.

El componente del complemento 5 (C5) también tiene un papel clínico y biológico importante en el LES (45), ya que deficiencias de alguna de las proteínas de la vía clásica del complemento están asociadas con susceptibilidad al desarrollo del LES. Estas proteínas están ampliamente aumentadas en el plasma de pacientes con LES, y más principalmente en tejidos inflamados en donde se depositan. La segmentación de C5 genera la anafilatoxina pro-inflamatoria C5a, y también C5b, lo cual da lugar a la formación del complejo de ataque a la membrana. Este complejo y la molécula C5a parecen ser los mediadores claves del daño inducido por el sistema del complemento (Figura 2.27). Se ha observado que la inhibición de C5 en modelos animales de LES mejoran la enfermedad y que anticuerpos anti-C5 monoclonales reducen la glomerulonefritis y aumentan el periodo de vida en ratones NZB/W (210, 211).

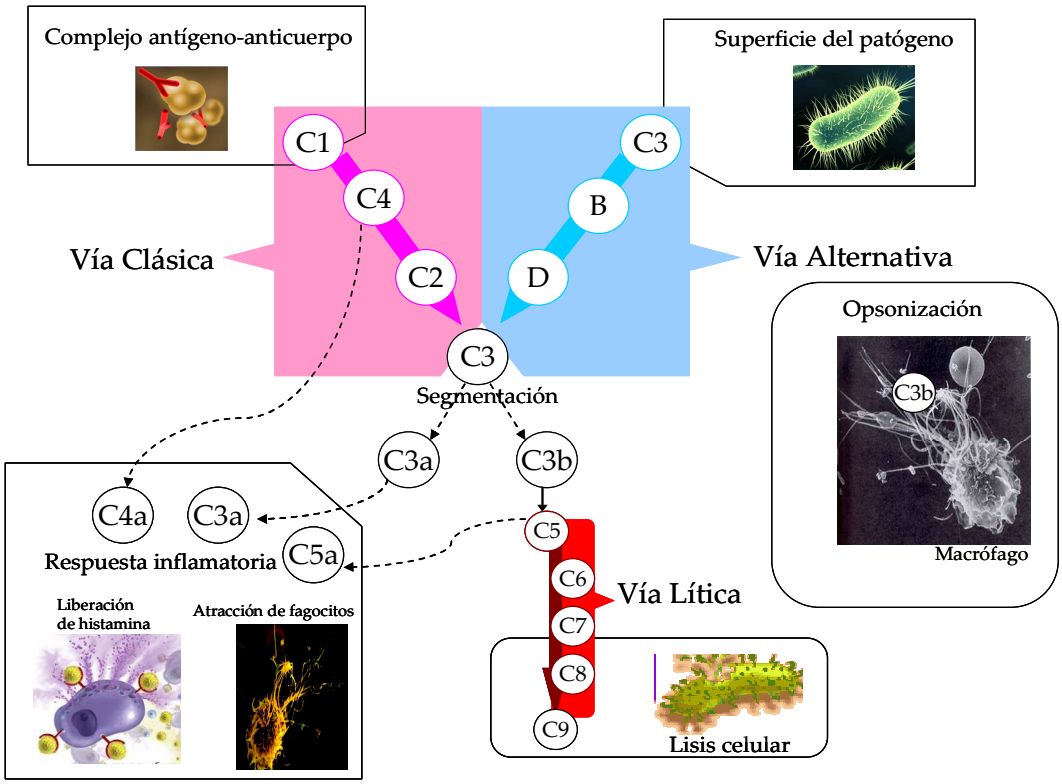

Figura 2.27. Vías de activación del complemento.

Ya que tanto *TRAF1* como *C5* son genes implicados en inflamación y en la respuesta inmune, y que la represión de ambos representa una poderosa herramienta en el tratamiento del LES, decidimos explorar el papel de ambos genes en el LES.

3. JUSTIFICACIÓN Y OBJETIVOS

El lupus eritematoso sistémico es una enfermedad compleja, inflamatoria y crónica de etiología desconocida. Aunque poco a poco se van dilucidando los mecanismos que conducen a la enfermedad, aún quedan numerosas cuestiones por resolver. Entre ellas, las causas exactas que desencadenan el LES y los mecanismos fisiopatológicos que lo controlan. Las lagunas que aún existen con respecto al LES, hacen que no haya un tratamiento específico y que su diagnóstico sea complicado.

Los estudios genéticos podrían ayudar a dilucidar los mecanismos que intervienen en la enfermedad. Así pues, los estudios genéticos encaminados a la identificación de nuevos marcadores de susceptibilidad al LES, se han convertido en una herramienta de creciente importancia en la medicina moderna. El descubrimiento de una asociación entre un polimorfismo genético y la progresión de la enfermedad podría ayudar a dilucidar la patogenia de la enfermedad, así como a diseñar nuevas diana terapéuticas, faciliar el diagnóstico e incluso predecir las caracteristicas clínicas que presentara cada paciente.

El objetivo principal de esta tesis doctoral fue la identificación de nuevos marcadores genéticos de predisposición al LES. Para ello se siguió una estrategia basada en estudios de asociación caso-control de genes candidatos. En concreto, nos planteamos los siguientes objetivos:

1. Estudiar el posible papel de los genes *TLR2*, *TLR4*, *TLR5* y *TLR7* en la predisposición a sufrir LES.

2. Evaluar la posible asociación del gen *NFKB1* en la susceptiblidad al LES en nuestra población.

3. Comprobar en nuestra población la relevancia de los polimorfismos del gen *FCRL3* en la susceptibilidad al LES.

4. Establecer si los polimorfismos más estudiados de los genes de la familia de la IL-12: *IL12B*, *IL12RB1* e *IL23R* están implicados en el LES. Y si la subunidad p19 del gen *IL23* era polimórfica en nuestra población.

5. Comprobar el papel en la genética del LES del gen *MIF*.

6. Estudiar el papel de los polimorfimos localizados en la región promotora del gen *IL18* en la susceptibilidad al LES, y evaluar la relevancia funcional que pueden tener en la regulación de la expresión de la IL-18.

7. Investigar la implicación de los genes de la región MHC, *MICA* y *BTNL2*, con el LES. Asi como comprobar la posible relación de estos con los genes *HLA-B* y *HLA-DR*, fuertemente asociados a LES.

8. Comprobar el papel del gen *PTPN22*, considerado un marcador genético común de autoinmunidad, en la predisposición al LES.

9. Analizar gen *CD24* como posible gen candidato de susceptibilidad al LES.

10. Evaluar el papel de los genes *BANK1*, *STAT4* y *TRAF1/C5* encontrados recientemente asociados con susceptibilidad a LES y a otras enfermedades autoinmunes, mediante estudios de asociación del genoma completo, en nuestra población de pacientes con LES.

4. ANEXO PUBLICACIONES

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Polymorphisms of toll-like receptor 2 and 4 genes in rheumatoid arthritis and systemic lupus erythematosus

Key words:
polymorphisms; rheumatoid arthritis; systemic lupus erythematosus; TLR2; TLR4

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Abstract: Human toll-like receptors (TLRs) participate in the innate response and signal the activation of adaptive immunity. Therefore, these TLRs may be important in autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). We investigated, by using a polymerase chain reaction restriction-fragment length polymorphism method, the possible association between the polymorphisms of TLR2 (Arg677Trp and Arg753Gln) and TLR4 (Asp299Gly and Thr399Ile) genes with the susceptibility or severity of RA and SLE. Our study population consisted of 122 patients with SLE, 224 patients with RA, and a control group of 199 healthy individuals. The TLR2 polymorphisms were very rare in our population; no individual carrying the TLR2-Arg677Trp polymorphism was observed, whereas the TLR2-Arg753Gln polymorphism was present in only 1% of the total population. We found no statistically significant differences in the TLR4-Asp299Gly and the TLR4-Thr399Ile genotype or allele distribution between SLE patients, RA patients, and control individuals. Similarly, no association was found with any of the demographic and clinical parameters tested either in RA or in SLE patients. In conclusion, a case-control study was used to analyze, for the first time, the influence of TLR2 and TLR4 gene polymorphism on the predisposition and clinical characteristics of SLE and RA but provided no evidence for association of TLR2 or TLR4 gene polymorphism with either disease in the population under study.

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Rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are common complex diseases that are thought to have an immunological origin because of the abundance of immune complex, presence of autoantibodies, association with human leucocyte antigen specificities, and accumulation of lymphocytes, monocytes, and macrophages within pathological lesion (1, 2).

The toll-like receptor (TLR) family play a central role in the initiation of cellular innate immune responses (3, 4). To date, 10

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members of the TLR family have been characterized. TLR2 is activated primarily by peptidoglycan and lipoproteins, and TLR4 is predominantly activated by lipopolysaccharide (LPS) and lipoteichoic acid (5). TLR2 and TLR4 can activate nuclear factor-κB and induce expression of inflammatory cytokines and costimulatory molecules, suggesting that human TLRs participate in the innate response and signal the activation of adaptive immunity (6).

Two cosegregating missense polymorphisms in TLR4 gene have described an adenine for guanine substitution at 896 nucleotide from the start codon, which results in replacement of an aspartic acid residue for glycine at amino acid 299 (Asp299Gly), and a second point mutation that leads a threonine for isoleucine substitution at amino acid 399 (Thr399Ile) (7). These TLR4 polymorphisms appeared to be correlated with endotoxin hyporesponsiveness in human (7), susceptibility to gram-negative sepsis shock (8), and with the development of atherosclerosis (9). As regards TLR2, a polymorphism has been described as leading to an exchange of arginine by glutamine at position 753 (Arg753Gln), which is correlated with the incidence of sepsis caused by gram-positive bacteria in human (10). Recently, another polymorphism in the TLR2 gene at position 677 (Arg677Trp) was associated with susceptibility to lepromatous leprosy (11). Therefore, in the present study, we assessed the possible contribution of the TLR2 and TLR4 gene polymorphism to susceptibility and clinical outcome of RA an SLE.

Patients and methods

Subjects

Our study population consisted of 122 patients with SLE and 224 patients with RA from the Hospital Virgen de las Nieves, Granada, Spain. A control group consisted of 199 healthy individuals from the same geographic area. The mean age of controls at analysis was 45 ± 12; 75% were female and 25% were male. The patients and controls were Spanish Caucasians and were matched for age and sex. The SLE and RA were diagnosed according to the Criteria Committee of the American College of Rheumatology (12, 13). The mean age of SLE patients at analysis was 38 ± 9 and at diagnosis was 32 ± 11.6; 111 SLE patients were female (91%) and 11 were male (9%). The SLE clinical manifestations studied were articular involvement, renal affectation, cutaneous lesions, hematopoiteic alterations, neurological disease, and serositis. In addition, clinical activity or severity was performed determining the Systemic Lupus Erythematosus Disease Activity Index score, every six months. Furthermore, this study included 224 RA patients whose characteristics are described in Table 2.

TLR2 and TLR4 genotyping

DNA was isolated from anticoagulated peripheral blood mononuclear cells using standard methods. We determined TLR4 genotypes by a PCR-based method, as previously described (14). Regarding TLR2 Arg677Trp and Arg753Gln genotyping, we designed the following primers: forward 5′-CCTTCAAGTTGTGTCTTCATAAC-3′ and reverse 5′-GGCCACTCCAGGTAGGTCTT-3′. The forward primer was modified to create a *Hpa* II (TLR2 Arg677Trp) that recognized the mutant allele. The TLR2 Arg753Gln polymorphism was detected taking advantage of the presence of natural restriction sites for *Aci* I that recognizes the wild-type allele and *Pst* I that recognizes the mutant allele, which was used to confirm the results. Fifteen microliters of the PCR products was digested by appropriate restriction enzymes, and digests were run out on a 4% agarose gel. The PCR conditions were as follows: 4 min at 95°C, then 35 cycles were performed at 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s and thereafter 7 min at 72°C. All mutations were confirmed by direct sequencing using the ABI PRISM 377 automatic sequencer (Applied Biosystems, Foster City, CA, USA).

Statistical methods

For association studies, P values were calculated by the χ^2 method or Fisher's exact test as appropriate. Odds ratios (ORs) with 95% CI were calculated according to Woolf's method. A P value below 0.05 was considered statistically significant. For non-parametric data analysis, the Mann–Whitney U-test was used for ordinal variables and Fisher's exact test for dichotomous variables.

Results and discussion

The distribution of the TLR4 genotypes in RA and SLE patients and control individuals is summarized in Table 1. We observed that the Asp299Gly and the Thr399Ile mutations are in linkage disequilibrium, although does not seem to be complete. The observed allele frequencies in our control population for the TLR4 mutations were in good agreement with allele frequencies found in other European and North American Caucasian populations (7, 8, 15, 16). On the contrary, these polymorphims appear to be very rare amongst Japanese (17). No statistically significant differences were observed when the Asp299Gly and the Thr399Ile genotypes and allele distribution in RA patients, SLE patients, and healthy controls were compared. In addition, no differences in the Asp299Gly and the Thr399Ile genotypes and allele distribution were found when RA patients and controls were stratified by the presence or absence of the shared

Toll-like receptor 4 genotype and allele frequencies in rheumatoid arthritis patients, systemic lupus erythematosus patients and in healthy controls

Toll-like receptor 4	Controls ($n=199$) (%)	Rheumatoid arthritis ($n=224$) (%)	Systemic lupus erythematosus ($n=122$) (%)
Amino acid 299			
Genotypes			
Asp/Asp	171 (86)	203 (91)	106 (87)
Asp/Gly	26 (13)	21 (9)	16 (13)
Gly/Gly	2 (1)	0	0
Alleles			
Asp	0.92	0.95	0.93
Gly	0.08	0.05	0.07
Amino acid 399			
Genotypes			
Thr/Thr	173 (87)	202 (90)	105 (86)
Thr/Ile	24 (12)	22 (10)	17 (14)
Ile/Ile	2 (1)	0	0
Alleles			
Thr	0.93	0.95	0.93
Ile	0.07	0.05	0.07

We determined toll-like receptor 4 (TLR4) genotypes by a polymerase chain reaction (PCR)-based method, as previously described (14). The following primers were used: for TLR4 Asp299Gly, forward 5′-GATTAGCATACTTAGACTACTACTACCTCCATG-3′ and reverse 5′-GATCAACTTCTGAAAAAGCATTCCCAC-3′; for TLR4 Thr399Ile, forward 5′-GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA-3′ and reverse 5′-ACCTGAAGACTGGAGAGTGAGTTAAATGCT-3′. The forward primers are modified to create *Nco* I (TLR4 Asp299Gly) and *Hinf* I (TLR4 Thr399Ile) restriction sites in the mutant alleles and allow distinction between wild-type and mutant TLR4. The PCR conditions were as follows: 4 min at 95°C, then 30 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 30 s and thereafter 7 min at 72°C.

Table 1

epitope (Table 2). The presence of shared epitope plus alleles was increased in RA patients compared with controls (50.7 *vs* 37.7%, $P=0.007$, OR = 1.70, 95% CI 1.13–2.55). Next, we analyzed demographic and clinical characteristics of RA patients according to their TLR4 genotypes, and no associations were found with any of the parameters tested (Table 2). Similarly, no correlation was observed between TLR4 polymorphism and the clinical status of SLE patients (data not shown).

Regarding the TLR2 mutations, no individual in either RA-SLE patients group or control population was identified carrying the Arg677Trp polymorphism, which is in concordance with a recent report of a German population (18), suggesting that this polymorphism, previously reported in Asia (11), does not occur amongst White Caucasian populations. In addition, we observed a very low frequency of the Arg753Gln polymorphism in our study population; only four out of 224 (1.8%) RA patients, two out of 122 (1.6%) SLE

Distribution of clinical and laboratory characteristics amongst toll-like receptor 4 genotypes in rheumatoid arthritis patients

	Shared epitope (%)	Rheumatoid factor (%)	Rheumatic nodules (%)	Extra-articular disease (%)	Female sex (%)	Mean age of onset (%)
TLR4 Asp299Gly						
Asp/Asp ($n=203$)	102 (50.2)	146 (71.9)	48 (23.6)	43 (21.2)	159 (78.3)	50.2 ± 13.5
Asp/Gly ($n=21$)	13 (61.9)	15 (71.4)	2 (9.5)	5 (23.8)	20 (95.2)	53.5 ± 8.2
TLR4 Thr399Ile						
Thr/Thr ($n=202$)	100 (49.5)	147 (72.8)	48 (23.8)	43 (21.3)	158 (78.2)	50.2 ± 13.5
Thr/Ile ($n=22$)	14 (63.6)	15 (68.2)	2 (9.1)	5 (22.7)	21 (95.4)	53.5 ± 8.2

This study included 224 patients with rheumatoid arthritis (RA) which at onset had mean age of 50 ± 13 and at analysis was 53 ± 10; 179 were female (80%) and 45 men (20%). Seventy-eight percent of the RA patients were positive for rheumatoid factor, 24% of the patients presented nodular disease, 23% of them presented additional extra-articular manifestations and 50% were positive for the shared epitope.

Table 2

patients, and two out of 199 (1%) controls were carried of the Arg753Gln mutation. The low presence of this polymorphism in Caucasian population has been previously described (10), although a recent report found a higher frequency amongst whites, around 9%; this may be due to ethnic variations or due to the different typing methods used (18).

Through the recognition of pathogens or their products, TLRs can induce the production of proinflammatory cytokines that can contribute to the perpetuation of the inflammatory response. It is accepted that activation of the innate immune system is an important feature in the pathogenesis of RA (19). In this sense, an increased expression of TLR2 has been shown in synovial tissues of patients with RA (20). Moreover, a recent report provides evidence that TLRs response to bacterial DNA could be involved in triggering B cells to produce the autoreactive rheumatoid factor in a T-cell-independent manner (21). Therefore, it is tempting to speculate that genetic variation in the innate immune genes, TLR, may play a role in determining susceptibility, not only to infectious diseases but also to chronic inflammatory human diseases such as RA and SLE. The TLR4 polymorphisms appeared, specially the Asp299Gly mutation, to be associated with differences in LPS responsiveness in humans (7) in addition with susceptibility to gram-negative infection (8, 22); however, other authors did not observe an association of TLR4 polymorphism with susceptibility or severity of meningococcal disease (23). In concordance with our data, a very recent report showed no influence of TLR4 polymorphism on the incidence, progression, and inflammatory parameters of multiple sclerosis (16).

In summary, a case-control study was used to analyze for the first time the influence of TLR2 and TLR4 gene polymorphism on the predisposition and clinical characteristics of SLE and RA but provided no evidence for association with either disease in the population under study.

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Investigation of *TLR5* and *TLR7* as candidate genes for susceptibility to systemic lupus erythematosus

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Running title: *TLR5* and *TLR7* polymorphisms in SLE

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ABSTRACT

Objective: The aim of this study was to evaluate the relevance of genetic variants of *TLR5* (rs5744168) and *TLR7* (rs179008) gene in systemic lupus erythematosus (SLE) in a Spanish population.

Material and methods: Our study population consisted of 752 SLE patients and 1107 healthy controls. All individual were of Spanish Caucasian origin. The *TLR5* and *TLR7* polymorphisms were genotyped using a PCR system with pre-developed TaqMan allelic discrimination assay.

Results: No statistically significant differences were observed when the allele and genotype distribution of *TLR5* rs5744168 and *TLR7* rs179008 polymorphisms was compared between SLE patients and healthy controls. A significant increase frequency in the CC genotype of the *TLR5* rs5744168 polymorphism among SLE patients without nephritis was found (93.4% vs 87% in SLE patients with nephritis, P= 0.03, OR= 2.11 95%CI 0.93-3.51). However, this difference did not reach statistical significance in the allele frequencies (*P*=0.08).

Conclusion: These results suggest that the tested variations of *TLR5* and *TLR7* genes do not confer a relevant role in the susceptibility or severity of SLE in the Spanish population.

INTRODUCTION

Systemic lupus erythematosus (SLE) is the prototypic systemic autoimmune disease and is characterized by B-cell hyperreactivity and the production of autoantibodies (1). Autoantibodies to DNA, RNA and associated proteins are common targets of the autoimmune response in SLE (2). The presence of these antinuclear antibodies has been detected in the serum of a majority of patients with SLE, and these antibodies result in the formation of immune complexes (ICs) that deposit in tissues and induce inflammation, thereby contributing to disease pathology. In fact, DNA and/or RNA can behave as autoantigens because they have the capacity to stimulate the innate immune system directly via Toll-like receptors (TLRs) or indirectly via Fcγ receptors (FcγR) and thereby promote the self-directed immune response, potentially leading to tolerance (3). The TLR family constitutes an important group of pattern-recognition receptors that play an essential role in the activation and regulation of innate and adaptive immunity through the recognition of specific molecular patterns of pathogens (4, 5). Currently, 11 TLR subtypes have been identified in humans, with each having specific ligands, cellular localization and expression profiles. Stimulation of the TLR pathway culminates in NFκB activation and transcription of immune response genes, such as cytokines and chemokines (5-7). Because of their central role in the regulation of inflammation and the immune response to pathogens, TLRs are excellent candidate genes in genetic susceptibility studies for autoimmune diseases, such as SLE.

TLR5 gene is known to recognize the bacterial flagellin and it located at 1q41 (8), a chromosome region liked with susceptibility to SLE in different populations (9, 10). A stop codon polymorphism in the ligand-binding domain of *TLR5* (*TLR5* rs5744168 also called Arg392Stop or C1174T) is unable to mediate flagellin signalling (11) and has been found associated with susceptibility to Legionnaries´disease (11) and resistance to Crohn´ disease (12) and SLE (13). These findings suggest that *TLR5* may be considered both a biological and a positional candidate gene for SLE.

TLR7 has recently been described as a potential functional relevance gene in SLE (14). TLR7 is involved in the recognition of singled-stranded viral RNA (15). Recent studies in congenic mice bearing the Y-linked autoimmune accelerator (*yaa*) lupus susceptibility locus, have showed that

differences in expression of the *TLR7* gene as well as environmental factors that induce TLR7 responses may result in increased B cell sensivity to RNA-containing self-antigens (16, 17). In addition, TLR7 has the ability to induce the release of interferon-α (IFNα), a cytokine that has been shown to have a relevant role in SLE (18).

Due to the central role of these *TLRs* (*TLR5* and *TLR7*) genes within the innate immune system, the aim of this study was to determine the role of genetic variations in these genes with SLE in a Spanish population.

MATERIAL AND METHODS

Patients: Peripheral blood samples were obtained after written informed consent from 752 SLE patients meeting the American College of Rheumatology criteria for SLE (19). These patients were recruited from nine Spanish hospitals: Hospital Virgen de las Nieves and Hospital Clínico (Granada), Hospital Virgen del Rocío (Seville), Hospital Carlos-Haya and Hospital Virgen de la Victoria (Málaga), Hospital Central (Oviedo), Hospital Parc Taullí (Sabadell), Hospital La Fe (Valencia) and Hospital Xeral-Calde (Lugo). Similarly, blood was taken from 1107 blood bank and bone marrow donors of the corresponding cities were included as healthy individuals. Both patient and control groups were of Spanish Caucasian origin and were matched for age by mean age and sex by frequency matching. Informed consent was provided by each individual included in the study. The samples were collected according to the Helsinki declaration. The study was approved by all local ethical committees from the corresponding centers. Demographic characteristics of the cases and controls in each population have been described previously (20).

Genotyping of TLR5 and TLR7 polymorphisms: DNA was obtained from peripheral blood, using QIAamp DNA Blood Maxi Kit (Qiagen, Hilden, Germany). *TLR5* and *TLR7* genotyping were performed using a TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, CA). The PCR reaction was carried out in a total reaction volume of 4 µl, containg 50 ng genomic DNA as template, 2 µl of Taqman genotyping master mix, 0.1 µl of 20x assay mix and ddH_2O up to 4 µl of final volume. The amplification protocol used was: initial denaturation at 95ºC for 10 min followed by 40 cycles of denaturation at 92ºC for 15 s, and annealing/ extension at 60ºC for 1 min. After PCR, the genotype of each

sample was automatically attributed by measuring the allele-specific fluorescence in the ABI Prism 7900 Sequence Detection System, using the SDS 2.2.2 software for allele discrimination (Applied Biosystems).

Statistic analysis: Allele and genotype frequencies were obtained by direct counting and for the statistical analysis to compare allelic and genotypic distributions we used the χ^2 test. We assessed the quality of the genotype data by testing for Hardy-Weinberg equilibrium in the case and control samples, using Fisher´s exact test (P>0.05). Odds ratio (OR) with 95% confidence intervals (95%CI) were calculated according to Woolf´s method. The software used was StatCalc program (Epi Info 2002; Centers of Disease Control and Prevention, Atlanta, GA, USA). *P* values below 0.05 were regarded as statistically significant. The power of each study was computed as the probability of detecting an association between *TLR5* and *TLR7* polymorphisms and SLE at the 0.05 level of significance, assuming an OR of 1.5 (small effect size). Power analysis was estimated using the Quanto v 0.5 software (Department of Preventive Medicine University of Southern California, CA, USA).

RESULTS

All the genotype frequencies in cases and healthy controls were not significantly different from those predicted by Hardy-Weinberg equilibrium.

Table 1 shows the distribution of genotypes and alleles of the *TLR5* rs5744168polymorphism studied in SLE patients and controls. No statistically significant differences were observed when the allele and genotype distribution was compared between SLE patients and healthy controls. The allele frequencies found in our controls population are in good agreement with allele frequencies observed in other Caucasian populations (21). We next considered whether the *TLR5* stop codon showed a preferential association with particular clinical manifestations of SLE (Table 2). A significant increase frequency in the CC genotype among SLE patients without nephritis was found (93.4% vs 87% in SLE patients with nephritis, P= 0.03, OR= 0.47 95%CI 0.28-1.07). However, this difference did not reach statistical significance in the allele frequencies (*P*=0.08).

No *TLR7* polymorphisms have been described to date that influence the course of human diseases. Nevertheless, a recent study detected a variants with a frequency over 5% (rs179008), which results in an

aminoacid change from glutamine to leucine at codon 11 (Q11L) (22). We analyzed the *TLR7* Q11L polymorphism in our cohort of SLE patients. No statistically significant differences were observed between allele frequencies of SLE patients and healthy controls (Table 3). In addition, we found no association of this polymorphism and genotype frequencies in female patients with SLE (data not shown). We also estimated the allele frequencies in male with SLE and no deviation in the distribution compared with allele frequencies in male controls was observed. In addition, available clinical features of patients with SLE were analyses for possible association with the different alleles or genotypes of *TLR7* polymorphism. However, when we stratified SLE patients according to the presence of renal involvement, no statistically significant differences were observed in the distribution of this polymorphism between SLE patients with or without lupus nephritis (data not shown). Similarly, no significant differences were observed between this genetic variant and the following variables, age at onset, articular involvement, cutaneous lesions, photosensivity, hematological alterations, neurological disorders and serositis (data not shown).

DISCUSSION

Due to the central role of TLRs within the innate immune system, genetic variation in this gene family may alter susceptibility to some diseases. Genetic variations in the TLRs genes have been associated with many inflammatory and/or autoimmune diseases (13, 23-26). Accumulating evidence indicates a role of TLRs in the recognition of endogenous ligands which might be involved in these disorders (3, 27).

In the present study we analysed a large cohort of SLE patients and healthy controls to assess the role of *TLR7* gene in the susceptibility to SLE, and tried to replicate the previously association reported by Hawn *et al*. between the *TLR5* rs5744168 polymorphism and SLE (13). Our data revealed no evidence of association of these variants with SLE in a Caucasian cohort from Spain. These findings contradict a recent family based report, which found that *TLR5* 1174C allele confers protection from developing SLE (13). However, an in agreement with our data a study in two Caucasians populations from North-America could not replicate the initial association (21). Several reasons could be underlying this discrepancy. The first result could emerge as a consequence of a type I error (false

positive), as a result of examining the statistical power of the study using allelic OR results, and it concluded that it is underpowered to detect an association (<40%). On the contrary, the existence of a type II error (false negative) in our and Demirci *et al*. studies is unlikely because of the high statistical power of these studies (97% and 73%, respectively) to detect an effect similar to that observed in the first study.

Although *TLR7* genetic variants are largely unexplored, Pisitkun *et al*. have reported that a genomic segmental duplication, which included the murine *Tlr7* gene, and the translocation of this segment to the *Yaa* locus were associated with autoreactive B cell responses to RNA-related antigens. (16). However, the genomic increase in *Tlr7* in a murine model of lupus cannot be translated directly to humans with SLE, since no significant concordance between the relative number of gene copies and the SLE phenotype was found (28). Although these variants in the *TLR7* seem not play a relevant role in human SLE, several evidences show a role of this gene in the development of the disease. Since *TLR7* is located on chromosome X and there is an increase prevalence of SLE in women (29), it is suggested an X-linked genetic component in SLE. In addition, IFN-α, a cytokine critically involved in the pathogenesis of SLE (18, 30), markedly increases B cell expression of TLR7 by plasmocytoid dendritic cells (31).

In the present study we investigated for the first time a *TLR7* polymorphism (Q11L) to test SLE susceptibility, and we found no evidence of association. The possibility that this lack of association could have arisen due to type II error seems unlikely, since we estimated that our cohort has enough power (>99%) to detect the effect of the polymorphism, taking into consideration an odds ratio of 1.5 at a 5% significance level. Furthermore, allele frequencies in our control population are similar to those reported in the SNP database (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=179008) in other Caucasian-European populations. However, we cannot excluded the possibility that other polymorphisms of the *TLR7* gene that are not in linkage disequilibrium with the alleles tested may contributed to the development of SLE. In addition, this SNP is located a region of known copy number variation, which should be taken with caution in genotyping studies since the alleles may differ according to the number of copies carried.

TLR5 and TLR7 signalling involves the adaptor protein myeloid differentiation

factor 88 (MyD88), since mice rendered MyD88-deficient are unresponsive to ligands for these TLRs (32) and that activation leads to the production of proinflammatory cytokines such as TNF-α, IL6, IL1β and IL12. In addition, TLR stimulation generally leads to the production of IL12 and IL23 and thereby favours a Th1-type response (33). However, in previous studies, we failed to find an association between genetic variants in these cytokines and other TLRs with SLE in our population (34-37).

In conclusion, although the clinical relevance of *TLR5* rs5744168 and *TLR7* rs179008 polymorphisms indicates the possible physiological effect of other polymorphisms in chronic inflammatory diseases, these variants seems not play a relevant role in SLE in our population. However, this finding cannot rule out a possible role of TLR5 and TLR7 in SLE pathogenesis therapeutic targets (38).

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Table 1. Genetic and allelic distribution of *TLR5* rs5744168 polymorphism in SLE patients and healthy controls.

TLR5 rs5744168	**SLE patients n= 752 (%)**	**Healthy controls n= 1107 (%)**	P_{value}	**OR (95% CI)**
Genotypes				
CC	673 (89.5)	1009 (91.1)	0.2	0.83 (0.60-1.14)
CT	74 (9.8)	96 (8.7)	0.4	1.15 (0.83-1.60)
TT	5 (0.7)	2 (0.2)	0.1	3.70 (0.64-27.54)
Alleles	**2n= 1504**	**2n= 2214**		
C	1420 (94.4)	2114 (95.5)	0.1	1.12 (0.93-1.34)
T	84 (5.6)	100 (4.5)	0.1	0.89 (0.74-1.07)

Table 2. Distribution of *TLR5* rs5744168 genotypes (%) by clinical features of the SLE patients.

SLE feature		CC	CT	TT	C	T
Nephritis	+	0.87*	0.13	0	0.93**	0.07
	-	0.93	0.6	0.08	0.97	0.03
Rash Malar	+	0.93	0.07	0	96.4	3.6
	-	0.88	0.10	0.02	93.3	6.7
Discoide	+	0.87	0.1	0.03	0.92	0.08
	-	0.91	0.08	0.01	0.095	0.05
Oral Ulcer	+	0.88	0.12	0	0.94	0.06
	-	0.93	0.06	0.01	0.96	0.04
Photosensivity	+	0.92	0.08	0.01	0.96	0.04
	-	0.89	0.10	0.01	0.94	0.06
Arthritis	+	0.92	0.09	0.01	0.95	0.05
	-	0.093	0.06	0.01	0.96	0.4
ANA	+	0.91	0.08	0.01	0.95	0.5
	-	1.00	0	0	1.00	0
Anti-dsDNA Ab	+	0.90	0.09	0.01	0.95	0.5
	-	0.96	0.03	0.01	0.97	0.03

*P= 0.03, OR= 0.47 95%CI 0.28-1.07

**P= 0.08

Table 3. Allelic distribution of *TLR7* rs179008 polymorphism in SLE patients and healthy controls.

TLR7 **rs179008**	**SLE patients n= 752 (%)**	**Healthy controls n= 1107 (%)**	P_{value}	**OR (95% CI)**
Alleles				
C	1104 (81.2)	1439 (80.6)	0.7	1.04 (0.87-1.23)
T	256 (18.8)	346 (19.4)	0.7	0.97 (0.80-1.54)

Brief communication

Analysis of the functional *NFKB1* promoter polymorphism in rheumatoid arthritis and systemic lupus erythematosus

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Key words:
–94ins/delATTG polymorphism; CA microsatellite polymorphism; genetics; nuclear factor-κB; rheumatoid arthritis; systemic lupus erythematosus

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Abstract: Nuclear factor (NF)-κB plays an important role in inflammatory diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). A functional insertion/deletion polymorphism (−94ins/delATTG) has been identified in the promoter of the *NFKB1* gene. In addition, a polymorphic dinucleotide repeat (CA) has been identified in proximity to the coding region of the human *NFKB1* gene. The aim of the present study was to investigate the influence of both the −94ins/delATTG and the (CA) microsatellite *NFKB1* polymorphisms in the susceptibility/severity of RA and SLE. We analyzed the distribution of −94ins/delATTG and the multiallelic $(CA)_n$ repeat in 272 RA patients, 181 SLE patients, and 264 healthy controls from Southern Spain, in both cases using a polymerase chain reaction-fluorescent method. No statistically significant difference in the distribution of the −94delATTG *NFKB1* genotypes and alleles between RA patients, SLE patients, and control subjects was observed. Similarly, we found no statistically significant differences in the $(CA)_n$ microsatellite allele frequency between controls and RA patients or SLE patients. In addition, no association was found between the above mentioned *NFKB1* polymorphisms with any of the demographic and clinical parameters tested either in RA or in SLE patients. From these results, it seems that the −94ins/delATTG and the $(CA)_n$ repeat of *NFKB1* gene may not play a relevant role in RA and/or SLE in our population.

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Rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are chronic inflammatory autoimmune disorders whose etiology remains unknown, but genetic and environmental factors are both important in the development of the diseases. The transcription factor nuclear factor (NF)-κB is particularly important in the regulation of inflammation (1, 2). NF-κB is found overactivated in human RA synovium, and the number of inducers and targets of NF-κB resembles the profile of mediators of inflammation in RA, suggesting a role in the control of inflammation (3–6). By contrast, the activation of NF-kB activity in SLE patients was significantly decreased in

*These authors have contributed equally to this study.

lupus patients when compared with normal cells (7, 8). The above observations suggest that the pathologic mechanism for NF-κB regulation differs between these two autoimmune diseases, which may be due, in part, to genetic factors.

Recently, a novel functional *NFKB1* promoter polymorphism has been identified, consisting of a common insertion/deletion (−94ins/delATTG) located between two putative key promoter regulatory elements, showing functional effects on the transcription of the *NFKB1* gene (9). The presence of a 4-bp deletion results in the loss of binding to nuclear proteins and reduced promoter activity of *NFKB1* promoter-luciferase reporter constructs in transient transfection experiments. Furthermore, this deletion increases the risk of ulcerative colitis (UC) in a North American population (9). The −94ins/delATTG seems to be the first potential functional *NFKB1* polymorphism.

Besides the important NF-κB role in the control of inflammation and the functional relevance of the above mentioned −94ins/delATTG polymorphism, *NFKB1* gene is located at chromosome 4q and recent whole-genome scans of RA- and SLE-affected sibling pairs have suggested a linkage to the 4q region (10, 11). Therefore, *NFKB1* seems to be a functional and positional candidate gene in RA and SLE. In addition, a polymorphic dinucleotide repeat $(CA)_n$ has been identified in proximity to the human *NFKB1* gene (12). This polymorphism has recently been suggested to be associated with susceptibility to an autoimmune disease, such as type 1 diabetes mellitus (T1DM) (13).

Taking into account these findings, the aim of this study is firstly to assess the possible influence of the functional −94ins/delATTG and $(CA)_n$ *NFKB1* gene polymorphisms in the susceptibility and/or severity of RA and SLE in a Spanish population. On other hand, we were interested in investigating the possibility that variation in the functional *NFKB1* gene polymorphism may explain the different NF-κB activity observed in RA and SLE.

The present study includes 272 RA patients, 181 SLE patients, and 264 healthy volunteer blood donors from the Granada area. RA and SLE patients were recruited from the Virgen de las Nieves Hospital in Granada, Spain. The patients and controls were of Spanish Caucasians origin. The mean age of controls at analysis was 45 ± 12, 75% were female, and 25% were male. All the RA and the SLE patients satisfied the Criteria Committee of the American College of Rheumatology (14, 15). Written consent was obtained from all the participants. 79.3% of the RA patients were women; the mean age at onset was 50.7 ± 13 years; 50.2% carried the shared epitope; 78% were positive for the rheumatoid factor; 25% presented nodular disease, 24% of them presented additional extra-articular manifestations; 92% presented hand and wrist X-rays, erosion or joint space narrowing, and 80% presented X-ray erosion or joint space narrowing joint other than hands or wrists. 89% of the SLE patients were women; the mean age-onset was 32 ± 15; 60% presented articular involvement, 37% renal affectation, 88% cutaneous lesions, 62% hematopoietic alterations, 50% neurological disease, and 27% presented serositis. Antinuclear antibodies were presented in 97% of the patients, with antidouble-stranded DNA antibodies (anti-ds DNA) found in 54% of patients. In addition, clinical activity or severity was performed determining the SLEDAI score every 6 months. For association studies, *P*-values were calculated by the χ^2 method or the Fisher's exact test when appropriate. Odds ratios (ORs) with 95% confidence intervals were calculated according to Woolf's method. A *P*-value below 0.05 was considered statistically significant. For non-parametric data analysis, the Mann–Whitney *U*-test was used for ordinal variables and Fisher's exact test for dichotomous variables.

Table 1 summarizes the genotype and allele distribution frequencies of the −94ins/delATTG *NFKB1* promoter polymorphism in RA patients, SLE patients, and healthy controls. The observed allele frequencies in our control population for the −94ins/delATTG variation were in good agreement with allele frequencies found in other North-American Caucasian population (9). No statistically significant differences were observed when the −94ins/delATTG genotypes and allele distribution between RA patients, SLE patients, and healthy controls were compared. We found the del/del genotype in 14% of the healthy subjects, in 10.7% of the RA patients, and in 10.5% of the SLE patients, but this slight difference did not reach statistical significance. Similarly, no differences in the −94ins/delATTG genotype and allele distribution were found when RA patients and

Frequencies of −94ins/delATTG *NFKB1* promoter polymorphism genotypes and alleles in rheumatoid arthritis (RA) patients, systemic lupus erythematosus (SLE) patients, and healthy subjects

−94ins/delATTG	RA patients $n = 272$ (%)	SLE patients $n = 181$ (%)	Controls $n = 264$ (%)
Genotypes			
del/del	29 (10.7)	19 (10.5)	37 (14)
del/ins	131 (48.2)	89 (49.2)	113 (42.8)
ins/ins	112 (41.2)	73 (40.3)	114 (43.2)
Alleles			
del	0.35	0.35	0.35
ins	0.65	0.65	0.65

del, −94ATTG deletion; ins, −94ATTG insertion.

−94ins/delATTG and $(CA)_n$ *NFKB1* genotyping: DNA was isolated from anticoagulant-treated peripheral blood mononuclear cells using standard methods. We determined the −94ins/delATTG genotypes using a polymerase chain reaction (PCR)-based method as described (9). Briefly, a 289-bp PCR fragment was amplified from genomic DNA using the forward primer 5′-TTTAAT CTG TGAAGA GAT GTG AAT G-3′ and the reverse primer 5′-CTCTGGCTTCCTAGCAGG G-3′. The forward primer was 5′ labeled with the fluorescent dye 6-FAM. The presence or absence of the 4-bp deletion was determined by the size of the labeled PCR product on an ABI 3100 sequencer, using GENESCAN 672 software (Applied Biosystems, Foster City, CA).

Table 1

controls were stratified by the presence or absence of the shared epitope (data not shown). In order to address clinical heterogeneity, analysis of more phenotypically homogeneous subgroups of patients may be undertaken. Therefore, we analyzed demographic and clinical characteristics of RA patients according to their −94ins/delATTG genotypes, and no association was found with any of the parameters tested (data not shown).

With regard to SLE patients, the distribution of −94 delATTG allele was similar in patients with (27%) and without lupus nephritis (31%). No correlation was observed between −94ins/delATTG polymorphism and the clinical status of SLE patients. Neither we observed significant deviation in the distribution of this polymorphism and autoantibody profile (data not shown).

The distribution of alleles of the *NFKB1* $(CA)_n$ repeats in the control individuals, in the RA patients, and in the SLE patients is summarized in Table 2. In our population, 12 alleles of the *NFKB1* $(CA)_n$ repeat microsatellite were detected, with the allele size ranging from 122 to 144 bp. No statistically significant differences were observed in the genotype and allele distribution between RA patients, SLE patients, and controls. We further examined the *NFKB1* $(CA)_n$ alleles with regard to clinical and laboratory parameters of RA and SLE patients, and no significant differences were observed. In addition, the −94ins/delATTG and the $(CA)_n$ microsatellite *NFKB1* polymorphsims were evaluated for linkage disequilibrium, and we found that these two variants do not show any linkage.

Distribution of *NFKB1* CA microsatellite alleles in patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and healthy controls

NFKB1 alleles CA microsatellite repeat number	Base pair	Controls (%) $n=200$	RA patients (%) $n=197$	SLE patients (%) $n=181$
16 CA	122	0	0.5	0.4
17 CA	124	0.76	0.26	0.3
18 CA	126	21.83	17.56	19.27
19 CA	128	9.64	8.25	9.04
20 CA	130	2.28	2.21	2.24
21 CA	132	4.57	7.25	6.35
22 CA	134	6.09	8.75	7.34
23 CA	136	11.68	10.2	10.8
24 CA	138	32.99	36.28	34.67
25 CA	140	5.08	3.5	4.27
26 CA	142	4.82	4.74	4.92
27 CA	144	0.25	0.5	0.4

$(CA)_n$ microsatellite typing: Polymerase chain reaction (PCR) amplification was performed as previously described (13), using a forward primer 5′ labeled with fluorescent dye 6-FAM 5′-CTTCAGTATCTAAGAGTATCCT-3′ and a reverse primer 5′-CAAGTAAGACTCTACGGAGTC-3′. The size of the labeled PCR fragment was determined on an ABI 3100 sequencer, using GENESCAN 672 software. Selected samples were sequenced on the ABI 3100 sequencer. The sequence results confirmed quite accurately the molecular weight determined by fluorescence labeling.

Table 2

There could be several possible explanations why the association found between the −94ins/delATTG *NFKB1* with UC was not replicated in RA and SLE. With regard to the functional relevance of the *NFKB1* gene variation, the −94ins/delATTG *NFKB1* alleles appear to affect promoter activity of the *NFKB1* gene and differential nuclear protein binding (9). Nonetheless, caution should be exercised in extrapolating the results of *in vitro* experiments to the individual patient, because other factors within the disease environment may affect the NF-κB production and the biologic activity. In addition, further detailed molecular promoter studies using cell lines of different origins are needed to define the overall functional importance of −94ins/delATTG *NFKB1* polymorphism, bearing in mind that other polymorphisms in linkage disequilibrium might also be influencing the promoter activity. It is clear that NF-κB plays an important role in autoimmunity and inflammation, but the pathologic processes involved are complex and further genetic studies are required to assess the relative importance of *NFKB1* polymorphism in relation to the genetic predisposition to autoimmunity.

Although complex autoimmune diseases, such as UC, RA, and SLE, may share common pathogenic and genetic mechanisms (16), the effects of genetic, population, and clinical heterogeneity in addition to different gene–environment interaction may explain the discrepant results obtained with regard to the influence of −94ins/delATTG *NFKB1* polymorphism in UC, RA, and SLE. The lack of agreement between the different studies could also be due to the presence of false negatives because of the relatively lower power of our study. However, this is unlikely because the study had 80% power to detect the relative risk of the polymorphism (considering an OR 1.66–2.00) at the 5% significance level. In addition to this, genotype frequencies did not differ from Hardy–Weinberg expectations in the control population, and allele frequencies in our Spanish Caucasian population are similar to those reported by Karban et al. (9).

With regard to the $(CA)_n$ microsatellite polymorphism, the allele frequency distribution of the *NFKB1* $(CA)_n$ repeats observed in our study is in strong contrast with those reported by Hegazy et al. (13). We suggest that the strong differences in the allelic distribution of control population observed between the study by Hegazy et al. (13) and other studies (17–19) might be due to different molecular weight assignation, leading to result stratification. Because of this fact, a significant association with T1DM was described (13), while in concordance with our data and allele distribution, no association between *NFKB1* $(CA)_n$ allele and multiple sclerosis was found (18). Of note, another report could not confirm the highly significant

association to T1DM of *NFKB1* $(CA)_n$ alleles in a transmission disequilibrium test study (17).

Our group has previously studied several polymorphisms related to the NF-κB-signaling pathway, such as +738T/C and −62A/T, both of them mapping on the *IκBL* gene, and we found no association with RA (20, 21). The lack of association between RA and +738T/C and −62A/T on *IκBL* gene and −94ins/delATTG and $(CA)_n$ on *NFKB1* gene suggests a non-critical role of the NF-κB-signaling pathway gene polymorphism in the development of RA.

In summary, in the present study, we have analyzed the *NFKB1* promoter polymorphism −94ins/delATTG and the $(CA)_n$ repeat polymorphism in RA and SLE, and our results appear to rule out the relevance of these polymorphisms in the susceptibility or clinical features of RA and SLE in our population.

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Patients' own ability to assess activity of their rheumatoid arthritis

Sir, Increasingly patients are able to access their Rheumatology Department via nurse-led Rheumatology helplines and, due to the over-booking of our clinics and the long distances patients have to travel to attend, there clearly is a potential for departments to develop a telephone follow-up service for patients with rheumatoid arthritis (RA). Published work on Rheumatology telephone follow up concentrates on a doctor- rather than nurse-led service [1] and we have been unable to find any literature on patients' ability to assess their own disease activity on the telephone, although there is work on patients' assessment of Disease Activity Score (DAS) using a mannequin [2]. We therefore undertook a pilot study to assess this fact. We chose DAS28 as a measure of disease activity as it is a validated score for both the early and the established RA [3] and has been shown to be sensitive to change [4].

Patients attending the nurse consultant, nurse specialist and the specialist registrar clinics at Worcestershire Royal Hospital over the summer of 2005 with a diagnosis of RA were invited to participate in this study. They were asked to count the number of their tender and swollen joints using only verbal clues as to which joint, and then gave a numerical global health assessment from 0–100. The health care practitioner (HCP) then undertook a standard DAS28 assessment with global health assessment measured on a visual analogue scale and later calculated both the scores. Changes in medication and investigations requested were also noted.

A total of 50 patients were recruited (of which 32 were female; age: mean and median 59 yrs; range 31–83). The disease duration ranged from 6 months to 32 yrs with a mean of 12 and a median of 11 yrs. Of the 50 patients, 39 were on one disease modifying drug (DMARD), six were on two and five on none. Two patients were also taking an anti-TNF therapy.

The results of verbal DAS28 (vDAS28) and standard DAS28 were distributed normally with a mean vDAS28 score of 4.2 with a range of 0.46–8.54, and a mean DAS28 of 3.99 with a range of 0.76–6.68. The correlation between the scores was good with an R value of 0.895. Bland Altman plot analysis did not suggest whether the patients were more or less likely to overestimate DAS28 score at differing levels of disease activity.

Interestingly vDAS28 correlated best with verbal tender joint count, $R = 0.729$, and least well with actual swollen joint count, $R = 0.294$, whereas DAS28 correlated best with global health assessment, $R = 0.681$, and least well with actual swollen joint count, $R = 0.46$. Verbal tender joints correlated relatively poorly with actual tender joints, $R = 0.57$, as did verbal swollen joints with actual swollen joints, $R = 0.46$.

Ten patients were prescribed an increase in medication at the clinic, seven were to start, restart or increase methotrexate, two were to start leflunomide and one to start sulphasalazine. One patient was advised a reduced dose of oral steroid and one, a reduced dose of methotrexate due to mildly deranged liver function tests. An abdominal ultrasound and a pulmonary function test were requested.

These results suggest that it may be possible for patients to assess their own disease activity and that vDAS could form a part of a nurse-led telephone follow-up consultation. There are limitations to this study in that patients might have had non-verbal clues and the HCP may not have looked at inter-observer error. We also recognize the limitations of DAS28 as an assessment tool, particularly in patients who have a predominantly lower limb disease. However, we intend to assess the vDAS28 further by contacting patients the day before they attend our nurse-led anti-TNF clinic and if there is a good correlation between the vDAS and standard DAS at the clinic visit, we will aim to offer the alternate mode of consultation through the telephone. Such a strategy would require dedicated clinic slots for patients who are identified as needing more detailed assessment, investigations or a change in therapy. Funding for such clinics would also need to be identified by healthcare commissioners.

The authors have declared no conflicts of interest.

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Polymorphisms of the *FCRL3* gene in a Spanish population of systemic lupus erythematosus patients

Sir, Receptors for the Fc portion of IgG (FcγRs) are essential mediators of the inflammatory effect of immune complexes and cytotoxic antibodies [1, 2]. FcγRs are candidate genes to the susceptibility to autoimmune disease. A new family of FcRs, FcR-like (FcRL) or FcR homologous (FcRH) genes, with similarity in structure and sequence to the classical FcR genes, has been recently identified [3]. They map in the chromosomal region 1q21–32, which has showed evidence of linkage with systemic lupus erythematosus (SLE) and other autoimmune diseases [4, 5]. A very recent study reported an association of the *FCRL3* gene with several autoimmune diseases [6]. The aim of this study was to investigate the association of the *FCRL3* and SLE in a large cohort of SLE Spanish patients.

We analysed a Spanish Caucasian case-control panel consisting of 520 SLE patients meeting the American College of Rheumatology (ACR) criteria for SLE [7, 8], and recruited from five Spanish hospitals. Samples were obtained from subjects after they provided written informed consent. The study was approved by all local ethical committees of the corresponding hospitals. A total of 540 matched blood and bone marrow donors were included as healthy controls. Among the patients, 59.9% had anti-dsDNA antibodies, 35.9% developed lupus nephritis and 37.5% were DRB1*03 positive. No significant differences in the frequency of the different alleles of the three polymorphisms studied were observed among the patient groups or the control groups from different cohorts. Hence, we combined all cohorts to form a SLE case-control group, which was used in further analyses. The control study population was found to be

in the Hardy–Weinberg equilibrium for all the polymorphisms studied.

Table 1 shows the distribution of genotypes and alleles of the three *FCRL3* polymorphisms studied in SLE patients and controls. As described previously [6], the concordance between the polymorphisms fcr3_3 and fcr3_6 was almost total, and so they both are referred to as fcr3_3 Single Nucleotide Polymorphism (SNP) hereafter. There was a significant deviation in the distribution of the fcr3_3 genotypes between the patient and the control groups ($P = 0.047$ by chi-square test on a 3×2 contingency table). We tested the hypothesis of a recessive model of inheritante for the proposed causal allele fcr3_3 C. Frequency of homozygous CC was higher in SLE patients (18.5 *vs* 14.3% in the control group), but the difference did not reach statistical significance ($P = 0.06$). No statistically significant differences in the distribution of fcr3_4 genotypes were detected to compare SLE patients and controls ($P = 0.8$ by chi-square test on a 3×2 contingency table). Also no significant differences in the distribution of the allelic frequencies were observed to compare SLE patients and controls in any case.

Table 1 shows data for the three most common fcr3_3/4/6 haplotypes (frequency $> 5\%$) found in our population. A significantly higher frequency of the CGA haplotype was found among patients (15.7 *vs* 12.4%, $P = 0.04$, OR = 1.32, 95% CI 1.00–1.75).

No significant differences in the distribution of these polymorphisms were observed when comparing individuals with *vs* without anti-dsDNA antibodies, having *vs* not having lupus nephritis and DRB1*03 positive *vs* negative (data not shown).

Validation of genetic association studies requires replication using independent data set in order to search for functional variants relevant to disease ethiology [9]. Results of the present work cannot completely confirm the recent finding that *FCRL3* is associated with SLE [6]. We found a different genotype distribution of the proposed causal variant among SLE patients and controls. Although our results for CC genotype were not statistically significant (statistical power $< 78\%$ to detect an OR = 1.49), they showed the same trend as the Japanese study.

Table 1. Frequency of *FCRL3_3* and *FCRL3_4* polymorphisms and distribution of the most frequent *FCRL3_3/4/6* haplotypes in Spanish SLE and healthy controls

	SLE	Healthy controls	*P*	OR (95% CI)
FCRL3_3	$n = 520$ (%)	$n = 540$ (%)		
T/T	185 (35.6)	177 (32.8)		
C/T	239 (46.0)	286 (53.0)		
C/C	96 (18.5)	77 (14.3)	0.06	1.36 (0.97–1.91)
T	609 (58.6)	640 (59.3)		
C	431 (41.4)	440 (40.7)	NS	
FCRL3_4	$n = 495$ (%)	$n = 459$ (%)		
G/G	264 (51.3)	236 (51.4)		
G/A	188 (38.0)	178 (38.8)		
A/A	43 (8.7)	45 (9.8)		
G	725 (72.3)	650 (70.8)		
A	274 (27.7)	268 (29.2)	NS	
Fcr3/4/6 Haplotypes	$2n = 922$ (%)	$2n = 848$ (%)		
1 T-G-G	523 (56.7)	497 (58.6)	NS	
2 C-A-A	245 (26.6)	220 (26.0)	NS	
3 C-G-A	145 (15.7)	105 (12.4)	0.04	1.32 (1.00–1.75)

FCRL3_3, 3_4 and *3_6* genotyping was carried out in genomic DNA obtained by standard methods using *Taq*man assays. The estimation of the frequency of the haplotypes taking into account the three studied positions was performed using Haploview, version 3.11 (available at the web site: http://www.broad.mit.edu/mpg/haploview/download.php). The software used to compare genotypic and allelic frequencies and to calculate OR and 95% CI was Statcalc program (Epi Info 2002; Centers for Disease Control and Prevention, Atlanta, GA, USA). *P*-values below 0.05 were considered statistically significant.

In fact, odds ratios (ORs) (1.36 *vs* 1.49) were very similar in both studies. The finding that no significant differences in the fcr3_3 allelic frequencies does not discard a recessive model as that proposed by Kochi *et al.* [6]. To analyse the haplotype distribution, we found that the CGA haplotype was the only *FCRL3* haplotype that seemed to be associated with SLE. Of note, the study by Kochi *et al.* [6] did not perform haplotype analysis in SLE, but both haplotypes bearing the fcr3_3 C allele were associated with RA with similar OR. According to our results, the presence of the fcr3_3 C allele in neutral and risk haplotypes would discard the fcr3_3 C allele as the only SLE associated variant. Genetic heterogeneity, due to variability not only in the frequency of alleles but also in diverse effects of linkage disequilibrium for other important genetic markers, seems to be the most likely cause of the discrepancies between ours and the previous results. In fact, the frequency of CAA haplotype was higher in Caucasian (31% in European American and 26% in Spanish) than in Japanese controls (19% $P < 0.0001$ in both cases), whereas the frequency of the CGA haplotype was similar in Spanish (12.4%), Japanese (14%) and European American (14%, $P > 0.05$) populations. In conclusion, our results suggest that the FCRL3_3 SNP does not play a major role in SLE susceptibility in Spanish population. Potential association of the *FCRL3* gene cannot be excluded.

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Deficient activity of von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura in the setting of adult-onset Still's disease

Sir, Adult-onset Still's disease (AOSD) is a systemic autoimmune disorder of unknown aetiology and pathogenesis, characterized by high spiking fever, a salmon-pink evanescent rash and polyarthritis. Although the aetiology and pathogenesis of this disease are not fully understood, several lines of evidence suggested that immunological mechanism play important roles in the pathogenesis [1].

Thrombotic thrombocytopenic purpura (TTP) is a potentially life-threatening disorder characterized by haemolytic anaemia, consumptive thrombocytopenia, disturbance of consciousness, fever and renal damage. A major breakthrough in the understanding of the pathogenesis of TTP is the discovery of deficient activity of the von Willebrand factor-cleaving protease (vWF-CP), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-13 [2–4]. Severe ADAMTS13 deficiency is an important pathogenetic factor for many cases of classical TTP. TTP is occasionally associated with various systemic autoimmune diseases such as systemic lupus erythematosus and Sjogren's syndrome [5, 6]. However, TTP is rarely reported occurring with AOSD. Here, we describe the first case report of AOSD that developed TTP with the detection of diminished ADAMTS-13 activity.

A 23-yr-old woman with a 4-yr history of AOSD was admitted to our hospital complaining of nausea, vomitting and gross haematuria. Since her onset of AOSD, the patient had had three exacerbations of spiking fever and polyarthritis in the past. She was administered prednisolone for recurrent spiking fever and polyarthrits at another hospital. Though D-penicillamine and methotrexate were also used at one point, they were discontinued owing to adverse effects. At the time of this current admission, renal function (serum creatinine was 0.7 mg/dl) and platelet count ($36 \times 10^4/\mu$l) were normal. However, her disease activity and polyarthritis had not been well-controlled with prednisolone only. She first visited our clinic in 1998, and three weeks after prednisolone was tapered from 15 to 14 mg/day, severe polyarthritis and low-grade fever appeared, and she was admitted to Tokyo Women's Medical University Aoyama Hospital in May 2000 (Fig. 1).

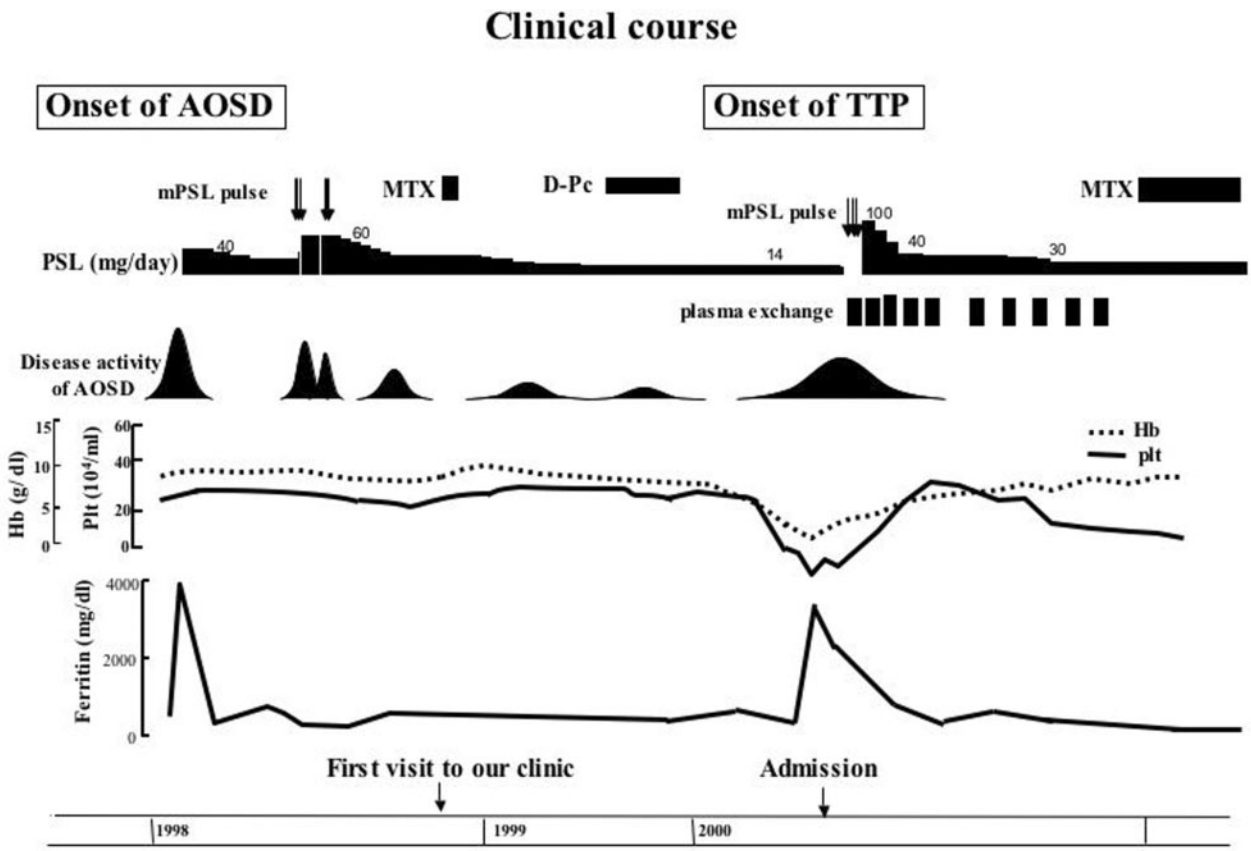

Fig. 1. Clinical course. Onset of AOSD: spiking fever, salmon-pink evascent rash and polyarthritis repeatedly appeared as the dose of corticosteroid was decreased. These symptons were ameliorated with increasing dose of corticosteroid. Just before the onset of TTP, prednisolone dose was tapered from 15 to 14 mg/day, symptoms of AOSD appeared and serum ferritin level increased. Onset of TTP: after the treatment with high-dose corticosteroid and plasma exchange, anaemia, thrombocytopenia, liver function and renal dysfunction were rapidly ameliorated. PSL: prednisolone; MTX: methotrexate; D-Pc: D-penicillamine; Hb: haemoglobin; Plt: platelet; LDH: lactic acid dehydrogenase.

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Concise Report

Interleukin 12 (*IL12B*), interleukin 12 receptor (*IL12RB1*) and interleukin 23 (*IL23A*) gene polymorphism in systemic lupus erythematosus

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***Objective*. The aim of this study was to assess the possible association between the interleukin-12B (*IL12B*) and interleukin-12 receptor beta 1 (*IL12RB1*) gene polymorphisms with systemic lupus erythematosus (SLE). In addition, we have undertaken a systematic search for genetic variants of interleukin 23 (*IL23A*).**

***Methods*. The study was conducted on 559 SLE patients and 603 ethnically matched healthy controls. Genotyping of the *IL12B* [*IL12Bpro* and *IL12B* 3′ untranslated region (UTR)] and *IL12RB1* (641A→G, 1094T→C and 1132G→C) polymorphisms was performed with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and PCR-fluorescent methods, whereas *IL23A* genetic variants were realized with direct sequencing.**

***Results*. No statistically significant differences in the distribution of the *IL12B* and the *IL12RB1* genotypes and alleles were observed when comparing SLE patients and control subjects. Additionally, no differences in the genotype and allele distribution were found when SLE patients were stratified according to the presence or absence of lupus nephritis. Despite an extensive analysis in 30 individuals, variations located in the exons and in the 5′ and 3′ UTR regions of *IL23A* gene were not found in any case.**

***Conclusions*. These results suggest that polymorphisms located in *IL12B*, *IL12RB1* and *IL23A* genes may not play a relevant role in the susceptibility or severity of SLE in the Spanish population.**

KEY WORDS: Systemic lupus erythematosus (SLE), Interleukin-12 (IL-12), Interleukin-12 receptor (IL-12R), Interleukin-23 (IL-23), Polymorphism, Susceptibility.

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with a complex pathogenesis involving multiple genetic and environmental contributions [1]. The disease is characterized by autoantibody production and inflammatory manifestation such as nephritis, vasculitis, arthritis and lymphadenopathy [2, 3]. Therefore, genetic polymorphisms of inflammatory mediators and immunological modulators, such as cytokines, are attractive genetic factors that may predispose to the development of SLE [4, 5].

Interleukin-12 (IL-12) is a pro-inflammatory cytokine that induces the production of interferon-γ (IFN-γ), favours the differentiation of T helper 1 (Th1) cells and it is a connecting point for innate and adaptive immunity [6]. IL-12 is a heterodimer formed by a 35 kDa chain (p35 or IL-12α) and a 40 kDa chain (p40 or IL-12β). IL-12β is encoded by the *IL12B* gene located on 5q31–33 and because of its pro-inflammatory and immunoregulatory activities it might be an important functional candidate gene for autoimmune diseases such as SLE. In addition, excessive IL-12 production has been found in patients with SLE [7–9]. Differences observed in IL-12 production between SLE patients and controls could be caused by variations in the regulatory regions of the *IL12B* gene. Two polymorphisms which may have a biological significance have been previously found in *IL12B* gene: an insertion/deletion of 4 bp in the promoter region and an A→C single nucleotide polymorphism (SNP) located in the 3′ untranslated region (UTR) at position 1188 [10, 11].

The biological activities of IL-12 are mediated through high-affinity binding to the IL-12 receptor (IL-12R), which is composed of two subunits: IL-12Rβ1, encoded by *IL12RB1* and IL-12Rβ2, encoded by *IL12RB2* [6]. Three SNPs have been described in *IL12RB1* (641A→G, 1094T→C and 1132G→C), causing three missense variants (Q214R, M365T, G378R) [12], which may influence IL-12-induced signalling [13].

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Interleukin-23 (IL-23) is a recently discovered cytokine that bears structural and functional resemblance to IL-12. IL-23 is a heterodimer composed of a subunit identical to IL-12 p40 and a novel IL-12 p35-related protein, p19. IL-23 shares its receptor structure and signalling pathways with IL-12. IL-23 affects IFN-γ production by T and natural killer (NK) cells, activates memory T cells and stimulates Th1-cell responses. IL-23 may enhance inflammation through stimulating the production of pro-inflammatory cytokines [14, 15]. Therefore, IL-23 may have important implications for the pathogenesis of chronic inflammatory diseases such as SLE.

The aim of this work was to examine the possible influence of the previously described *IL12B* and *IL12RB1* gene polymorphisms on the development of SLE and lupus nephritis, and also to undertake a systematic search for common genetic polymorphisms in the *IL23A* gene and to analyse the possible influence of these putative variants in the development and outcome of SLE.

Patients and methods

Subjects

A total of 559 SLE patients, meeting the American College of Rheumatology (ACR) criteria for SLE [16], were recruited from four Spanish hospitals: Hospital Virgen de las Nieves and Hospital Clínico San Cecilio (Granada), Hospital Virgen del Rocio (Seville) and Hospital Xeral-Calde (Lugo). The control group consisted of 603 healthy individuals from the same geographical areas. Both patient and control groups were Spanish Caucasians matched for age and sex. Eighty-seven per cent of the SLE patients were women, the mean age of SLE patients at diagnosis was 43 ± 13.3 yr, the mean age at onset was 32 ± 15 yr. The SLE clinical manifestations studied were articular involvement, renal effects, cutaneous lesions, haematopoietic alterations, neurological disease and serositis. All study subjects included in this study gave written informed consent. We obtained approval for the study from all local ethical committees of the corresponding hospitals.

Genotyping

IL-12 promoter insertion/deletion and 3′ UTR 1188 SNP. Genomic DNA was isolated from anticoagulant-treated peripheral blood mononuclear cells (PBMCs) using standard methods. Genotyping of the *IL12B* promoter was performed by a polymerase chain reaction (PCR)-based method as previously described [11]. Briefly, forward primer was labelled at its 5′ end with the fluorescent dye 6-FAM and the lengths of the fragments were analysed in an ABI PRISM 3100 genetic analyser using Genescan 672 software (Applied Biosystem, Foster City, CA, USA). The 3′ UTR genotypes were determined using a PCR-restriction fragment length polymorphism (RFLP)-based method as previously described [17]. DNA samples were amplified using the forward primer 5′-TTCTATCTGATTTGCTTTA-3′ and the reverse primer 5′-TGAAACATTCCATACATCC-3′. The presence of the A→C polymorphism in the 3′ UTR of the gene generates a *Taq*I site. PCR products were digested overnight at 65°C, and 3′ UTR alleles were detected after separation on ethidium bromide-stained 4% agarose gels. Samples showing one 233 bp band were typed as homozygous AA, samples displaying 165 bp and 69 bp bands were typed as homozygous CC, and samples exhibiting 233 bp, 165 bp and 69 bp bands were typed as heterozygous.

IL12RB1 641A→G, 1094 T→C and 1132 G→C polymorphisms. The polymorphisms within the *IL12RB1* gene were genotyped as previously described [18]. Briefly, forward primer 5′-AGCCAGGACTTGAACTGAGG-3′ and reverse primer 3′-TTTCTAATGCCTGCCCTGT-5′ were used to amplify exon 7 where the polymorphic position 641 is found. PCR products were digested with *Pvu*II (641A→G). Exon 10, containing polymorphisms located in 1094 and 1132 positions, was amplified using the forward primer 5′-CCCTGTAGGGT CAGGGGTA-3′ and the reverse primer 3′-CAACACCTCTC TGGGCCTTA-5′. PCR products were digested with *Nla*III and *Hpa*II to type 1094T→C and 1132G→C, respectively. The digested PCR products were analysed by electrophoresis on 3% agarose gels.

Search for polymorphisms in the IL23 gene. The detection of genetic variants in the *IL23A* gene was performed by direct sequencing. We analysed a fragment of 4.5 kb of the *IL23A* gene, including the promoter region (1000 bp upstream of the start codon), four exons, three introns and the 3′ UTR (1000 bp) of 30 healthy controls. The primers designed to amplify each of the exons and 5′ and 3′ UTR regions of the *IL23A* gene are listed in Table 1. PCR products were purified using the Millipore Montage PCR kit (Millipore Corporation, Bedford, MA, USA) This purified DNA was sequenced using the ABI PRISM 3100 genetic analyser (Applied Biosystem), and sequence data were analysed using two different sequence editor software suites: Chromas version 1.43 and DNASIS MAX version 2.0.

Statistical analysis

Allele and genotype frequencies of *IL12B* and *IL12RB1* were obtained by direct counting. Statistical analysis, to compare allele and genotype distributions, was performed by the χ^2 test. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated according to Wolf's method. using the Statcalc program (Epi Info 2002; Centers for Disease Control and Prevention, Atlanta, GA, USA). A P value of less than 0.05 was considered statistically significant. We used the UNPHASED software created for case–control analysis of haplotypes. Statistical power was estimated using Quanto 0.5 software (Department of Preventive Medicine, University of Southern California, CA, USA).

Results

IL12B typing

Table 2 shows the allele and genotype distribution of the *IL12B* promoter and the *IL12B* 3′ UTR polymorphisms in 559 Spanish SLE patients and 603 controls. In both patient and control groups, genotype and allele frequencies did not deviate significantly from those expected from the Hardy–Weinberg equilibrium. No significant differences in the allele and genotype frequencies

Table 1. *IL23A* primer sequences and expected PCR product sizes

Designation	Sequence 5′–3′	Product size (bp)	T_m (°C)
IL23A-1F	GGATTTTACCCATGGATGCA	485	54
IL23A-1R	AACGGGCTTCTTTTTCTCAG	485	52
IL23A-2F	TTCCTTCATGCGCATTCTCT	739	54
IL23A-2R	CAAATCTGGCTGGCTCTGC	739	55
IL23A-3F	TCACCTGCTGGTATAAAGGGC	831	55
IL23A-3R	CATTCACTGAAGACTCACCCA	831	52
IL23A-4F	AAGGAGAGGGGACTGAGAACA	640	54
IL23A-4R	TGTTGGCTCACAGGTGCCT	640	56
IL23A-5F	TAAAGGCAGCAGCTCAAGGA	787	55
IL23A-5R	GGCAACAGAATGAGACTCCAT	787	53
IL23A-6F	TGGGACTTTCCTCTCAAAGGA	602	55
IL23A-6R	GTGCTGGGAGCTATATATGCA	602	52

T_m, melting temperature.

of the *IL12B* promoter and *IL12* 3′ UTR variants were found between SLE patients and controls.

In addition, available clinical characteristics of patients with SLE were analysed for possible association with the different alleles or genotypes of the *IL12B* polymorphisms. No correlation was observed between *IL12B* variants and the following variables: sex, age at onset, articular involvement, cutaneous lesions, haematopoietic alterations, neurological disease and serositis (data not shown). Of interest, the frequency of homozygous individuals for the *IL12* 3′ UTR A allele was slightly increased among patients with lupus nephritis compared with patients without it, although this difference did not reach statistical significance (data not shown).

IL12RB1 typing

Genotyping of *IL12RB1* SNPs A641G, T1094C and G1132C was performed in a group of 559 SLE patients and 603 controls (Table 2). Results of this study confirm a complete linkage disequilibrium among these polymorphisms in our population and allows us to establish two haplotypes: haplotype 1 Q214–M365–G378 and haplotype 2 R214–T365–R378 [13]. No statistically significant differences were observed when the *IL12RB1* haplotype distribution was compared between SLE patients and healthy controls. In addition, no statistically significant differences were observed in the distribution of *IL12RB1* haplotypes between SLE patients with and without lupus nephritis (data not shown) or with regard to other SLE clinical manifestations (data not shown).

IL23A polymorphism screening

We analysed the *IL23A* gene to search for variants. Despite an extensive analysis in 30 individuals no sequence variations in any of the exons or 5′ and 3′ UTR regions were found. Indeed, neither of the *IL23A* polymorphisms previously described in the SNP database (http://www.ncbi.nlm.nih.gov/SNP/) could be confirmed. This is in concordance with the low frequency reported for these SNPs, indicating that the *IL23A* gene is highly conserved in humans.

Table 2. Allele and genotype frequencies of *IL12B* polymorphisms and *IL12RB1* haplotypes in SLE patients and healthy controls

	Genotype or allele	SLE	Healthy controls
IL12B promoter:		$n=559$ (%)	$n=603$ (%)
Genotype	280/280	118 (21.2)	123 (20.5)
	280/276	319 (57)	347 (57.5)
	276/276	122 (21.8)	133 (22)
		$2n=1118$ (%)	$2n=1206$ (%)
Allele	280	555 (49.6)	593 (49)
	276	563 (50.4)	613 (51)
IL12B 3′ UTR		$n=559$	$n=603$
Genotype	AA	341 (61)	394 (65.3)
	AC	202 (36)	177 (29.4)
	CC	16 (3)	32 (5.3)
		$2n=1118$	$2n=1206$
Allele	A	884 (79)	965 (80)
	C	234 (21)	241 (20)
IL12RB1		$n=559$	$n=603$
Genotype	11	276 (49.4)	267 (44.2)
	12	235(42.1)	291 (48.3)
	22	48 (8.5)	45 (7.5)
		$2n=1118$	$2n=1206$
Allele	1	787 (70.4)	824 (68.3)
	2	331 (29.6)	382 (31.7)

Discussion

The present study constitutes the first attempt to analyse the *IL12B* and *IL12RB1* polymorphisms in SLE, but provided no evidence for an association of the polymorphisms investigated in these genes with predisposition to SLE or with the development of lupus nephritis. This lack of association of the polymorphisms tested with SLE cannot be due to a lack of statistical power, because our sample size had 90% power for *IL12* 3′ UTR and *IL12RB1* and 80% for *IL12B* promoter to detect the effect of a polymorphism, conferring an OR of 1.5 at the 5% significance level. However, our study was underpowered to detect a smaller effect. The genotype frequencies of the *IL12B* promoter and 3′ UTR polymorphisms in our population were comparable with those described in other European and Australian populations [17, 19, 20].

Although a primary role for the *IL12B* 3′ UTR gene polymorphism in type 1 diabetes (T1D) has been proposed [21], several large case–control and familial studies have failed to replicate the association between the *IL12B* 3′ UTR SNP and T1D [22–24]. Similarly, a lack of association with the *IL12B* gene has been observed in a range of autoimmune diseases, such as coeliac disease [20], multiple sclerosis [25] and Crohn's disease [26], suggesting that the *IL12B* gene has a negligible effect on susceptibility to autoimmune diseases. In addition to the lack of agreement in the role that the *IL12B* 3′ UTR may play in the susceptibility to autoimmune diseases, the biological significance of this polymorphism has been called into question. While several studies observed differences in *IL12B* gene expression regarding the *IL12B* 3′ UTR genotypes [21, 26], other authors have not observed any correlation between *IL12B* genotype and IL-12 expression [23, 27].

To further test the involvement of the IL-12 pathway in susceptibility to SLE, a gene encoding another component of this pathway, *IL12RB1*, was chosen for analysis. We found no association between the *IL12RB1* (641A→G, 1094T→C and 1132G→C) gene polymorphisms and susceptibility to SLE and development of lupus nephritis. Our findings are in agreement with others reporting no association of these polymorphisms and T1D [18, 28]. As previously reported, these three *IL12RB1* polymorphisms were in a high linkage disequilibrium forming two major haplotypes in our population [12]. The haplotype frequency of these *IL12RB1* polymorphisms was similar to that described in other Caucasian populations [18]. Further analysis to observe functional differences between *IL12RB1* haplotypes is important to establish their significance in Th1-mediated immune diseases. We cannot excluded the possibility that other genes in the IL-12 pathway that are not in linkage disequilibrium with the alleles tested may contribute to the development of SLE.

Having found no association with these polymorphisms in *IL12B* and *IL12RB1* genes and the pathogenesis of SLE, we considered a new member of the IL-12-related cytokine family, IL-23, as a candidate for involvement in SLE. However, no SNPs were found among 30 samples from healthy donors, suggesting that an important role for an *IL23A* polymorphism in SLE is unlikely. Our results are in concordance with the reported low frequency of the *IL23A* SNPs in other populations of European ancestry (see http://www.ncbi.nlm.nih.gov/SNP/ and http://www.innateimmunity.net/IIPGA2/PGAs/InnateImmunity). In conclusion, our data do not provide a direct role for the tested polymorphisms in *IL12B* and *IL12RB1* genes in predisposition to SLE nor in the development of lupus nephritis in our population. In addition, we have confirmed the high level of conservation of the *IL23A* gene.

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Analysis of interleukin-23 receptor (*IL23R*) gene polymorphisms in systemic lupus erythematosus

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Key words
IL23R gene; polymorphism; susceptibility; systemic lupus erythematosus

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Abstract

The aim of this study was to evaluate the association between systemic lupus erythematosus (SLE) and polymorphisms in the interleukin-23 receptor (*IL23R*) gene, which have been previously found to be associated with two autoimmune diseases: inflammatory bowel disease and psoriasis. Our study includes 224 SLE patients and 342 healthy controls. The genotyping of *IL23R* variants was carried out using a polymerase chain reaction system with predeveloped TaqMan allelic discrimination assays. No statistically significant differences were observed between SLE patients and healthy controls with any of the *IL23R* genetic variants. In addition, we did not find any significant differences when we stratified SLE patients according to their clinical and demographic features. These results suggest that *IL23R* polymorphisms do not appear to play an important role in the susceptibility or severity of SLE in the Spanish population.

Introduction

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease with a complex pathogenesis, which involves multiple genetic and environmental factors. The disease is characterized by autoantibody production, abnormalities of immune-inflammatory system functions and inflammatory manifestations in several organs. Although the pathogenesis of SLE is unknown, there is an evident familial tendency for disease expression, such as the higher concordance of SLE in monozygotic twins and familial clustering, which provides evidence on the role of genetic factors within this disorder (1). The genetic background of SLE is complex and involves multiple genes, which encode different molecules with significant functions in the regulatory pathway of the immune system (1–4). In this respect, several studies support that cytokines and their receptors play an important role in the development and progression of the autoimmune diseases, such as SLE (5, 6).

Interleukin-23 (IL-23) is a covalently linked heterodimeric cytokine, which is composed of a p19 subunit in addition to a p40 subunit, which is shared with IL-12. IL-23 also has one receptor subunit in common to IL-12, IL12Rβ1, and a unique subunit called IL-23R. This receptor is absent during the first stages of the activation of naïve T cells, suggesting that IL-23 can only influence the function of more mature cells. Although IL-12 and IL-23 share common p40 subunits, IL-23, rather than IL-12, seems to drive the pathogenesis of various experimental models of autoimmune disease (7–9). Furthermore, in contrast to IL-12, IL-23 activates a subset of T cells characterized by the

production of the cytokine IL-17, called Th17 cells (10–12). These Th17 cells express the master transcription factor RORγt and mediate chronic inflammatory and autoimmune diseases in animal models (13). IL-23 may enhance inflammation by stimulating the production of pro-inflammatory cytokines (14, 15). All these findings suggest that the IL-23R pathway may have important implications in the pathogenesis of chronic inflammatory diseases such as SLE.

Recently, a genome-wide study found that the *IL23R* gene, which maps on chromosome 1p31, is associated with susceptibility to inflammatory bowel disease (IBD) in two independent cohorts (16). An uncommon coding variant (rs11209026, Arg381Gln) confers strong protection against IBD, and additional non-coding *IL23R* variants are independently associated.

There are increasing evidences that autoimmune diseases share a common background of genetic risk (17), as suggested by the familial aggregation of autoimmunity, the overlap of the chromosomal regions linked to autoimmune diseases and the sharing of established genetic risk factors, such as *CTLA4* (18–20) and *PTPN22* (21, 22). The aim of this study was to investigate the role of *IL23R* variants in SLE susceptibility.

Patients and methods

Patients

Peripheral blood samples were collected after obtaining written informed consent from 224 SLE patients, meeting the American College of Rheumatology criteria for SLE (23, 24) and 342 healthy individuals. Both SLE patients and donors were recruited from Hospital Virgen de las Nieves and Hospital Clínico San Cecilio in Granada. Demographic characteristics of the subjects have been described previously (25). The study was approved by all local ethical committees from the corresponding hospitals.

IL23R genotyping

Eight single-nucleotide polymorphisms (SNPs) (rs1004819, rs7517847, rs10489629, rs11209026, rs1343151, rs10889677, rs11209032 and rs1495965) located in intronic, coding and 3′-untranslated regions (UTRs) have been selected. Although 10 SNPs of the previously reported SNPs were associated with IBD genetic predisposition by Duerr et al. (16), by using the tagger algorithm our group determined that rs2201841 and rs11465804 SNPs were in absolute linkage disequilibrium (LD) with rs10889677 and rs11209026 SNPs, respectively ($r^2 = 1$, Hapmap CEU data set), and were not tested (26). The eight SNPs tested in our study were genotyped by predeveloped TaqMan allelic discrimination assays (Applied Biosystems, Foster City, CA). The polymerase chain reaction (PCR) was carried out in a total reaction volume of 5 μl using the following amplification protocol: denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 92°C for 15 s, and annealing and extension at 60°C for 60 s. Post-PCR, the genotype of each sample was automatically attributed by measuring the allele-specific fluorescence in the ABI Prism 7500 Sequence Detection Systems, using the SDS 1.3.1 software for allele discrimination (Applied Biosystems). To confirm the genotype obtained by TaqMan allelic discrimination assay, direct sequencing of the selected samples of each genotype was carried out using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems). To verify the consistency of genotyping, 96 control samples for each SNP were typed twice, showing 99% identical genotypes.

Statistical analysis

Allele and genotype frequencies were obtained by direct counting. Hardy–Weinberg equilibrium (HWE) was carried out using the FINETTI (T. F. Wienker and T. M. Strom, unpublished data, http://ihg.gsf.de/cgi-bin/hw/hwa2.pl) program. In the case of no departure from HWE, the significance was calculated by 2×2 contingency tables and Fisher's exact test, and the odds ratio (OR) with 95% confidence intervals was calculated in accordance with Woolf's method. The software used was StatCalc and logistic regression programs (Epi Info 2002; Centers of Disease Control and Prevention, Atlanta, GA). When the distribution of genotypes deviated from Hardy–Weinberg proportions, we carried out an Armitage's trend test, which considered the individual genotypes rather than the alleles, used in the FINETTI software. Although it has been suggested that a deviation from HWE increases the chance of a false-positive result, the Armitage's trend test bases itself on genotype remains, which are valid even in the case of departure from HWE (27, 28). *P* values below 0.05 were regarded as statistically significant. The power of each study was calculated as the probability of detecting an association between *IL23R* polymorphisms and SLE at the 0.05 level of significance, assuming an OR between 1.5 and 2 (small effect size). Power analysis was estimated using the Quanto v 0.5 software (Department of Preventive Medicine, University of Southern California). The Haploview software was used to obtain LD pairwise values.

Results

We estimated HWE for the eight SNPs in our population. Although no departure from HWE was observed for all eight SNPs, in the cases of the rs10489629, rs11209026 and rs1495965 polymorphisms a deviation from HWE was observed in the control population ($P = 0.02$, $P = 0.001$ and $P = 0.005$, respectively). Despite this finding, allelic

frequencies for all SNPs in our control population were very similar to those reported for other White populations (16, 29). Similar to the other populations tested, we observed a strong LD among the eight SNPs under study ($D' = 0.7–1$).

Table 1 shows the minor allele frequency and genotype distribution of the *IL23R* polymorphisms in SLE patients and healthy controls, after applying the appropriate statistical analysis for each genetic variant according to HWE estimations. A significant deviation in the distribution of the rs10489629 polymorphism was found. This deviation corresponds to an increase in frequency in the GG genotype among SLE patients (31.7% *vs* 22.8% in the control group). However, this difference did not reach statistical significance after carrying out Armitage's trend test ($P = 0.2$). Similarly, no statistically significant differences were observed when allele and genotype distribution was compared between SLE patients and healthy controls with any other *IL23R* genetic variants. In addition, available clinical features of patients suffering from SLE were analysed for possible association with the different alleles or genotypes of *IL23R* polymorphisms. However, once we stratified SLE patients according to the presence of renal involvement, no statistically significant differences were observed in the distribution of *IL23R* polymorphisms between SLE patients with or without lupus nephritis (data not shown). Similarly, no significant differences were observed between these genetic variants and the following variables: sex, age at onset, articular involvement, cutaneous lesions, photosensivity, haematological alterations, neurological disorders and serositis (data not shown).

Discussion

This study constitutes the first attempt to determine the contribution of *IL23R* polymorphisms in SLE. These variants in the *IL23R* gene have been recently associated with protection against an autoimmune inflammatory disease, IBD (16), and this association has been replicated in different populations (26, 29–31). Because of the fact that an *IL23R* haplotype has also been identified as a psoriasis-risk marker, *IL23R* gene was postulated as a possible common genetic marker for autoimmunity.

No evidence of association of *IL23R* rs1004819, rs7517847, rs10489629, rs11209026, rs1343151, rs10889677, rs11209032 and rs1495965 genetic variants was found with SLE in a Spanish population. The possibility that this lack of association could have arisen because of type II error (false negative) seems to be unlikely because our sample size has >70% power to detect the effect of the polymorphisms considering an OR between 1.5 and 2. The allele and genotype frequencies observed in our study were similar to those described previously in other White populations (16, 29). There are two possible reasons for this lack of association with a systemic autoimmune disease, such as SLE. First, it is known that IL-23 is critical for the pathogenesis of organ-specific autoimmune diseases, including experimental autoimmune encephalomyelitis (7), collagen-induced arthritis (8) and IBD (11), but not for the developed lupus-like autoimmune disease in mice models (32). Therefore, it is possible that IL-23R may play a more important role in regulating local inflammation than systemic inflammation. Second, IL-23 preferentially induces T cells to produce cytokines such as IL-17, IL-6 and tumour necrosis factor-α, but IL-23 does not induce the production of type I interferons, which play a relevant role in the development and maintenance of the disease process in SLE (33–34).

Interestingly, in a previous study, we failed to find an association between *IL12B* and *IL12RB1* gene polymorphisms and SLE susceptibility (35), as well as rheumatoid

Table 1 Minor allele frequency and genotype distribution of *IL23R* polymorphisms in SLE patients and healthy controls

			SLE patients ($n = 224$)				Healthy controls ($n = 342$)					
	Alleles		Genotypes				Genotypes					
IL23R SNPs	1	2	11	12	22	MAF	11	12	22	MAF	Allele *P* value	OR (95% CI)
rs1004819	G	A	120	90	14	0.26	170	137	35	0.30	0.1[a]	0.83 (0.63–1.10)
rs7517847	A	C	69	106	49	0.45	121	153	68	0.42	0.3[a]	1.14 (0.9–1.45)
rs10489629	G	A	71	103	50	0.45	78	192	72	0.49	0.2[b]	0.86
rs11209026	G	A	194	28	2	0.07	302	34	6	0.07	0.8[b]	1.08
rs1343151	G	A	71	119	34	0.42	136	157	49	0.37	0.1[a]	1.21 (0.94–1.53)
rs10889677	C	A	112	95	17	0.29	167	134	41	0.32	0.3[a]	0.88 (0.67–1.13)
rs11209032	G	A	110	95	19	0.30	161	137	44	0.33	0.2[a]	0.86 (0.66–1.11)
rs1495965	A	G	89	98	37	0.38	135	139	68	0.40	0.6[b]	0.93

MAF, minor allele frequency.
[a] Chi-squared test *P* value.
[b] Armitage's trend test *P* value.

arthritis susceptibility (36). Furthermore, we searched for common genetic polymorphisms in the *IL23A* gene by direct sequencing. We analysed a fragment of 4.5 kb including the promoter region, four exons, three introns and the 3′UTR in 30 individuals; however, no sequence variations were found (35). These current data, together with what has been found previously in other IL-12 cytokine gene family members (*IL12*, *IL23* and *IL12RB1*), suggest that these cytokines do not appear to play an important role in genetic predisposition to SLE.

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SHORT COMMUNICATION

Evidence of association of macrophage migration inhibitory factor gene polymorphisms with systemic lupus erythematosus

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*The aim of this study was to evaluate the potential association of functional polymorphisms of macrophage migration inhibitory factor with systemic lupus erythematosus. Our study includes 711 systemic lupus erythematosus (SLE) patients and 755 healthy controls. We genotyped the migration inhibitory factor (MIF) −173G/C using a polymerase chain reaction (PCR) system with predeveloped TaqMan allelic discrimination assay and the MIF −794 $CATT_n$ microsatellite polymorphism using a PCR-fluorescent method. A statistically significant difference in the distribution of the MIF −173*C allele between SLE patients and controls (P = 0.004, OR = 1.34, 95% CI = 1.05–1.27) was observed. In addition, the frequency of the MIF −173*C/C genotype was higher in SLE patient (P = 0.002, OR = 2.58, 95% CI = 1.32–5.10). No differences in the distribution of $CATT_n$ were found. However, the haplotypes analyses showed that only the $CATT_7$-MIF −173*C haplotype was associated with a higher susceptibility to SLE (P = 0.001, OR 1.84, 95% CI 1.35–2.79). No association with clinical features was detected in any case. These results suggest that both, MIF −173*C allele and $CATT_7$-MIF −173*C haplotype, confer susceptibility to SLE in our population.*

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Keywords: *polymorphism; systemic lupus erythematosus; macrophage migration inhibitory factor; inflammation*

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with a complex pathogenesis involving multiple genetic and environmental factors. The genetic background of SLE is complex and involves multiple genes encoding different molecules with significant functions in the regulation of the immune system.[1–4] In this respect, several studies support that cytokines play an important role in the development and progression of the autoimmune diseases.[5,6]

Macrophage migration inhibitory factor (MIF) is an immunoregulatory cytokine that has proinflammatory, hormonal and enzymatic activities, and it is expressed in a wide variety of cell types including macrophages, B and T cells.[7,8] There are several lines of evidence supporting the *MIF* gene as a good candidate in inflammatory disorders, such as SLE.[9] *MIF* plays a critical role in regulation of T-cell activation, and it has been demonstrated that alterations in this pathway lead to the development of SLE in animal models[10] *MIF* may provide signals for B-cell proliferation that could maintain the hyperactivity of B cells showed in SLE patients.[11] *MIF* might also contribute to the chronic inflammatory injury in SLE, due to, induction of proinflammatory cytokines, such as interleukin (IL)-1β, tumor necrosis factor α (TNF-α), IL-2, IL-6, IL-8 and interferon γ (IFN-γ), which seem to be associated with the severity of SLE.[7] *MIF* is able to increase the nitric oxide (NO) production, which can directly mediate the inflammatory process. In addition, *MIF* inhibited p53 expression,[12] and it has been demonstrated that p53 has apoptotic effects *in vitro* and *in vivo*, which is consistent with the possible impairment of apoptosis in SLE. Finally, MIF serum levels are significantly increased in SLE patients[13] and functional polymorphisms of the human *MIF* gene have been associated with increased susceptibility to inflammatory and autoimmune diseases.[14]

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The *MIF* gene maps to chromosome 22q11.2. Four polymorphisms have been reported in the human MIF gene,[15,16] including a microsatellite consisting of a different number of CATT repeats located in the promoter region of the gene (beginning at position −794) and three single-nucleotide polymorphism (SNP) at positions −173 (rs755622), +254 (rs2096525) and +656 (rs2070766). The rs2096525 and rs2070766 are located in introns whereas rs755622 is located in the position −173 within the promoter region of the gene and the −173C allele (MIF −173*G/C) has been associated with a higher production of MIF protein.[16] On the other hand, the allele $CATT_7$ of the −794 microsatellite polymorphism has been associated with higher levels of MIF gene transcription *in vitro*.[16,17] The aim of this study was to investigate the potential association of the functional *MIF* −173G/C and −794 $(CATT)_n$ polymorphisms with the susceptibility and/or clinical features of SLE.

Our study includes 711 SLE patients and 755 blood donors. Both patients and donors were recruited from five Spanish hospitals: Hospital Virgen de las Nieves and Hospital Clinico (Granada), Hospital Virgen del Rocio (Seville), Hospital Carlos-Haya (Malaga) and Hospital Xeral-Calde (Lugo). The patient and control groups were of Spanish Caucasian origin and were matched for age and sex. Eighty-seven percent of the SLE patients were women, the mean age of SLE patients at diagnosis was 43 ± 13.3 years and the mean age at disease onset of SLE symptoms was 32 ± 15 years. The SLE clinical manifestations studied were articular involvement (76%), renal affectation (37%), cutaneous lesions (62%), hematopoietic alterations (73%), photosensivity (51%), neurological disease (17%) and serositis (28%). The study was approved by all local ethical committees from the corresponding hospitals.

Results and discussion

The genotype frequencies were not found to be significantly different from those predicted by the Hardy–Weinberg equilibrium in healthy controls and SLE patients for the polymorphisms studied. The homogeneity between five Spanish populations was calculated using a Pearson χ^2 goodness-of-fit test, then we decided combine the groups.

With regard to the *MIF −173G/C* polymorphism, the genotype and allele distribution in SLE patients and healthy controls are shown in Table 1. Statistically significant differences in the distribution of the genotypes were observed comparing SLE patients and healthy subjects ($P = 0.005$ by χ^2 on 2×3 contingency table). Frequency of the C/C genotype was higher in the SLE patient group than in the healthy control group (4.6 versus 1.8%, $P = 0.002$, OR = 2.58, 95% CI = 1.32–5.10). Differences in the distribution of the allele frequencies were also observed, being the −173*C allele overrepresented in SLE patients (17 versus 13.2%, $P = 0.004$, OR = 1.34, 95% CI = 1.05–1.27).

Table 2 shows the genotype and allele distribution of the CATT repeat polymorphism in SLE patients and healthy controls. Four alleles having from five to eight repeats were detected in our control population with similar frequencies to those reported in other white

Table 1 Frequency of *MIF −173G/C* alleles and genotypes among SLE Spanish patients and healthy controls

MIF −173G/C Genotype	*SLE* n = *711 (%)*	*Controls* n = *755 (%)*	P-*value*	*OR (95% CI)*
G/G	503 (70.8)	570 (75.5)	NS	
G/C	175 (24.6)	171 (22.7)	NS	
C/C	33 (4.6)	14 (1.8)	0.002	2.58 (1.32–5.10)
Allele	2n = *1422 (%)*	2n = *1510 (%)*		
G	1181 (83)	1311 (86.8)		
C	241 (17)	199 (13.2)	0.004	1.34 (1.05–1.27)

MIF −173 genotyping was performed using a pre-developed TaqMan allelic discrimination assay (part number: C_2213785_10, Applied Biosystems, Foster City, CA, USA). The PCR was carried out with mixes consisting of 8 ng of genomic DNA, 2.5 μl of Taqman master mix, 0.125 μl of 20 × assay mix and ddH_2O up to 5 μl of final volume. The amplification protocol used was 50°C for 2 min and initial denaturation at 95°C for 10 min followed by 50 cycles of denaturation at 92°C for 15 s, and annealing/extension at 60°C for 1 min. After PCR, the genotype of each sample was automatically attributed by measuring the allele-specific fluorescence in the ABI Prism 7900 Sequence Detection System, using the SDS 2.2.2 software for allele discrimination (Applied Biosystems, Foster City, CA, USA). To confirm the genotype obtained by predeveloped TaqMan allelic discrimination assay, direct sequencing using ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) had been performed. For Hardy–Weinberg equilibrium and statistical analysis to compare allelic and genotypic distributions, we used the χ^2-test. Odds ratio (OR) with 95% confidence intervals (95% CI) were calculated using the software StatCalc program (Epi Info 2002; Centers of Disease Control and Prevention, Atlanta, GA, USA).

Table 2 Frequency of *MIF −794 $(CATT)_{5–8}$* alleles and genotypes among SLE Spanish patients and healthy controls

MIF −794 $(CATT)_{5–8}$ Genotype	*SLE* n = *711 (%)*	*Controls* n = *755 (%)*
5/5	67 (9.4)	55 (7.3)
5/6	212 (29.8)	254 (33.6)
5/7	29 (4.1)	37 (5)
5/8	0	1 (0.1)
6/6	305 (42.9)	318 (42.1)
6/7	84 (11.8)	81 (10.7)
7/7	14 (2)	9 (1.2)
Allele	2n = *1422 (%)*	2n = *1510 (%)*
5	375 (26.4)	402 (26.6)
6	906 (63.7)	971 (64.3)
7	141 (9.9)	136 (9)
8	0	1 (0.1)

MIF CATT repeats genotyping was carried out by PCR using a primer labeled with a fluorescent dye as previously described.[18] In brief, we used the following primers: 5′-TTG CAC CTA TCA GAG ACC-3′ as forward primer 5′ labeled with 6-FAM and 5′-TCC ACT AAT GGT AAA CTC G-3′ as reverse. After capillary electrophoresis on an automated DNA sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems), alleles of the CATT repeat element were identified using Genotyper 3.7 software (Applied Biosystems). Some selected samples were sequenced to confirm the length of each allele.

Table 3 Frequencies of estimated CATT-MIF −173 haplotypes among SLE Spanish patients and healthy controls[a]

Haplotypes	*SLE* 2n = *1090 (%)*	*Controls* 2n = *1098 (%)*
$CATT_5$-MIF −173*G	259 (23.7)	276 (25.1)
$CATT_6$-MIF −173*G	631 (58)	669 (60.9)
$CATT_7$-MIF −173*G	18 (1.6)	25 (2.3)
$CATT_5$-MIF −173*C	17 (1.5)	27 (2.5)
$CATT_6$-MIF −173*C	69 (6.3)	55 (5)
$CATT_7$-MIF −173*C	96 (8.8)	52 (4.7)

[a]Comparison of $CATT_7$-MIF −173*C haplotype versus all other haplotypes combined in SLE patients and controls, $P = 0.001$, OR = 1.94, and 95% CI = 1.35–2.79. The haplotype analysis was constructed using the UNPHASED software.

populations.[14] No statistically significant differences between SLE patients and healthy controls were observed when the −794 $(CATT)_{5–8}$ genotype and allele distributions were compared.

Table 3 shows data of haplotype frequencies in SLE patients and healthy controls. After analysis only four common haplotypes with frequencies >4% in our control population were found: $CATT_5$-MIF −173*G, $CATT_6$-MIF −173*G, $CATT_6$-MIF −173*C and $CATT_7$-MIF −173*C. When the haplotype frequencies were compared, a significant deviation was found ($P = 0.007$) (the results were not corrected for multiple comparisons). This significant deviation corresponded mainly to an increased frequency of the haplotype $CATT_7$-MIF −173*C in SLE patients (8.8 versus 4.7%, $P = 0.001$, OR = 1.94, 95% CI = 1.35–2.79).

In addition, available clinical features of patients with SLE were analysed for possible association with the different alleles or genotypes of these *MIF* polymorphisms. When we stratified SLE patients according to the presence of renal involvement, no statistically significant differences were observed in the distribution of *MIF* polymorphisms between SLE patients with and without lupus nephritis. Similarly, no significant differences were observed between −173G/C, −794CATT and haplotypes variants and the following variables: sex, age at onset, articular involvement, cutaneous lesions, photosensitivity, hematological alterations, neurological disorders and serositis (data not shown).

This study constitutes the first attempt to determine the potential implication of the *MIF* gene polymorphisms in SLE. Our results show that in Spanish population the *MIF* −173*C allele was associated with an increased risk of SLE (OR = 1.34). In addition, we demonstrated that homozygosity for the *MIF* −173*C risk allele increased significantly the susceptibility to SLE (OR = 2.58). This implies that the effect of *MIF* gene on SLE predisposition is dose-dependent. Furthermore, the MIF −173*C haplotype with the – $CATT_7$ allele was found to confer twofold increased risk of SLE susceptibility. This haplotype has been found to be associated with juvenile idiopathic arthritis (JIA),[16] rheumatoid arthritis (RA)[17] and psoriasis.[18]

The allele frequencies found in the Spanish population are similar to those reported in other white population from North America and Europe,[14] and differ significantly from those observed in other populations of different ethnic origin.[19,20] Variations in the distribution of *MIF* alleles among racial groups may suggest the existence of selective pressure acting on the *MIF* locus. Therefore, it might be of major interest to investigate the role of the *MIF* gene variants in other populations, such as African-American patients with SLE.

Several studies have reported a functional significance of *MIF* promoter polymorphism. A study performed in healthy individuals found significantly higher MIF protein serum levels in individuals carrying the *MIF* −173*C allele.[16] In addition, using a human T lymphoblast cell line a higher reporter luciferase activity for both the *MIF* −173*C allele and the *MIF* −173*C-$CATT_7$ haplotype was found, although cell type-specific differences were also reported in this study.[16] These results provide biological support for the observed association of both the *MIF* −173*C allele and the *MIF* −173*C $CATT_7$ haplotype with SLE.

Elevated levels of MIF have been correlated with indices of organ damage in SLE patients.[13] We do not currently have sufficient data to exclude the influence of the *MIF* polymorphism in the severity of SLE. In other autoimmune diseases, the influence of *MIF* polymorphism in disease severity is controversial. In this regard, the *MIF* −794 $CATT_n$ was reported to be associated with disease severity in patient with RA in the United States.[17] Recently, a correlation of *MIF* variants with high levels of radiological joint damage in RA was also reported.[21] However, a study on RA patients in the UK population supported association with susceptibility but not with disease severity.[22] In yet other studies, association of MIF with susceptibility to JIA and psoriasis is not restricted to the most severe clinical subgroups.[16,18] These observations suggest that the MIF gene may be associated with predisposition rather than with severity. These discrepancies could be explained, at least in part, by the small sample size of some clinical subgroups resulting in the lack of power to detect an association. Alternatively, *MIF* may be involved in the persistence, but no in the magnitude of the inflammatory response. Further studies using SLE clinical cohorts assessed early after the onset of the disease should be undertaken to determine the potential effect of *MIF* on SLE outcome.

There is accumulating evidence to suggest the presence of common genetic factor that predispose to autoimmunity.These findings show another piece of evidence that support the hypothesis that different genetics components may be shared between autoimmune diseases. Furthermore, the association of regulators of T-cell activation, *CTLA4*,[23] *PTPN22*[24,25] and *MIF*,[14] with autoimmune diseases indicates the importance of the regulation of T-cell response in the development of the autoimmune response. The data support the development of novel anti-MIF therapies in SLE patients[26] like the use of anti-TNF and anti-IL-6 currently being used in other autoimmune diseases.

In conclusion, our findings support that both the MIF −173*C allele and in particular the MIF −173*C-$CATT_7$ haplotype confer risk in the susceptibility to SLE.

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A new functional variant within the *IL18* promoter gene confers susceptibility to systemic lupus erythematosus

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ABSTRACT

Interleukin-18 (IL-18) is a proinflammatory cytokine that plays an important role in chronic inflammation and autoimmune diseases. In this study we aimed to determine the potential role of three polymorphisms (-137, -607, -1297) within the *IL18* gene promoter in SLE. We analysed three independent cohorts: 736 SLE and 510 healthy controls from Spain, 330 SLE and 366 healthy controls from Italy, and 276 SLE and 262 healthy controls from Argentina. The *IL18* polymorphisms were genotyped by PCR, using a predeveloped TaqMan allele discrimination assay. Quantitative real-time PCR was performed to assess *IL18* mRNA expression in PBMC from subjects with different *IL18* -1297 genotypes. We tested the effect of the *IL18* -1297 polymorphism on the transcription of *IL18* by electrophoretic mobility shift assay (EMSA) and western blot. No significant differences were observed in the distribution of the *IL18* -137 and -607 polymorphisms after correction by multiple testing comparisons (combined *P*c= 0.3 and *Pc*= 0.1, respectively). There was, however, a significant association of the -1297C allele with susceptibility to SLE (Pooled OR= 1.28, 95%CI 1.13-1.46, *combined P*= 0.00002, *Pc*= 0.00006,). We found a significant increase in the relative expression of *IL18* mRNA in individuals carrying the -1297C risk allele (*P*= 0.012), and we show that the polymorphism creates a binding site for the transcriptional factor Oct-1. These findings suggest that the novel functional *IL18* -1297 variant plays an important role in determining the susceptibility to SLE and it could be a key factor in the expression of the *IL18* gene.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease with a complex pathogenesis involving multiple genetic and environmental factors. The disease is characterized by enhanced autoantibody production, abnormalities of immune-inflammatory system function and inflammatory manifestation in several organs. Although the pathogenesis of SLE is unknown, a strong genetic component has been supported by studies on twins and families (1). Like most autoimmune diseases, the HLA genes make an important contribution, although other somewhat weaker but well established associations have been found with non-HLA genes (2).

Interleukin-18 (IL-18) is an important proinflammatory cytokine, member of the IL-1 cytokine family, which has been shown to exert innate and acquired immune responses (3-5). IL-18 is expressed by a wide range of immune cells (6) and has been found to have multiple biological functions. The IL-18 has recently been shown to be a pleiotropic cytokine that can mediate both Th1 and Th2 driven immune responses (7, 8). In

combination with IL-12, IL-18 induces IFN-γ production in Th1 cells, B cells, and natural killer cells, promoting Th1-type immune responses (9, 10), but it can also stimulate Th2 immune responses in the absence of IL-12 (11, 12). Abnormalities in the production of Th1 and Th2 cytokines have been shown in SLE patients (13). In addition, IL-18 can accelerate spontaneous autoimmune disease in MRL/*lpr* mice, characterized by glomerulonephritis, vasculitis, and symmetrical malar rash, suggestin that it is an important mediator of lupus-like disease (14). Likewise, elevated serum levels of IL-18 have been described in SLE patients compared to controls (15-17) and the elevation of IL-18 was positively correlated to SLE disease activity index (15). Because of its multiple functions in inflammation and immunological responses, a potential pathological role in the development of chronic inflammation has been suggested for IL-18, including autoimmune diseases such as SLE.

The *IL18* gene is located on chromosome 11q22.2-22.3 (18), a close linkage region with SLE in European populations (19, 20). In addition, several polymorphisms within the *IL18* promoter gene have been associated with different inflammatory and autoimmune diseases (21-28).

These findings suggest that *IL18* is a candidate proinflammatory cytokine gene involved in the susceptibility to autoimmune diseases, such as SLE. The aim of this study was to determine the relationship between the *IL18* promoter polymorphisms and their susceptibility to SLE.

RESULTS

IL18 is associated with susceptibility to SLE

Similar to the HapMap database, we observed a weak linkage disequilibrium among the three polymorphisms studied (-137 *vs* -607, *D'*= 0.21, r^2= 0.02; -137 *vs* -1297, *D'*= 0.74, r^2= 0.49; -607 *vs* -1297, *D'*= 0.20, r^2= 0.01).

In all populations, genotype frequencies were in Hardy-Weinberg equilibrium in patients and controls for the three polymorphisms analyzed. Table 1 shows the *IL18* -137 (rs187238), -607 (rs1946518) and -1297 (rs360719) genotype and allele frequencies in SLE patients and controls for the three independent cohorts from Spain, Argentina and Italy. The success rate of genotyping (that is the percentage of samples that could be analyzed) was >95% for all polymorphisms in both SLE cases and controls.

After analyzing these three promoter variants, we did not find any significant association between the *IL18* -137 and -607 polymorphisms and SLE after correcting by multiple testing comparisons in the three populations (combined *Pc*= 0.3 and *Pc*= 0.1, respectively). A significant association with susceptibility to SLE was, however, exhibited

for -1297C allele in the Spanish population (*P*= 0.00003, *P*c= 0.00009, OR= 1.45 95%CI 1.22-1.73). Interestingly, in Argentinean and Italian populations, we found that the frequencies of the -1297CC genotype were slightly increased in SLE patients compared with healthy controls (*P*= 0.02, OR= 1.88 95%CI 1.07-3.31 in Italians and *P*= 0.09, OR= 1.70 95%CI 0.89-3.23 in Argentineans), although these differences did not reach statistical significance in the allele frequencies (*P*= 0.2, OR= 1.17 95%CI 0.92-1.47 in Italians and *P*= 0.4, OR= 1.11 95% CI 0.85-1.45 in Argentineans).

Using homogeneity and combinability test according to the Breslow-Day method, we carried out a meta-analysis comprising 2480 individuals. We then used the Mantel-Haenszel test to calculate pooled OR for *IL18* -1297 polymorphisms (Table 1), and corroborated the genetic association with SLE for the *IL18* -1297 polymorphism (pooled OR= 1.28, 95%CI 1.13-1.46, *P*= 0.00002, Pc= 0.00006).

In addition, the clinical and demographic features of patients with SLE were analyzed for possible association with the different alleles or genotypes of the three *IL18* polymorphisms. No statistically significant differences were observed in the distribution of these variants (data not shown).

In view of these interesting results, we decided to perform functional experiments in order to confirm the potential role of the *IL18* -1297 polymorphism in the pathogenesis of SLE.

Expression analysis

A relative quantification of mRNA was performed in total RNA from 23 healthy individuals carrying different genotypes for *IL18* -1297 polymorphism (Figure 1). A statistically significant deviation was observed when we compared the relative expression of the *IL18* in samples from healthy subjects stratified according to their *IL18* -1297 genotypes, showing an increased expression in individual carriers of the C allele (CC+CT: *n*= 11, *vs* TT: *n*= 12; *P*= 0.012).

EMSA

Our in silico analysis of the wild-type and variant sequences (http://www.cbrc.jp/research/db/TFSEARCH.html) indicates that transcription factor Oct-1 binds to the protective allele (T) but not to the risk allele (C) at position -1297. To investigate the effect of this polymorphism on transcription factor binding, we performed an EMSA analysis. We observed a higher level of biding of protein to the *IL18* -1297T allele than to the -1297C allele (Figure 2), which support the sequence based prediction of Oct-1 binding to the -1297 alleles of *IL18* gene.

Western blot analysis

To test the interaction between IL-18 and Oct-1 we analysed the expression patterns of both proteins in controls and stimulated Jurkat cells. Western blot analysis showed an

increased expression of the IL-18 after activation of Jurkat cells with LPS (Figure 3). Conversely, we observed that the Oct-1 expression is decreased after stimulation.

DISCUSSION

Although the role of IL-18 in inflammatory and autoimmune processes has been well established (14), the available genetic data are largely contradictory, reflecting the small samples used, the different diseases analyzed and the ethnic groups investigated (29). The aim of this study was to investigate the role of *IL18* gene variations in SLE For this purpose we performed the most powerful genetic study to date. Regarding the *IL18* -137 and -607 polymorphisms, we could not confirm the previously reported association with SLE (28) in our three independent Caucasian cohorts from Spain, Italy and Argentina. These contradictory data could be due to genetic or environmental ethnical heterogeneity, which is clearly present, since allele and genotype frequencies are significantly different between Chinese and Spanish populations. Similarly, these ethnic differences in allele frequency of autoimmune disease-associated polymorphisms have been found in other susceptibility genes to SLE, such as *PDCD1* and *PTPN22* (30).

We have identified a novel functional variation determining the expression of the *IL18* gene (*IL18* -1297), which could be the key genetic variant in the role of the *IL18* gene in autoimmune diseases. Our results have shown that, in the combined analysis of the three populations, including 1342 SLE patients and 1138 healthy controls, the *IL18* -1297C allele was associated with an increased risk of SLE (OR 1.28). Interestingly, we observed an increased expression of IL-18 levels in correlation with the *IL18* -1297 polymorphism.

An important issue is how this *IL18* variant affects the expression of the gene. Several transcription factor binding sites that may be involved in the gene regulation of *IL18* were identified by Kalina et al (31). The -1297 polymorphism in *IL18* leads to loss of the Oct-1 transcription factor binding site. Oct-1 is known as a ubiquitously expressed factor and is involved in the regulation of several genes. It can also repress the expression of certain genes, including some cytokines (32-36). We have shown that the presence of the T allele at position -1297 is critical for transcriptional suppression of the *IL18* gene and that such suppression is mediated through Oct-1 binding. This suppression would result in reduced IL-18 production and potential protection against IL-18 overexpression in disorders such as SLE, in which a persistent inflammatory response appears to be an underlying pathogenic process. Such a functional explanation would be consistent with the observation that the -1297C allele is associated with SLE, in which

IL-18 overexpression is generally observed (15-17). Thus, the functional experiments that we describe not only unequivocally demonstrate differences in the transcriptional activity of the -1297T and -1297C alleles and differential DNA binding but also identify a novel major repressor site in the *IL18* promoter (the -1297T allele).

Studies using animal models that develop spontaneous lupus-like autoimmune disease have provided further evidence that *IL18* is involved in the pathology (14, 37). Interestingly, MRL/*lpr* mice have significantly elevated serum levels of IL-18 compared with MLR/++ controls, and MRL/*lpr* mice treated with IL-18 or IL-18 plus IL-12 resulted in accelerated proteinuria, glomerulonephritis, vasculitis, and increased levels of proinflammatory cytokines. These data together with our findings suggest that IL-18 is a possible novel therapeutic target in the treatment of autoimmune SLE.

In conclusion, we have identified a novel functional variant within the *IL18* promoter region that seems to have a very important role in IL-18 expression associated with susceptibility to SLE.

MATERIALS AND METHODS

Patients

Three independent case-control cohorts from Spain, Italy and Argentina were analysed. The study includes, 736 SLE patients and 510 controls from Spain, 330 SLE patients and 366 controls from Italy and 276 SLE patients and 262 controls from Argentina. The Spanish, Italian and Argentinean SLE cases have all been previously described (38-40). Both patient and control groups were matched for age and sex in each geographic region. All cases fulfil the American College of Rheumatology (ACR) criteria for the classification of SLE (41). The samples were collected according to the Helsinki Declaration. All subjects provided informed consent for this study. The study was approved by the various institutional review boards and ethical committees at each of the participating locations.

IL18 polymorphisms selection

Several studies have re-sequenced the *IL18* gene; however, no non-synomymous variations or polymorphisms that may interfere with mRNA splicing have been discovered (42, 43). Nevertheless, there are variations within the *IL18* promoter region, that may cause alterations in transcription rate (42, 44). In this study we selected two functional promoter polymorphisms (*IL18* -137 and -607) previously associated with SLE, which were suggested to alter the *IL18* promoter activity. To make a more exhaustive examination of genetic variants within the *IL18* promoter region, we referred to the database (http://www.ncbi.nlm.nih.gov/sites/entrez) and found a variant in this region that could have a potential role in the IL-18 expression (*IL18* -1297 or rs360719). We selected this polymorphism based on the minor allele frequency and its ability to bind the transcription factor Oct-1. Location of the polymorphisms site was based

on the GenBank Accession Nos. AB015961 and BC007461 as the reference sequence.

IL18 genotyping

DNA was obtained from peripheral blood mononuclear cells (PBMC), using standard methods. The genotyping of the three *IL18* polymorphisms was performed using a predevelopment TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA). The PCR reaction was carried out in a total reaction volume of 5 μl, containing 50 ng genomic DNA as template, 2 μl of TaqMan genotyping master mix, 0.1 μl of 20x assay mix and ddH_2O up to 5 μl of final volume. The amplification protocol used was: initial denaturation at 95ºC for 10 min followed by 40 cycles of denaturation at 92ºC for 15 s and annealing/ extension at 60ºC for 1 min. After PCR, the genotype of each sample was automatically attributed by measuring the allele-specific fluorescence in the ABI Prism 7900 Sequence Detection System, using the SDS 2.2.2 software for allele discrimination (Applied Biosystems).

Real-time quantitative PCR

To analyze constitutive *IL18* mRNA expression, PBMCs from 23 selected healthy individuals were isolated by Ficoll density gradient centrifugation. Total RNA was isolated with Trizol according to the manufacture's protocol (Invitrogen, Carlsbad, CA). RNA integrity was verified both electrophoretically and by the 260/280 nm absorption ratio. Reverse-transcription was performed in a total volume of 20 μl with Superscript™ First-Strand Synthesis System for RT-PCR (Invitrogen) as recommended by the manufacturer. Real-time quantitative PCR was performed on an ABI PRISM 7500 Fast SDS (Applied Biosystems) using a TaqMan gene expression assay (Applied Biosystems) in a total volume of 20 μl using 10 μl of TaqMan Fast Universal PCR Master Mix, 1 μl of each probe and 200 ng of cDNA. Cycle conditions were 95ºC for 20 sec followed by 40 cycles at 95ºC for 3 sec and 60ºC for 30 sec. Each sample was tested in triplicate and a sample without template was included as a negative control. Relative expression levels of *IL18* mRNA were normalized according to β-actin expression using the $\Delta\Delta C_T$ method (45).

Cell culture and treatment

Jurkat cells were purchased from American Type Culture Collection (ATCC) and were cultured in RPMI (Rosweli Park Memorial Institute) 1640 medium (PAA Laboratories GmbH) supplemented with 10% fetal bovine serum (FBS, Gibco), 1 mM glutamine and 1% penicillin/streptomycin. They were kept at 37 °C in a humidified 5% CO2/95% air incubator. Cells were maintained at a cell density of 5 X 10^5 cells/ml and were then treated with 10ng/ml LPS for 24 h.

Electrophoretic mobility-shift assay (EMSA)

Nuclear extract from Jurkat cells was prepared by the mini-extraction procedure as described previously (46). The double-stranded oligonucleotides (50ng) spanning the *IL18* -1297 polymorphism were as follows: top-strand T allele oligonucleotide 5′-CACTTCGTGCTTTCATGTTATTGGCCCAAT-3′and top-strand C allele oligonucleotide 5′-CACTTCGTGCTTTCACGTTAT

TGGCCCAAT-3′. A pairof oligonucleotides corresponding to the Oct-1 consensus binding sequence (47) (5′-TGTCGAATGCAAATCACTAGAA-3′ and 3′-TTCTAGTGATTTGCATTCGACA-5′) was end-labeled with (γ-^{32}P)adenosime 5′-triphosphate (ATP) by using T4 polynucleotide kinase (Promega corporation, Madison, WI). For EMSAs with nuclear extract, 20.000-50.000 cpm double-stranded oligonucleotides corresponding to ~0.5 ng were used for each reaction. The binding-reaction mixtures were set up containing 15 pmol DNA probe, 5 μg nuclear extract, 2 μg poly(dI-dC).poly(dI-dC), and binding buffer 2x (40 mM HEPES pH 7.5, 200 mM ClNa, 4 mM Cl_2Mg, 4 mM DTT, 10% glycerol, 200 μg/ml BSA) up to 20 μl. The mixtures were incubated on ice for 15 min before adding the probe, followed by another 20 min at room temperature. Samples were loaded onto 7% nondenaturing polyacrylamide gels and electrophoresed in 0.5xTBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA pH 8.0) buffer at 100 V, followed by transfer to Whatman paper and visualized by phosphorimager analysis.

Western blot

The protein levels of IL-18 and Oct-1 were determined by western blot analysis with specific polyclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Twenty micrograms of nuclear proteins was separated on a 7.5% (for Oct-1) or 12% (for IL-18) SDS-PAGE and transferred to PVDF membrane (Immobilon P, Millipore). The membrane was blocked in washing solution (0.01 M Tris, 0.1 M NaCl, 0.1% Tween 20; pH 7.5) with 5% nonfat dried milk, for 1 hour at room temperature. It was first incubated overnight with 1μg/ml (for IL-18) or 10μg/ml (for Oct-1) of primary antibody at 4ºC and then with a peroxidise-conjugated secondary antibody for 1 hour at room temperature. The bands were detected with a chemiluminescent system (ECL, Amersham, Arlington Heights, IL) and exposed to X-ray film.

Data analysis

Allele and genotype frequencies were obtained by direct counting. We used the χ^2 test for statistical analysis to compare allelic and genotypic distributions. We assessed the quality of the genotype data by testing for Hardy-Weinberg equilibrium for all samples using Fisher´s exact test and found no differences. Odds ratios (OR) with 95% confidence intervals (95%CI) were calculated according to Woolf´s method. All statistics described above were performed using Statcalc (Epi Info 2002; Centers for Disease Control and Prevention, Atlanta, GA, USA). *P* values below 0.05 were regarded as statistically significant. The Breslow-Day test of combinability and the Mantel-Haenszel test were carried out using the StatsDirect software v2.4.6. The pooled OR was calculated according to a fixed-effects model (Mantel-Haenszel meta-analysis) as well as a random-effects model (DerSimonian-Laird). Genotypic ORs were calculated using the Unphased software with homozygosity for non-associated allele as reference with OR=1. The power was computed as the probability of detecting an association between these polymorphisms and SLE at the

0.05 level of significance, assuming an OR of 1.5 (a small effect size).

Results relative to mRNA expression are shown as mean ± standard deviation. Because the variances were homogeneous (Bartlett's test P> 0.05), a statistical analysis of the mean of relative expression of the IL-18 was performed using the ANOVA test included in Epi Info 2002.

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Table 1. Individual and pooled* genetic association analysis of *IL18* polymorphisms -137, -607 and -1297 in three sets of SLE cases and controls.

	Population		GG	GC	CC	P-Value	0dds Ratio (95%CI)	Allele G	Allele C	P-Value	0dds ratio (95%CI)
IL18 -137	Spain SLE	Cases (721)	357 (49.5%)	310 (43%)	54 (7.5%)	0.8	GC: 0.97 (0.77-1.22)	1024 (71%)	418 (29%)		
		Controls (502)	251 (50%)	220 (43.8%)	31 (6.2%)	0.4	CC: 1.21 (0.77-1.91)	722 (72%)	282 (28%)	0.6	1.04 (0.87-1.25)
	Italy SLE	Cases (330)	180 (54,5%)	120 (63.4%)	30 (9.1%)	0.6	GC: 1.08 (0.79-1.47)	480 (72.7%)	180 (27.3%)		
		Controls (355)	215 (60.6%)	123 (34.6%)	17 (4.8%)	0.03	CC: 1.94 (1.06-3.56)	553 (78%)	157 (22%)	0.03	1.32 (1.03-1.69)
	Argentina SLE	Cases (258)	111 (43%)	126 (48.8%)	21 (8.1%)	0.4	GC: 1.16 (0.81-1.65)	348 (67.4%)	168 (32.6%)		
		Controls (244)	113 (46.3%)	110 (45.1%)	21 (8.6%)	0.8	CC: 0.94 (0.50-1.75)	336 (69%)	152 (31%)	0.6	1.07 (0.81-1.40)
	Pooled	Cases (1309)	648 (49.5%)	556 (42.5%)	105 (8%)	0.5	GC: 1.06 (0.89-1.25)	1852 (70.7%)	766 (29.3%)		
		Controls (1101)	579 (52.6%)	453 (41.1%)	69 (6.3%)	0.09	CC : 1.30 (0.94-1.81)	1611 (73.2%)	591 (26.8%)	0.09§	1.11 (0.98-1.26)
	Population		**CC**	**AC**	**AA**	**P-Value**	**0dds Ratio (95%CI)**	**Allele C**	**Allele A**	**P-Value**	**0dds ratio (95%CI)**
IL18 -607	Spain SLE	Cases (734)	212 (28.9%)	380 (51.8%)	142 (19.3%)	0.8	AC: 0.98 (0.78-1.23)	804 (54.8%)	664 (45.2%)	0.03	1.20 (1.02-1.42)
		Controls (500)	166 (33.2%)	261 (52.2%)	73 (14.6%)	0.03	AA: 1.40 (1.02-1.93)	593 (59.3%)	407 (40.7%)		
	Italy SLE	Cases (347)	99 (28.5%)	178 (51.3%)	70 (20.2%)	0.3	AC: 1.15 (0.86-1.55)	376 (54.2%)	318 (45.8%)	0.5	1.06 (0.86-1.31)
		Controls (352)	112 (31.8%)	168 (47.7%)	72 (20.5%)	0.9	AA: 0.98 (0.86-1.31)	392 (55.7%)	312 (44.3%)		
	Argentina SLE	Cases (260)	74 (28.5%)	142 (54.5%)	44 (17%)	0.05	AC: 1.41 (1.00-1.99)	290 (55.8%)	230 (44.2%)		
		Controls (259)	74 (28.5%)	119 (46%)	66 (25.5%)	0.02	AA: 0.60 (0.39-0.91)	267 (51.5%)	251 (48.5%)	0.02	0.84 (0.66-1.08)
	Pooled‡	Cases (1081)	311 (28.8%)	558 (51.6%)	212 (19.6%)	0.06	AC: 1.05 (0.88-1.26)	1180 (54.6%)	982 (45.4%)		
	(Spain+Italy)	Controls (852)	278 (32.7%)	429 (50.3%)	145 (17%)	0.1	AA: 1.19 (0.94-1.51)	985 (57.8 %)	719 (42.2%)	0.04¶	1.15 (1.00-1.30)
	Population		**TT**	**TC**	**CC**	**P-Value**	**0dds Ratio (95%CI)**	**Allele T**	**Allele C**	**P-Value**	**0dds ratio (95%CI)**
IL18 -1297	Spain SLE	Cases (714)	315 (44.1%)	305 (42.7%)	94 (13.2%)	0.5	TC: 1.07 (0.85-1.35)	935 (65.5%)	493 (34.5%)		
		Controls (507)	268 (52.9%)	208 (41%)	31 (6.1%)	0.00006	CC: 2.28 (1.50-3.47)	744 (73.4%)	270 (26.6%)	0.00003	1.45 (1.22-1.73)
	Italy SLE	Cases (348)	186 (53.4%)	127 (36.5%)	35 (10.1%)	0.4	TC: 0.88 (0.65-1.19)	499 (71.7%)	197 (28.3%)		
		Controls (364)	200 (55%)	144 (39.5%)	20 (5.5%)	0.02	CC: 1.88 (1.07-3.31)	544 (74.7%)	184 (25.3%)	0.2	1.17 (0.92-1.47)
	Argentina SLE	Cases (274)	131 (47.8%)	115 (42%)	28 (10.2%)	0.4	TC: 0.86 (0.61-1.22)	377 (68.8%)	171 (31.2%)		
		Controls (245)	118 (48.2%)	112 (45.7%)	15 (6.1%)	0.09	CC: 1.70 (0.89-3.23)	348 (71%)	142 (29%)	0.4	1.11 (0.85-1.45)
	Pooled	Cases (1336)	632 (47.3%)	547 (41%)	157 (11.7%)	0.7	TC: 0.97 (0.83-1.15)	1811 (67.8%)	861 (32.2%)		
		Controls (1116)	586 (52.5%)	464 (41.6%)	66 (5.9%)	0.0000006	CC: 2.12 (1.55-2.89)	1636 (73.3%)	596 (26.7%)	0.00002‖	1.28 (1.13-1.46)

‡ Pooled OR with random effects 1.04 95%CI 0.85-1.27, *P*= 0.7,

§*Pc*= 0.3, ¶*Pc*= 0.1, ‖*Pc*= 0.00006.

Figure 1. Relative quantification of *IL18* mRNA expression in PBMCs from 23 individuals (TT, n= 12; CC+CT, n= 11).

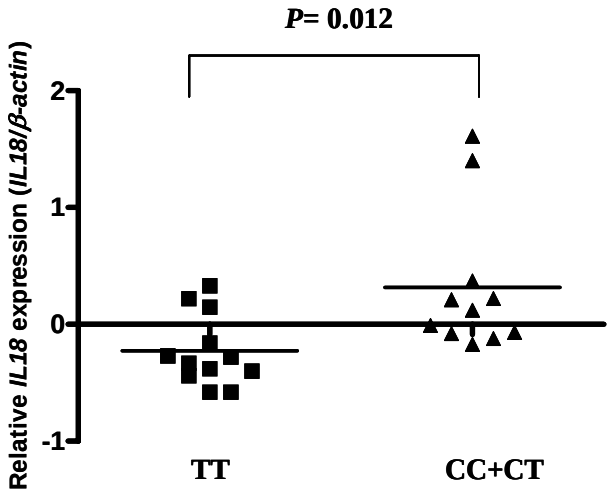
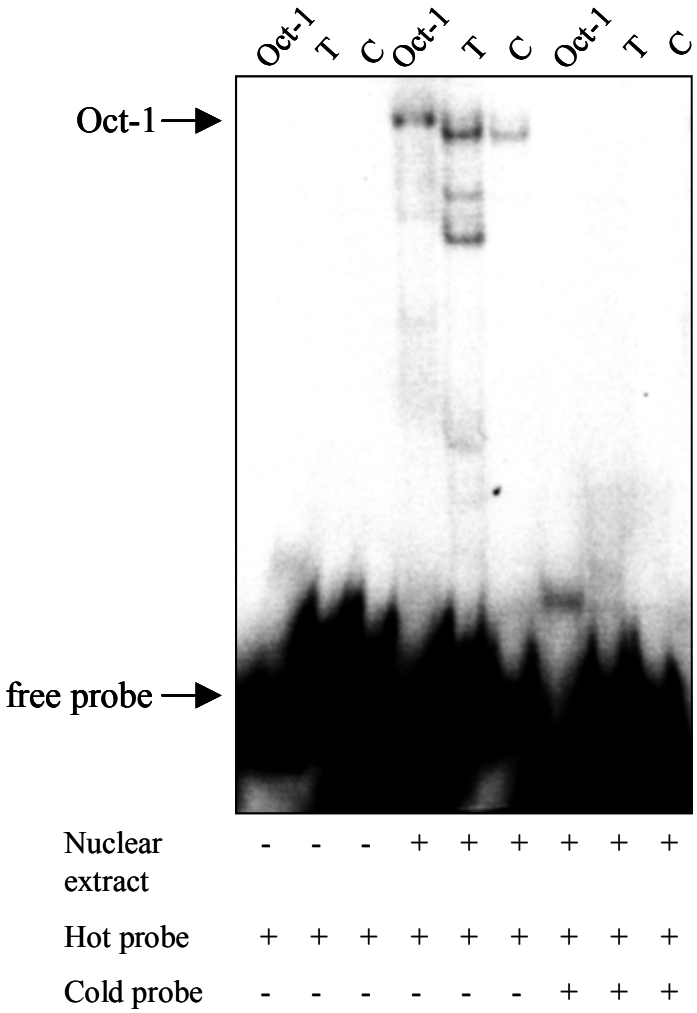


Figure 2. a) Binding abilities of the *IL18* -1297 variant in nuclear proteins from Jurkat cells analyzed by EMSA. b) Proposed model of interaction of the polymorphic site with the Oct-1 transcription factor in the *IL18* gene.

a)



b)

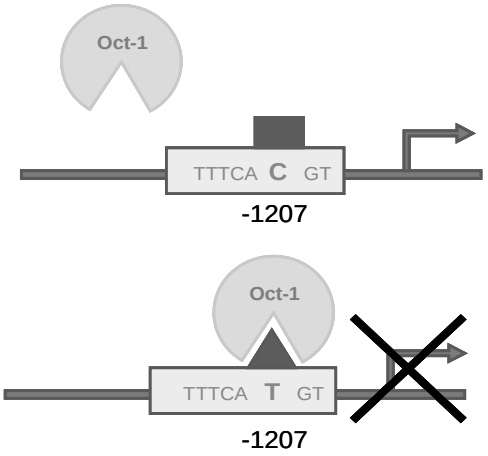
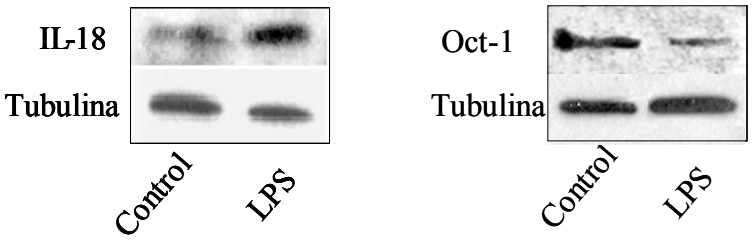


Figure 3. Western blot analysis of Oct-1 and IL-18 in Jurkat cells. Proteins from cells incubated in medium alone (controls) or LPS for 24 h were separated on a 7.5% (for Oct-1) or 12% (for IL-18) SDS-PAGE gel, transferred to a PVDF membrane and hybridized with an anti-Oct-1 or anti-IL-18 antibody. The molecular weights are 97 KDa for Oct-1 and 18 KDa for IL-18.



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No primary association of MICA polymorphism with systemic lupus erythematosus

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***Objective*. To replicate the described association between MHC class I chain-related A (MICA) gene polymorphism and susceptibility to systemic lupus erythematosus (SLE).**

***Methods*. MICA transmembrane microsatellite polymorphism was genotyped using a polymerase chain reaction (PCR)-based method. Genotyping of HLA-B* and DRB1* was performed using PCR and detection with a reverse sequence-specific oligonucleotide (SSO) probe system. Combined data for these three loci (HLA-B*, DRB1* and MICA) were obtained from a total of 333 patients and 361 healthy controls.**

***Results*. Significant association with B*08 [$P < 10^{-7}$, odds ratio (OR) 3.17, 95% confidence interval (CI) 2.02–5.00], DRB1*0301 ($P < 10^{-7}$, OR 2.07, 95% CI 1.59–2.68) and MICA5.1 ($P = 0.01$, OR 1.23, 95% CI 1.04–1.46) was observed. The combinations DRB1*0301-MICA5.1-B8 and HLA-DRB1*0301-B*08-positive and MICA5-1-negative were more frequent among SLE patients (11.4 *vs* 3.3% in healthy controls, $P = 3.9 \times 10^{-5}$, OR 3.76, 95% CI 1.85–7.73, and 6.9 *vs* 1.7%, $P = 0.0007$, OR 4.32, 95% CI 1.68–13.10, respectively). Additionally, individuals who were HLA-DRB1*0301-B*08-negative and MICA5-1-positive were less frequent among patients (22.2 *vs* 31.3% in healthy controls, $P = 0.007$, OR 0.63, 95% CI 0.44–0.89) and the magnitude of the OR was similar to that obtained in individuals negative for all the three factors (OR 0.69, 95% CI 050–0.94). Further analysis performed to detect independent association strongly suggested that the association between MICA5.1 and SLE is secondary to the linkage disequilibrium of this allele with B*08.**

***Conclusions*. Our results do not support an independent association of MICA gene polymorphism with susceptibility to SLE.**

KEY WORDS: MICA, HLA-DRB1, HLA-B, SLE, Susceptibility.

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with a complex pathogenesis involving multiple genetic and environmental factors. Production of multiple autoantibodies and immune complex deposits causing tissue damage is a characteristic of this disease. Human major histocompatibility (HLA) class I and class II molecules have been described as genetic risk factors for many autoimmune diseases [reviewed in 1–3]. Two HLA class II haplotypes, DRB1*0301-DQA1*0501-DQB1*0201 and DRB1*1501-DQA1*0102-DQB1*0602, have been consistently associated with susceptibility to SLE in different Caucasian populations [4–6]. Additionally, several studies have described an association of HLA-B8 with susceptibility to SLE [7], and other genes located in the HLA region, such as TNFA, C2 and C4 have also been associated with susceptibility to the disease [reviewed in 8, 9].

A family of non-classical HLA genes known as major histocompatibility complex class I chain-related gene A (MICA) has been located 46 kb centromeric to the HLA-B gene. The MICA gene has a full length of 11.7 kb and encodes a stress-inducible molecule with three extracellular domains (α1, α2 and α3), a transmembrane (TM) region and a cytoplasmic tail (CY) [10, 11], and it shows preferential expression in fibroblasts and epithelial cells. The MICA chain folds similarly to typical class I chains and may have the capacity to bind peptides or other short ligands. A microsatellite polymorphism consisting of a variable number of GCT repeats encoding 4, 5, 6, 7, 9 or 10 alanine residues and located on the MICA exon 5 (TM segment) has been identified. Additionally, the MICA A5.1 allele presents a nucleotide insertion (G) between the second and third triplet repeats, resulting in a premature stop codon [12–14].

The location of the MICA gene in the HLA region and its association with other autoimmune diseases suggests the MICA gene as a possible candidate gene for the development of SLE. A recent study reported an independent contribution of the MICA TM polymorphism to susceptibility to SLE in an Italian population [15]. The aim of the present study was to investigate whether the MICA TM polymorphism, as well as HLA class I (locus B) and class II (locus DRB1*), influences genetic predisposition to SLE in a large Spanish cohort of patients.

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TABLE 2. Contribution of B*08/MICA5.1, DRB1*0301/MICA5.1 and B*08/DRB1*0301 to the risk of SLE, comparing patients and controls in a 2 × 2 analysis

B*08	MICA5.1	SLE patients $n=396$ (%)	Healthy controls $n=364$ (%)	P	OR (95% CI)
+	+	56 (14.1)	21 (5.8)	0.0001	2.69 (1.55–4.70)
+	−	31 (7.8)	6 (1.7)	7.7×10^{-5}	5.07 (2.05–15.01)
−	+	116 (29.3)	124 (34.1)	NS	
−	−	193 (48.7)	213 (58.5)	0.007	0.67 (0.50–0.91)
MICA5.1	**DRB1*03**	**SLE patients $n=333$ (%)**	**Healthy controls $n=361$ (%)**	**P**	**OR 95% CI**
+	+	55 (16.5)	27 (7.5)	0.0002	2.45 (1.47–4.10)
+	−	83 (24.9)	119 (33.0)	0.02	0.68 (0.48–0.95)
−	+	65 (19.5)	47 (13.0)	0.02	1.62 (1.06–2.49)
−	−	130 (39.0)	168 (46.5)	0.05	0.74 (0.54–1.01)
B*08	**DRB1*0301**	**SLE patients $n=388$ (%)**	**Healthy controls $n=386$ (%)**	**P**	**OR (95% CI)**
+	+	69 (17.8)	19 (4.9)	$<10^{-7}$	4.18 (2.40–7.36)
+	−	71 (18.3)	59 (15.3)	NS	
−	+	18 (4.6)	10 (2.6)	NS	
−	−	230 (59.3)	298 (77.2)	10^{-7}	0.43 (0.31–0.59)

NS, not significant.

TABLE 3. Contribution of B*08/MICA5.1/DRB1*0301 to susceptibility to SLE

DRB1*0301	MICA5.1	B*08	SLE patients $n=333$ (%)	Healthy controls $n=361$ (%)	P	OR (95% CI)
+	+	+	38 (11.4)	12 (3.3)	3.9×10^{-5}	3.76 (1.85–7.73)
+	+	−	17 (5.1)	15 (4.2)	NS	
+	−	+	23 (6.9)	6 (1.7)	0.0007	4.32 (1.68–13.10)
+	−	−	41 (12.3)	41 (11.4)	NS	
−	+	+	9 (2.7)	6 (1.7)	NS	
−	+	−	74 (22.2)	113 (31.3)	0.007	0.63 (0.44–0.89)
−	−	+	7 (2.1)	2 (0.6)	NS	
−	−	−	123 (36.9)	166 (46.0)	0.01	0.69 (0.50–0.94)

NS, not significant.

$P=0.02$, OR 1.62, 95% CI 1.06–2.49). Nevertheless, frequencies of individuals who were B*08-negative-MICA5.1-positive and individuals who were DRB1*0301-negative-MICA5.1-positive were lower among patients (29.3 *vs* 34.1% in the control group, $P>0.05$, and 24.9 *vs* 33.0 in the control group, respectively, $P=0.02$, OR 0.68 95% CI 0.48–0.95).

Table 3 shows data for the eight possible phenotypic combinations of the three risk factors studied. The highest OR (4.32, 95% CI 1.68–13.10) found was for the combination B*08 positive–MICA5.1-negative–DRB1 0301-positive, whereas the lowest OR found was for the combination B*08-negative–MICA5.1-positive–DRB1*0301-negative (0.63, 0.44–0.89). Next, we investigated the association of DRB1*0301-MICA5.1-B*08 to evaluate which of these factors are likely to be the responsible of the association, using the test recommended by Sverjgaard and Ryde [19]. In this analysis, MICA5.1 did not show any association with SLE in B*08-positive ($P=0.2$) or in B*08-negative ($P=0.8$) individuals or in DR*0301-positive ($P=0.2$) or DRB1*0301-negative ($P=0.6$) individuals, suggesting that MICA5.1 is not an independent risk factor for SLE. On the contrary, B*08 was associated with SLE in MICA5.1-positive [$P(6)=0.0012$, OR 2.57, 95% CI 1.57–5.20] and negative [$P(6)=0.00012$, OR 5.70, 95% CI 2.07–17.03] individuals and DRB1*0301 was also associated in both MICA5.1-positive [$P(6)=0.0042$, OR 2.92, 95% CI 1.65–5.20] and MICA5.1-negative [$P(6)=0.05$, OR 1.79, 1.13–2.84] individuals. Regarding the independent contribution of B*08 and DRB1*0301, the frequency of B*08 was not increased in DRB1*0301-positive individuals ($P=0.1$) and was probably only increased in DRB1*0301-negative [$P=0.02$, $P(6)=0.12$, OR 1.56 95% CI 1.04–2.34] individuals. Conversely, the frequency of DRB1*0301 was increased in B*08-positive individuals [$P(6)=0.0018$, OR 2.98, 95% CI 1.55–5.77] and probably only increased in B*08 negative individuals [$P=0.03$, $P(6)=0.18$, OR 2.33, 95% CI 1.00–5.54]. Using the test recommended by Sverjgaard and Ryde [19], associations with SLE of B*08 and DRB1*0301 were stronger than the association of MICA5.1 [$P(6)=0.00036$ and $P(6)=0.024$ respectively]. Nevertheless, no significant differences in the strength of the association of B*08 and DRB1*0301 was observed ($P=0.4$). Finally, strong linkage disequilibrium between B*08 and both MICA5.1 and DRB1*0301 was observed also in the patient group [$P(9)=0.000072$ and $P(9)<10^{-6}$, respectively]. Nevertheless, no significant linkage disequilibrium between MICA5.1 and DRB1*0301 was observed among patients ($P=0.2$) or among controls ($P=0.4$), even after stratification for B*08 [$P(9)>0.05$ in all cases].

Additionally, there was no evidence for an association of MICA5.1 with SLE among B*08 positive–DRB1*0301-positive

Materials and methods

Subjects

A total of 574 SLE patients (13% men and 87% women), meeting the American College of Rheumatology (ACR) criteria for SLE [16, 17], were recruited from five Spanish hospitals: Hospital Virgen de las Nieves and Hospital Clínico San Cecilio (Granada), Hospital Virgen del Rocio (Seville), Hospital Xeral-Calde (Lugo) and Hospital Carlos-Haya (Malaga). The mean (S.D.) age of the SLE patients at diagnosis was 43 ± 13.3 yr and the mean age at onset was 32 ± 15 yr. A total of 934 blood bank and bone marrow donors from the corresponding cities were included as healthy controls. The patient and control groups were both of Spanish Caucasian origin and were matched for age and sex. Samples were obtained from subjects after they had given written informed consent. The study was approved by all local ethical committees of the corresponding hospitals.

MICA genotyping

Genomic DNA was isolated from anticoagulant-treated peripheral blood mononuclear cells using standard methods. Genotyping of the MICA TM microsatellite polymorphism was performed using a polymerase chain reaction (PCR)-based method as previously described [18]. Briefly, primer sequences were MICA 5F 5'-CCT TTT TTT CAG GGA AAG TGC-3' and MICA 5R 5'-CCT TAC CAT CTC CAG AAA CTG C-3'. The reverse primer was labelled at the 5' end with 6-FAM dye. PCR products were electrophoresed in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and their sizes were determined using the Genescan 672 software (Applied Biosystems).

HLA genotyping

Genotyping for HLA-B* and DRB1* was carried out using a reverse dot-blot kit with sequence-specific oligonucleotide (SSO) probes (Dynal RELI^{TM} SSO HLA-B and DRB1* typing kits; Dynal Biotech, Bromborough, UK). When necessary, high resolution typing of HLA-DRB1*03 samples was performed using Dynal AllSet^{TM} SSP DRB1*03.

Genotyping of the three loci HLA-B*, MICA and DRB1* was performed in 333 SLE patients and 361 healthy controls.

Statistical analyses

Allelic and phenotypic frequencies of the HLA markers studied were obtained by direct counting. Statistical analysis to compare distributions was performed with the χ^2 test. Odds ratios (OR) and 95% confidence intervals (CIs) were calculated according to Wolf's method. The software used was Statcalc program (Epi Info 2002; Centers for Disease Control and Prevention, Atlanta, GA, USA). *P*-values below 0.05 were considered statistically significant. The *P*-values were corrected (P_c) according to the Bonferroni method for the MICA TM alleles ($n = 5$). To detect the strongest association for data in Table 2, the test described by Svejgaard and Ryder was used and the *P*-values were corrected as recommended by these authors [19]. The correction factor used in each case is shown in parentheses.

Results

No significant differences in the frequency of the different alleles of the three genes studied were observed among patient groups or among control groups from the five cohorts. Hence we combined the five cohorts to form a SLE case–control group, which was used in further analyses. The control study population was found to be in Hardy–Weinberg equilibrium for all the loci studied.

TABLE 1. Frequencies of the MICA alleles in Spanish SLE patients and healthy controls

Allele	SLE patients $2n = 1148$ (%)	Controls $2n = 1868$ (%)	*P* value	P_c[a]	OR (95% CI)
A4	145 (12.6)	203 (10.9)	NS	NS	
A5	125 (10.9)	264 (14.1)	0.009	0.045	0.74 (0.59–0.94)
A5.1	322 (28.1)	449 (24.0)	0.01	0.05	1.23 (1.04–1.46)
A6	411 (35.8)	726 (38.9)	NS	NS	
A9	145 (12.6)	226 (12.1)	NS	NS	

[a] *P*-value corrected by the Bonferroni method. NS, not significant.

Table 1 shows the distribution of the MICA alleles in SLE patients and controls. As has been found in other Caucasian populations [15, 18], in our population we found five alleles: A4, A5, A5-1, A6 and A9; the MICA allele displaying the highest frequency in both patient and control groups was A6 (35.8 and 38.9% respectively). There was a significant deviation in the distribution of the alleles of MICA that distinguished between patient and control groups ($P = 0.007$, χ^2 test with a 5×2 contingency table). This significant deviation corresponded to a higher frequency of MICA5.1 [28.1 *vs* 24.0% in the control group; $P = 0.01$, $P(5) = 0.05$, OR 1.23, 95% CI 1.04–1.46] and a lower frequency of MICA5 among SLE patients [10.9 *vs* 14.1% in the control group; $P = 0.009$, $P(5) = 0.045$, OR 0.74, 95% CI 0.59–0.94]. No significant differences in the distribution of individuals homozygous for MICA5.1 were observed when patient and control groups were compared (12.0 *vs* 12.3%, $P > 0.05$).

Regarding HLA typing, the HLA-B* gene was genotyped in 399 SLE patients and 385 control subjects. The allelic frequency of HLA-B*08 was significantly higher in patients (11.0 *vs* 3.8% in the control group, $P < 10^{-7}$, OR 3.17, 95% CI 2.02–5.00). Additionally, the HLA-DRB1* was genotyped in 459 SLE patients and 522 healthy controls and, as in other studies, the allelic frequency of HLA-DRB1*0301 was higher in patients (20.4 *vs* 11.0% in controls, $P < 10^{-7}$, OR 2.07, 95% CI 1.59–2.68). The allelic frequency of HLA-DRB1*15 was higher in SLE patients (12.0 *vs* 9.4% in the control group) but the difference did not reach statistical significance ($P = 0.06$). The distribution of HLA-DRB1*08 was similar in patients and controls (3.4 *vs* 3.1%, $P = 0.7$).

Strong linkage disequilibrium between B*08 and both MICA5.1 and DRB1*0301 was observed in our control population [$P(9) = 0.00018$ and $P(9) < 10^{-6}$, respectively]. Nevertheless, no significant linkage disequilibrium between MICA5.1 and DRB1*0301 was observed in our control group ($P = 0.4$). No significant linkage disequilibrium between MICA5.1 and DRB1*15 was observed among B*08- or DRB1*0301-negative controls [$P(9) > 0.05$ in all cases].

We performed further analysis to better define the contribution of each marker to susceptibility to SLE. Table 2 shows data for the four phenotypic combinations of the three factors studied two by two. The highest OR, taking into account two positive risk factors, was for the combination B*08-positive–DRB1*0301-positive ($P < 10^{-7}$, OR 4.18, 95% CI 2.40–7.36) and the lowest OR was for the combination B*08-negative–DRB1*0301-negative ($P < 10^{-7}$, OR 0.43, 95% CI 0.31–0.59). Additionally, frequencies of individuals who were B*08 positive–MICA5.1-negative and individuals who were DRB1*0301-positive–MICA5.1-negative were higher in patients (7.8 *vs* 1.7% in controls, $P = 7.7 \times 10^{-5}$, OR 5.07 95% CI 2.05–15.01, and 19.5% *vs* 13.0% in controls,

individuals ORs were very similar for MICA5.1-negative and MICA5.1-positive individuals (4.32 *vs* 3.76).

Discussion

The MHC region has been associated with susceptibility to SLE in many studies. However, the literature concerning association with HLA-SLE is often in disagreement regarding the genes involved in the susceptibility [20, 21]. This could be due to confounding factors caused by strong linkage disequilibrium in the region.

Recently, a study suggesting an independent contribution of the MICA TM polymorphism to susceptibility to SLE in an Italian population was reported [15]. This case–control study was performed in a relatively small group of individuals (48 patients and 158 controls) and the authors reported a positive association of susceptibility to SLE with both MICA5.1 and MICA5 alleles and a negative association with MICA9. Gambelunghe *et al.* [15] reported an association between HLA-B8 and SLE in the Italian population. They also found strong linkage disequilibrium between HLA-B8 and MICA5.1, indicating that HLA-B8 was not significantly and independently associated with SLE. However, they did not perform any analysis to detect which of these genes showed the strongest association. We think this may explain the discrepant results obtained in the present work. Our study failed to confirm an independent association between MICA and SLE. The MICA5.1 allele showed an association with SLE susceptibility in the one-marker analysis, although the OR (1.23) found was lower than those obtained for B*08 (3.17) and DRB1*0301 (2.07). Tables 2 and 3 show that the presence of MICA5.1 is neutral if B*08 is absent, or even protective if DRB1*0301 or both B*08 and DRB1*0301 are absent. The magnitude of the OR (0.63), as given by the simultaneous presence of MICA5.1 and absence of both B*08 and DRB1*0301, was similar to that given by the absence of these three factors (0.69). Additionally, analysis performed to detect independent association strongly suggests that the association described between MICA5.1 and SLE is secondary to the linkage disequilibrium of this allele with B*08. Regarding MICA5, we observed a lower the frequency of this allele among patients. This contradiction with the previous study could be due to a difference in linkage disequilibrium between HLA-B* and MICA in Italian and Spanish populations and, in any case, the contrary effect found for MICA5 in both populations does not support any association of this allele. No differences in the distribution of the allele MICA9 were observed to allow comparison between patient and control groups.

Our results confirm previous findings regarding the association of HLA-B*08 and DRB1*0301 with susceptibility to SLE [reviewed in 1–5]. Data obtained in the present study are not conclusive enough to clarify which of these two markers is more strongly associated with the disease. Tables 2 and 3 show a strong positive association of susceptibility to the disease with the presence of both factors and a strong negative association with their absence. Nevertheless, individuals positive only for one of these factors are in the minority, although they are not significantly increased among patients. Additional analysis performed to detect independent association did not provide strong evidence supporting any hypothesis because several critical *P*-values are not significant after correction, both markers show a similar strength of association and they are in strong linkage disequilibrium. Regarding other class II specificities associated with SLE in different populations, as already described in other Caucasian populations, only a weak increase in the frequency of HLA-DRB1*1501 was observed in our series of patients and the distribution of DRB1*08 was very similar in both patients and controls.

In conclusion, our results do not support an independent association between MICA and SLE and are in agreement with the previous finding reporting an association with B*08 and DRB1*0301.

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Clinical Vignette

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The patient, a 69-yr-old man, had seropositive erosive rheumatoid arthritis (RA) diagnosed in 1977. He failed on the conventional disease-modifying agents of salazopyrin and methotrexate. Complications of Felty's syndrome (splenomegaly, neutropenia) and osteoporosis developed during his disease course. In 1997 he developed skin ulceration on the right first web space, left medial malleolus and right medial malleolus.

Clinically, his lower limb pulses were palpable with no venous insufficiency. Peripheral nerves were intact. Arterial pulse pressures were normal. Blood glucose and cholesterol were within normal limits. Anti-neutrophil cytoplasmic antigens were negative. Serum inflammatory markers were elevated, with erythrocyte sedimentation rate (ESR) at 90 mm/h and C-reactive protein (CRP) at 67 mg/dl. While pyoderma granulosum was considered, following a dermatological review a clinical diagnosis of vasculitic ulceration of the skin associated with Felty's syndrome was made in the light of his previous features. Treatment was high-dose corticosteroids and oral cyclophosphamide. There was an initial improvement in the size of the ulcers. After 6 months of therapy, cyclophosphamide was switched to azathioprine. With reduction in corticosteroid, the ulcers again deteriorated. Treatment was adjusted with the administration of intravenous prostaglandins and pulse cyclophosphamide. Subsequent maintenance therapy was oral cyclophosphamide.

In 2001, due to inefficacy, treatment was changed to infliximab (dose 3 mg/kg every 8 weeks). Over the subsequent months, there was a dramatic improvement at the right medial malleolus and complete healing at the other two sites. The patient's joint symptoms also diminished, although ESR remained elevated at 30 mm/h and neutropenia persisted. Due to the development of a septic prosthetic joint, infliximab therapy was discontinued. During this time there was a significant deterioration in the areas of ulceration. Improvement was noted again when anti-TNF therapy, this time adalimumab, was reinstituted with prophylactic antibiotic cover.

Anti-TNF therapy can be an effective treatment for systemic large- and medium-vessel vasculidites. Cutaneous manifestations also respond with pyoderma gangrenosum healing in patients with inflammatory bowel disease. There appears to be increased blood flow due to improved endothelial vasomotor responses following anti-TNF therapy [1]. Adverse event reporting has shown paradoxically that these agents can be associated with the development of new-onset vasculitis [2]. The mechanism of this is unclear. Our case highlights a role for anti-TNF therapy in the treatment of cutaneous ulceration secondary to RA/Felty's syndrome.

The authors have declared no conflicts of interest.

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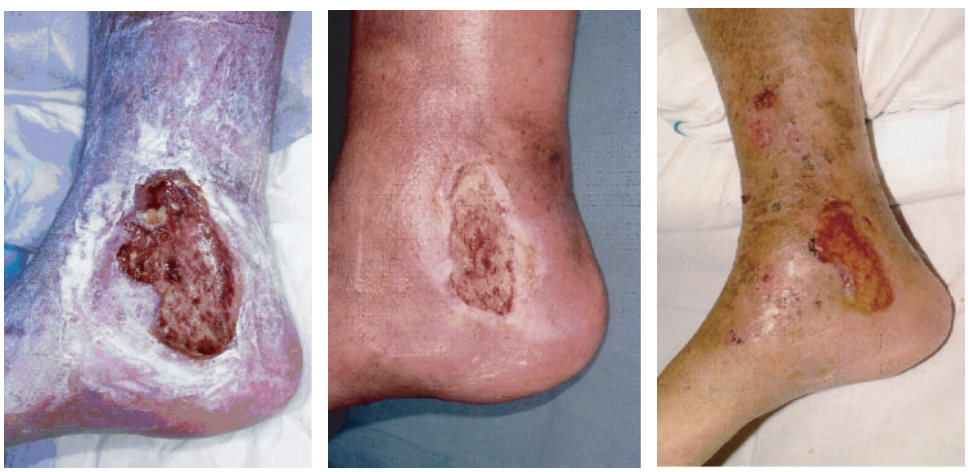

Fig. 1. Serial photographs of the ulcer at the right medial malleolus in a patient with rheumatoid arthritis following commencement on infliximab therapy.

ELSEVIER

Analysis of a Functional *BTNL2* Polymorphism in Type 1 Diabetes, Rheumatoid Arthritis, and Systemic Lupus Erythematosus

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ABSTRACT: The aim of this study was to test whether the functional variant rs2076530 of the *BTNL2* gene confers susceptibility to the autoimmune diseases type 1 diabetes (T1D), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE). Our study populations consisted of 326 patients with T1D and 351 healthy subjects, 808 patients with RA and 1137 healthy controls, and 372 patients with SLE and 280 healthy controls. Genotyping of the *BTNL2* gene rs2076530 polymorphism was performed by real-time polymerase chain reaction technology, using the TaqMan 5′-allele discrimination assay. We observed statistically significant differences in the distribution of *BTNL2*rs2076530 alleles between patients with T1D, RA, and SLE and healthy controls ($p = 0.0035$, 0.000003, and 0.00002, respectively), but in two divergent ways: the G allele was associated with T1D and RA, and the A allele was associated with SLE. However, the polymorphism exhibited strong linkage disequilibrium with *HLA DQB1–DRB1* haplotypes previously identified as predisposing to the diseases. When the *BTNL2* polymorphism was tested conditional on *HLA DQB1–DRB1*haplotypes, the *BTNL2* effect was no longer significant in all three study populations. The *BTNL2* rs2076530 polymorphism is associated with T1D, RA, and SLE because of its strong linkage disequalibrium with predisposing *HLA DQB1–DRB1* haplotypes in Caucasian populations. *Human Immunology 66, 1235–1241 (2005).* © American Society for Histocompatibility and Immunogenetics, 2006. Published by Elsevier Inc.

KEYWORDS: type 1 diabetes; rheumatoid arthritis; systemic lupus erythematosus; *BTNL2*; polymorphism

ABBREVIATIONS

BTNL2	butyrophilin-like 2
CI	confidence interval
HLA	human leukocyte antigen
LD	linkage disequilibrium
MHC	major histocompatibility complex
OR	odds ratio
PCR	polymerase chain reaction
RA	rheumatoid arthritis
SLE	systemic lupus erythematosus
SNP	single-nucleotide polymorphism
T1D	type 1 diabetes
TDT	transmission disequilibrium test

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INTRODUCTION

Type 1 diabetes (T1D), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) are among the most common autoimmune diseases [1]. These diseases share etiological pathways, which supports the recently proposed hypothesis of a common genetic basis for autoimmunity [2].

The B7-1/B7-2−CD28/CTLA-4 T-cell costimulatory pathway is vital for the initiation and termination of the immune response and plays a key role in the control of T-cell activation [3]. This pathway seems to be of great importance in the development of autoimmune diseases because of the relevance of the T-cell response in such traits [4]. Accordingly, several reports have reported exacerbated autoimmune disease in B7-1/B7-2-deficient mice [5–7].

An association between a functional polymorphism of the butyrophilin-like 2 (*BTNL2*) gene and susceptibility to sarcoidosis has been demonstrated [8]. The associated single-nucleotide polymorphism (SNP), rs2076530, constitutes a G⟶A transition that leads to a premature stop codon in the spliced mRNA of the risk-associated allele transcripts. This results in a truncated protein and the loss of its membrane location [8].

BTNL2 belongs to the immunoglobulin superfamily and its biological function is not completely understood yet. However, the homology between BTLN2 and B7-1 has suggested a costimulatory role in the T-cell activation pathway [8, 9]. Therefore, BTLN2 may play a major role in the outcome of autoimmune diseases such as T1D, RA, and SLE. *BTNL2* resides in the class II major histocompatibility complex (MHC) region of chromosome 6p [10], a region in strong linkage disequilibrium (LD) with HLA DRB1 and DQB1 genes.

As the contribution of MHC class II genes to the development of T1D, RA, and SLE is well established [11–13], any analysis of BTNL2 has to be performed conditional on MHC class II genes [14]. Because of the possible role of BTNL2 in the development of autoimmunity, the functional relevance of the rs2076530 polymorphism, and its chromosomal location, the aim of the present study was to test whether the SNP confers susceptibility to T1D, RA, and SLE independent of MHC class II.

MATERIALS AND METHODS

Patients

Type 1 diabetes. The juvenile-onset T1D cohort consisted of 326 cases and 351 controls from the southwestern part of The Netherlands. Patients had been, on average, 8.75 (0.78–16.72) years old at diagnosis, and in 88% of the study families, both parents were of native Dutch descent. The epidemiological representativeness of the cohort was ascertained to be 74% with the capture–recapture method; cases among members of the patient organization, the Dutch Diabetes Association, and those from a separate incidence study in the area were independent sources.

Rheumatoid arthritis. A total of 808 patients meeting American College of Rheumatology (ACR) 1982 revised criteria for RA [15] were recruited from four Spanish hospitals: Hospital Virgen de las Nieves (Granada), Hospital Universitario Virgen del Rocio (Seville), Hospital Xeral-Calde (Lugo), and Hospital Universitario La Paz (Madrid). A total of 1137 blood bank donors and bone marrow donors from the corresponding cities were included as healthy controls. Patients with RA were genotyped for HLA-DRB1. Among the patients with RA, 75.3% were women; the mean age of onset was 50.3 ± 14; 55.7% carried the shared epitope; 75.8% were positive for the rheumatoid factor; 20% had nodular disease; and 27% manifested additional extraarticular manifestations.

Systemic lupus erythematosus. In the present study, 372 Spanish patients meeting ACR criteria for SLE [16] were recruited from Hospital Universitario Virgen de las Nieves (Granada), Hospital Clínico Universitario San Cecilio (Granada), and Hospital Universitario Virgen del Rocio (Seville). A total of 280 blood bank donors and bone marrow donors from the corresponding cities were included as healthy controls. The mean age of patients at diagnosis was 43 ± 13.3, and the mean age at onset was 32 ± 15. The clinical manifestations of SLE studied were articular involvement, renal effects, cutaneous lesions, hematopoietic alterations, neurologic disease, and serositis. In addition, clinical activity or severity was assessed by determining the SLEDAI score every 6 months.

All subjects, cases and controls, were Caucasian and were included in this study after providing written informed consent. We obtained approval for the study from the local ethics committees in the different cities.

Genotyping

DNA from patients and controls was obtained from peripheral blood using standard methods. Samples were genotyped for the *BTNL2* G⟶A transition of rs2076530 using a TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA, USA). Minor groove binding probes were labeled with the fluorescent dyes VIC and FAM, respectively. Polymerase chain reaction (PCR) was carried in a total reaction volume of 8 μl with the following amplification protocol: denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 92°C for 15 seconds and annealing and extension at 60°C for 1 minute. After

PCR, the genotype of each sample was determined automatically by measuring allelic-specific fluorescence on the ABI PRIM 7000 Sequence Detection Systems using the SDS 1.1 software for allelic discrimination (Applied Biosystems).

Statistical analysis

Allelic and genotypic distributions were statistically analyzed with the χ^2 test. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated according to Woolf's method. The software used was the Statcalc program (Epi Info 2002; Centers for Disease Control and Prevention, Atlanta, GA, USA). *P* values less than 0.05 were considered statistically significant. For nonparametric data analysis, the Mann–Whitney *U*test was used for ordinal variables, and Fisher's exact test was used for dichotomous variables. For haplotype analysis, pairwise LD measures were investigated, and DQDR_BTNL2 haplotypes constructed with the expectation–maximization (EM) algorithm implemented in UNPHASED software [17].

Our sample sizes were sufficient to detect association given previously reported allele frequencies (minor allele frequencies of 42% [17]) and odds ratios of 1.5–2.0 at the 5% significance level, assuming a dominant inheritance model with 80% power, as calculated using Quanto 0.5 software (Department of Preventive Medicine, University of Southern California, Los Angeles, CA, USA).

RESULTS

BTNL2 rs2076530 genotype frequencies were in Hardy–Weinberg equilibrium across all the populations included in this study. Genotype and allele frequencies were similar to those reported previously for Caucasian populations [8, 18] (Table 1).

When allele and genotype frequencies of healthy controls and patients were compared, an association of the *BTNL2* rs2076530 polymorphism with T1D, RA, and SLE was noted (Table 1). The G allele of the polymorphism was more frequent among patients with T1D and RA than in healthy controls ($P = 0.0035$ and $P = 0.000003$, respectively). However, the A allele was more frequent in patients with SLE than in healthy controls ($P = 0.00002$). The distribution of genotypes between patients and controls also yielded statistically significant differences between T1D, RA, and SLE ($P = 0.0085$, 0.000047, and 0.0003, respectively), but in two diverging pathways: the AA genotype predisposed to SLE ($P = 0.00009$), whereas it was protective with respect to T1D and RA ($P = 0.002$ and 0.00009, respectively). In addition, we carried out a transmission disequilibrium test (TDT) study of patients with T1D,

TABLE 1 Allele and genotype frequencies of the *BTNL2* rs2076530 polymorphism among patients with T1D, RA, and SLE and healthy controls

	T1D				RA				SLE			
	No. (%) of patients $n = 326$	No. (%) of healthy controls $n = 351$	p^a	OR (% CI)	No. (%) if patients $n = 808$	No. (%) of healthy controls $n = 1137$	p^b	OR (% CI)	No. (%) of patients $n = 372$	No. (%) of healthy controls $n = 280$	p^c	OR (% CI)
G	320 (49.1)	289 (41.2)	0.0035	1.38 (1.10–1.72)	837 (51.8)	1006 (44.2)	0.000003	1.35 (1.19–1.54)	257 (34.5)	283 (46)	0.00002	0.62 (0.5–0.78)
A	332 (50.9)	413 (58.8)	0.0035	0.73 (0.58–0.91)	779 (48.2)	1268 (55.8)	0.000003	0.74 (0.65–0.84)	487 (65.5)	333 (54)	0.00002	1.61 (1.29–2.02)
GG	74 (22.7)	61 (17.4)	0.08	1.40 (0.94–2.07)	232 (28.7)	248 (21.8)	0.0005	1.44 (1.17–1.79)	61 (16.4)	75 (24.3)	0.009	0.61 (0.41–0.90)
GA	172 (52.8)	167 (47.6)	0.18	1.23 (0.9–1.68)	373 (46.2)	510 (44.9)	0.56	1.05 (0.88–1.27)	135 (36.3)	133 (43.2)	0.06	0.75 (0.54–1.03)
AA	80 (24.5)	123 (35.0)	0.002	0.60 (0.43–0.85)	203 (25.1)	379 (33.3)	0.00009	0.67 (0.55–0.82)	176 (47.3)	100 (32.5)	0.00009	1.87 (1.35–2.59)

[a] Global 3 × 2 *p* value = 0.0085.
[b] Global 3 × 2 *p* value = 0.000047.
[c] Global 3 × 2 *p* value = 0.0003.

TABLE 2 BTNL2 allele distribution in T1D stratified for high-risk DQDR haplotypes

DQDR genotype	BTNL2-A/G Controls	(%A) Patients	*p* value conditional on DQDR genotype	OR (95% CI)
DQ2DR17–DQ2DR17	16/0 (100)	64/2 (97.0)	0.48	0.78 (0.04–17.1)
DQ2DR17–DQ8DR4	2/8 (20.0)	104/90 (53.6)	0.44	1.51 (0.52–4.36)
DQ8DR4–DQ8DR4	1/5 (16.7)	1/37 (2.6)	0.12	0.15 (0.01–1.67)
DQ2DR17–X[a]	112/26 (81.2)	80/30 (72.7)	0.11	0.62 (0.34–1.13)
DQ8DR4–X	39/71 (35.5)	51/127 (28.7)	0.23	0.73 (0.44–1.21)
X–X	236/176 (57.3)	32/34 (48.5)	0.18	0.70 (0.42–1.18)
Overall	406/223 (64.5)	332/320 (50.9)	8.06*E*-07	0.57 (0.46–0.71)

[a] X denotes all HLA-DQDR haplotypes except DQ2DR17 and DQ8DR4.

and the result of this analysis was also significant, with the G allele of the SNP preferentially transmitted to the affected offspring ($P = 8.5 \times 10^{-5}$) (data not shown). The difference in results for SLE versus T1D and RA probably reflects the effect of the *HLA-DRB1* and *HLA-DQB1* genes. Because of the close proximity of *BTNL2* to *HLA-DRB1* and *HLA-DQB1,* which harbor alleles for susceptibility to T1D, RA, and SLE, and strong LD, stratification for the presence of MHC class II alleles was necessary.

With respect to T1D, after stratification for *HLA-DR* and *HLA-DQ,*the difference was not significant ($P = 0.92$ and 0.94, respectively), implying that the BTNL2 association is due to LD with the nearby MHC class II genes. When T1D patients were subdivided into the different high-risk *HLA-DQDR* haplotypes, a strong correlation between *BTNL2* and *HLA-DQDR* indicative of LD was observed (Table 2).

With respect to RA, we stratified the patients according to the presence of the shared epitope (SE) [19] or DERAA-bearing *HLA-DRB1* alleles [20], and we noted no statistically significant differences in the distribution of *BTNL2* rs2076530 alleles (Table 3). BTNL2 alleles were again strongly correlated with SE and DERAA classes. Because we subsequently included only those patients who had HLA class II high-resolution typing, we had to exclude the Lugo cohort. It was necessary to analyze separately the Granada and Seville cohorts to avoid Simpson's paradox, because healthy controls differ significantly with respect to the frequencies of *DQ*, *DR,* and *DQDR*. When the *BTNL2* polymorphism is tested relative to DQDR, the *BTNL2* effect is no longer significant in the Granada and Seville case–control settings ($P = 0.88$ and 0.44, respectively).

We observed the same effect for SLE: When we tested the *BTNL2* polymorphisms with relation to *HLA-DR* and *HLA-DQ* no significance was found.

To determine haplotypes of *BTNL2*and *HLA-DQDR*, co-transmission of these loci was evaluated and *HLA-DQDR_BTNL2* haplotypes were assigned for controls and patients with T1D, RA, and SLE (Table 4). It became apparent that the observed unconditional association of *BTNL2* with the different diseases was due to strong LD with *HLA-DQDR*.

DISCUSSION

This study constitutes the first attempt to test the possible role of the *BTNL2* rs2076530 polymorphism in susceptibility to three of the most common autoimmune diseases: T1D, RA, and SLE. It was determined that the

TABLE 3 Distribution of BTNL2 rs2076530 alleles in patients with RA patients according the presence of SE- or DERAA-bearing *HLA-DRB1* alleles

	Granada				Seville			
	BTNL2-A/G Controls	(%A) Patients	*p* value	OR (95% CI)	BTNL2-A/G Controls	(%A) Patients	*p* value	OR (95% CI)
SE+[a]	98/100 (49.5%)	132/158 (45.5%)	0.39	0.85 (0.59–1.22)	37/67 (35.6%)	71/89 (44.4%)	0.16	1.44 (0.87–2.39)
SE-[b]	164/110 (59.9%)	96/83 (53.6%)	0.19	0.78 (0.53–1.13)	135/105 (56.3%)	43/33 (56.6%)	0.96	1.01 (0.60–1.70)
DERAA+[c]	58/58 (50.0%)	45/51 (46.9%)	0.65	0.88 (0.52–1.51)	70/68 (50.7%)	25/33 (43.1%)	0.33	0.74 (0.40–1.37)
DERAA-[d]	204/152 (57.3%)	182/188 (49.2%)	0.028	0.72 (0.54–0.96)	101/101 (50%)	99/101 (49.5%)	0.92	0.98 (0.66–1.45)

[a] CMH test Granada and Seville populations pooled: $p = 0.38$.

[b] CMH test Granada and Seville populations pooled: $p = 0.30$.

[c] CMH test Granada and Seville populations pooled: $p = 0.33$.

[d] CMH test Granada and Seville populations pooled: $p = 0.07$.

TABLE 4 Haplotype risk estimation for separated populations[a]

			The Netherlands			Granada					Seville		
DQB1	DRB1	BTNL2	Controls (%)	T1D (%)	OR (95% CI)	Controls (%)	RA (%)	OR (95% CI)	SLE (%)	OR (95% CI)	Controls (%)	RA (%)	OR (95% CI)
0501	0101	**G**	**76.2 (10.95)**	**47 (7.37)**	**1.0**	**29.9 (6.34)**	**49.6 (10.54)**	**1.0**	**16.4 (5.47)**	**1.0**	**20 (5.81)**	**21.8 (9.16)**	**1.0**
		A	2.8 (0.4)	0.0 (0)		9.1 (1.92)	1.4 (0.31)	0.1 (0–0.6)	5.6 (1.87)	1.1 (0.4–3.7)	0 (0)	3.2 (1.34)	
0501	0102	G	1.2 (0.17)	0 (0)		3.5 (0.73)	1.1 (0.23)		0 (0)		0 (0)		
		A	4.9 (0.7)	4 (0.63)		21.6 (4.57)	20.9 (4.45)	0.6 (0.3–1.2)	4 (1.33)	0.4 (0.1–1.2)	12 (3.49)	8 (3.36)	0.6 (0.2–1.8)
0301	04	G	36.2 (5.19)	8 (1.25)	0.4 (0.2–0.9)	11 (2.33)	28.1 (5.99)	1.5 (0.7–3.4)	11 (3.66)	1.8 (0.7–4.9)	9 (2.62)	14.9 (6.27)	1.5 (0.5–4.1)
		A	3.9 (0.55)	0 (0)		0 (0)	1.9 (0.4)		2 (0.68)		0 (0)	2.1 (0.87)	
0302	04	G	60.9 (8.74)	207.4 (32.51)	5.5 (3.5–8.7)	38 (8.05)	62.8 (13.36)	1 (0.5–1.18)	19.4 (6.48)	0.9 (0.4–2.1)	30 (8.71)	34.2 (14.35)	1 (0.5–2.3)
		A	7.1 (1.03)	16.7 (2.61)	3.6 (1.4–9.2)	10 (2.12)	16.2 (3.44)	1 (0.4-2.4)	6.6 (2.19)	1.2 (0.4–3.7)	1 (0.31)	8.8 (3.71)	
02	07	G	46.4 (6.66)	20.2 (3.16)	0.7 (0.4–1.3)	52.8 (11.18)	48.6 (10.33)	0.6 (0.3–1)	36.5 (12.17)	1.3 (0.6–2.6)	56.4 (16.39)	19.5 (8.19)	0.3 (0.1–0.7)
		A	4.6 (0.67)	4.8 (0.75)		10.3 (2.17)	5.4 (1.16)	0.3 (0.1–1)	3.5 (1.16)	0.7 (0.2–2.4)	5.6 (1.63)	1.5 (0.63)	
0402	08	G	1.8 (0.26)	0.2 (0.04)		4.9 (1.05)	0 (0)		1 (0.34)		4 (1.16)	4 (1.68)	
		A	16.2 (2.32)	19.8 (3.1)	2 (0.9–4.1)	11.1 (2.34)	7 (1.49)	0.4 (0.1–1.1)	10 (3.33)	1.6 (0.6–4.6)	1 (0.29)	1 (0.42)	
0501	1001	G	0 (0)	0 (0)		0 (0)	2.3 (0.48)		0.6 (0.21)		0 (0)	3.9 (1.65)	
		A	12 (1.72)	2 (0.31)	0.3 (0.1–1.3)	9 (1.91)	20.7 (4.41)	1.4 (0.6–3.3)	7.4 (2.46)	1.5 (0.5-4.6)	0 (0.58)	8.1 (3.39)	3.1 (0.7–14.6)
0301	11	G	8.7 (1.24)	3 (0.47)	0.6 (0.2–2.2)	10.9 (2.31)	9.6 (2.03)	0.5 (0.2–1.4)	1.9 (0.62)	0.4 (0.1-1.7)	11.4 (3.32)	4.4 (1.83)	0.4 (0.1–1.3)
		A	38.4 (5.51)	5 (0.78)	0.2 (0.1–0.6)	42.1 (8.92)	31.5 (6.69)	0.5 (0.2–0.9)	30.1 (10.04)	1.3 (0.6-2.8)	33.6 (9.76)	17.7 (7.42)	05 (0.2–1.1)
0603	1301	G	25.9 (3.73)	5.4 (0.85)	0.4 (0.1–0.9)	8.2 (1.75)	6.2 (1.33)	0.5 (0.2–1.4)	9.4 (3.13)	2 (0.7-6)	5.8 (1.67)	4.6 (1.95)	0.8 (0.2-2.8)
		A	11.1 (1.59)	2.6 (0.41)	0.4 (0.1–1.6)	7.8 (1.64)	6.8 (1.44)	0.5 (0.2–1.6)	4.6 (1.53)	1.1 (0.3-3.9)	24.3 (7.05)	4.4 (1.83)	0.2 (0.1-0.6)
06	1302	G	2.4 (0.34)	3.7 (0.58)		6.9 (1.46)	1.5 (0.32)		0.8 (0.26)		6.9 (2.01)	2.1 (0.89)	
		A	41.6 (5.98)	28.3 (4.44)	1.1 (0.6–2)	9.1 (1.93)	4.5 (0.96)	0.3 (0.1–1)	5.2 (1.74)	1.1 (0.3-3.5)	6.1 (1.77)	3.9 (1.63)	
0301	1303/5	G	5.3 (0.77)	1 (0.16)		8.3 (1.77)	2.1 (0.45)	0.2 (0–0.8)	1.5 (0.5)		0.5 (1.45)	3 (1.26)	
		A	1.7 (0.24)	0 (0)		2.7 (0.56)	1.9 (0.4)		0.5 (0.16)		0 (0)	0 (0)	
0503	14	G	3.8 (0.54)	1 (0.16)		1.8 (0.38)	2.2 (0.48)		0 (0)		1.8 (0.51)	1.1 (0.47)	
		A	21.2 (3.05)	2 (0.31)	0.2 (0–0.7)	14.2 (3.01)	10.8 (2.29)	0.5 (0.2-1.1)	6 (2)	0.8 (0.3-2.4)	4.3 (1.24)	2.9 (1.21)	
0602	15	G	1.7 (0.24)	0 (0)		7.3 (1.55)	0 (0)		1.1 (0.37)		1.5 (0.42)	0 (0)	
		A	89.3 (12.83)	2 (0.31)	0 (0–0.2)	35.7 (7.56)	20 (4.26)	0.3 (0.2-0.7)	30.9 (10.3)	1.6 (0.7-3.3)	22.5 (6.55)	11 (4.62)	0.5 (0.2-1.2)
0201	0301	G	1.7 (0.25)	8.7 (1.36)	6.6 (1.5–29.8)	2.8 (0.6)	1.2 (0.26)		0 (0)		4.4 (1.26)	3 (1.27)	
		A	90.3 (12.97)	210.3 (33)	3.8 (2.4–5.8)	51.2 (10.84)	46.8 (10)	0.6 (0.3-1)	69 (23)	2.4 (1.2-4.9)	31.7 (9.2)	14 (5.9)	0.4 (0.2-1)
Global D′			0.83	0.86		0.64	0.73		0.84		0.76	0.7	

[a]DQB1–DRB1–BTNL2 haplotypes are given. For some haplotypes (partial) subtypes of the alleles are given; otherwise, serological types are given. Controls: count of haplotypes in controls, T1D, RA, SLE: count of haplotypes in patients. Odds ratios and confidence intervals calculated relative to the DQB*0501–DRB1*01–BTNL2*G reference haplotype. Only the most frequent haplotypes are listed. Odds ratios are given for total counts >10.

SNP was associated with the three diseases. However, when the polymorphism was tested conditional on *HLA-DRB1* and *HLA-DQB1* haplotypes, we observed that the effect was due to strong LD with the MHC class II alleles previously described as disease-predisposing alleles. Considering the small number of observations in some *HLA-DR/DQ* genotype categories, it remains possible that the *BTNL2* variant has a small effect on the risk of certain HLA genotypes. Nevertheless, our results suggest that BTNL2 does not contribute significantly to susceptibility to these three autoimmune diseases.

Previous studies concerning *BTNL2* polymorphisms and susceptibility to sarcoidosis have shown that *BTNL2* risk effects are independent of *HLA-DRB1* associations in Caucasian individuals [8, 18]. On the contrary, we have found that the effect of *BTNL2* on susceptibility to T1D, RA, and SLE is dependent on HLA class II alleles. It is worth noting that in the present study, the *BTNL2* allele associated with T1D and RA was the G allele, whereas the A allele was associated with SLE. However, this effect is the result of the different *HLA-DQDR_BTNL2* haplotypes, as outlined in Tables 3 and 4.

The association between T1D, RA, and SLE and HLA class II was stronger for HLA class II than for BTNL2 (3.3*E*-71, 8.0*E*-6, 1.3*E*-4, and 1.2*E*-8 for HLA-DQDR vs 0.0033, 0.037, 0.62, and 0.0056 for *BTNL2,* respectively) On the contrary, Valentonyte *et al*. observed that the *P* value for *BTNL2* was stronger than the values for *HLA-DQB1* and *HLA-DRB1*alleles in sarcoidosis. In the latter case the observed association of BTNL2 cannot be fully explained by LD with putative "causal" HLA class II alleles unless not all disease-associated class II haplotypes are observed. To prevent this observation bias, we used only HLA class II high-resolution typing and stratified HLA class II according to the established alleles for susceptibility to T1D, RA, and SLE, respectively.

Although a functional role for the *BTNL2* rs2076530 SNP has been proposed, it is necessary to investigate this hypothesis further. It is possible that the functionality of this variation is contributing to the effect of the extended or ancestral MHC haplotype [14]. However, the physiologic role of BTNL2 has not yet been elucidated, and, therefore, the role of the protein in the pathogenesis of autoimmune diseases remains to be revealed. Therefore, we conclude from our results presented here that there is probably no major role for BTNL2 in the pathogenesis of T1D, RA, and SLE.

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Association of a Functional Single-Nucleotide Polymorphism of *PTPN22*, Encoding Lymphoid Protein Phosphatase, With Rheumatoid Arthritis and Systemic Lupus Erythematosus

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Objective. **To assess the possible association between the *PTPN22* gene 1858C→T polymorphism and the predisposition and clinical expression of 2 systemic autoimmune diseases, rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE).**

Methods. **Our study population consisted of 826 RA patients, 338 SLE patients, and 1,036 healthy subjects. All subjects were of Spanish Caucasian origin. Genotyping of the *PTPN22* gene 1858C→T polymorphism was performed by real-time polymerase chain reaction technology, using the TaqMan 5′-allele discrimination assay.**

Results. **The overall distribution of genotypes in the RA patients was significantly different from that in the controls ($P = 0.005$, by chi-square test with 2×3 contingency tables). We observed a statistically significant difference in the distribution of the *PTPN22* 1858T allele between healthy subjects (7.4%), and RA patients (10.4%) ($P = 0.001$, odds ratio [OR] 1.45 [95% confidence interval (95% CI) 1.15–1.83]). In addition, *PTPN22* 1858 C/T and T/T genotypes were present at a significantly higher frequency in SLE patients than in controls ($P = 0.02$, OR 1.55 [95% CI 1.05–2.29]). Differences were also observed when allele frequencies were compared, with the *PTPN22* 1858T allele being present at a higher frequency among SLE patients ($P = 0.03$, OR 1.45 [95% CI 1.01–2.09]).**

Conclusion. **These results suggest that the *PTPN22* 1858T allele may confer differential susceptibility to RA and SLE in the Spanish population.**

Rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are common complex rheumatic diseases that are thought to have an immunologic origin because of the abundance of immune complexes, presence of autoantibodies, association with HLA specificities, and accumulation of lymphocytes, monocytes, and macrophages within the pathologic lesions (1–3). Although the pathogenesis of RA and SLE is unknown, the higher concordance of RA and SLE in monozygotic twins and familial clustering provide evidence for the role of genetic factors in these disorders (4,5). The genetic background of systemic autoimmune diseases, such as RA and SLE, is complex and likely involves multiple genes encoding proteins with significant functions in the regulation of the immune system. One crucial point in the pathogenesis of RA and SLE is the regulation of the T cell response (6,7).

Protein tyrosine phosphatases (PTPs) are critical regulators of T cell signal transduction (8). In conjunction with protein tyrosine kinases, PTPs regulate the reversible phosphorylation of tyrosine residues and thereby play important roles in many different aspects of

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T cell physiology (9). Abnormalities in tyrosine phosphorylation have been shown to be involved in the pathogenesis of numerous human diseases, from autoimmunity to cancer (10). Thus, T cells displaying dysregulated tyrosine phosphorylation would be expected to mediate the pathologic process in autoimmunity. In this regard, increased protein tyrosine phosphorylation patterns have been observed in T cells from patients with SLE (11,12). Due to their potential etiologic and pathogenic roles in human disease, PTPs can be considered good candidate genes in the study of autoimmune diseases.

The *PTPN22* (protein tyrosine phosphatase nonreceptor 22) gene, located on chromosome 1p13, encodes a lymphoid-specific phosphatase (Lyp). Lyp is an intracellular PTP with a molecular weight of 110 kd that contains an N-terminal catalytic domain and a noncatalytic C-terminus with 4 proline-rich domains (13). Lyp is physically bound through 1 proline-rich motif (referred to as P1) to the SH3 domain of the Csk kinase (14). The ability of Csk and Lyp to inhibit T cell receptor signaling requires their physical association (15). Recent findings have demonstrated that a single-nucleotide polymorphism (SNP) of *PTPN22* (1858C→T; National Center for Biotechnology Information dbSNP no. rs2476601; R620W), located at the P1 motif, disrupts the interaction between Lyp and Csk, avoiding the formation of the complex and, therefore, the suppression of the T cell activation (16). Furthermore, Bottini et al (16) also reported an association of the T variant of the polymorphism with the autoimmune disease type 1 diabetes mellitus. Of note, the chromosome region 1p13, where *PTPN22* maps, has shown linkage to RA and SLE (17,18).

Taking into account these findings, the aim of this study was to assess the role of the 1858C→T polymorphism in the *PTPN22* gene in the predisposition and clinical expression of RA and SLE.

PATIENTS AND METHODS

Patients. For the RA patient group, 826 patients who met the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1982 revised criteria for RA (19) were recruited from 4 Spanish hospitals: Hospital Virgen de las Nieves in Granada, Hospital Virgen del Rocío in Seville, Hospital Xeral-Calde in Lugo, and Hospital La Paz in Madrid. A total of 1,036 blood bank donors and bone marrow donors from the same cities were included as healthy controls. All RA patients had been genotyped for HLA–DRB1. The patients' mean ± SD age at RA onset was 50.3 ± 14 years; 75.3% of the RA patients were women, 55.7% carried the shared epitope, 75.8% were positive for rheumatoid factor, 20% had nodular disease, and 27% had additional extraarticular manifestations.

For the SLE patient group, 338 patients who met the ACR criteria for SLE (20) were recruited from 3 Spanish hospitals: Hospital Virgen de las Nieves in Granada, Hospital Clínico San Cecilio in Granada, and Hospital Virgen del Rocío in Seville. A total of 512 blood bank donors and bone marrow donors from the same cities were included as healthy controls. The mean ± SD age of the SLE patients at the time of diagnosis was 43 ± 13.3 years, and the mean age at disease onset was 32 ± 15 years. The clinical manifestations of SLE studied were articular involvement, renal involvement, cutaneous lesions, hematopoietic alterations, neurologic disease, and serositis. In addition, clinical activity or severity was evaluated by determining the SLE Disease Activity Index every 6 months.

All study subjects were of Spanish Caucasian origin and were included in the study after giving their written informed consent. The RA and SLE patients were matched with their respective healthy control group for age and sex. We obtained approval for the study from all the local ethics committees.

Genotyping. DNA from patients and controls was obtained from peripheral blood, using standard methods. Samples were genotyped for *PTPN22* 1858C→T variants using a TaqMan 5′-allele discrimination Assay-By-Design method (Applied Biosystems, Foster City, CA). The primer sequences were 5′-CCAGCTTCCTCAACCACAATAAATG-3′ (forward) and 5′-CAACTGCTCCAAGGATAGATGATGA-3′ (reverse). The TaqMan minor groove binder probe sequences were 5′-CAGGTGTCCATACAGG-3′, and 5′-CAGGTGTCCGTACAGG-3′; the probes were labeled with the fluorescent dyes VIC and FAM, respectively.

The polymerase chain reaction (PCR) was carried out in a total reaction volume of 12.5 μl using the following amplification protocol: denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 92°C for 15 seconds,

Table 1. Frequency of *PTPN22* 1858C→T alleles and genotypes in RA patients and healthy controls*

	No. (%) in RA patients	No. (%) in healthy controls
PTPN22 1858C→T genotypes		
No. of genotypes	826	1,036
C/C	663 (80.3)	890 (85.9)
C/T	155 (18.8)	139 (13.4)
T/T	8 (0.9)	7 (0.7)
PTPN22 1858C→T alleles		
No. of alleles	1,652	2,072
C	1,481 (89.6)	1,919 (92.6)
T	171 (10.4)	153 (7.4)

* For comparison of genotype frequencies in rheumatoid arthritis (RA) patients and controls, using a 2 × 3 contingency table, $\chi^2(2df) = 10.57$, $P = 0.005$. For comparison of C/T + T/T versus C/C genotype frequencies in RA patients and controls, $\chi^2(1df) = 10.57$, $P = 0.001$; odds ratio 1.49, and 95% confidence interval 1.16–1.92. For comparison of allele T versus allele C frequencies in RA patients and controls, $\chi^2(1df) = 10.19$, $P = 0.001$, odds ratio 1.45, and 95% confidence interval 1.15–1.83.

Table 2. Frequency of *PTPN22* 1858C→T alleles and genotypes among SLE patients and healthy controls*

	No. (%) in SLE patients	No. (%) in healthy controls
PTPN22 1858C→T genotypes		
No. of genotypes	338	512
C/C	274 (81)	445 (87)
C/T	62 (18.3)	63 (12.3)
T/T	2 (0.6)	4 (0.8)
PTPN22 1858C→T alleles		
No. of alleles	676	1,024
C	610 (90.2)	953 (93)
T	66 (9.8)	71 (7)

* For comparison of C/T + T/T versus C/C genotype frequencies in systemic lupus erythematosus (SLE) patients and controls, $\chi^2(1df) = 5.34$, $P = 0.02$, odds ratio 1.55, and 95% confidence interval 1.05–2.29. For comparison of allele T versus allele C frequencies in SLE patients and controls, $\chi^2(1df) = 4.40$, $P = 0.03$, odds ratio 1.45, and 95% confidence interval 1.01–2.09.

and annealing and extension at 60°C for 1 minute. After PCR, the genotype of each sample was automatically attributed by measuring the allele-specific fluorescence in the ABI Prism 7000 Sequence Detection System, using the SDS 1.1 software for allele discrimination (Applied Biosystems).

To confirm the genotype obtained by the TaqMan 5′-allele discrimination assay, PCR–restriction fragment length polymorphism was performed on representative samples from each genotype, as previously described (16). Briefly, we used the forward primer 5′-TCACCAGCTTCCTCAACCACA-3′ and the reverse primer 5′-GATAATGTTGCTTCAACGG-AATTTA-3′. The genotypes were identified by *Xcm* I restriction endonuclease digestion, which recognizes its target sequence only when the *PTPN22* 1858T allele is present. Digestion products were resolved on 3% agarose gels.

Statistical analysis. Allele and genotype frequencies of *PTPN22* 1858C→T were obtained by direct counting. Statistical analysis to compare allele and genotype distributions was performed by chi-square test. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated according to Woolf's method. The software used was the Statcalc program (EpiInfo 2002; Centers for Disease Control and Prevention, Atlanta, GA). *P* values less than 0.05 were considered statistically significant. For nonparametric data analysis, the Mann-Whitney U test was used for ordinal variables, and Fisher's exact test was used for dichotomous variables.

RESULTS

Table 1 shows the *PTPN22* 1858C→T genotype and allele frequencies in RA patients and their healthy controls. The genotype frequencies were not found to be significantly different from those predicted by Hardy-Weinberg equilibrium testing in healthy controls. When genotype frequencies of healthy subjects and RA patients were compared in a 2 × 3 contingency table, a statistically significant association was observed ($P = 0.005$). When genotypes in 2 × 2 contingency tables were compared, an association with RA susceptibility was observed in T allele–carrying genotypes (C/T + T/T genotypes versus C/C genotypes, $P = 0.001$, OR 1.49 [95% CI 1.16–1.92]). This statistically significant skewing was also seen when T allele frequencies were compared in RA patients and controls ($P = 0.001$, OR 1.45 [95% CI 1.15–1.83]).

PTPN22 1858C→T genotype and allele frequencies in SLE patients and their healthy controls are shown in Table 2. The control population was found to be in Hardy-Weinberg equilibrium. When the overall genotype distribution of the control subjects was compared with that of the SLE patients in a 2 × 3 contingency table, we observed a similar trend as in the RA patients, with an increased frequency of T-carrying genotypes in SLE patients; however, the association was within the limit of statistical significance ($P = 0.05$). The C/T and T/T genotypes were present at significantly higher frequencies in SLE patients than in controls ($P = 0.02$, OR 1.55 [95% CI 1.05–2.29]). Differences were also observed when allele frequencies were compared, with the T allele being present at a higher frequency in SLE patients ($P = 0.03$, OR 1.45 [95% CI 1.01–2.09]).

Next, we analyzed the demographic and clinical characteristics of the RA patients according to their *PTPN22* 1858C→T genotypes (Table 3). No significant differences were observed for the clinical features, except for extraarticular disease and female sex. We found differences in the presence of extraarticular disease in RA patients carrying C/C genotypes and those carrying

Table 3. Distribution of *PTPN22* 1858C→T genotypes by clinical and demographic features of the rheumatoid arthritis patients

	Shared epitope, %	Rheumatoid factor, %	Rheumatoid nodules, %	Extraarticular disease, %*	Female, %†	Age at onset, mean ± SD years
C/C	51.5	74	21.1	24.8	80.6	50.9 ± 13.6
C/T + T/T	50.9	82.4	27.2	10.2	67.6	49.8 ± 12.1

* For genotypes C/T + T/T versus C/C, $P = 0.04$, odds ratio 2.97, 95% confidence interval 0.91–10.47, and corrected $P = 0.24$.
† For genotypes C/T +T/T versus C/C, $P = 0.01$, odds ratio 2.04, 95% confidence interval 1.11–3.72, and corrected $P = 0.06$.

Table 4. Relationship between *PTPN22* 1858C→T and the presence of nephritis in patients with systemic lupus erythematosus (SLE)

	No. (%) in SLE patients with nephritis	No. (%) in SLE patients without nephritis
PTPN22 1858C→T genotypes		
No. of genotypes	86	173
C/C	70 (81.4)	138 (79.8)
C/T	15 (17.4)	35 (20.2)
T/T	1 (1.2)	0 (0)
PTPN22 1858C→T alleles		
No. of alleles	172	346
C	155 (90.1)	311 (89.9)
T	17 (9.9)	35 (10.1)

C/T or T/T genotypes (24.8% versus 10.2%; $P = 0.04$, OR 2.97 [95% CI 0.91–10.47]). The C/C genotype was increased in female patients (80.6% C/C versus 67.6% C/T + T/T; $P = 0.01$, OR 2.04 [95% CI 1.11–3.72]). Both these differences turned out to be nonsignificant after correction for the number of stratifications performed, using the Bonferroni inequality correction.

One of the most severe clinical features of SLE is nephritis. We stratified SLE patients with regard to renal involvement, and found no statistically significant differences in the distribution of *PTPN22* 1858C→T genotypes or alleles between SLE patients with and those without lupus nephritis (Table 4). Similarly, no significant differences were observed when *PTPN22* 1858C→T genotypes were analyzed with regard to other clinical manifestations of SLE.

DISCUSSION

Due to its functional relevance as a negative regulator of T cell activation and its positional mapping to a chromosome region showing linkage to RA and SLE (17,18), *PTPN22* is a good candidate as a genetic marker for systemic autoimmune diseases, such as RA and SLE. In the present study, we found an association between the functional 1858C→T polymorphism of the *PTPN22* gene and susceptibility to RA and SLE in 2 large Spanish cohorts. The *PTPN22* 1858T allele was more frequent in RA and SLE patients than in their matched healthy controls, suggesting that the *PTPN22* 1858T allele may predispose individuals to the development of RA and SLE. Our sample size is large enough to detect an association at an odds ratio between 1.5 and 2, since it had 80% power at the 5% significant level. We found no association of the *PTPN22* variation with most of the clinical and laboratory parameters analyzed.

Current evidence suggests that abnormalities in tyrosine phosphorylation in SLE T cells contribute to T cell effector dysfunction and, ultimately, to the immunopathogenesis of SLE (21,22). For example, mutations in the protein kinase A gene have been identified in SLE T cells (23,24). In addition, we found that the *PTPN22* 1858T allele was associated with SLE, suggesting that down-regulation of T cell activation plays a role in T cell effector dysfunction in SLE. Two recently published seminal reviews discussed the criteria for inclusion of genes as putative biomarkers for SLE (25,26). Based on these criteria, the *PTPN22* gene could be included as another susceptibility locus for SLE. Furthermore, since it is unlikely that a single defect in the *PTPN22* gene is solely responsible for the diverse T cell dysfunction observed in SLE, it will be of interest to evaluate systematically the possible implications of other members of the PTP gene family in autoimmune diseases (10).

During the course of this work, a similar study showing a statistically significant association between the *PTPN22* 1858C→T polymorphism and RA was published (27). Those investigators observed an increased frequency of the 1858T allele among RA patients (13.8%) compared with healthy controls (8.8%) in a North American population. Those authors were also able to replicate their findings using a different cohort of individuals as well as in a family study (27). Furthermore, they confirmed the functional effect of the *PTPN22* 1858 variation in the binding of Lyp to Csk, as previously reported by Bottini et al (16), which suggests that the association of the polymorphism with autoimmunity may be due to the role of the *PTPN22* gene in the negative regulation of T cell activation (16,27). Begovich et al (27) also reported an association between the *PTPN22* SNP and the presence of rheumatoid factor–positive disease. We did not find this association in our Spanish RA patients, but we observed a higher frequency of the 1858T-bearing genotypes in male patients and in patients without extraarticular disease, although this skewing did not reach statistical significance after correction for multiple tests. These discrepancies may reflect the clinical heterogeneity present in rheumatoid arthritis patients across populations. Kyogoku et al (28) recently reported an association of the R620W SNP with SLE in a North American population.

Results obtained in the present study confirm previous findings in independent cohorts and therefore reinforce the role of the *PTPN22* 1858C→T polymorphism as a genetic risk marker for RA and SLE. With regard to the association between *PTPN22* variants and susceptibility to RA and SLE found in previous studies

(27,28), it was suggested that there was a dose effect, with the homozygous T alleles being associated with greater risk than the heterozygous C/T alleles. We did not observe such a trend, although this may be due to the limited number of T/T genotypes found in our study population.

The T allele was present in 14% of individuals, at an allele frequency of 7.4%, in our Spanish control population. These allele frequencies are similar to those reported in other Caucasian populations (16,27) and differ significantly from those observed in other populations of different ethnic origins, such as Mexican Americans (3.5%) and African Americans (2.4%), as noted by Begovich et al (27). It might be worth examining a possible contribution of the *PTPN22* 1858 SNP to autoimmune diseases in these populations, where the distribution of the T allele is lower than that in Caucasians. It might also be of major interest to analyze the possible role of the *PTPN22* gene in African American patients with SLE, since we observed an association between the functional 1858C→T polymorphism of the *PTPN22* gene and susceptibility to SLE.

There is accumulating evidence to suggest the presence of common genetic factors that predispose to autoimmunity. Nonrandom clustering of disease susceptibility loci has been observed both in rodent models of autoimmune disease and in linkage studies of autoimmune diseases in humans (29–32). The finding that the *PTPN22* 1858T allele is associated with RA and SLE as well as diabetes provides support for the idea that susceptibility to multiple autoimmune diseases may have some common susceptibility alleles or pathways. The involvement of *CTLA4* in susceptibility to Graves' disease, autoimmune hypothyroidism, type 1 diabetes mellitus, and SLE (33,34) is another piece of evidence that supports the above-mentioned hypothesis. The association of 2 negative regulators of T cell activation, such as *PTPN22* and *CTLA4*, with autoimmune disease indicates the importance of the regulation of T cell responses in the development of autoimmunity.

In conclusion, this study shows the possible implication of the *PTPN22* gene 1858C→T polymorphism in the development of rheumatoid arthritis and systemic lupus erythematosus.

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Association of a *CD24* Gene Polymorphism With Susceptibility to Systemic Lupus Erythematosus

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Objective. **To determine the potential role of the *CD24* A57V gene polymorphism in systemic lupus erythematosus (SLE).**

Methods. **We studied 3 cohorts of Caucasian patients and controls. The Spanish cohort included 696 SLE patients and 539 controls, the German cohort included 257 SLE patients and 317 controls, and the Swedish cohort included 310 SLE patients and 247 controls. The *CD24* A57V polymorphism was genotyped by polymerase chain reaction, using a predeveloped TaqMan allele discrimination assay. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated.**

Results. **In the Spanish cohort there was a statistically significant difference in the distribution of the *CD24* V allele between SLE patients and controls (OR 3.6 [95% CI 2.13–6.16], $P < 0.0001$). In addition, frequency of the *CD24* V/V genotype was increased in SLE patients compared with controls (OR 3.7 [95% CI 2.16–6.34], $P < 0.00001$). We sought to replicate this association with SLE in a German population and a Swedish population. A similar trend was found in the German group. The *CD24* V/V genotype and the *CD24* V allele were more frequent in SLE patients than in controls, although this difference was not statistically significant. No differences were observed in the Swedish group. A meta-analysis of the Spanish and German cohorts demonstrated that the *CD24* V allele has a risk effect in SLE patients (pooled OR 1.25 [95% CI 1.08–1.46], $P = 0.003$). In addition, homozygosity for the *CD24* V risk allele significantly increased the effect (pooled OR 2.19 [95% CI 1.50–3.22], $P = 0.00007$).**

Conclusion. **These findings suggest that the *CD24* A57V polymorphism plays a role in susceptibility to SLE in a Spanish population.**

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease with a complex pathogenesis involving multiple genetic and environmental factors. The disease is characterized by enhanced autoantibody production, abnormalities of immune/inflammatory system function, and inflammation in several organs. Al-

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though the pathogenesis of SLE is unknown, there is evident familial clustering and a high concordance of SLE in monozygotic twins, providing evidence of the role of genetic factors in this disorder (1). The genetic background of SLE is complex and involves multiple genes encoding different molecules with significant functions in the regulatory pathway of the immune system (1–4). During the past few years, genome-wide linkage studies have identified several loci as being associated with SLE (3). In this respect, findings of several studies support the notion that regulatory T and B cell genes play an important role in the pathogenesis of SLE.

One of the chromosome regions suggested to be associated with SLE and other autoimmune diseases is 6q21–25 (5,6), where the *CD24* gene maps. CD24 (heat-stable antigen) is a glycosyl phosphatidylinositol (GPI)–linked protein that is anchored to the cell surface and is expressed in a wide variety of cell types, including activated T cells, B-lineage cells, mature granulocytes, macrophages, and dendritic cells, among others (7–10). The biologic function of CD24 is unclear, but it has been shown that CD24 is a ligand for P-selectin on tumor cells (11). This binding could be important in the dissemination of tumor cells and could be a key factor in recruiting leukocytes into inflamed tissue. CD24 has also been implicated in the activation and differentiation of B lymphocytes (12) and has been identified as an important mediator in a CD28-independent costimulatory pathway in the activation of both CD4 and CD8 T cells (9). In addition, CD24 plays an important role in binding the adhesion molecules very late activation antigen 4 and vascular cell adhesion molecule 1 (13). These adhesion molecules are important in lymphocyte costimulation in specific tissue and sites of inflammation in SLE patients (14,15).

A recent study has identified a coding polymorphism of the *CD24* gene (226C→T, single-nucleotide polymorphism [SNP] no. rs8734), which results in the replacement of an alanine (*CD24* A) amino acid by valine (*CD24* V) (A57V) (16). This nonconservative amino acid change at a position that precedes the putative cleavage site for the GPI anchor has been found to be associated with susceptibility to and progression of multiple sclerosis (MS) (16). Taking into account these findings, in this study we aimed to investigate the potential implication of the functional *CD24* polymorphism for susceptibility to SLE.

PATIENTS AND METHODS

Patients. Peripheral blood samples were obtained from 3 unrelated cohorts of European Caucasian patients with SLE and from healthy controls. The Spanish cohort consisted of 696 SLE patients and 539 healthy controls, the German cohort consisted of 257 SLE patients and 317 healthy controls, and the Swedish cohort consisted of 310 SLE patients and 247 healthy controls. All 1,263 SLE patients met the American College of Rheumatology criteria for SLE (17). Patients in the Spanish cohort were recruited from the following 5 Spanish hospitals: Hospital Virgen de las Nieves, Hospital Clínico San Cecilio, Hospital Virgen del Rocío, Hospital Carlos-Haya, and Hospital Xeral-Calde. Patients in the German cohort were recruited from a North German multicenter study on the genetics of SLE. Members of the German Systemic Lupus Erythematosus Study Group are shown in Appendix A. Patients in the Swedish cohort were recruited from 3 centers in central and southern Sweden (Stockholm, Eskilstuna, and Lund). Control samples were obtained from a total of 1,103 healthy blood bank or bone marrow donors in the corresponding cities. Patients and controls were all Caucasian and were matched for age (by mean age) and for sex (by frequency matching). Written informed consent was obtained from all subjects, and the study was approved by the local ethics committee of each center. Demographic characteristics of the patients and controls in each population have been described previously (5,18,19).

Genotyping. DNA was obtained from peripheral blood, using standard methods. Samples were genotyped for the *CD24* A57V variant using a TaqMan 5′-allele discrimination method (Sigma-Aldrich, St. Louis, MO). The primer sequences were 5′-CCC-AAA-TCC-AAC-TAA-TGC-C-3′ and 5′-TAA-GAG-TAG-AGA-TGC-AGA-AGA-G-3′. The TaqMan minor groove binder probe sequences were 5′-ACC-AAG-GCG-GCT-GGT-GGT-G-3′ and 5′-ACC-AAG-GTG-GCT-GGT-GGT-G-3′; the probes were labeled with the fluorescent dyes FAM and JOE, respectively.

The polymerase chain reaction (PCR) was carried out in a total reaction volume of 10 μl using the following amplification protocol: denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 92°C for 15 seconds, and annealing and extension at 60°C for 90 seconds. Following PCR, the genotype of each sample was automatically determined by measuring the allele-specific fluorescence in the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA), using SDS, version 1.1 software (Applied Biosystems) for allele discrimination. To confirm the genotype obtained by the TaqMan 5′-allele discrimination assay, direct sequencing using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems) was performed. The German samples were typed at Uppsala University using the Custom TaqMan SNP genotyping assay (Applied Biosystems). To verify the genotyping consistency, 96 of the Swedish control samples were typed in both centers (Uppsala University and Instituto de Parasitología y Biomedicina López-Neyra, CSIC), and for 95 of the 96 samples (99%) the 2 genotypes were identical.

Statistical analysis. Allele and genotype frequencies were obtained by direct counting. Hardy-Weinberg equilibrium was assessed using the chi-square test, and statistical analysis was performed to compare allele and genotype distributions. Odds ratios (ORs), with 95% confidence intervals (95% CIs), were calculated by Woolf's method, using StatCalc and logistic regression software programs (Epi Info 2002; Centers for Disease Control and Prevention, Atlanta, GA). The pooled OR was calculated according to a fixed-effects

Table 1. Genotype and allele frequencies of the *CD24* A57V polymorphism in SLE patients and healthy controls*

	Spanish cohort				German cohort				Swedish cohort			
	SLE patients (n = 696)	Healthy controls (n = 539)	P†	OR (95% CI)	SLE patients (n = 257)	Healthy controls (n = 317)	P†	OR (95% CI)	SLE patients (n = 310)	Healthy controls (n = 247)	P†	OR (95% CI)
Genotype												
CD24 A/A	356 (51.1)	305 (56.6)	0.4	1 (referent)	129 (50.2)	161 (50.8)	0.4	1 (referent)	141 (45.5)	117 (47.4)	0.7	1 (referent)
CD24 A/V	269 (38.7)	211 (39.1)	0.7	1.05 (0.83–1.32)	105 (40.9)	138 (43.5)	0.5	1.15 (0.70–1.70)	142 (45.8)	108 (43.7)	0.9	1.01 (0.72–1.41)
CD24 V/V	71 (10.2)	23 (4.3)	<0.00001	3.7 (2.16–6.34)	23 (8.9)	18 (5.7)	0.2	1.54 (0.70–3.40)	27 (8.7)	22 (8.9)	0.9	1.03 (0.58–1.81)
Allele												
CD24 A	981 (70.5)	821 (76.2)	–	1 (referent)	363 (70.6)	460 (72.6)	–	1 (referent)	424 (68.4)	342 (69.2)	–	1 (referent)
CD24 V	411 (29.5)	257 (23.8)	<0.0001	3.6 (2.13–6.16)	151 (29.4)	174 (27.4)	0.3	1.45 (0.67–3.13)	196 (31.6)	152 (30.8)	0.9	1.03 (0.58–1.76)

* Values are the number (%) of genotypes or alleles. OR = odds ratio; 95% CI = 95% confidence interval.
† *P* values indicate the difference between systemic lupus erythematosus (SLE) patients and healthy controls.

model (Mantel-Haenszel meta-analysis) as well as a random-effects model (DerSimonian-Laird), using StatsDirect software (StatsDirect, Cheshire, UK). *P* values less than 0.05 were considered significant. Homogeneity of ORs among cohorts was calculated by the Breslow-Day test and Woolf Q method, using StatsDirect software. The power of each analysis was computed as the probability of detecting an association between the *CD24* polymorphism and SLE at the 5% significance level, assuming an OR of 1.5 (small effect size). Power was estimated using Quanto, version 0.5 (Department of Preventive Medicine, University of Southern California, Los Angeles, CA).

RESULTS

CD24 genotypes were in Hardy-Weinberg equilibrium in patients and in controls in the 3 populations studied. We first analyzed the cohorts individually and then combined the samples in a meta-analysis. Table 1 shows the *CD24* A57V allele and genotype distributions in SLE patients and healthy controls in all 3 cohorts.

An association was found when we compared genotype frequencies in Spanish patients with SLE with those in Spanish controls ($P = 0.0004$ by chi-square test using a 2×3 contingency table), with a higher frequency of the *CD24* V/V genotype in the SLE group (10.2% versus 4.3%) (OR 3.7 [95% CI 2.16–6.34], $P < 0.00001$). Analyses of allele frequencies produced similar results, showing an increased frequency of the *CD24* V allele in SLE patients compared with healthy controls (29.5% versus 23.8%) (OR 3.6 [95% CI 2.13–6.16], $P < 0.0001$).

Additionally, we sought to replicate the association of the *CD24* A57V polymorphism with SLE in 2 different populations from Germany and Sweden. The same trend was found in the German cohort, in which the frequencies of the *CD24* V/V genotype and *CD24* V allele were slightly increased in SLE patients compared with healthy controls (8.9% versus 5.7% for the *CD24* V/V genotype and 29.4% versus 27.4% for the *CD24* V allele). Although this difference did not reach statistical significance (OR 1.54 [95% CI 0.70–3.40], $P = 0.2$ for the *CD24* V/V genotype and OR 1.45 [95% CI 0.67–3.13], $P = 0.3$ for the *CD24* V allele), meta-analysis of the ORs in the Spanish and German cohorts demonstrated that the *CD24* V allele has a risk effect in SLE patients (pooled OR 1.25 [95% CI 1.08–1.46], $P = 0.003$) and the *CD24* V/V genotype has an increased risk effect (pooled OR 2.19 [95% CI 1.50–3.22], $P = 0.00007$) (Table 2). However, we did not find any significant difference in genotype and allele frequencies between SLE patients and healthy controls in the Swedish cohort.

Estimation of OR homogeneity between the cohorts showed the combinability of all 3 cohorts at the allele level (*CD24* V), but only of the Spanish and German cohorts at the genotype level (*CD24* V/V). Consequently, meta-analysis of the *CD24* V/V genotype under a fixed-effects model should not include the Swedish cohort, but analysis of the *CD24* V allele can include all 3 cohorts. Meta-analysis of all 3 cohorts using the Mantel-Haenszel test revealed a significant association of SLE with the *CD24* V allele (pooled OR 1.20 [95% CI 1.05–1.36], $P = 0.007$), although a separate analysis of the Spanish and German cohorts showed the strongest association (Table 2).

Table 2. Meta-analysis using fixed-effects and random-effects models*

CD24 A57V meta-analysis	Spanish and German cohorts		All 3 cohorts	
	Pooled OR (95% CI)	*P*	Pooled OR (95% CI)	*P*
Fixed-effects model†				
CD24 V versus *CD24* A	1.25 (1.08–1.46)	0.003	1.20 (1.05–1.36)	0.007
CD24 V/V versus *CD24* A/V + *CD24* A/A	2.19 (1.50–3.22)	0.00007	NA	NA
Random-effects model‡				
CD24 V versus *CD24* A	1.24 (1.03–1.50)	0.02	1.18 (1.00–1.39)	0.046
CD24 V/V versus *CD24* A/V + *CD24* A/A	2.15 (1.40–3.29)	0.0004	1.63 (0.92–2.89)	0.098

* 95% CI = 95% confidence interval; NA = not applicable.
† Odds ratios (ORs) determined by Mantel-Haenszel test.
‡ ORs determined by DerSimonian-Laird test.

Clinical features of Spanish patients with SLE were analyzed for possible association with the different alleles or genotypes of the *CD24* A57V polymorphism. When we stratified Spanish SLE patients according to the presence or absence of renal involvement, no statistically significant differences were observed in the distribution of *CD24* polymorphism between SLE patients with and without lupus nephritis (Table 3). Similar results were found after comparisons of other demographic and clinical features (data not shown).

DISCUSSION

This is the first study to attempt to determine the potential role of the *CD24* A57V polymorphism in SLE. Our results showed that in the Spanish population the *CD24* V allele was associated with an increased risk of SLE (OR 3.6). These results were further supported by analysis of a separate SLE cohort from Germany. The trend toward an increase in *CD24* V frequency was repeated in the German cohort, although this trend did not reach statistical significance. This could be explained by the sample size, which was smaller in the German cohort (n = 257) than in the Spanish cohort (n = 696), resulting in a lack of power to detect a susceptibility association. In addition, we demonstrated that the frequency of the *CD24* V/V genotype in Spanish SLE patients is more than twice that in the control population. This implies that the effect of the *CD24* gene on SLE predisposition is dose dependent, since homozygosity for the *CD24* V allele confers a 2-fold increased risk of SLE (OR 3.7).

Table 3. Relationship between the *CD24* A57V polymorphism and the presence of nephritis in Spanish patients with SLE*

	SLE with nephritis (n = 271)	SLE without nephritis (n = 425)
Genotype		
CD24 A/A	142 (52.4)	211 (49.6)
CD24 A/V	96 (35.4)	169 (39.8)
CD24 V/V	33 (12.2)	45 (10.6)
Allele		
CD24 A	380 (70.1)	591 (69.5)
CD24 V	162 (29.9)	259 (30.5)

* Values are the number (%) of genotypes or alleles. SLE = systemic lupus erythematosus.

Similar results have been found for MS in other Caucasian populations (16), and the *CD24* A57V polymorphism has also recently been shown to be associated with MS in a Spanish population (20). However, a recent replication study of *CD24* in 2 large, unrelated cohorts of Caucasian patients with MS living in Belgium and the UK failed to demonstrate the reported association (21). The lack of replication in that study was probably not due to insufficient power to detect an association, since the sample sizes (n = 334 patients living in Belgium and n = 846 patients living in the UK) were large enough to reach >90% statistical power to detect a relative risk similar to that found in the original study.

Population heterogeneity may explain the conflicting results of these studies, since Swedish, Belgian, and British populations showed different allele frequencies compared with Spanish and German populations. Northern European populations had a higher frequency of the T allele (*CD24* V) than did the other groups, exhibiting a clear north–south gradient. For example, the frequency of the T allele in the original study (20) (26.8%) was very similar to that found in controls from northern Spain (25.3%) and our controls from southern Spain (23.8%), but was slightly different from that found in controls from Germany (27.4%), and differed to a

greater extent from those found in controls from Belgium (30%), Sweden (30.8%), and the UK (34%).

However, in the present study the frequencies of the *CD24* V allele among SLE patients in all 3 cohorts were similar (29.5% in the Spanish cohort, 29.4% in the German cohort, and 31.6% in the Swedish cohort), suggesting the existence of clines among controls, with an association of the *CD24* V allele with SLE. In fact, the same trend has been observed in MS, where a difference has been found among controls but not among MS patients in 4 independent cohorts. The frequency of the *CD24* V allele was 33.2% in American Caucasian patients with MS, 32% in Belgian patients with MS, 30% in British patients with MS, and 30.7% in Spanish patients with MS (16,20,21). Regarding SLE, a study of the *PDCD1* gene (22) showed a north–south gradient in the distribution of the PD1.3A allele in healthy individuals. This gradient of frequencies in European Caucasians in relation to their geographic origin has not been observed in SLE patients, causing divergent results when different populations are analyzed. This pattern of variation has been characterized in a large panel of SNPs, defining primarily 2 groups of European subpopulations, from southern Europe and from northern Europe (23).

We believe that there could be 3 possible explanations for the observed phenomenon. First, *CD24* V might be associated with a gene for SLE not in linkage disequilibrium with *CD24* and with which *CD24* interacts. Second, *CD24* V could be associated with an environmental factor which confers selection of this allele in patients with SLE. Finally, there may be a founder effect of *CD24* V in SLE.

Alternatively, lack of replication in different populations may be considered evidence of a Type I error, but this requires the implicit assumption that the variant that causes disease is shared and has an equal effect in all populations, i.e., that it is ancestral before divergence of the populations. However, regional founder effects may underlie differences even within European populations, as has been suggested for *NOD2* and *DLG5* (24,25). Furthermore, allele frequencies of *NOD2* risk alleles have been reported to vary significantly between European populations (26). Therefore, the inconsistent results of *CD24* A57V association studies may indicate that allelic heterogeneity, regional founder effects, or both, underlie the conflicting results. Analysis of more European subjects from other southern and eastern regions is needed to determine the gradient effect of this allele.

There is also the possibility that a second functional variant of *CD24* plays a role in disease risk in populations other than the Spanish population because of allelic heterogeneity. Complete analysis of the *CD24* gene would be required to clarify the existence of 1 or several risk haplotypes.

The observation that an inherited variation in *CD24* predisposes to both MS and SLE is intriguing, and supports current hypotheses that common cellular and molecular pathogenetic mechanisms might be involved in multiple inflammatory diseases. The general observation that other inflammatory and autoimmune diseases are associated with the same genes (e.g., *CTLA4* [27], *PTPN22* [28–30], and *PDCD1* [31–34]) suggests it may be useful to evaluate the role of *CD24* in other diseases.

The role of CD24 in autoimmunity has not been clearly elucidated. Bai et al (35) previously reported the critical role of CD24 in the development of a mouse model of MS (experimental autoimmune encephalomyelitis). CD24 has been demonstrated to function as an important regulator during the early stages of B and T cell lymphopoiesis (36). Murine CD24 is expressed during the initial stages of T and B cell development and its expression is reduced in CD4+,CD8+ thymocytes. Current evidence suggests that CD24 could affect both adhesion and signaling (37). To date, P-selectin is the only ligand that has been identified that exhibits increased levels of expression in response to acute mediators of inflammation in SLE (15). It is, however, unlikely that this CD24–P-selectin interaction accounts for all of the biologic activities attributed to CD24. Since CD24 is localized in lipid rafts, the absence or presence of CD24 may influence membrane raft composition and thereby affect important signaling pathways within the cell (37). It has also been reported that CD24 is involved in intracellular signaling by direct binding to tyrosine kinases (38).

In conclusion, it appears that the *CD24* A57V polymorphism plays a role in susceptibility to SLE in a Spanish population. The evidence of association of the *CD24* genetic variation with SLE was strongest in the Spanish cohort, although broadly similar allele frequencies were observed in the German cohort. Nevertheless, conflicting results have been found in different populations, suggesting that allele frequency clines are common in other polymorphisms and that their impact should be carefully considered in genetic epidemiology studies. Therefore, it might be of interest to further investigate the role of the *CD24* gene variant in additional ethnic groups to confirm this hypothesis.

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AUTHOR CONTRIBUTIONS

Dr. Martín 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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APPENDIX A: THE GERMAN SYSTEMIC LUPUS ERYTHEMATOSUS STUDY GROUP

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Functional variants in the B-cell gene *BANK1* are associated with systemic lupus erythematosus

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Systemic lupus erythematosus (SLE) is a prototypical autoimmune disease characterized by production of autoantibodies and complex genetic inheritance[1–3]. In a genome-wide scan using 85,042 SNPs, we identified an association between SLE and a nonsynonymous substitution (rs10516487, R61H) in the B-cell scaffold protein with ankyrin repeats gene, *BANK1*. We replicated the association in four independent case-control sets (combined $P = 3.7 \times 10^{-10}$; OR = 1.38). We analyzed *BANK1* cDNA and found two isoforms, one full-length and the other alternatively spliced and lacking exon 2 (Δ2), encoding a protein without a putative IP3R-binding domain. The transcripts were differentially expressed depending on a branch point–site SNP, rs17266594, in strong linkage disequilibrium (LD) with rs10516487. A third associated variant was found in the ankyrin domain (rs3733197, A383T). Our findings implicate *BANK1* as a susceptibility gene for SLE, with variants affecting regulatory sites and key functional domains. The disease-associated variants could contribute to sustained B cell–receptor signaling and B-cell hyperactivity characteristic of this disease.

We genotyped 279 Swedish individuals with SLE and 515 control individuals using the 100K Affymetrix SNP array. As our purpose was to identify non-MHC genes and, if possible, important functional polymorphisms involved in SLE pathogenesis, we carried out an analysis of the genomic location of the associated SNPs, focusing on nonsynonymous substitutions. Among all associated SNPs, one (rs10516487) led to a substitution of arginine to histidine at amino-acid position 61 (R61H) of the BANK1 protein (allelic association, $P = 6.4 \times 10^{-3}$; genotypic association, $P = 2.01 \times 10^{-2}$). This SNP was ranked 679th in the allelic and 2,148th in the genotypic tests across the genome scan, with estimated false-discovery rates of 71.1% and 77.5%, respectively[4]. Four other SNPs in *BANK1* also showed association (**Supplementary Table 1** online). Because of the described B cell–specific expression of *BANK1* and its potential role in B cell receptor–mediated activation, we pursued this gene[5,6].

To provide better SNP coverage and refine the association signal, we genotyped 30 SNPs spanning the 284-kb *BANK1* genomic region (including the scan SNPs) in the Swedish SLE case and control samples. Two SNPs were not polymorphic, and nine SNPs were associated (**Table 1** bold). All associated SNPs were located between introns 1 and 7 (**Table 1**, **Supplementary Table 2** and **Supplementary Fig. 1** online).

Next, we carried out a detailed analysis of *BANK1* expression and structure. We observed that *BANK1* is indeed primarily expressed in $CD19^+$ B cells, with very low expression in other cell populations (**Fig. 1a**). To clone *BANK1* for functional analysis, we amplified full-length cDNA using distal primers. Of note, we detected two main bands following gel electrophoresis of the PCR products (**Fig. 1b**). *BANK1* is known to have two full-length alternative isoforms containing exon 1A or exon 1B[5]. Through subsequent cloning and sequencing, we identified a previously unknown isoform with an in-frame deletion of the entire exon 2 (Δ2 isoform). We analyzed cDNA from 83 healthy individuals and 30 individuals with SLE and found that this isoform was present in every sample, indicating that it is constitutively spliced. Moreover, we detected this isoform in cDNA from chimpanzee and mouse spleen (data not shown), suggesting that it is conserved across species.

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Table 1 Association analysis of *BANK1* SNPs in Swedish SLE cases and controls

SNP	Associated allele	χ^2	*P*
rs7675129	T	0.155	0.6933
rs11726012	G	0.361	0.5479
rs11097755	C	1.433	0.2313
rs4522865	A	6.757	**0.0093**
rs4496585	A	2.618	0.1057
rs4572885	T	5.113	**0.0238**
rs10516487	G	8.091	**0.0044**
rs10516486	C	9.62	**0.0019**
rs17200824	A	4.265	**0.0389**
rs6849308	C	7.268	**0.007**
rs10516482	C	8.625	**0.0033**
rs10516483	C	11.437	**0.0007**
rs10516484	A	0.595	0.4404
rs4493533	C	3.184	0.0744
rs3733197	G	0.692	0.4054
rs2631271	G	6.356	**0.0117**
rs2850390	C	1.225	0.2684
rs2631265	C	0.016	0.8997
rs2631267	G	0.1	0.7524
rs2631268	T	1.446	0.2292
rs10516491	C	2.424	0.1195
rs1872701	G	1.842	0.1747
rs2850393	T	0.061	0.8053
rs2850396	C	0.682	0.4088
rs10516490	G	0.329	0.5665
rs10516489	T	0.338	0.5609
rs10516488	G	0.561	0.4538
rs1395306	T	1.97	0.1604

We carried out quantitative analysis of isoform expression in peripheral blood mononuclear cells. As exon 1B transcript was present at very low concentrations (data not shown), we continued the analysis, measuring common (exon 1A and exon 1B) full-length isoform concentrations. We noticed that the ratio of the full-length isoform to Δ2 (FL/Δ2) was not constant, which would be expected if Δ2 were equally expressed regardless of the genotypes of the samples. On the contrary, samples could be divided into groups according to the FL/Δ2 isoform ratio. After closely examining the genomic sequences surrounding exon 2, where putative signals affecting splicing could be located, we identified one SNP, rs17266594, located in the branch-point site. When we re-grouped the expression data, we observed a clear difference between genotypes (**Fig. 1c**). Individuals homozygous for the T allele and having the classical structure of the branch-point site[7] (YNY*T*G**A**YYN) showed higher expression of the full-length isoform; this expression was significantly suppressed (up to 40%) in homozygotes for the minor allele C, with concomitant upregulation of the Δ2 isoform expression. Total *BANK1* expression was not significantly affected by the SNP (**Fig. 1d**).

To determine whether other polymorphisms might contribute to the alternative splicing of exon 2, we sequenced the proximal promoter regions, exon 1A, exon 1B and exon 2 and 500 bp upstream and downstream of these exons, in 24 individuals with SLE and 8 controls. However, we found no previously unidentified SNPs in these regions that could be functional. Next, we identified five nonsynonymous substitutions in *BANK1* from the SNP databases. Although most were nonpolymorphic in our samples, we identified one polymorphic SNP, rs3733197, causing an alanine to threonine substitution at amino acid position 383 (A383T) in exon 7, which encodes the ankyrin repeat-like motif (**Supplementary Table 3** online).

To extend our association analyses, we genotyped rs10516487 (R61H), rs17266594 (branch-point variant) and rs3733197 (A383T) in four additional sets of SLE cases and controls (**Table 2**). We corroborated the genetic association with SLE for all three SNPs, although there were differences between individual populations. Using homogeneity and combinability tests according to the Breslow-Day method, we carried out a meta-analysis comprising 3,971 individuals. We then used the Mantel-Haenszel test to calculate pooled odds ratios of 1.38 ($P = 3.74 \times 10^{-10}$), 1.42 ($P = 4.74 \times 10^{-11}$) and 1.23

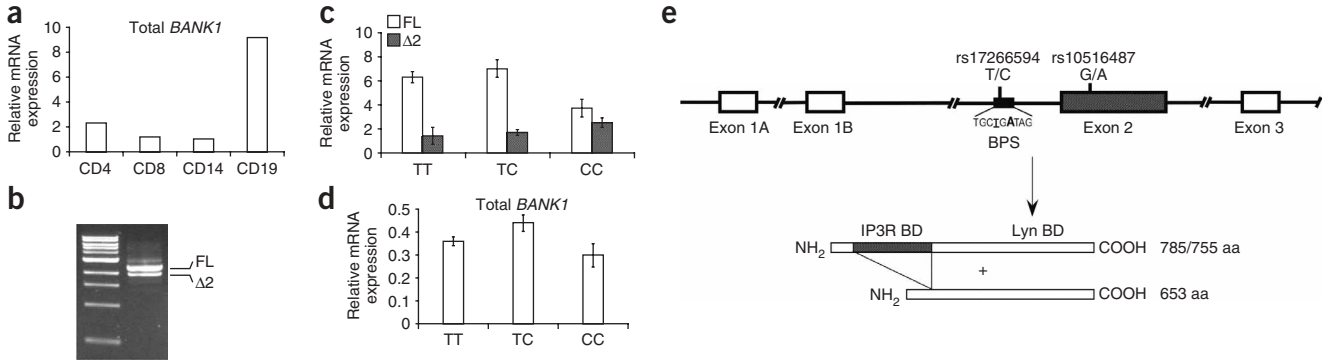

Figure 1 Correlation of rs17266594 genotypes with differences in FL/Δ2 isoform ratio of *BANK1*. (**a**) Expression of total *BANK1* mRNA in Clontech human blood fractions: $CD4^+$ and $CD8^+$ T cells, $CD14^+$ myeloid cells and $CD19^+$ B cells. (**b**) RT-PCR of the coding sequence of *BANK1* amplified from total human spleen cDNA shows two bands on a gel. Ladder (1 kb, New England Biolabs) is shown on the left. We confirmed the identity of both bands (2.3-kb upper band and 1.9-kb smaller band) by sequencing. (**c**) Relative mRNA expression of the full-length and Δ2 isoforms, as determined by quantitative real-time RT-PCR on total RNA purified from human PBMCs of healthy controls and cases. Data represent mean ± s.d. We analyzed 39 individuals with TT for the branch point–site SNP, 34 with TC and 10 with CC. Full-length transcript: TT versus CC, $P = 0.0004$ (Student's *t*-test); Δ2 transcript: TT versus CC, $P = 0.0088$. (**d**) Total *BANK1* expression did not correlate with genotypes of rs17266594. TT versus CC, $P = 0.229$. (**e**) Schematic structure of the 5′ end of *BANK1*. rs17266594, located in the branch point site of intron 1, alters splicing efficiency of the full-length and Δ2 transcripts. rs10516487 results in nonsynonymous substitution R61H. Alternative splicing gives rise to two isoforms, full-length and Δ2 with an in-frame deletion of entire exon 2. The short protein isoform lacks the putative domain for IP3R binding. IP3R BD, inositol 1,4,5-triphosphate receptor binding domain; Lyn BD, tyrosine kinase Lyn binding domain.

Table 2 Genotypic and allelic association of rs10516487 (R61H), rs17266594 and rs3733197 in five sets of SLE cases and controls and joint analysis with Mantel-Haenszel test

	Population	*n*	GG	GA	AA	χ^2	*P*	Odds ratio (CI)[a]	Allele G	Allele A	*P*	Odds ratio (CI)[b]
rs10516487	Scandinavia	Cases (536)	309 (57.6%)	200 (37.3%)	27 (5.0%)	11.7874	0.0028	GG: 2.12 (1.29–3.47)	818 (76.3%)	254 (23.7%)	7.3×10^{-4}	1.39 (1.14–1.68)
		Controls (565)	276 (48.8%)	238 (42.1%)	51 (9.0%)			GA: 1.59 (0.96–2.63)	790 (69.9%)	340 (30.1%)		
	Argentina	Cases (255)	164 (64.3%)	75 (29.4%)	16 (6.3%)	3.8013	0.1495	GG: 1.41 (0.73–2.72)	403 (79.0%)	107 (21.0%)	0.0564	1.31 (0.98–1.74)
		Controls (337)	190 (56.4%)	121 (35.9%)	26 (7.7%)			GA: 1.01 (0.51–2.00)	499 (74.3%)	173 (25.7%)		
	Germany	Cases (312)	181 (58.0%)	118 (37.8%)	13 (4.2%)	11.8503	0.0027	GG: 2.60 (1.32–5.14)	480 (76.9%)	144 (23.1%)	8.13×10^{-4}	1.52 (1.18–1.95)
		Controls (360)	166 (46.1%)	163 (45.3%)	31 (8.6%)			GA: 1.73 (0.87–3.44)	495 (68.8%)	225 (31.2%)		
	Italy	Cases (279)	166 (59.5%)	100 (35.8%)	13 (4.7%)	7.5139	0.0234	GG: 2.49 (1.22–5.09)	432 (77.4%)	126 (22.6%)	0.0078	1.46 (1.09–1.94)
		Controls (245)	123 (50.2%)	98 (40.0%)	24 (9.8)			GA: 1.88 (0.91–3.91)	344 (70.2%)	146 (29.8%)		
	Spain	Cases (702)	414 (59.0%)	243 (34.6%)	45 (6.4%)	11.3579	0.0034	GG: 1.26 (0.77–2.06)	1,071 (76.3%)	333 (23.7%)	0.0065	1.30 (1.07–1.58)
		Controls (446)	219 (49.1%)	197 (44.2%)	30 (6.7%)			GA: 0.82 (0.50–1.35)	635 (71.2%)	257 (28.8%)		
	Pooled	Cases (2,003)	1,187 (59.3%)	706 (35.2%)	110 (5.5%)				3,080 (76.9%)	926 (23.1%)	3.74×10^{-10}[c]	1.38 (1.25–1.53)
		Controls (1,968)	974 (49.9%)	817 (41.8%)	162 (8.3%)				2,763 (70.8%)	1,141 (29.2%)		

	Population		TT	CT	CC	χ^2	*P*	Odds ratio (CI)[a]	Allele T	Allele C	*P*	Odds ratio (CI)
rs17266594	Scandinavia	Cases (511)	296 (57.9%)	189 (37.0%)	26 (5.1%)	9.4399	0.0089	TT: 2.17 (1.28–3.66)	781 (76.4%)	241 (23.6%)	0.0036	1.36 (1.10–1.68)
		Controls (416)	210 (50.5%)	166 (39.9%)	40 (9.6%)			CT: 1.75 (1.03–2.99)	586 (70.4%)	246 (29.6%)		
	Argentina	Cases (274)	188 (68.6%)	77 (28.1%)	9 (3.3%)	14.1697	8.38×10^{-4}	TT: 3.26 (1.51–7.06)	453 (82.7%)	95 (17.3%)	1.06×10^{-4}	1.73 (1.30–2.31)
		Controls (346)	192 (55.5%)	124 (35.8%)	30 (8.7%)			CT: 2.07 (0.93–4.59)	508 (73.4%)	184 (26.6%)		
	Germany	Cases (241)	132 (54.8%)	98 (40.7%)	11 (4.6%)	7.7164	0.0211	TT: 2.46 (1.19–5.09)	362 (75.1%)	120 (24.9%)	0.0080	1.43 (1.09–1.87)
		Controls (335)	151 (45.1%)	153 (45.7%)	31 (9.3%)			CT: 1.81 (0.87–3.76)	455 (67.9%)	215 (32.1%)		
	Italy	Cases (231)	130 (56.3%)	87 (37.7%)	14 (6.1%)	10.1706	0.0062	TT: 2.42 (1.19–4.93)	347 (75.1%)	115 (24.9%)	0.0016	1.59 (1.18–2.14)
		Controls (219)	92 (42.0%)	103 (47.0%)	24 (11.0%)			CT: 1.45 (0.71–2.97)	287 (65.5%)	151 (34.5%)		
	Spain	Cases (678)	404 (59.6%)	231 (34.1%)	43 (6.3%)	14.8617	5.93×10^{-4}	TT: 1.04 (0.62–1.76)	1,039 (76.6%)	317 (23.4%)	0.010	1.29 (1.06–1.56)
		Controls (458)	225 (49.1%)	208 (45.4%)	25 (5.5%)			CT: 0.65 (0.38–1.09)	658 (71.8%)	258 (28.2%)		
	Pooled	Cases (1,856)	1,102 (59.4%)	655 (35.3%)	99 (5.3%)				2,859 (77.0%)	853 (23.0%)	4.74×10^{-11}	1.42 (1.28–1.58)
		Controls (1,774)	870 (49.0%)	754 (42.5%)	150 (8.5%)				2,494 (70.3%)	1,054 (29.7%)		

	Population		GG	GA	AA	χ^2	*P*	Odds ratio (CI)[a]	Allele G	Allele A	*P*	Odds ratio (CI)
rs3733197	Scandinavia	Cases (419)	167 (39.9%)	192 (45.8%)	60 (14.3%)	1.2365	0.5389	GG: 1.04 (0.69–1.58)	526 (62.8%)	312 (37.2%)	0.5832	1.06 (0.87–1.29)
		Controls (444)	163 (36.7%)	220 (49.6%)	61 (13.7%)			GA: 0.89 (0.59–1.33)	546 (61.5%)	342 (38.5%)		
	Argentina	Cases (287)	177 (61.7%)	97 (33.8%)	13 (4.5%)	9.6496	0.0080	GG: 2.36 (1.20–4.66)	451 (78.6%)	123 (21.4%)	0.0018	1.15 (0.95–1.40)
		Controls (363)	184 (50.7%)	147 (40.5%)	32 (8.8%)			GA: 1.62 (0.81–3.25)	515 (70.9%)	211 (29.1%)		
	Germany	Cases (272)	128 (47.1%)	112 (41.2%)	32 (11.8%)	4.1431	0.1260	GG: 1.65 (1.01–2.69)	368 (67.6%)	176 (32.4%)	0.0382	1.28 (1.00–1.63)
		Controls (362)	148 (40.9%)	153 (42.3%)	61 (16.9%)			GA: 1.40 (0.85–2.28)	449 (62.0%)	275 (38.0%)		
	Italy	Cases (253)	131 (51.8%)	102 (40.3%)	20 (7.9%)	8.2595	0.0161	GG: 1.74 (0.92–3.29)	364 (71.9%)	142 (28.1%)	0.0097	1.42 (1.08–1.87)
		Controls (251)	98 (39.0%)	127 (50.6%)	26 (10.4%)			GA: 1.04 (0.55–1.98)	323 (64.3%)	179 (35.7%)		
	Spain	Cases (588)	307 (52.2%)	234 (39.8%)	47 (8.0%)	3.4580	0.1775	GG: 1.14 (0.72–1.82)	977 (72.1%)	379 (27.9%)	0.1474	1.50 (1.15–1.96)
		Controls (455)	212 (46.6%)	206 (45.3%)	37 (8.1%)			GA: 0.89 (0.56–1.43)	630 (69.2%)	280 (30.8%)		
	Pooled	Cases (1,819)	910 (50.0%)	737 (40.5%)	172 (9.5%)				2,686 (70.4%)	1,132 (29.6%)	4.67×10^{-5}	1.23 (1.11–1.36)
		Controls (1,875)	805 (42.9%)	853 (45.5%)	217 (11.6%)				2,463 (65.7%)	1,287 (34.3%)		

[a]Genotypic odds ratio calculated using homozygosity for the protective allele as reference with OR = 1. [b]Calculated using the Robins, Breslow and Greenland method. [c]Calculated using the Mantel-Haenszel χ^2 with fixed effects.

Table 3 Effect sizes of individual SNPs and 2- and 3-SNP haplotypes of *BANK1*[a]

SNP or haplotype	Allele or haplotype	Frequency	OR	95% CI	Effect
rs17266594 (branch point)	T	0.738	1.18	1.09–1.23	Risk
	C	0.262	0.70	0.63–0.78	Protection
rs10516487 (R61H)	G	0.739	1.16	1.07–1.26	Risk
	A	0.261	0.72	0.66–0.80	Protection
rs3733197 (A383T)	G	0.680	1.13	1.04–1.23	Risk
	A	0.320	0.80	0.73–0.89	Protection
2-SNP haplotype[b]	TG	0.744	1.16	1.07–1.25	Risk
	CA	0.256	0.70	0.63–0.77	Protection
3-SNP haplotype	TGG	0.636	1.16	1.06–1.27	Risk
	CAA	0.211	0.69	0.62–0.77	Protection
	TGA	0.108	0.98	0.84–1.14	Neutral
	CAG	0.045	0.73	0.54–0.92	Protection

[a]Effect sizes were calculated using WHAP, and ORs were estimated using R language.
[b]2-SNP = rs17266594 + rs10516487; 3-SNPs are in the order rs17266594, rs10516487 and rs3733197.

($P = 4.67 \times 10^{-5}$) for rs10516485, rs17266594 and rs3733197, respectively, for the allelic association, supported by genotypic association (**Table 2**).

rs17266594 and rs10516487 are separated by 153 bp and are in strong LD ($D' = 0.95$; $r^2 = 0.90$ calculated for all sets jointly; **Supplementary Fig. 2** online). rs3733197 is 88 kb away from rs10516487 ($D' = 0.72$; $r^2 = 0.39$) and rs17266594 ($D' = 0.68$; $r^2 = 0.27$), and could segregate with a risk haplotype in some individuals (**Supplementary Figs. 2** and **3** online). To better define the relative contribution of each SNP, we carried out conditional logistic regression analyses using the three SNPs. We found that none of the SNPs is independent of the others, as a result of the LD between them (colineality in the multiple logistic regression analysis). Through haplotype-based logistic regression analysis using WHAP[8], we did not find any differences in the effect sizes (OR) of the individual SNP alleles or the 2- or 3-SNP haplotypes (**Table 3**). Thus, linkage disequilibrium, haplotype and conditional regression analyses suggested that all three SNPs, either individually or as haplotypes, confer susceptibility for SLE.

BANK1 is a B-cell adaptor protein[9,10]. The two full-length isoforms of 785 and 755 amino acids differ by 30 amino acids at the N terminus, encoded by the alternative exon 1A (**Fig. 1e**), and contain ankyrin repeat motifs and coiled-coil regions, structures very similar to other adaptor proteins[11]. B-cell activation through the B-cell receptor leads to tyrosine phosphorylation of BANK1, which in turn promotes its association with the tyrosine kinase Lyn and the calcium channel IP3R, facilitating phosphorylation and activation of IP3R by Lyn and release of Ca^{2+} from endoplasmic reticulum stores[5,12]. IP3R associates with the N-terminal domain of BANK1 encoded by exon 2, whereas Lyn interacts with the C-terminal portion[5]. Our own analysis predicts a pleckstrin homology domain in the N terminus, which could also participate in phosphatidylinositol-mediated signaling. rs10516487 lies within the region essential for binding of IP3R. We speculate that R61, being highly protonated under conditions of physiological pH, could potentially alter the affinity of BANK1 for IP3R, favoring stronger binding, although this has yet to be tested.

rs17266594 may affect the relative splicing efficiency, but not splicing *per se*, of the full-length and Δ2 isoforms of *BANK1*. Mutations affecting the thymidine of the branch point consensus sequence and altering splicing efficiency have been previously described[13]. Through more efficient splicing of a full-length transcript containing the arginine residue in the IP3R-binding domain, a more 'active' protein would be expected in individuals at risk. On the contrary, given that the Δ2 isoform lacks the entire exon 2 and thus the IP3R-binding and PH domains (**Fig. 1e**), it possibly functions as a dominant-negative or rather, a dose-dependent isoform attenuating BANK1-mediated signaling. This is supported by the observation of a strong protective genetic effect in individuals with the CC and CT genotypes of rs17266594 (CC: OR = 0.52, 95% CI = 0.0–0.67; CT: OR = 0.68, 95% CI = 0.59–0.78) that show increased concentrations of the Δ2 isoform relative to the full-length isoform (**Fig. 1c**). Experimental evidence for a dominant-negative effect of the Δ2 isoform is needed to validate this proposed mechanism.

The importance of mutations in ankyrin motifs for interactions with IP3R was recently highlighted by a discovery linking single amino acid substitutions in the adaptor protein ankyrin-B with cardiac arrhythmia and sudden cardiac death[14]. Although the A383 variant is associated with SLE, the minor allele 383T of rs3733197 might create a site for threonine kinases[15].

B cells are the primary cell type affected in SLE. Novel therapies are aimed at depleting hyperactivated B cells that may function as autoantibody-producing cells and as important regulators of innate and adaptive immune responses through antigen presentation and cytokine-mediated signaling[16]. Functional and expression abnormalities of signaling molecules in B cells have been described in individuals with lupus. Of note, Lyn, a binding partner of BANK1, is of key importance in both human and mouse lupus disease models[17–22].

Increased binding of BANK1 to downstream effector proteins may lead to a steady state marked by B-cell hyperresponsiveness or deregulated B-cell activation. The precise role of BANK1 in B cell receptor–mediated signaling remains unclear, as two reports published to date contain conflicting data regarding the stimulatory or inhibitory role of BANK1 on B-cell activation[5,6]. Given the previously unreported existence of the alternative splicing of exon 2, we can speculate that the negative role for BANK1 assigned from the knockout model was a result of, in part, the residual expression of the Δ2 isoform, as this exon was targeted by the knockout construct[6]. Further experiments are required to fully understand if, and how, *BANK1* polymorphisms lead to B-cell hyperactivity, breakage of B-cell tolerance and production of autoantibodies, which are the principal hallmarks of SLE.

METHODS

Clinical samples. In our initial scan, we genotyped 279 Swedish SLE cases and 515 controls using the Affymetrix 100K array. Of these, 279 cases and 352 controls were available for the additional genotyping of *BANK1* SNPs (**Table 1**). For the functional polymorphisms, we genotyped an additional 185 Swedish SLE cases; 465 of the controls were available for genotyping of rs17266594 and rs3733197. For the final Mantel-Haenszel analysis and OR estimation, we added 84 Danish SLE cases to the Swedish cases, comprising the Scandinavian set (**Table 2**). The replication sets included 384 North German SLE cases and 374 controls, 288 Argentine SLE cases and 372 controls, and 286 Italian SLE cases and 252 controls. The Spanish cohort included 799 SLE cases and 542 controls from several regions in Spain. Of these, we genotyped 707 SLE cases and 469 controls for rs10516487 and rs3733197, and 678 SLE cases and 457 controls for rs17266594, as DNA from a number of controls was not available. The German, Spanish and Argentine SLE cases have all been previously described[23]. The Italian cases are a multicenter collection of affected individuals and their matched controls from Rome, Siena, Milan and Naples (North- and Mid-Italy). All cases fulfill the 1982 American College of Rheumatology (ACR) criteria for

the classification of SLE[24]. All participating subjects provided informed consent for this study. The study was approved by the various institutional review boards and ethical committees at each of the participating locations.

Genotyping. We carried out genotyping using the 100K Affymetrix array according to the manufacturer's instructions. We carried out fine-mapping and replication for SNPs rs10516487, rs17266594 and rs3733197 using TaqMan SNP genotyping assays (Applied Biosystems). The Affymetrix genotyping and fine-mapping were done at Serono Genetics Institute (now MerckSerono SA). The functional polymorphism replications were done at Uppsala University. For verification, 106 samples were genotyped twice, showing 100% concordance. Genotyping success rate for all the samples was over 92%.

Statistical analysis. For the 100K Affymetrix whole-genome scan analysis, we applied the following pre-processing filters. Specifically, SNPs were discarded if: (i) the proportion of missing genotypes was higher than 5%, (ii) the relative minor allele frequency was lower than 1%, or (iii) the probability that the observed genotype distribution results from sampling a SNP which follows the Hardy-Weinberg equilibrium was lower than 0.02. After filtering, we used data from 85,042 SNPs. We retained only SNPs from autosomal chromosomes for the sake of homogeneity between male and female subjects. SNP sequences were mapped onto NCBI 36 human genome assembly, and SNPs with multiple localizations were discarded. For each remaining SNP, we calculated genotypic and allelic frequencies in cases and controls and computed the corresponding probability values using exact (non-asymptotic) and unbiased algorithms[25]. Detailed results from the scan will be published elsewhere. The false-discovery rate (FDR) was then estimated using a method previously described[4].

For fine-mapping analyses, we estimated genetic association, haplotype estimation, LD and r^2 using Haploview (v4.0RC2). The Breslow-Day test of combinability and the Mantel-Haenszel test were carried out using the StatsDirect software (v2.4.6). As the Breslow-Day test showed combinability of the strata, the Mantel-Haenszel test for fixed effects was used in the analysis. Haplotypes were constructed using the PHASE software (v2.1)[26,27]. Genotypic odds ratios were calculated using the Unphased software (v3.0.9)[28].

We carried out logistic regression analysis and conditional multiple logistic regression analysis using R language glm routines. Haplotype-based logistic regression analysis was done using WHAP[8]. Coefficients were estimated with WHAP, and ORs and confidence intervals (**Table 3**) were calculated using R language.

Sequencing. DNA fragments for sequencing were amplified with the corresponding primers (**Supplementary Table 3**), purified from agarose gel with QIAquick gel extraction kit (Qiagen) and sequenced using BigDye Terminator 3.1 (Applied Biosystems) at the Uppsala Genome Center.

RNA purification and *BANK1* expression analysis. Total RNA was purified with TRIZOL Reagent (Invitrogen) from peripheral blood mononuclear cells (PBMCs) obtained with agreed consent from healthy donors and SLE cases. 2 μg of RNA were reverse-transcribed with 2 U of MultiScribe transcriptase in PCR buffer II containing 5 mM $MgCl_2$, 1 mM dNTPs, 0.4 U of RNase inhibitor and 5 μM oligo-dT. All reagents were purchased from Applied Biosystems. cDNA synthesis was done at 42 °C for 80 min, and then the reaction was terminated at 95 °C for 5 min. All cDNA samples were diluted to 15 ng/μl.

BANK1 expression was determined by real-time PCR on an ABI PRISM 7700 Sequence Detector (Applied Biosystems) with SDS 1.9.1 software. Total *BANK1*, both alternative full-length isoforms and the Δ2 isoform were quantified with SYBR Green and relevant primers (**Supplementary Table 3**). We carried out initial denaturation at 95 °C for 5 min followed by 45 cycles of PCR (95 °C for 15 s, 62 °C for 15 s and 72 °C for 30 s). PCR buffer provided with enzyme was supplemented with 3 mM $MgCl_2$, 200 μM of each of dNTPs, primers, SYBR Green (Molecular Probes), 15 ng of cDNA and 0.5 U of Platinum Taq polymerase (Invitrogen). Expression levels were normalized to the levels of TBP in the same samples amplified with commercial reagents (Applied Biosystems). All experiments were run in triplicate. Independent cDNA synthesis was carried out twice.

Expression levels for total *BANK1*, both full-length isoforms and Δ2 isoform in separated blood cell populations ($CD4^+$, $CD8^+$, $CD14^+$ and $CD19^+$ cells) were determined using Human Blood Fractions MTC Panel (Clontech). Statistical calculations were performed with available online GraphPad Software using two-tailed *t*-test.

Cloning of human, mouse and chimpanzee Δ2 isoform. Purification of total RNA from mouse spleen and cDNA synthesis were conducted as described above for the human PBMCs. Total RNA from chimpanzee (*Pan troglodytes*) spleen was provided by T. Bergström and L. Cavelier (Uppsala University). The human gene was amplified from Human Spleen BD Marathon-Ready cDNA (Clontech). After initial denaturation at 95 °C for 5 min, 35 cycles (95 °C for 20 s, 60 °C for 15 s and 72 °C for 2 min 30 s) were performed in PCR buffer containing 2 mM $MgSO_4$, 200 μM of each of dNTPs, 0.4 μM of each of the corresponding primers (**Supplementary Table 3**) and 0.5 U of Platinum Taq-High Fidelity enzyme (Invitrogen). Chimpanzee cDNA was amplified with human-specific primers. PCR products were purified from agarose gel and cloned in pCR 4-TOPO vector (Invitrogen) according to the manufacturer's instructions. Plasmid DNA from positive clones was purified with QIAprep Spin Miniprep kit (Qiagen) and verified by sequencing.

Accession codes. GenBank: full-length isoforms of *BANK1* containing exon 1A or exon 1B, NM_017935 and AB063170, respectively. Δ2 transcript sequences have been deposited with the following accession codes: EU051376, human; EU051377, chimpanzee and EU051378, mouse.

URLs. Haploview, http://www.broad.mit.edu/mpg/haploview/; GraphPad Software, http://www.graphpad.com; protein analysis, http://www.ebi.ac.uk/saps/, http://smart.embl-heidelberg.de/, http://ca.expasy.org/prosite/, and http://www.cbs.dtu.dk/services/NetPhos/; WHAP, http://pngu.mgh.harvard.edu/~purcell/whap/.

Note: Supplementary information is available on the Nature Genetics website.

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M.E.A.-R., S.V.K. and H.A. designed the experiments. S.V.K., A.-K.A., J.W., A.Z., M.V.P.L.R. and E.S. performed the experiments. M.E.A.-R., S.V.K., A.-K.A., J.W. and H.A. performed the analyses. I.G., E.S., G.S., L.T., A.J., T.W., S.D., N.B., M.G.D., C.G., A.S., P.J., H.L., B.A.P.-E., M.F.G.-E. and J.M. and their multicenter collaborators provided the samples. M.E.A.-R., S.V.K. and A.-K.A. wrote the manuscript.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturegenetics/.

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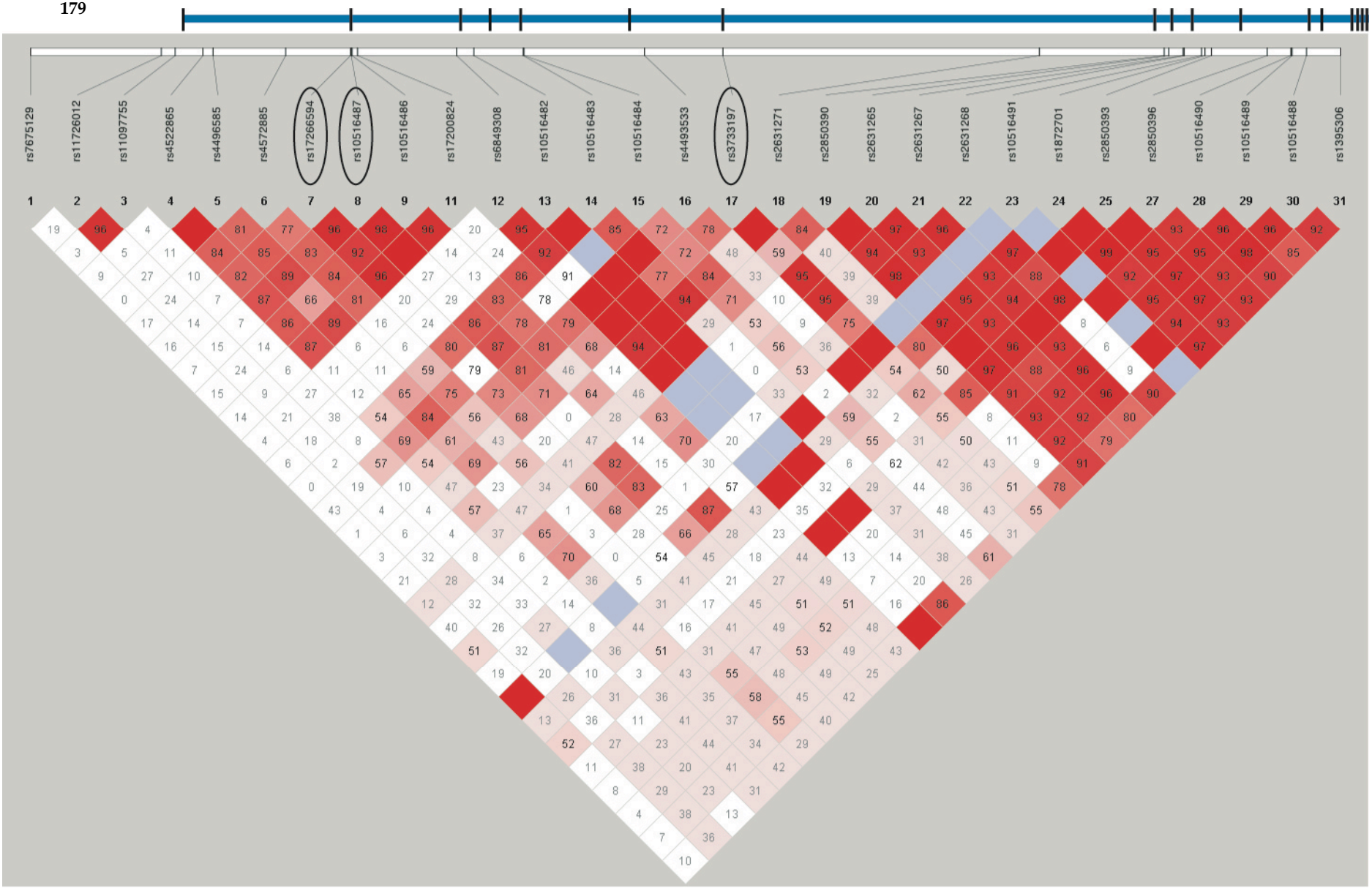
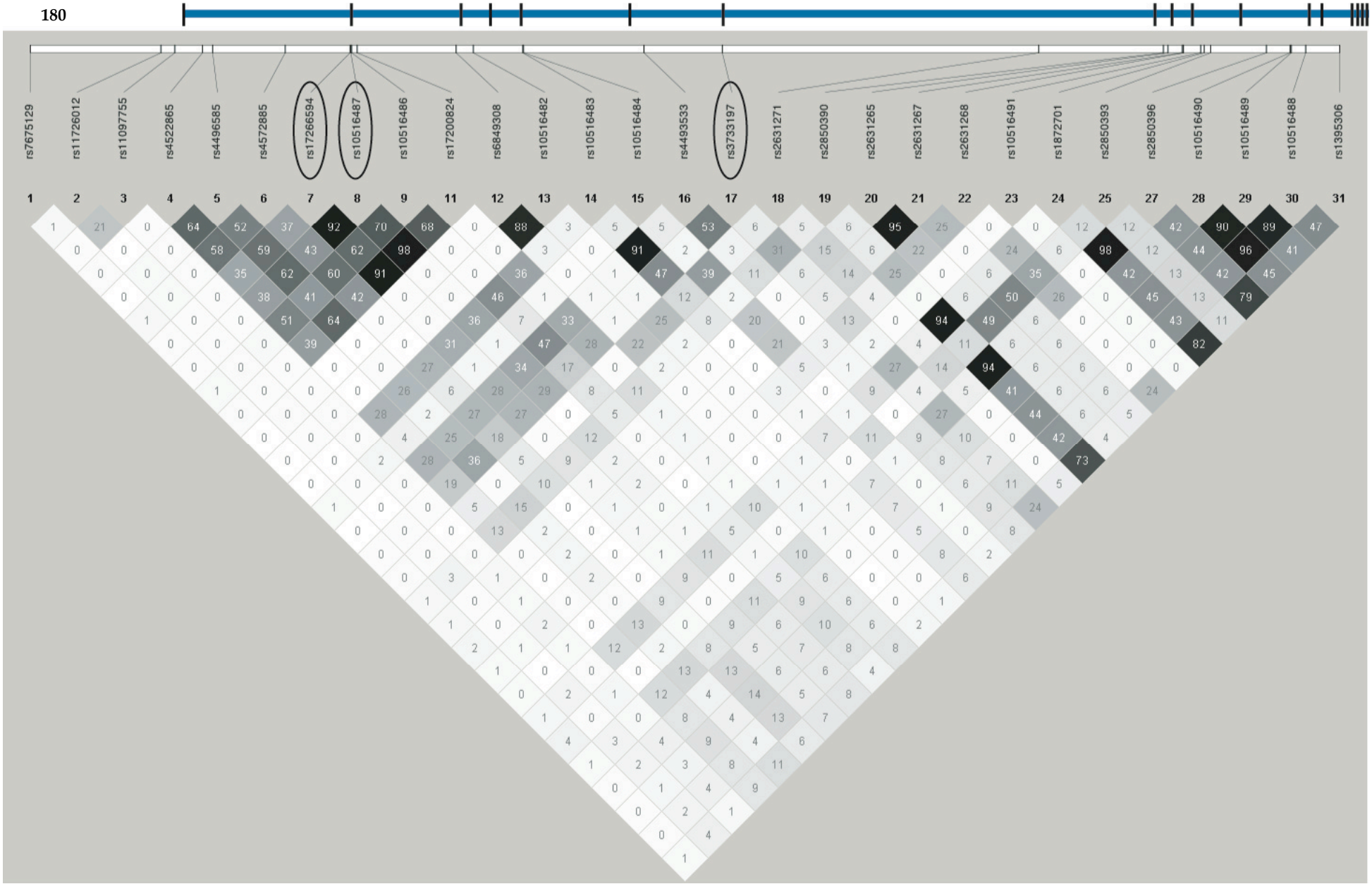
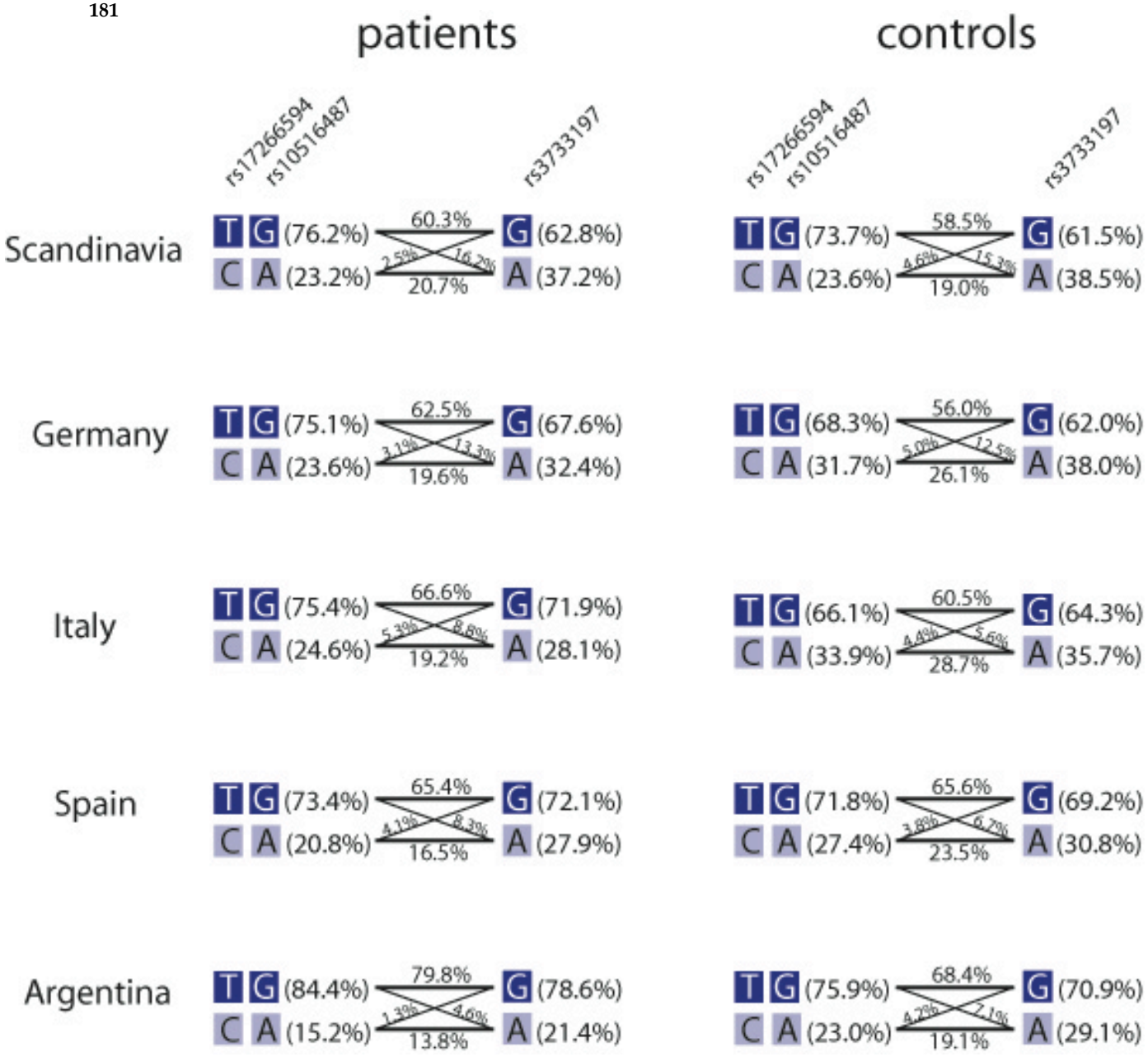


Supplementary Figure 1
Linkage disequilibrium structure (D') across *BANK1*. Data calculated with Haploview using the Swedish cases and controls run for 30 SNPs across the gene. In top of the figure, the genomic structure of *BANK1* is shown in blue, with exons shown as bars, being 5'-3' direction from left to right.



Supplementary Figure 2
Estimation of the R^2 between SNPs across *BANK1* using Haploview and data from the Swedish cases and controls. The genomic structure of *BANK1* is also shown in the top as in supplementary figure 1.



Supplementary Figure 3
Frequency and structure of the 2-SNP (rs17266594 and rs10516487) and 3-SNP haplotypes (including rs3733197) for each of the populations and cases and controls, separately.

Supplementary Table 1. BANK1 SNPs in the 100k Array			
SNP	**rs number**	**Position**	**(-log) P value**
SNP_A-1701374	rs10516487	103108254	2.27
SNP_A-1701494	rs10516486	103108454	2.79
SNP_A-1664926	rs6849308	103133261	2.22
SNP_A-1706628	rs10516482	103137348	2.52
SNP_A-1744756	rs10516483	103149083	3.25
SNP_A-1683131	rs2631271	103271574	n.s.
SNP_A-1697391	rs10516489	103331537	n.s.

Supplementary Table 2. Location of BANK1 SNPs

SNP rs number	Position**	Location in BANK1
rs7675129	102894046	intergenic, 36.8 kb upstream
rs11726012	102925041	distal promoter, 5.8 kb upstream
rs11097755	102928331	promoter, 2.5 kb upstream
rs4522865	102934911	intron 1
rs4496585	102937309	intron 1
rs4572885	102954536	intron 1
rs10516487	102970099	exon 2 coding (NS)*
rs10516486	102970299	exon 2 (synonymous)
rs17200824	102971612	intron 2
rs6849308	102995106	intron 2
rs10516482	102999193	intron 3
rs10516483	103010928	intron 5
rs10516484	103011108	intron 5
rs4493533	103039707	intron 6
rs3733197	103058310	exon 7 coding (NS)
rs2631271	103133419	intron 7
rs2850390	103163019	intron 8
rs2631265	103164099	intron 8
rs2631267	103167495	intron 9
rs2631268	103167753	intron 9
rs10516491	103171889	intron 10
rs1872701	103172704	intron 10
rs2850393	103174239	intron 10
rs2850396	103187471	intron 11
rs10516490	103193084	intron 11
rs10516489	103193382	intron 11
rs10516488	103196800	intron 11
rs1395306	103204873	intron 13

*NS: non-synonymous substitution

**Ensembl release 46

Supplementary Table 3. Primer sequences

Gene /gene fragment/isoform	Forward	Reverse
hBANK cDNA amplification	CACCTCAACCGCCACAATGCTGCCAGCA	ATAATAACCTTCTTTAATGATCTTTCTTGC
Total BANK1 qRT-PCR	AGAGGAAACTACACCTTACATAGCTC	GATGAGTTCTTCCTGACCATCAG
Total full-length isoforms	TCAAAGCAGATGGGAGATCTCAAC	
Δ2 isoform	CAGCGCCCCCAGATTCTGAAG	
Exon1A full-length isoform	CAGCGCCCCCAGGAAATACA	
Alternative exon1 full-length isoform	GCCTATTCTTTGTTTTGGAAATACA	
		Common reverse primer for all isoforms for qRT-PCR
		CACATGGAATTTCAGTGGGAAGCAC
		Common reverse primer for gel-analysis
		ATCACAGTAGACATTGACATGGAC
FOR GENOMIC SEQUENCING:		
promoter, exon 1A and 5'-part of intron1	TTGGAGAGGGTATTTAGAGCCATA	AAGCAGGGCTACCAATTCACCAG
Alternative exon1B	CTATGATACTGGAAATACTGTCAGT	AGCATATGACCAGCTGATCAG
Exon2	TTGATTTACTATGAAAATATCAAGC	TTACATAAGAAACCAGCTTCCAG
Mouse BANK1 cDNA	ACCTCCCGCAATGCTTCCTGT	ACATGGAATTTCCCCAGGAAGCAC

STAT4 Associates with SLE Through Two Independent Effects that Correlate with Gene Expression and Act Additively with *IRF5* to Increase Risk

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ABSTRACT

To confirm and define the genetic association of *STAT4* and systemic lupus erythematosus we genotyped 30 tag SNPs in an independent set of Spanish cases and controls. Four SNPs were still significant after fine mapping of the *STAT4* gene and three appeared as independent peaks from rs7574865 after which the effect of rs3821236 was the strongest. All SNPs surviving correction for multiple tests were genotyped in 5 new sets of cases and controls for replication. After conditional regression analyses, two major independent signals represented by SNPs rs3821236 and rs7574865, remained significant across the sets and after meta-analyses including adjustment for population-specific confounding effects. These two SNPs are not correlating and are in separate haplotype blocks. High levels of *STAT4* expression correlated with SNPs rs3821236, rs3024866 (in the same haplotype block as rs3821236) and rs7574865 but not with other SNPs. 5′-RACE PCR analysis revealed transcription of alternative tissue-specific exons 1, indicating presence of tissue-specific promoters that could be of importance in the expression of *STAT4*. When we analyzed if there was interaction with associated SNPs from the *IRF5* gene, we observed no interaction using regression analysis, which suggested an additive effect between the genes. The C-statistic was significantly increased when rs7574865 and the SNP rs2070197 tagging the *IRF5* risk haplotype were included in the model as compared to either SNP alone. These data confirm *STAT4* as a susceptibility gene for SLE and suggest the presence of at least two functional variants affecting levels of *STAT4*. Our results also suggest that both genes *STAT4* and *IRF5* act additively to increase risk for SLE.

INTRODUCTION

The identification of genetic susceptibility for systemic lupus erythematosus (SLE) as well as for other complex diseases is now approachable through dense SNP association analyses [1, 2]. SLE has a strong genetic component supported by studies on twins and families and a high familial aggregation[3]. Like most autoimmune diseases, the HLA has an important contribution [1, 2, 4] and we recently showed that the HLA is indeed the genetic factor in individuals of European ancestry followed by *IRF5* and *ITGAM* [1, 2, 4, 5]. Other somewhat weaker but well established associations have been found to *FCGRIIA* [6], *PTPN22* [7], *PDCD1* [8], *TNFSF4* [9], *BLK* [1, 2] and most recently *BANK1* [10]. Recently a genetic association with the signal transducer and activator of transcription 4 (*STAT4)* was identified in rheumatoid arthritis (RA) with SNP rs7574865 and this association was also

found in SLE [11]. From the RA studies, the genetic association was defined to the region covering the 3rd intron of *STAT4*. Both replication and dense genotyping of the genes in different populations is required to define whether there are more than one independent signal that could be important in susceptibility and if the genetic association indeed applies through ethnic and geographic barriers. Indeed, most of the results published to date, have also a bias towards North European populations. Thus, our aim with the present study has been to revise the *STAT4* genetic association using independent sets from other populations and a dense set of tag SNPs to define if indeed only rs7574865 and thus, the 3rd intron signal is the sole genetic contributor to susceptibility in *STAT4*.

The product of the *STAT4* gene is a critical regulator of immune responses, is primarily induced by the dendritic cell-produced IL-12 leading to the development of Th1 cells, cells with the capacity to secrete high levels of IFN-γ. *STAT4* is activated after IL-12 ligation to each of the two subunits of the IL-12 receptor, IL12RB1 and IL12RB2 that respectively associate with the tyrosine kinases Tyk2 and Jak2 [12, 13]. These are expressed in activated T and B cells and particularly NK cells. Activation of *STAT4* leads to the formation of homodimers of *STAT4* that translocate into the nucleus and induce transcription of IFN-γ. In addition, *STAT4* activation is also induced by IFNα/β stimulation, however this stimulation does not appear to lead to Th1 development, but only to an acute IFN-γ secretion by CD4+ T and NK cells where IL-18 is also required. Indeed, IFNα/β induces *STAT4* phosphorylation through direct interaction of *STAT4* with the IFNαR2 subunit.

Here, we fine mapped a Spanish set of cases and controls. We find evidence for another peak of association beyond the intron 3 SNP rs7574865, which was replicated in 4 independent sets of cases and controls. We also find evidence for a correlation between associated SNPs and high expression levels of *STAT4*. When we analyzed the possibility of genetic interaction between *STAT4* and *IRF5*, we found no interaction, however *STAT4* and *IRF5* do act additively to increase the risk for SLE [3].

MATERIAL AND METHODS

Patients and controls

The following samples have been used in this study, all with complete data for the SNPs analyzed, in total 1581 cases and

1844 controls: from Spain 390 cases and 620 controls, 247 cases and 220 controls from Germany, 221 patients and 207 controls from Italy, 171 patients and 171 controls from Argentina and 231 cases and 250 controls from Mexico (adults) along with a set of 321 pediatric patients and 383 adult controls. The patient and control sets studied here have been described previously [10, 14]. All patients fulfilled the 1982 ACR criteria for the classification of SLE.

Selection of Tag SNPs

29 Tag SNPs covering the *STAT1* and *STAT4* genes, and the intergenic region, were selected using Haploview version 3.32 from the HapMap-CEU population genotype data. Aggressive tagging mode was used to select a set of tag SNPs with a minor allele frequency of at least 5%, with an r2 threshold ≥0.8. The SNP rs7574865 was added to the tag list after it was reported as associated with RA [11]. The SNPs associated in the Spanish fine mapping, after quality control and correction for multiple testing, were typed in the German, Italian, Argentinean and both sets of Mexican samples.

Genotyping

The Spanish samples used for fine mapping were genotyped in Granada using the Custom TaqMan® 5′ exonuclease assay service from ABI (Foster City, CA). German, Italian, as well as the Latin American samples were genotyped at the University of Uppsala. The Mexican pediatric samples were genotyped at the Instituto Nacional de Medicina Genómica using the same method. Genotyping consistency between the centers was established to be near to 100% [15].

Statistical Analyses

The Spanish genotyping data was processed using Haploview version 4.0 [16], PLINK version 1.02 [17], and R language. Quality control filters were applied to remove SNPs when they presented more than 10% of missing data in either cases or controls (1 SNP excluded), a genotype distribution in controls not following the Hardy-Weinberg equilibrium proportions ($p < 0.001$, 2 SNPs excluded), or too low minor allele frequency in controls (MAF < 5%) (2 SNPs excluded). After frequency and genotyping pruning, there were 25 SNPs left. Subjects with an individual missing genotyping rate > 10% were also removed (n=42). The genotyping rate in remaining individuals was 97.4%. Pairwise linkage disequilibrium (LD) measures (*D′* and *r2*) between SNPs and maximumlikelihood haplotype frequencies were estimated by the EM algorithm, and then allele,

genotype and haplotype frequencies in cases and controls were compared. A *p* value below 0.05 was considered significant for all the statistical tests and multiple testing was corrected by both Bonferroni and false discovery rate (FDR) methods [18]. The SNPs remaining associated at the significance level after data pruning were chosen for replication in German, Italian, Argentinean and both Mexican sample sets and only subjects with individual genotyping rates equal to 100% were included for analysis. Both the DerSimonian-Laird and Cochran-Mantel-Haenszel methods implemented in StatsDirect and PLINK were used to estimate the pooled odds ratio for all populations assuming random and fixed effects models on the alleleic association, respectively. The heterogeneity test based on partitioning the chi-square statistic as implemented in PLINK was used to test for between-population differences in the association. Logistic regression was used to estimate univariate odds ratios considering genotypes [19]. The obtained pooled odds ratios were adjusted by adding the stratification variable "Population" to the logistic regression model already containing the genotype as the exposure variable. Day and Byar [20] have shown that this test is identical to the 1 degree of freedom Mantel-Haenszel test of the hypothesis that the stratum specific odds ratios are 1. Conditional logistic regression was used to determine if the additional associated SNPs were independent or not from rs7574865, a well established associated variant for SLE. All the logistic regression analysis was done with R. Multiple logistic regression was used to evaluate if additive or interaction effects were present between SNPs within *STAT4* and with SNPs in *IRF5*. Test for interaction included both SNPs and the interaction effect between them in the model. If no significant interaction was present an additive model was assumed if both SNPs remained significant in a model with the interaction term removed. To measure the ability to discriminate between SLE cases and controls, the area under the receiveroperating characteristic (ROC) curve (C-statistic) was calculated. To statistically compare the C-statistics we applied the method by DeLong et al. [21]

5′-RACE and 3′-RACE PCR

Marathon-Ready cDNA from different tissues (Clontech) was used as template for amplification of tissue-specific 5′- and 3′-UTRs. The following gene-specific primers were used for 5′-RACE PCR: 5′-GAAATTCTACTGAGAGACTCCCATTG-3′ in exon 13 and nested primer in exon 3

5′GAATCGTTGCCATGGTTTCATTGTTA G-3′ and for 3′-RACE PCR: 5′-CTAAACTATCAGGTAAAGGTTAAGGC ATC-3′ in exon 11 and nested primer in exon 21 5′-GGTAAACACTACAGCTCTCAGCCTTG-3′. Adapter primers Ap1 and Ap2 were provided with cDNA. Nested PCR was carried out using 1/30 of the first round PCR products. 35 cycles of PCR (95oC for 20 s, 60oC for 15 s and 72oC for 3 min) were performed after initial denaturation at 95oC for 5 min in buffer containing 1.5 mM MgCl2, 200 μM of each of dNTPs, 0.4 μM of each of the corresponding primers, and 0.5 U of Platinum Taq high fidelity enzyme (Invitrogen). PCR products were cloned and analysed by sequencing: PCR products were purified from agarose gel with QIAquick gel extraction kit (Qiagen) and either sequenced directly using BigDye Terminator 3.1 (Applied Biosystems) or after subcloning into pCR4-TOPO vector (Invitrogen).

RNA purification and STAT4 expression analysis

Total RNA was purified as described elsewhere [10] from peripheral blood mononuclear cells (PBMCs) obtained with agreed consent from healthy volunteers. Single strand cDNA was transcribed from 2 μg of RNA with 2 U of MuLV reverse transcriptase (Applied Biosystems) in buffer containing 5 mM MgCl2, 1 mM dNTPs, 0.4 U of RNase inhibitor and 5 μM oligo-dT. Synthesis was performed at 42oC for 80 min followed by termination at 95oC for 5 min.

STAT4 expression was determined by real-time PCR on an ABI PRISM 7700 Sequence Detector (Applied Biosystems) with SDS 1.9.1 software. We performed initial denaturation at 95oC for 5 min followed by 45 cycles of PCR (95oC for 15 s, 60oC for 10 s and 72oC for 20 s). PCR buffer provided with enzyme was supplemented with 1.5 mMgCl2, 200 μM of each of dNTPs, primers, SYBR Green (Molecular Probes), cDNA and 0.5 U of Platinum Taq polymerase (Invitrogen). α-isoform was detected with the following primers: forward 5′-CATCTCAACAATCCGAAGTGATTCA-3′ and common reverse primer 5′-GTCAGAGTTTATCCTGTCATTCAGCAG -3′. β-isoform-specific forward primer was 5′-TGACCTTGTTATCTCTTTAAGCCGA-3′. Expression levels were normalized against TATA-binding protein (TBP) gene expression amplified with commercial reagents (Applied Biosystems). All experiments were run in triplicate.

Statistical analysis of gene expression

Statistical calculations were performed with available on-line GraphPad Software using two-tailed *t*-test. ANOVA and F-test were used to determine the difference in the mRNA expression level in relation to each of the SNPs, taking the three possible genotypes as factor levels. Significance of gene expression was also tested with linear regression using ΔT values as a continuous trait and WHAP for regression with individual SNP alleles [22].

RESULTS

29 tag SNPs selected from HapMap were genotyped as well as SNP rs7574865 recently reported to be associated with RA and SLE [11]. Of these, 25 passed our quality control filters. The results showed strong evidence of association in this set of patients for several SNPs across *STAT4* and the *STAT1-STAT4* intergenic region. We observed the strongest association with SNPs rs3821236 (p = 7.07x10-8), rs7574865 (p = 9.37x10-6), rs3024866 (p = 3.83x10-7) and rs1467199 (p = 7x10-5), which remained significant after correction for multiple tests (**Table 1).** The region LD structure defined six haplotype blocks, two located in *STAT1* (blocks 1-2), one located in the intergenic region (block 3) and three in *STAT4* (blocks 4-6) **(Figure 1 and Supplementary Figure 1**). All the haplotype blocks were associated with disease susceptibility except the ones located in *STAT1*. According to previous studies, a haplotype located in the 3rd intron and containing the SNP rs7574865 was strongly associated with RA and SLE. However, two more haplotype blocks were also associated: block 3, which contains rs1467199, and block 4 harboring both SNPs rs3821236 and rs3024866. SNP rs7574865 is located in block 6. **(Figure 1)**. In order to further replicate the genetic associations found and increase the statistical power of our analyses, we genotyped the associated SNPs in 5 independent sets from Italy, Germany, Argentina, and Mexico (one adult and one pediatric set). The homogeneity test showed combinability of the odds ratios for SNPs rs1467199 and rs3821236, while SNPs rs3024866 and rs7574865 had some heterogeneity across the strata. None of the SNPs provided association with the German population except for a weak borderline association with rs7574865 (**Tables 2 and Suplementary Table 1**). The Mexican pediatric set showed also no association for any of the SNPs except for rs1467199 (p = 0.008, **Supplementary Table 2**). However, as the heterogeneity was weak (p = 0.01) for the German set, it was included in the meta-analysis. The odds

ratios of the Mexican pediatric set were not combinable with the other sets and allele frequencies were statistically different (compare Supplementary Table 2 with Table 2) and therefore it was not included in the meta-analysis. Allelic and genotypic meta-analyses of the data were then performed using the tests described in materials and methods. Clearly, SNPs rs3821236 (p = 5.96 x 10-20) and rs7574865 (p = 4.44 x 10-23) showed the strongest association across all the strata in the allelic and genotypic tests, but rs3024866 was also associated (p = 2.31 x 10-12) (**Table 2**). Although SNP rs1467199 reaches significant association in the meta-analysis, at the individual population level it was only replicated in the Argentine set (rs1467199-CG p = 1.63 x10-2, rs1467199-GG p = 0.053) (**Supplementary Table 1**). We then analyzed the possibility that the SNPs were showing independent effects from each other and primarily from rs7574865. Importantly, the correlation between rs3024866 and rs7574865 was R2 =.29 and between rs3821236 and rs7574865 R2=.42, therefore these two SNPs located 62 kb and 42 kb from rs7574865, respectively, are not proxies of rs7574865. The same can be said for SNP rs1467199 which had an R2 =.30 with rs3821236, R2 =.18 with rs3024866 and R2 =.13 with rs7574865 (**Figure 1**). The low pairwise correlation coefficients (R2) suggest that the individual SNP associations might reflect independent effects, except for rs3024866 which has relatively high R2 with rs3821236 (R2=.64) (**Figure1**) and is in the same haplotype block. This was further confirmed by conditional logistic regression analysis: conditioning on rs7574865, we observed that rs3821236 remained significant. Likewise, when conditioning on rs3821236, rs7574865 was the only associated SNP (**Supplementary Table 3**). Thus, rs3821236 and rs7574865 seem to represent two independent genetic effects within *STAT4*, with the former located on intron 16th far from the 3rd intron of *STAT4* where the association has been confined in previous studies [11]. SNP rs3821236 and rs3024866 are tagging a 26kb haplotype block covering from intron 8 to 16th (**Figure 1 and Supplementary Figure 1**) that contains 3 of the 6 (50%) markers associated with SLE in the Spanish fine mapping after stringent multiple test correction (**Table 1**). We did one more test using the PLINK conditional haplotype-based association test. The haplotype effect of the phased SNPs rs3821236-rs932169 in this block, controlling for rs7574865, remains significant (p = 0.002277) further

confirming their independency. When we compare the SNP predictive abilities using the ROC curve and c-statistic, we observed that rs7574865 was a somewhat better risk predictor (c=0.590) compared to rs3821236 (c=0.577), but the small difference did not reach statistical significance, even when we considered the added predictive ability of these SNPs taken together (c=0.597) (**Supplementary Table 4**). Thus, each SNP alone provides the same level of prediction for risk for SLE.

Statistical analysis of the Interaction with *IRF5*

IRF5 was recently found as a consistent and well-established non-MHC associationwith SLE. We wanted to study if the effect of *STAT4* was independent of *IRF5*, test whether SNPs in both genes acted in a multiplicative (epistasis) or additive manner, and investigate if the added predictive ability when combining SNPs from both genes was unaffected or increased. These hypotheses were tested by multiple logistic regression analysis and the c-statistic using data of individuals already genotyped for *IRF5* from previous studies [5, 14] for the associated SNPs rs2004640, rs2070197, and rs10954213, that were also genotyped for *STAT4* SNPs rs1467199, rs3821236, rs3034866 and rs7574865. The analyses revealed no significant interaction effects between *STAT4* and *IRF5* but close to completely independent effects on SLE risk shown by a slight change in estimates when combined in a multivariate model, thus rejecting an interaction effect (data not shown).

The three SNPs of *IRF5* had c-statistics of 0.587 0.585 and 0.565, respectively (**Supplementary Table 5**). The lower predictive ability of rs10954213 was expected, as this SNP was shown to be, by itself, not strongly associated with SLE, while rs2070197 tags the major risk haplotype and is therefore strongest [14]. Addition of rs7574865 to the *IRF5* SNPs increased the predictive value of the models significantly (rs7574865 with rs2004640: c-statistic = 0.632, $P = 1.66 \times 10\text{-}5$; rs7574865 with rs2070197: C-statistic = 0.636, $P = 3.28 \times 10\text{-}11$ and rs7574865 with rs10954213: C-statistic = 0.624, $P = 9.62 \times 10\text{-}7$)). The results also support an additive effect between SNPs of both genes, particularly rs7574865 and rs2070197 to increase risk for SLE (**Figure 2**).

Tissue-specific alternative transcripts of *STAT4*

In order to investigate if differential splicing could be related to the genetic association of *STAT4*, we initiated the functional annotation of gene transcripts

expressed in different tissues. Until now, two isoforms have been described for *STAT4*, α and β [23]. We used a number of primer pairs matching to different coding exons in an attempt to find if there were any new isoforms present in the spleen, testis, kidney, lung, pancreas, small intestine and uterus. We detected only α and β isoforms. Further, since 5'- and 3'-UTRs may substantially affect gene expression [14], we performed more detailed analysis of non-coding 5'-exons and 3'-UTRs.

Interestingly, the pattern of 5'UTRs happened to be diverse in different tissues (**Table 3**). As shown, a series of alternative 5'UTRs are expressed in various tissues, but all of them lead to the two isoforms of *STAT4*. Further, all 3'UTRs matched to the previously described *STAT4* cDNA end. Small intestine and kidney cDNAs along with the α isoform expressed also the β isoform with retention of intron 22.

Analysis of *STAT4* expression levels

We next performed analysis of gene expression levels in human mononuclear cells (PBMCs) purified from 73 healthy individuals. First, the pattern of the gene transcripts was assessed in PBMCs by PCR with primers matching to different exons as described above. One major product identified as the α isoform was present in all PCRs. The β isoform was barely detectable along with other not entirely spliced transcripts containing partial introns (data not shown). Second, α and β isoform expression was measured by quantitative real time PCR and the values were normalized against TBP gene expression. Since expression of β transcript was much lower than that of the α transcript and its expression followed the trend of the α- transcript in all the samples, it was later excluded from the analysis. DNA from expression samples was genotyped for the SNPs rs7574865, rs3024866, rs3821236, and rs1467199. Using ANOVA to test differences in gene expression between genotypes, we found modest up regulation of *STAT4* mRNA and the risk genotype rs3024866-CC ($p = 0.0371$). By regression analysis with WHAP, risk alleles of SNPs rs3821236 and rs7574865 were correlated also with higher expression levels of *STAT4* but less strongly than rs3024866. Importantly, SNP rs1467199 did not correlate with *STAT4* expression (**Figure 3**).

DISCUSSION

In the present paper, we have confirmed the genetic association between *STAT4* and SLE and we have performed a fine mapping effort where we identify at least

one independent effect located in the vicinity of intron 16, suggesting that the contribution of *STAT4* to SLE derives from at least two independent risk haplotypes. The publication of Remmers, et al, [11] identifying *STAT4* as a susceptibility gene for RA and SLE came out while we were fine mapping *STAT4* in the Spanish set, after having identified a strong signal for this gene in a 100k scan in Argentine individuals (data not shown). The data from the Spanish tag map suggested at first the presence of several peaks independent of rs7574865. Further analyses using the larger set of joined samples confirmed the independent effect of rs3821236 located within intron 16. Conditional SNP and haplotype regression analysis using PLINK supports this result. Interestingly, we do observe differences between North and South European samples exemplified by the following: the German set contributed, albeit weakly to the genetic association only at rs7574865, but not the other peaks, while the association of rs3821236 appears to be contributed particularly from the Spanish set, but also the Italian set, while we observe the same for the Latin American sets. While part of the effect could be due to population stratification, we do not believe this to be the case, at least not for the Spanish and Italian sets for rs3821236. The Spanish and Italian cases and controls used here are all European Caucasian and have been used previously. In our view it is highly plausible that several independent risk haplotypes are involved in disease susceptibility with some having a stronger effect in some populations as compared to others, as it has been previously shown for several other genes, best exemplified by the HLA DRB1 gene. More work needs to be done to determine better the various contributions on genetic susceptibility in several populations, as there is a clear bias towards North European populations in the populations used for gene discovery in several complex diseases. The weak result of the German set could be also the result of a certain lack of power, however this does not explain the lack of association in this set for rs3821236, as the size of the other sets is comparable, in particular of the Italian set. We are approaching a phase in SLE and complex disease genetics where identification of the genes involved in disease susceptibility is becoming a reality. Therefore it is of interest that we begin to try to understand the relationship between the various genetic effects that we observe. Here we have attempted to understand if there are any genetic interactions between

SNPs described from *IRF5* and the *STAT4* SNP rs7574865. We observe no genetic interactions between *IRF5* and *STAT4* genetic variants, but we do observe that the predictability of the *STAT4* SNP is significantly increased when we add the *IRF5* SNPs to the model. To us, these results suggest that the *IRF5* and *STAT4* SNPs act additively to increase risk for SLE. Unfortunately we do not count with complete HLA data for the samples used here in order to analyze how this locus would fit with the data presented, but previous similar analyses have also shown the additive effect of the various genetic contributions in SLE [1]. We thus confirm this finding in independent sets of cases and controls.

Identification of the functional variants for *STAT4* should be the next step and this might prove to be relatively difficult considering the large size of this gene and the fact that rs7574865 is located in intron 3, the largest intron of this gene and an independent effect is found far away between introns 8 and 16. Complete resequencing of the gene and more genotyping will be needed to reveal the true functional variants. We determine here that no differences are observed in splicing of the gene at least in peripheral blood mononuclear cells, instead we do find a correlation with expression levels of *STAT4* in PBMCs and the three most associated SNPs, rs7574865, rs3024866 and rs3821236. We analyzed nearly 80 samples of PBMCs. It should be noted that these samples are expected to have varying numbers of T and NK cells, and therefore variation in gene expression is rather high showing therefore a weak correlation. Nevertheless, the fact that we observe this correlation in the three associated SNPs within *STAT4* and not for rs1467199 located in the *STAT1-STAT4* intergenic region provides strong support for our observation as being a true effect. Further, we also expect functional effects on genes of functional variants in complex diseases to be modest, most important at the tissue-specific or individual cell level and therefore difficult to assess [14].

How does a modest increase of expression of *STAT4* contribute to the risk for SLE? *STAT4* is a transcription factor through which the functional effects of IL12 are conducted and eventually expressed in the form of IFN-γ production. The effect of the *STAT4* pathway has been studied in mice and patients with SLE, but in general, the results appear to be contradictory. In two studies, mice deficient for *STAT4* show increased development of glomerulonephritis [24, 25], while in a

third study, the opposite is observed, deficiency of *STAT4* protects against glomerulonephritis. The latter is in line with what we find, namely that the risk allele of the *STAT4* SNPs are correlated with increased levels of *STAT4* [26], and higher levels of *STAT4* would be deleterious.

STAT4 is a ubiquitous transcription factor with variable expression in several different tissues. Although we detected only two isoforms of *STAT4*, one of which was expressed at extremely low levels (β), they have a number of tissue-specific promoters, which is reflected by the presence of several alternative 5′-UTR exon 1 specific for each tissue (**Table 3**). Given that such promoters could adjust transcription of the gene in a particular tissue, this poses an additional obstacle for defining the precise "breakage" in the gene. Using purified cell populations (e.g. NK cells, which constitute only 5% of the blood leukocytes but have high basal level of STAT4, kidney mesangial cells, etc) may be critical for correct assessment of the altered levels of *STAT4* gene expression as well as definition of the splicing isoforms that could be involved. On this regard, it was shown recently, that altered Lyn expression could be detected only in B cell-enriched populations, but not in total PBMCs [27]. This could explain to some extent, why only modest up-regulation of *STAT4* was seen in PBMCs.

Activation of *STAT4* leads to increased expression of IFN-γ. A study has shown that increased expression of IL-12 and IFN-γ in the kidneys of MRL-*lpr*/*lpr* mice precedes the development of glomerulonephritis [28, 29]. Thus, the localized action of the various genes in several tissues may be of importance. Even as we do not find correlation between splicing variants and genotypes, we cannot exclude the possibility that there might be differences in splicing of kidney-specific variants. This is also important to consider in view of the recent results showing a strong correlation between rs7574865 and end-organ disease, in particular kidney disease [30] There is still much more to be done to discern the role of *STAT4* in human SLE, but our results do support an important role of *STAT4* in SLE susceptibility, a role that appears to vary between different populations and deriving from two different and independent risk variants, whose functional nature needs to be addressed.

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Conflict of Interest

JW and HA are employees of Merck Serono Inc. and produced the Argentine 100k data on which our investigation and search for *STAT4* variants was first based.

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Table 1. Results from the fine mapping conducted in Spanish patients with SLE and matched controls.

Chr.	Position	SNP	Cases	Controls	Assoc	Frequency	Frequency	Genotypic	Allelic	OR	L95	U95	Multiple test correction	
			aa/Aa/AA	aa/Aa/AA	allele	Cases	Controls	test p-value	test p-value				Bonferroni	FDR
2	191546759	rs13395505	83/189/110	90/222/160	A	0,46	0,43	0,252	0,108	1,17	0,97	1,42	1,000	0,574
2	191553970	rs1547550	70/165/145	52/225/195	G	0,40	0,35	**9,55E-03**	**2,50E-02**	1,25	1,03	1,53	0,625	0,184
2	191554382	rs4327257	8/81/291	15/111/346	C	0,13	0,15	0,459	0,198	0,83	0,63	1,10	1,000	0,946
2	191558344	rs2280234	88/169/124	75/224/171	A	0,45	0,40	**3,07E-02**	**2,26E-02**	1,25	1,03	1,52	0,566	0,184
2	191558811	rs2280233	68/161/141	92/216/148	C	0,40	0,44	0,243	0,127	0,86	0,70	1,05	1,000	0,640
2	191559011	rs2280232	29/131/219	28/168/240	C	0,25	0,26	0,450	0,727	0,96	0,77	1,20	1,000	1,000
2	191568747	rs12693591	5/102/268	14/143/311	A	0,15	0,18	0,137	0,069	0,79	0,61	1,02	1,000	0,385
2	191574903	rs13029247	56/138/139	48/197/216	C	0,38	0,32	**2,76E-02**	**1,70E-02**	1,29	1,05	1,59	0,424	0,180
2	191577408	rs2030171	69/172/133	58/202/194	C	0,41	0,35	**2,99E-02**	**7,35E-03**	1,31	1,08	1,60	0,184	0,088
2	191588747	rs1467199	47/146/167	24/183/265	G	0,33	0,24	**7,45E-05**	**7,00E-05**	1,54	1,25	1,91	**1,75E-03**	**1,67E-03**
2	191604299	rs3024935	3/48/323	3/69/385	T	0,07	0,08	0,647	0,455	0,87	0,60	1,25	1,000	1,000
2	191605785	rs925847	50/171/158	46/196/230	T	0,36	0,31	0,076	**2,21E-02**	1,27	1,04	1,55	0,552	0,184
2	191611003	rs3821236	42/151/183	18/151/302	A	0,31	0,20	**5,90E-07**	**7,07E-08**	1,84	1,47	2,29	**1,77E-06**	**6,74E-06**
2	191613134	rs3024877	66/181/132	49/219/203	T	0,41	0,34	**3,50E-03**	**1,19E-03**	1,39	1,14	1,69	**2,96E-02**	**2,26E-02**
2	191625589	rs16833220	8/77/254	10/134/293	G	0,14	0,18	**4,39E-02**	**3,71E-02**	0,74	0,56	0,98	0,927	0,236
2	191631086	rs3024866	60/171/151	31/189/252	C	0,38	0,27	**1,95E-06**	**3,83E-07**	1,70	1,38	2,09	**9,58E-06**	**1,83E-05**
2	191637523	rs932169	3/55/321	5/59/382	C	0,08	0,08	0,781	0,815	1,04	0,73	1,50	1,000	1,000
2	191639709	rs1517352	102/159/103	75/241/137	A	0,50	0,43	**3,12E-04**	**6,88E-03**	1,31	1,08	1,59	0,172	0,088
2	191646845	rs7594501	0/37/335	3/65/403	A	0,05	0,08	0,057	**3,28E-02**	0,64	0,43	0,97	0,819	0,223
2	191651517	rs3024921	1/37/338	3/43/423	T	0,05	0,05	0,752	0,972	0,99	0,64	1,53	1,000	1,000
2	191662292	rs10931480	14/95/270	21/151/298	G	0,16	0,21	0,054	**2,35E-02**	0,75	0,58	0,96	0,586	0,184
2	191663097	rs10931481	58/174/150	42/207/221	G	0,38	0,31	**6,95E-03**	**2,43E-03**	1,36	1,12	1,67	0,061	**3,87E-02**
2	191664494	rs13011805	1/63/314	6/96/367	T	0,09	0,12	0,100	**4,88E-02**	0,72	0,52	1,00	1,000	0,291
2	191672878	rs7574865	41/153/181	18/170/284	T	0,31	0,22	**1,74E-05**	**9,37E-06**	1,64	1,31	2,03	**2,34E-04**	**2,98E-04**
2	191743288	rs10176621	29/146/205	33/148/262	C	0,27	0,24	0,294	0,212	1,15	0,92	1,44	1,000	0,961

The table shows the genotypes counts for the 25 SNPs that passed frequency and genotyping pruning, as well as the results for both allelic and genotypic association tests. The four SNPs highlighted where chosen to be replicated in independent sets of cases and controls since they had a significant p-value < 1.00E-05 and remained associated after multiple tests correction and were independent. OR: odds ratio, L95: lower limit of the 95% confidence interval, U95: upper limit of the 95% confidence interval; FDR: false discovery rate.

Table 2. Population-specific replication and general stratified allelic association analysis of the main associated SNPs in the fine mapping.

SNP	Risk Allele	Population/Test	Frequency Cases	Frequency Controls	Chi²	Df	P-value	OR
rs1467199	G	Germany	0.229	0.231	0.01	1	0.940	0.99
		Italy	0.235	0.229	0.04	1	0.842	1.03
		Spain	0.333	0.250	15.56	1	8.01E-0.5	1.50
		Argentina	0.345	0.284	2.97	1	0.085	1.33
		Mexico*	0.171	0.134	2.40	1	0.122	1.33
		Fixed effects			13.98	1	1.82E-04	1.27
		Random effects			6.03	1	1.41E-02	1.24
		Heterogeneity test			6.96	4	0.138	
rs3821236	A	Germany	0.213	0.186	1.05	1	0.305	1.18
		Italy	0.301	0.174	18.47	1	1.73E-05	2.04
		Spain	0.317	0.197	35.38	1	2.71E-09	1.89
		Argentina	0.506	0.354	15.94	1	6.53E-05	1.87
		Mexico*	0.318	0.458	18.79	1	1.46E-05	1.81
		Fixed effects			83.63	1	5.96E-20	1.77
		Random effects			42.03	1	8.98E-11	1.75
		Heterogeneity test			7.24	4	0.124	
rs3024866	C	Germany	0.251	0.258	0.06	1	0.807	0.96
		Italy	0.355	0.249	11.31	1	7.70E-04	1.66
		Spain	0.383	0.268	28.21	1	1.09E-07	1.70
		Argentina	0.477	0.383	6.07	1	1.38E-02	1.47
		Mexico*	0.395	0.520	14.41	1	1.47E-04	1.66
		Fixed effects			49.2	1	2.31E-12	1.51
		Random effects			14.64	1	1.30E-04	1.48
		Heterogeneity test			11.24	4	0.024	
rs7574865	T	Germany	0.265	0.210	3.81	1	0.051	1.35
		Italy	0.394	0.193	39.69	1	2.98E-10	2.70
		Spain	0.327	0.221	26.96	1	2.07E-07	1.72
		Argentina	0.415	0.319	6.80	1	9.11E-03	1.52
		Mexico*	0.547	0.364	30.70	1	3.01E-08	2.10
		Fixed effects			97.88	1	4.44E-23	1.82
		Random effects			28.85	1	7.81E-08	1.82
		Heterogeneity test			12.73	4	0.013	

Fixed effects: Cochran-Mantel-Haenszel SNP association test controlling for strata under fixed effects model; Random effects: DerSimonian-Laird SNP association test controlling for strata under random effects model; Heterogeneity test: Between-strata heterogeneity test.

*Only Mexican adult samples were used in this analysis.

Table 3. Usage and aplicing of 5′and 3′UTRs in *STAT4*.

Tissue	5′-terminal exons	Acc Number
Kidney	exon1B-exon2-exon3[B]	**NM_003151**
	exon1C-exon1D-exon2-exon3	EU304788
Spleen	exon1B-exon2-exon3[B]	EU304789
	exon1A-intron-exon1B-exon2-exon3	EU304790
	exon1C-D-E-F-exon2-exon3	EU304791
	exon1D-E-G-H-exon2-exon3	
Testis	exon1B-exon2-exon3[B]	**BC031212**
	exon1A-exon2-exon3[A]	EU304792
	exon1A-alt.spliced exon1B last 68pb-exon2-exon3	
Lung	exon1B-exon2-exon3[B]	
Pancreas	exon1B-exon2-exon3[B]	EU304793
	exon1A-intron-exon1B-297nt intron1-exon2-exon3	
Small intestine	exon1B-exon2-exon3[B]	
Uterus	exon1B-exon2-exon3[B]	EU304793
	exon1A-intron-exon1B-297nt intron1-exon2-exon3	

Figure 1. Fine mapping of *STAT1-STAT4* region. The physical position (top panel) of the SNPs typed in 390 patients and 480 controls from Spain covering the ~200kb *STAT1- STAT4* region is shown. The region LD structure defined six haplotype blocks (middle panel) of which three were associated with disease susceptibility (See block structure ad R2 values in Supplementary Figure 1). Risk haplotypes are shown in red and main single-marker hits replicated are underlined (See Tables 2 Supp Table 1). The bottom panel shows the significance of the association data presented as the –log10(P-value) for 25 tag SNPs passing genotyping quality control. The blocks have been defined using the solid spine of LD method in Haploview v4.0. The p values of the risk and protective haplotypes at each block in the Spanish population are as follows:

1 Block 3 risk haplotype p-value = 0.0037

2 Block 3 protective haplotype p-value = 7x10-4

3 Block 4 risk haplotype p-value = 2.64 x 10-6 4 Block

4 protective haplotype p-value = 2.24 x 10-5

5 Block 6 risk haplotype p-value=9.52 x 10-6

6 Block 6 protective haplotype p-value=0.0331

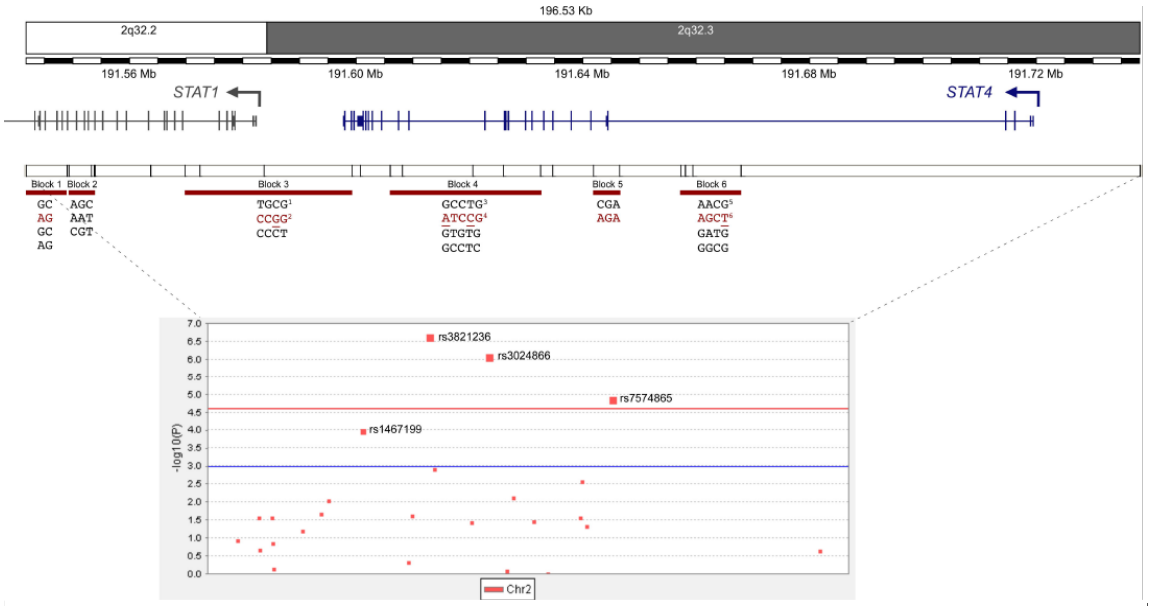


Figure 2. Predictive ability of *STAT4* and *IRF5* SNPs for SLE The predictive ability of the two genes for SLE was investigated using the c-statistic. For comparison of the c-statistics the test for comparing two dependent ROC curves is used. Within *STAT4*, the SNP rs7574865 is the strongest predictor for SLE and adds a significant fraction 30 to the predictive ability of the *IRF5* SNP rs2070197. Between genes the best combination is rs7574865+rs2070197 having an overall c-statistic of 0.636.

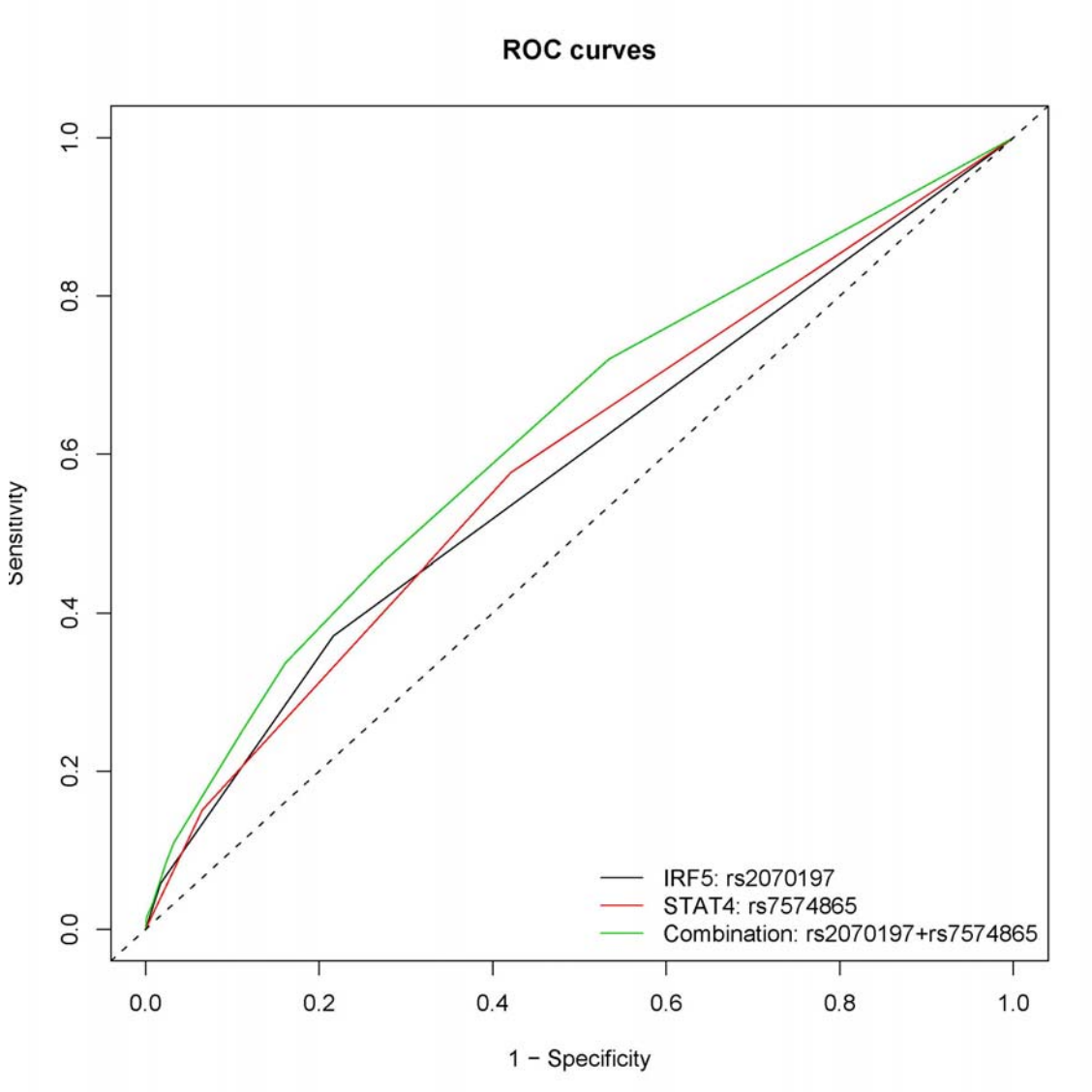
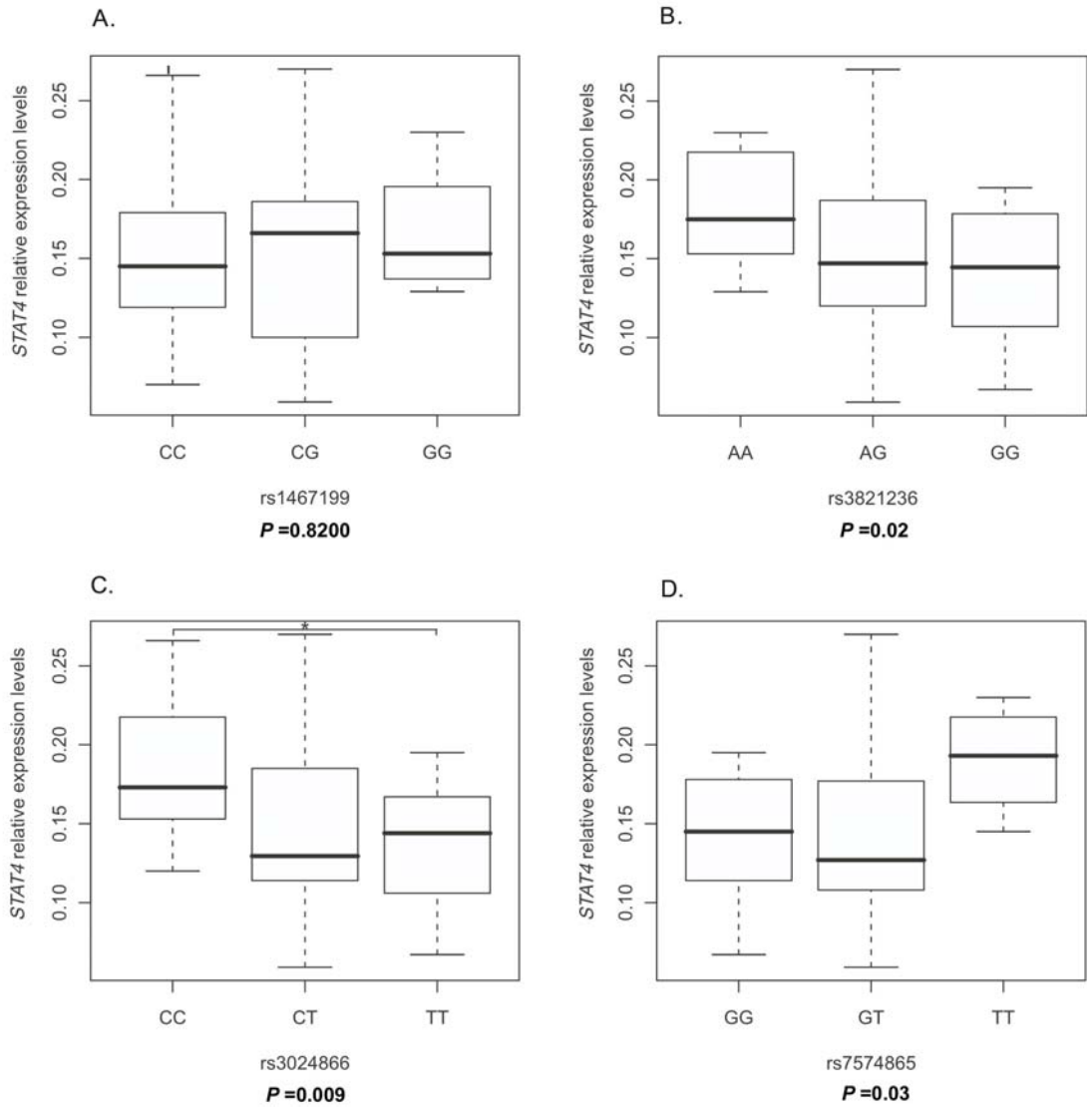


Figure 3. Association between *STAT4* polymorphism and expression levels ANOVA and F-test were used to determine difference in the mRNA relative expression levels between individuals carrying different genotypes, taking the three possible genotypes as factor levels and the major allele homozygous as reference. Multiple comparisons of means revealed that individuals with rs3024866-CC genotype have higher *STAT4* expression levels compared to the reference level (pvalue= 0.0281).



Supplementary Table 1. Population-specific replication and general stratified association analysis using genotypes rather than alleles.

	Population	Reference	Test	OR (95% CI)	z value	Pr(>|z|)
rs1467199	GERMANY	CC	CG	1.07 (0.72-1.58)	0,334	0,739
		CC	GG	0.85 (0.39-1.82)	-0,431	0,666
	ITALY	CC	CG	1.08 (0.72-1.6)	0,36	0,719
		CC	GG	0.96 (0.39-2.39)	-0,086	0,931
	SPAIN	CC	CG	1.25 (0.95-1.65)	1,619	0,105
		CC	GG	2.84 (1.75-4.60)	4,247	2,17E-05
	ARGENTINA	CC	CG	1.53 (1.08-2.17)	2,402	1,63E-02
		CC	GG	2.03 (0.99-4.15)	1,934	0,053
	MEXICO*	CC	CG	1.26 (0.83-1.92)	1,091	0,276
		CC	GG	2.21 (0.64-7.70)	1,247	0,212
	ALL	CC	CG	1.18 (1.01-1.38)	2,104	3,54E-02
		CC	GG	1.71 (1.25-2.34)	3,344	8,26E-04
	All adjusting for population	CC	CG	1.24 (1.06-1.45)	2,676	7,46E-03
		CC	GG	1.87 (1.36-2.57)	3,836	1,25E-04
rs3821236	GERMANY	GG	AG	1.23 (0.82-1.85)	1,017	0,309
		GG	AA	1.22 (0.54-2.78)	0,482	0,630
	ITALY	GG	AG	1.90 (1.26-2.87)	3,06	2,21E-03
		GG	AA	4.70 (1.84-11.99)	3,239	1,20E-03
	SPAIN	GG	AG	1.71 (1.30-2.26)	3,786	1,53E-04
		GG	AA	3.83 (2.27-6.46)	5,022	5,11E-07
	ARGENTINA	GG	AG	1.59 (1.08-2.33)	2,37	1,78E-02
		GG	AA	3.09 (1.88-5.11)	4,419	9,92E-06
	MEXICO	GG	AG	1.02 (0.60-1.73)	0,063	0,950
		GG	AA	2.55 (1.49-4.38)	3,394	6,90E-04
	ALL	GG	AG	1.38 (1.18-1.62)	3,955	7,65E-05
		GG	AA	2.58 (2.06-3.25)	8,139	3,98E-16
	All adjusting for population	GG	AG	1.53 (1.30-1.81)	4,995	5,87E-07
		GG	AA	3.18 (2.47-4.09)	8,981	<2.00E-16
rs3024866	GERMANY	TT	CT	0.96 (0.66-1.41)	-0,202	0,840
		TT	CC	0.94 (0.44-1.97)	-0,173	0,862
	ITALY	TT	CT	1.89 (1.26-2.83)	3,096	1,96E-03
		TT	CC	2.37 (1.16-4.81)	2,38	1,73E-02
	SPAIN	TT	CT	1.45 (1.10-1.92)	2,634	8,45E-03
		TT	CC	3.22 (2.08-4.99)	5,227	1,72E-07
	ARGENTINA	TT	CT	1.46 (1.00-2.15)	1,941	0,052
		TT	CC	2.03 (1.24-3.32)	2,836	4,56E-03
	MEXICO	TT	CT	1.24 (0.77-2.00)	0,884	0,376
		TT	CC	2.53 (1.51-4.25)	3,511	4,47E-04
	ALL	TT	CT	1.31 (1.12-1.54)	3,343	8,29E-04
		TT	CC	2.12 (1.69-2.66)	6,541	6,13E-11
	All adjusting for population	TT	CT	1.38 (1.17-1.62)	3,832	1,27E-04
		TT	CC	2.34 (1.85-2.97)	7,066	1,59E-12
rs7574865	GERMANY	GG	GT	1.42 (0.95-2.11)	1,73	0,084

	GG	TT	1.55 (0.76-3.18)	1,208	0,227
ITALY	GG	GT	2.66 (1.74-4.05)	4,528	5,95E-06
	GG	TT	5.69 (2.77-11.7)	4,724	2,31E-06
SPAIN	GG	GT	1.58 (1.20-2.08)	3,242	1,19E-03
	GG	TT	3.34 (2.01-5.56)	4,643	3,44E-06
ARGENTINA	GG	GT	1.30 (0.91-1.87)	1,432	0,152
	GG	TT	3.20 (1.80-5.69)	3,951	7,78E-05
MEXICO	GG	GT	1.47 (0.95-2.26)	1,734	0,083
	GG	TT	4.33 (2.54-7.38)	5,372	7,80E-08
ALL	GG	GT	1.52 (1.29-1.77)	5,156	2,52E-07
	GG	TT	3.40 (2.64-4.39)	9,424	<2.00E-16
All adjusting for population	GG	GT	1.58 (1.35-1.86)	5,592	2,24E-08
	GG	TT	3.56 (2.74-4.62)	9,507	<2.00E-16

***Only adult samples were used in this analysis**

Supplementary Table 2. Allelic association in Mexican pediatric patients with SLE

SNP	Assoc Allele	Freq case	Freq controls	P value
rs1467199	G	0,170	0,120	**0,008**
rs3821236	A	0,645	0,659	0,5717
rs3024866	C	0,573	0,529	0,0947
rs7574865	T	0,492	0,443	0,0628

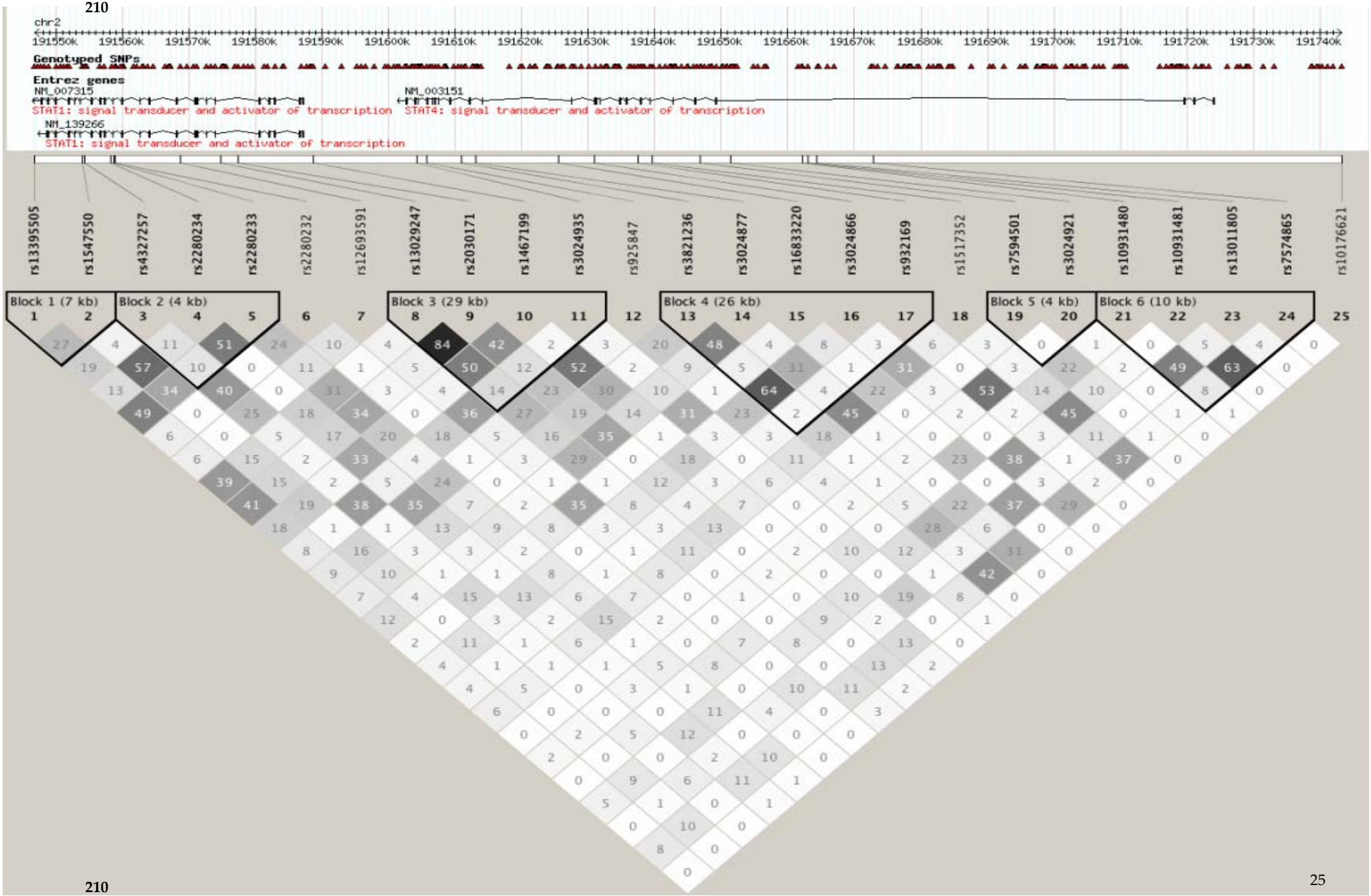
Supplementary Table 3. Conditional logistic regression of *STAT4* replicated variants in all the 1581 cases and 1844 controls studied*.

Test SNP	Reference	Test	P conditional on:			
			rs1467199	**rs3821236**	**rs3024866**	**rs7574865**
rs1467199	CC	CG	-	0,372	0,276	0,427
	CC	GG	-	0,143	0,053	0,111
rs3821236	GG	AA	3,83E-14	-	2,86E-07	**1,60E-02**
	GG	AG	7,63E-04	-	9,66E-03	0,440
rs3024866	TT	CC	4,27E-09	0,36051	-	0,947
	TT	CT	4,43E-03	0,76921	-	0,901
rs7574865	GG	GT	1,65E-06	**8,00E-04**	2,44E-05	-
	GG	TT	<2E-16	**3,s45E-09**	4,41E-13	-

*Of the Mexican samples, only the adult samples were used in this analysis

Supplementary Table 4. Predictive ability of individual SNPs and SNPs combinations

	Reference	C-statistic	Compared to	C-statistic	p-value
Within *STAT4* gene	rs7574865	0,590	rs3821236	0,577	0,0855
	rs7574865	0,590	rs7574865+rs3821236	0,597	0,0805
IRF5-STAT4	rs2004640	0,587	rs2004640+rs7574865	0,632	**1,66E-05**
	rs2070197	0,585	rs2070197+rs7574865	0,636	**3,28E-11**
	rs10954213	0,565	rs10954213+rs7574865	0,624	**9,62E-07**


chr2
191550k 191560k 191570k 191580k 191590k 191600k 191610k 191620k 191630k 191640k 191650k 191660k 191670k 191680k 191690k 191700k 191710k 191720k 191730k 191740k
Genotyped SNPs
Entrez genes
NM_007315
STAT1: signal transducer and activator of transcription
NM_003151
STAT4: signal transducer and activator of transcription
NM_139266
STAT1: signal transducer and activator of transcription
rs13395505
rs1547550
rs4327257
rs2280234
rs2280233
rs2280232
rs12693591
rs13029247
rs2030171
rs1467199
rs3024935
rs925847
rs3821236
rs3024877
rs16833220
rs3024866
rs932169
rs1517352
rs7594501
rs3024921
rs10931480
rs10931481
rs13011805
rs7574865
rs10176621
Block 1 (7 kb)
Block 2 (4 kb)
Block 3 (29 kb)
Block 4 (26 kb)
Block 5 (4 kb)
Block 6 (10 kb)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

The *TRAF1-C5* region on chromosome 9q33 is associated with multiple autoimmune diseases

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Genes and Immunity **2008 (submitted)**

ABSTRACT

The *TRAF1-C5* locus has recently been identified as a genetic risk factor for rheumatoid arthritis. Since genetic risk factors tend to overlap with several autoimmune diseases, we aimed to investigate whether this region is associated with Type I Diabetes (TID), Celiac Disease (CD), Systemic Sclerosis (SSc) and Systemic Lupus Erythematosus (SLE). We genotyped the most consistently associated SNP, rs10818488, in a total of 735 T1D, 1049 CD, 367 SSc, 746 SLE and 3494 ethnically and geographically matched healthy individuals. We detected significant association of the rs10818488 A allele with T1D (OR 1.14, p=0.027) and SLE (OR 1.16, p=0.016) which was replicated in 99 T1D, 272 SLE patients and 482 controls from Crete (OR 1.64, p=0.002; OR 1.43, p=0.002 respectively). Joint analysis of all T1D (N=961) and all SLE (N=1018) patients compared to 3976 healthy individuals yielded an allelic common OR of 1.19 (p=0.002) and 1.22 (P=2.6x10-4) respectively. We report here for the first time a role of the *TRAF1-C5* locus in multiple autoimmune diseases, particularly T1D and SLE, implying that this risk factor belongs to a common genetic pathway underlying these diseases.

INTRODUCTION

Type 1 Diabetes (T1D), celiac disease (CD), systemic sclerosis (SSc) and systemic lupus erythematosus (SLE) are common autoimmune diseases (AIDs), the aetiologies of which are largely unknown but are mostly thought to result from interaction of genes and environment. A large body of evidence exists to complement the idea that a common genetic basis to such diseases exists as seen by the prevalence of multiple autoimmune diseases within a given family as well as an individual. For example, 3-6% of first-degree relatives of rheumatoid arthritis (RA) probands have T1D (1;2) and 7-17% of relatives of SLE patients have RA(1;3). Patients with SSc may occasionally develop RA and SLE and patients with T1D sometimes develop CD(4;5). One major genetic risk factor for all four of these diseases is the distinct alleles in the *HLA* (Human Leucocyte Antigen) locus(1). Non-HLA risk factors that have also been confirmed in various autoimmune diseases are *PTPN22* (T1D, RA, SLE, as well as juvenile arthritis)(1;6), *CTLA4* (T1D, RA, SLE and CD)(7), *STAT4* (RA and SLE)(8) and *FCRL3* in the Japanese population (RA, SLE, autoimmune thyroid disease - AITD)(7).

The region encompassing *Tumour Necrosis Factor (TNF) receptor associated factor 1* (*TRAF1*) and *complement component 5* (*C5*) is contained in a 65kb block of linkage disequilibrium and we have recently reported it to be a genetic risk factor involved in RA(9). The robustness of this association is demonstrated by its prevalent risk in Dutch,

Swedish and American populations and is further corroborated by both a genome-wide association study(10) and an extensive fine-mapping study we have undertaken(11). Interestingly one consistent association signal defined by rs10818488 or its perfect proxy (rs3761847 and rs7021049, R2>0.98) has been identified in this region and has been further confirmed by a large European family-based study (Kurreeman et al, Arthritis Rheumatism, manuscript in press). Additionally, we and others have shown that this polymorphism is associated with juvenile arthritis(12;13).

TRAF1 is suggested to be a negative regulator of TNF-Receptor signaling(14) and *C5* is a central component of the complement pathway(15). Both molecules are potent immune mediators and so far the question remains whether this region is restricted to arthritis as such or whether it lies in a biological pathway common to autoimmune diseases. We therefore sought to investigate whether harboring the same risk allele would predispose patients to the development of T1D, CD, SSc and SLE.

RESULTS

T1D, CD, SSc and SLE samples were genotyped from both the Spanish and Dutch populations for the most informative SNP rs10818488. Since the Dutch study was largely underpowered to detect modest effect sizes (OR~1.2) we opted for a combined analysis which consisted of a total of 3494 controls and 735 T1D, 1049 CD, 367 SSc, 746 SLE patients. This combined dataset enhanced power to ≥85% to detect effect sizes of 1.2 at P<0.05 with the exception of SSc which only achieved 64% power to detect an odds ratio of 1.2.

The frequency of the rs10818488 A allele was significantly increased in T1D patients (OR 1.14; 95% CI 1.02- 1.28; p=0.027, Table 1). Similarly we found a significant difference in the prevalence of the A allele in SLE patients resulting in an OR of 1.16 (95% CI 1.03-1.31, p=0.016). Patients harboring two copies of the A allele had a 1.3 fold and a 1.4 fold increased risk for T1D and SLE respectively as compared to those who carried none (data not shown). We observed no association with CD (Allelic OR 1.07, 95% CI 0.97-1.18, p=0.18) and SSc (Allelic OR 1.02, 95% CI 0.87-1.19, p=0.84). While the possibility remains that the absence of association in SSc may be due to lack of power to detect OR of 1.2 or lower, the direction of association in the individual studies was opposite (Allelic OR, 95% CI; Spanish 1.16, 0.94-1.43; Dutch 0.85, 0.65-1.09), suggesting that the absence of association may be more likely.

We then proceeded to replicate our significant findings in T1D and SLE in the

genetically homogeneous population of Crete. Since this largest island of Greece consists of 650,000 inhabitants who share the same genetic and cultural background as well as a common environment, it represents a "geographically isolated" gene pool which may enhance the detection of risk alleles that may be diluted in larger continental populations(16). We observed an 11% increase and an 8% increase in the A allele in T1D and SLE patients, respectively, when compared to controls (Table 2). This resulted in a 1.6 fold increased risk for T1D (p=0.002) and a 1.4 fold increased risk for SLE (p=0.002) in the Crete population. Interestingly, we also observed a much lower frequency of the A allele in the healthy individuals from Crete (29%) as compared to either the Dutch (44%) or Spanish controls (36%), indicating that there are population specific differences in the prevalence of this allele. Overall analysis of T1D and SLE in all three datasets (Spanish, Dutch and Greek) reveals a common OR of ~1.2 (p=0.002 and p=2.6x10-4 respectively) for both diseases.

DISCUSSION

We report here for the first time reproducible association of the *TRAF1-C5* region with T1D and SLE complementing the already consistent finding of this variant with RA and juvenile arthritis. Remarkably, the same allele that predisposes to rheumatoid arthritis and juvenile arthritis also predisposes to these autoimmune diseases lending support to the hypothesis that this region may contribute to a shared pathway involved in RA, T1D and SLE. However, the possibility also remains that other additional alleles at this locus may still be involved in any of these diseases. Complementary studies undertaking further sequencing and fine-mapping will yield further insight into the most likely causal alleles at this locus.

We also observed a difference in allele frequencies in healthy populations of Dutch (44%), Spanish (36%) and Greek (29%) origins. To address this difference in population, each patient-control sample set was geographically and ethnically matched. Since the the Breslow and Day test of heterogeneity across sample sets for the allelic OR did not show significance (P>0.05), we conclude that the effect sizes across populations is similar providing further support for our observations. However, underlying population stratification is difficult to account for as panels of markers characterizing each population thoroughly have not as yet been described. In our study, association of the *TRAF1-C5* locus has been observed in more than one sample set considerably reducing the chances of false

positive findings due to population stratification.

The association of the *TRAF1-C5* region has not been reported in the recently published genome-wide association studies (GWAs) in SLE(17-19) and the GWA in either RA or T1D in WTCCC case-control study(20). However, it is not uncommon that modest variants are not detected in such largescale studies as exemplified by the association of *TNFSF4*, a gene identified by family-based association studies(21), that did not surface in the recently performed GWAs. Likewise *BANK1*, a gene identified in the GWA scan by Kozyrev *et al* was not identified by the other two GWA studies that employed 500K SNPs. It is at present difficult to speculate the specific reasons behind this lack of association.

In summary, we report here for the first time an association of the *TRAF1-C5* locus with T1D and SLE which, in combination with previous findings of an increased risk to RA and juvenile arthritis, indicates that this region is likely to be involved in a shared mechanism underlying several autoimmune diseases. However, while our study provides considerable evidence of the role of the TRAF1/C5 locus in autoimmune diseases, further sequencing, fine-mapping and functional studies will be required to identify the causal allele(s) and how this allele disrupts the function of either *TRAF1* and/or *C5* to lead to disease.

MATERIALS AND METHODS

DNA was obtained from cohorts of T1D, CD, SSC and SLE from The Netherlands and Spain as well as T1D and SLE from Crete.

T1D

556 Dutch Patients were included from two T1D studies. The first group of patients (N=337) was included from Netherlands Kolibrie study group of childhood diabetes. The details of the study have been explained elsewhere(22). In brief, the Kolibri T1D cohort includes 350 patients with juvenile onset T1D (median 8.7 yrs, range 1-17 yrs). The cohort was collected consecutively after diagnosis by pediatricians in the Southwestern part of The Netherlands between 1995 and 1999. The diagnosis was made according to International Society of Pediatric and Adolescent Diabetes (ISPAD) criteria.

The second group of T1D patients (N=109) was retrieved from the T1D Cohort for Growth study, a prospective cohort of 150 T1D Dutch families. Families have been selected on the basis they have at least 1 T1D proband and 1 non-affected sib, and were collected from 5 hospitals in the Netherlands between 2002 and 2005. For the present study, only 1 T1D proband per each family from 136 families were available who were

genotyped for the TRAF1-C5 SNP. Patients had a clinical onset at 4-15 years of age with an average of 8.3 years. The diagnostic criteria of T1D was similar to that of Kolibrie T1D Cohort as described earlier(23) .

306 white unrelated Spanish T1D patients diagnosed according to the criteria of the American Diabetes Association (ADA)(24) were also included. The age at onset for the consecutively recruited T1D patients ranged from 1 to 55 years old (mean: 17.3 ± 10.0).

Celiac Disease

496 Dutch and 553 Spanish CD patients were included and were diagnosed following the European Society for Pediatric Gastroenterology and Nutrition (ESPGAN) criteria (25). The Dutch patients were both adults and children whereas the Spanish patients were on average 2.7 (± 2.7) years at the time of inclusion. More than 90% of the Dutch and Spanish patients were HLA-DQ2 positive.

Systemic Sclerosis

138 Dutch SSc patients originated from the department of Rheumatology of the Radboud University Nijmegen Medical Center and fulfilled the preliminary criteria of the American college of Rheumatology for SSc and gave informed consent(26). SSc was classified as either a limited subtype or a diffuse subtype according to the extent of the skin involved, as proposed by Leroy *et al*(27). Involvement of the lungs was assessed according to the international guidelines(28). Pulmonary fibrosis was investigated by a computed tomography scan. Restrictive syndrome and diffusion capacity of the lungs was defined as a forced vital capacity < 75% of the predicted value and a diffusion capacity for carbon monoxide of less than 75% of predicted. The main clinical and serological characteristics of SSc patients were: 72% female sex, 78% limited versus 22% diffuse involvement, 24% presence of antitopoisomerase I antibodies, 27% positive for anti-centromere Ab and 28% pulmonary involvement.

229 Systemic Sclerosis patients included in the study were recruited at five Spanish hospitals (Hospital Clinico San Cecilio, Granada; Hospital Reina Sofia, Cordoba; Hospital Virgen de la Victoria and Hospital Carlos Haya, Málaga; Hospital Virgen del Rocio, Sevilla) and diagnosed according to the preliminary criteria of the American College of Rheumatology for the disease(26). The main clinical and serological characteristics of SSc patients were: 82.4 % female sex, 70.2 % limited vs 29.7 % diffuse involvement, 28.2 % presence of anti-topoisomerase I antibodies, 25 % positive for anti-centromere Ab and 63.2 % pulmonary involvement.

Systemic Lupus Erythematosus

DNA was available from 161 consecutive Caucasian SLE patients at the rheumatology outpatient department of the Leiden University Medical Center. SLE cohort from Spain originated from the following hospitals: Hospital Clínico San Cecilio, Granada; Hospital Virgen de las Nieves, Granada; Hospital Virgen de la Victoria and Hospital Carlos Haya, Málaga. Clinical data of the SLE Spanish patients have been described previously.(29). All SLE patients met the revised criteria for the classification of SLE (30).

Controls

All control sample sets consisted of healthy unrelated Caucasian individuals. Genotypes were available for 511 Dutch controls out of the 524 DNA samples which have been described before(9). This sample set was expanded by including an additional 535 Dutch healthy unrelated individuals from the Leiden region , 602 Dutch unrelated healthy individuals from the Utrecht region (Utrecht controls) and 450 controls from the Dutch GWA study in amyotrophic lateral sclerosis (ALS controls)(31-33). Genotypes were obtained from the ALS controls for SNP rs3761847 which is in absolute LD with rs10818488 (R2 rs3761847G and rs10818488A > 0.98). Since the two SNPs were genotyped on different platforms (rs3761847 on the Illumina platform and rs10818488 on the Sequenom and RFLP, as previously described(9;32)) and there was an overlap of 113 samples between the ALS and the Utrecht controls, we compared the genotypes which yielded >99% concordance. These 113 samples were excluded from the Utrecht controls. Spanish controls consisted of 1396 individuals, comprising of 547 from the Madrid region and 849 from Granada.

Replication sample set

The replication sample set consisted of two sets of controls (N=223 and N=259) with A allele frequencies of 30% and 28% respectively. Since we detected no significant differences in the A allele frequency between these two groups, we combined them to make one extended control population (N=482), consisting of healthy unrelated Caucasian individuals from the island of Crete, with the four grandparents of each individual being of Cretan origin. DNA was available from 272 SLE and 99 T1D patients. All SLE patients met the revised criteria for the classification of SLE (30). The T1D group comprised 99 individuals aged 4.96 to 22.9 (mean, 13.7) years. All of them were under regular follow-up at the Paediatric Diabetes Clinic at the University Hospital of Heraklion, the single clinic of this type on the island of Crete. T1D diagnosis followed the ADA guidelines(24). Informed consent was obtained from all subjects, and the study was

approved by the local ethics committee of each center.

Genotyping

All samples were genotyped using the Taqman assay (Applied Biosystems) according to the manufacturer's instructions. Each plate consisted of at least 8 positive and 8 negative controls. At least 10% of the samples were genotyped in duplo with no discrepancies observed. The Dutch control sets consists of 511 healthy individuals which were previously genotyped(9). To expand the Dutch control set an additional 535 healthy unrelated individuals were genotyped using allele specific kinetic PCR(34), 715 Dutch controls from Utrecht using the Taqman assay. To compare genotyping methods at least 50 samples were genotyped on each platform (Taqman, allele specific kinetic PCR and RFLP) and revealed a concordance rate of >99%.

Statistical analysis

In the controls, the frequencies were in Hardy-Weinberg equilibrium as determined by the observed versus expected genotype counts. Genotype counts were analysed using SPSS version 12.0. Odds ratios and confidence intervals were calculated using Statcalc. Combining odds ratios across sample sets was performed using the Cochran-Mantel-Haenszel test as implemented in EasyMA(35). No evidence of heterogeneity was observed using the Breslow and Day method (P>0.05) when combining OR. All power calculations were performed using Quanto version 1.2 (http://hydra.usc.edu/gxe). P values below 0.05 were considered significant.

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Conflict of Interest

The authors declare no conflict of interest.

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Table 1. Association of the *TRAF1-C5* locus with autoimmune diseases

	Spanish		Dutch		Combined		
	N	MAF*	N	MAF	N	OR (95% CI)	P
Controls	1396	36%	2098	44%	3494	-	-
T1D	306	39%	556	46%	862	1.14 (1.02-1.28)	0.027
CD	553	38%	496	45%	1049	1.07 (0.97-1.18)	0.181
SSC	229	39%	138	40%	367	1.02 (0.87-1.19)	0.842
SLE	585	41%	161	44%	746	1.16 (1.03-1.31)	0.016

* MAF- Minor Allele Frequency

Table 2. Replication of assocation of T1D and SLE in a homogeneous population originating from Crete

	Crete				Joint		
	N	MAF	OR (95% CI)	P	N	OR (95% CI)	P
Controls	482	29%	-	-	3976	-	-
T1D	99	40%	1.64 (1.18-2.28)	0.002	961	1.19 (1.07-1.32)	0.0016
SLE	272	37%	1.43 (1.14-1.80)	0.002	1018	1.22 (1.09-1.35)	$2.6x10^{-4}$

MAF- Minor Allele Frequency

5. DISCUSIÓN

5.1. Genes implicados en la respuestainflamatoria

5.1.1. Receptores tipo Toll (TLRs)

5.1.1.1. TLR2 y TLR4

TLR2 y TLR4 actúan como receptores de ligandos basados en lípidos procedentes de bacterias (212). Se cree que TLR4 reconoce, además de estos constituyentes microbianos, moléculas derivadas del hospedador (118). Recientemente se ha demostrado que células que expresan en superficie GP (un parálogo de la proteína humana de choque térmico 70, la cual se ha visto ligada a LES activo) es ligando de TLR2 y TLR4 dando lugar al desarrollo en ratones de lupus inducido de manera MyD88 dependiente (213). En el gen *TLR4* se han descrito dos polimorfismos codificantes (214), el Thr399Ile asociado a colitis ulcerosa (CU) (215) y el Asp299Gly que se ha visto asociado a la EII, a ateroesclerosis y a la AR (216-219), aunque el papel de estos polimorfismos en la AR no está claro ya que otros grupos no encuentran asociación (220, 221). Con respecto a *TLR2*, se ha encontrado un polimorfismo que conduce al cambio de una arginina por una glutamina en posición 753 de la proteína (Arg753Gln), que está asociado a sepsis por bacterias gram-positivas (222). Además, otro polimorfismo en *TLR2* (Arg677Trp) se ha visto asociado con lepra (223). En vista al importante papel que *TLR2* y *TLR4* parecen tener en el LES, debido a su importante función en la inmunidad innata y adquirida, decidimos estudiar estos cuatro polimorfismos por primera vez en una cohorte de LES (221).

En cuanto al gen *TLR4* observamos que ambos polimorfismos presentaban un alto grado de LD entre ellos aunque no completo, y que las

frecuencias alélicas encontradas en nuestra población control eran similares a las encontradas en otras poblaciones caucásicas (214, 224, 225). Sin embargo este polimorfismo parece ser raro en población japonesa (226, 227) y no detectable en población taiwanesa y coreana (228, 229). Cuando analizamos ambas variantes no observamos diferencias estadísticamente significativas al comparar la distribución de las frecuencias alélicas y genotípicas entre pacientes con LES y controles sanos. Tampoco encontramos ninguna relación entre estos polimorfismos de *TLR4* y las características clínicas y demográficas de los pacientes con LES. Con respecto al gen *TLR2*, la variante Arg677Trp resultó no ser polimórfica en nuestra cohorte, lo cual confirma los datos previos encontrados en alemanes y españoles (230, 231), sugiriendo que este polimorfismo que había sido descrito por primera vez en una población asiática (223) no está presente en población caucásica. En cuanto al SNP Arg753Gln, se encontró en una frecuencia muy baja, lo cual también se había descrito con anterioridad (222).

Por lo tanto, nuestros datos indican, que los polimorfismos Asp299Gly y Thr399Ile de *TLR4* y Arg677Trp y Arg753Gln de *TLR2* no son relevantes en el LES, aunque el análisis de estas y otras variantes génicas de los genes *TLR2* y *TLR4* en diferentes poblaciones y que contengan un mayor tamaño de muestra deben ser realizadas para poder confirmar estos datos.

5.1.1.2. TLR5 y TLR7

La presencia de autoanticuerpos frente ADN, ARN y proteínas asociadas son dianas comunes en la respuesta inmune del LES (25). Estas moléculas de ADN y ARN puedan comportarse como autoantígenos ya que tienen la capacidad de estimular el sistema inmune innato directamente a través de los TLRs o indirectamente vía FCγR y de ese modo promover la

respuesta inmune frente a lo propio, dando lugar a la perdida de la tolerancia (232).

El gen *TLR5* es el ligando de la flagelina bacteriana y se localiza en la región cromosómica 1q41 (233), una de las regiones asociadas con susceptibilidad a LES en diferentes poblaciones (188, 234). Recientemente se ha visto que un polimorfismo en la región codificante del gen, da lugar a un codon prematuro de parada en el dominio de unión al ligando (*TLR5* rs5744168, también llamado Arg392Stop ó C1174T), perdiendo de este modo la capacidad de señalización a través de la flagelina (235) y se ha encontrado asociado con susceptibilidad a la enfermedad del legionario (235) y resistencia a la enfermedad de Crohn (EC) (236) y al LES (237).

El gen *TLR7* tiene una gran relevancia funcional en el LES (238). TLR7 esta involucrado en el reconocimiento de ARNcs procedente de virus (239). Los últimos estudios en ratones deficientes en el acelerador autoinmune ligado al cromosoma Y (*Yaa*), muestran que diferencias en la expresión del gen *TLR7* y en los factores ambientales que inducen respuestas a través de TLR7, dan lugar a una mayor sensibilidad de las células B para contener antígenos propios de ARN (240, 241). Además, el TLR7 tiene la capacidad de inducir en las células B la liberación de IFN-α (242), una citoquina que posee un importante papel en el LES (243). Aunque se sabe poco acerca de las variantes genéticas del gen *TLR7*, Pisitkun y col (240) descubrieron una duplicación de un segmento genómico en el gen *Tlr7* de ratón, el cual se ha visto asociado con un mayor número de células B autoreactivas (240). Sin embargo, el incremento genómico en *Tlr7* en el modelo murino de lupus no puede ser trasladado directamente a humanos con LES, ya que no se ha encontrado una concordancia significativa entre el número relativo de copias de *TLR7* y el LES (244). Aunque estas variantes en el *TLR7* parecen no jugar un papel relevante

en el LES humano, numerosas evidencias muestran el papel de este gen en el desarrollo de la enfermedad. Debido a que *TLR7* esta localizado en el cromosoma X y que el LES es una enfermedad con una mayor prevalencia en mujeres, se sugiere la posible existencia de un componente genético ligado al cromosoma X en el LES.

Debido al importante papel de estos genes *TLR5* y *TLR7* en la regulación de la inflamación y en el sistema inmune innato, decidimos estudiar el papel de diversas variantes genéticas de estos genes en pacientes de LES.

Con respecto al gen *TLR5* no se encontró ninguna diferencia estadísticamente significativa al comparar las frecuencias alélicas y genotípicas del polimorfismo rs5744168, entre pacientes de LES y controles sanos. Lo cual contradice un estudio familiar reciente, en el cual se observa que el alelo 1174C es protector frente al desarrollo del LES (237). Sin embargo y de acuerdo a nuestros datos, otro estudio realizado en dos poblaciones caucásicas procedentes de Ámerica del Norte (245), no pudieron tampoco replicar la asociación inicial encontrada (237). Varias razones podrían explicar estas discrepancias. El primer estudio podría tratarse de un error tipo I (falso positivo), ya que este tiene menos de un 40% de poder estadístico para detectar una asociación. Por el contrario la existencia de un error tipo II (falso negativo) en el estudio de Demirici y col (245) y en el nuestro es improbable debido al alto poder estadístico de ambos estudios (73% y 97% respectivamente) para detectar un efecto similar al observado en el primer estudio.

No habían sido descritas previamente variantes génicas en el gen *TLR7*, asociadas a ninguna EAI. Un estudio reciente encontró 3 variantes con una frecuencia superior al 5%, uno de estos SNPs (rs179008) da lugar a un cambio de aminoácido de una glutamina por una leucina en el codon 11 (Q11L). Así

que decidimos estudiar por primera vez el papel de este polimorfismo Q11L del gen *TLR7* en la susceptibilidad al LES, no encontrando ninguna evidencia de asociación. Debido a que este estudio presenta un alto poder estadístico (>99%) para detectar una OR de 1.5 y que la frecuencia encontrada en nuestra población control es similar a la establecida en la base de datos de SNPs de otras poblaciones caucásicas europeas, podemos establecer que este polimorfismo no juega un papel relevante en el LES en la población española. Sin embargo, no podemos excluir la posibilidad de que otros polimorfismos del gen *TLR7* que no estén en LD con el SNP analizado estén contribuyendo al desarrollo de la enfermedad.

De modo que podemos concluir que los polimorfismos rs5744168 del gen *TLR5* y rs179008 del gen *TLR7*, no parecen ser relevantes en el LES en nuestra población. Sin embargo, no podemos excluir un posible papel de TLR5 y TLR7 como posibles dianas terapéuticas en la patogénesis del LES (246).

5.1.2. *NFKB1*

Teniendo en cuenta la importancia del factor de transcripción NFκB en la patogénesis del LES, y que el gen *NFKB1* se localiza en la región cromosómica 4q23-24, la cual se ha visto asociada a LES mediante rastreos sistemáticos del genoma (172), nos propusimos analizar dos de los polimorfismos del gen *NFKB1*. Estos dos polimorfismos consisten en una variante funcional del gen *NFKB1* que consiste en una inserción/deleción (-94ins/delATTG) que se localiza entre dos elementos reguladores del promotor, lo cual tiene un efecto funcional en la transcripción del gen *NFKB1* (247). La presencia de una deleción de 4pb da lugar a la perdida de unión de una proteína nuclear lo cual reduce la actividad promotora de *NFKB1*, mientras que la inserción da lugar a una mayor actividad promotora, produciéndose así

un aumento en los niveles de expresión de la proteína (248). Además esta deleción se ha observado que aumenta el riesgo de sufrir CU en una población norteamericana (247). Por otro lado, se ha observado un microsatélite $(CA)_n$ potencial regulador de la expresión de *NFKB1* y localizado en la proximidad del gen asociado a DT1 (249). No encontramos ninguna diferencia estadísticamente significativa en las frecuencias alélicas y genotípicas de ambos polimorfismos entre pacientes con LES y controles sanos, lo cual sugiere que estos polimorfismos no influyen en el desarrollo del LES en nuestra población (250).

Hasta la fecha este es el único estudio que ha tratado de relacionar estos u otros polimorfismos del gen *NFKB1* con susceptibilidad al LES, de manera que habría que intentar replicar este resultado negativo en poblaciones diferentes para poder afirmar que este gen no está implicado en esta enfermedad autoinmune. En el caso de la CU, aunque varios estudios han tratado de replicar la asociación inicial de Karban y col, solo un grupo holandés lo ha conseguido (251). Por el contrario, el resto de estudios, incluyendo un estudio de nuestro grupo, no han conseguido encontrar una asociación entre esta variante -94ins/delATTG y la CU en diversas poblaciones europeas (252-254). Un reciente meta-análisis ha revelado que esta variante génica no parece tener especial relevancia en la predisposición a CU (255). Igualmente esta inserción/deleción tampoco se encontró asociada con susceptibilidad a otras enfermedades autoinmunes como enfermedad de Graves (256), AR (250), EC (247, 251, 254), artritis psoriásica (257), arteritis de células gigantes (ACG) (258), DT1 (259, 260) y enfermedad celíaca (261). Con respecto al microsatélite $(CA)_n$ encontramos que la asociación inicial encontrada por Hegazy y col no se ha podido replicar en otras poblaciones en DT1, EM, AR y enfermedad celíaca (250, 260, 262-264).

Podemos concluir que las variantes genéticas -94ins/delATTG y $(CA)_n$ del gen *NFKB1*, no parecen tener un papel relevante en la susceptibilidad y severidad del LES en nuestra población. Así como tampoco parecen estar influyendo en un gran número de EAI. Aunque está claro que NFκB juega un papel importante en inflamación y autoinmunidad, los procesos patológicos en los que está involucrado son muy complejos de manera que serían necesarios más estudios genéticos para poder ver la importancia relativa de polimorfismos del gen *NFKB1* en relación a la predisposición genética con autoinmunidad. Además de la importancia de examinar la posible implicación en estas enfermedades de polimorfismos de otros componentes de la familia de NFκB, tales como *NFKB2*, *RELA*, *c-REL* y *REL*.

5.1.3. *FCRL3*

Debido al relevante papel que juegan los genes de la familia *FcRs* en el desarrollo del LES, decidimos realizar un estudio replicativo de las variantes genéticas del gen *FCRL3* que previamente se habían visto asociadas con susceptibilidad a LES en población japonesa en nuestra población española. Los polimorfismos analizados fueron fcr3_3 (-169C/T), fcr3_4 (-110A/G) y el fcr3_6 (+1381A/G). No encontramos diferencias significativas cuando las frecuencias de estos SNPs fueron comparadas entre pacientes de LES y controles sanos (265). Aunque se observó un ligero aumento del genotipo CC del polimorfismo -169C/T en pacientes de LES, las diferencias no llegaron a ser significativas (P= 0.06). Igualmente se encontró un ligero aumento del haplotipo CGA entre pacientes de LES. Sugiriendo estos resultados que estas variantes genéticas del gen *FCRL3* no parecen tener un papel relevante en la susceptibilidad al LES en nuestra población.

Posteriores estudios replicativos de la variante funcional -169C/T del gen *FCRL3* en población coreana, china y noruega de LES (266-268), tampoco han conseguido replicar la asociación inicial encontrada en japoneses. Diversos estudios y meta-análisis sugieren que el SNP -169C/T de *FCRL3* parece ser importante en la susceptibilidad a AR en poblaciones asiáticas, pero no en poblaciones caucásicas (148, 269-271). Sin embargo este hecho parece no ocurrir en el caso de otras enfermedades como el LES, donde tampoco se ha replicado la asociación inicial en otra población asiática de diferente origen. Por tanto, esta falta de concordancia no parece estar basada en las diferencias étnicas ya que las frecuencias del alelo menor (MAF) en controles sanos de este polimorfismo eran muy similares entre coreanos (41%), noruegos (42%), chinos (42.2%) y españoles (40.7%) y sin embargo difieren de la MAF encontrada en japoneses (37%). Ya que las diferencias no parecen deberse a diferencias genéticas entre grupos étnicos quizás el factor relevante sean diferencias en la exposición a factores de susceptibilidad ambientales.

Aunque no se haya encontrado ninguna relación entre estas variantes de *FCRL3* en caucásicos con LES, no podemos descartar que estas u otras variantes dentro de la región 1q21-23 puedan estar influyendo en la susceptibilidad o desarrollo de la enfermedad en esta u otras poblaciones.

5.1.4. Citoquinas

5.1.4.1. Citoquinas de la respuesta innata

5.1.4.1.1. IL12 e IL12R

La IL-12 fue identificada como un citoquina heterodimérica compuesta por dos subunidades unidas covalentemente, la subunidad p35 y la p40 (272). Una primera caracterización de su actividad biológica reveló que la IL-12,

cuando se unía en linfocitos humanos de sangre periférica, inducía la producción de IFN-γ, aumentando la citotoxicidad de las células NK y la proliferación de células T. En 1993, Hsieh y col (273) descubrieron que la IL-12 es producida por macrófagos en respuesta a patógenos microbianos, y que tenia un papel clave en la diferenciación de células Th1. Este hallazgo permitió establecer el papel central de la IL-12 en la vía por la cual el sistema inmune innato desencadena una respuesta inmune adaptativa, diferenciando las células $CD4^+$ naïve hacia un fenotipo Th1. De modo general se predice que el papel biológico de IL-12 es actuar frente bacterias y parásitos intracelulares y también establecer la autoinmunidad órgano-específica (272). La IL-12 es producida por fagocitos (monocitos, macrófagos y neutrófilos) y por células dendríticas, y actúa como un regulador crítico en la respuesta mediada por células. El papel biológico de la IL-12 está mediado por el receptor de IL-12 (IL-12R) el cual está compuesto por dos cadenas (β1 y β2) (274). La unión a este receptor activa la ruta de señalización JAK-STAT, siendo STAT4 el mediador predominante de las respuestas celulares activadas por IL-12 (274) (Figura 5.1).

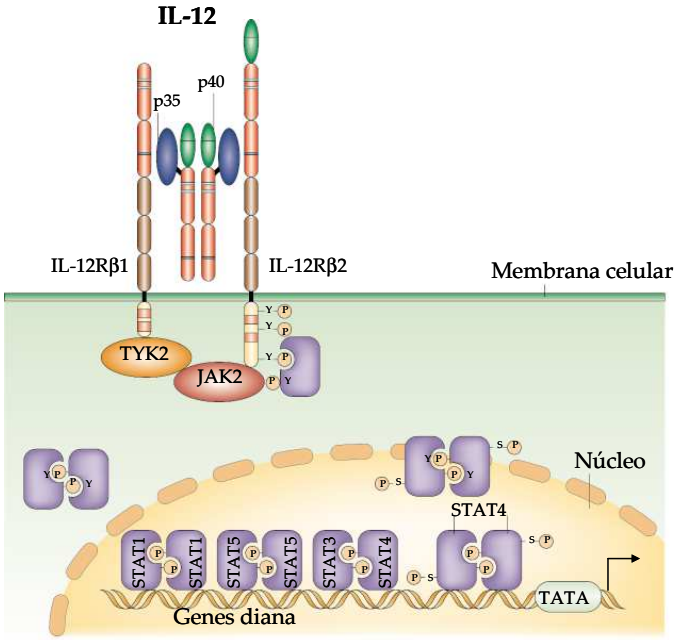


Figura 5.1. Interacción IL-12-IL12-R y señalización.

Debido a su conocida actividad pro-inflamatoria e inmunoreguladora, los genes de la *IL12* y su receptor quizás sean buenos candidatos en estudios de susceptibilidad al LES. Además se ha observado que en pacientes de LES se produce una excesiva producción de IL-12 (275-277). Estas variaciones en los niveles de IL-12 entre pacientes de LES y controles sanos podrían deberse a variaciones en las regiones reguladoras de dichos genes. En la subunidad p40 del gen de la *IL12* (también llamado *IL12B*) se han descrito dos variantes: una inserción/deleción de 4pb (*IL12Bpro*) en la región promotora y un SNP en la región 3´UTR (A1188C o rs3212227) que parecen tener una relevancia biológica (278, 279). Por otro lado en la subunidad β1 del IL-12R, la cual está codificada por el gen *IL12RB1* (272), se han descrito tres SNPs que dan lugar a un cambio de aminoácido en la región codificante del gen y que parecen estar influyendo en la señalización inducida a través de la IL-12 (280): 641A→C, 1094T→C y 1132G→C (Q214R, M365T, G378R) (281). De manera que decidimos estudiar por primera vez estas variantes genéticas de los genes *IL12B* e *IL12RB1* en pacientes de LES. No se encontraron evidencias de asociación entre estas variantes genéticas con predisposición a LES o con riesgo a desarrollar nefritis lúpica en nuestra población (282).

A pesar de que en un principio se propuso que el SNP A1188C del gen de la *IL12B* podría tener un papel importante en DT1 (283), posteriores estudios no han conseguido replicar estos resultados (284-286). Igualmente se ha observado que esta asociación tampoco se da en otras EAI como, enfermedad celiaca (287), AR (288), EM (289-291) y EC (292), sugiriendo que variantes genéticas dentro de este gen no parece tener ningún efecto en la susceptibilidad a EAI. Además, la relevancia biológica de estas variantes ha sido puesta en contradicho, ya que aunque varios estudios observaban diferencias en la expresión del gen de la *IL12B* con respecto a los distintos

genotipo del polimorfismo A1188C (283, 292), otros autores no han observado ninguna correlación (285, 293). Recientemente, se ha encontrado una segunda región de asociación situada 60Kb aguas arriba del sitio de inicio del ARNm (rs6887695) (294) asociada con protección a psoriasis y artritis psoriásica (295). Así mismo otra nueva variante, en la subunidad p35 del gen de la *IL12* (*IL12A*) se ha encontrado asociada a enfermedad celiaca en un reciente GWAS. Cuando analizamos el papel del *IL12RB1* en LES tampoco encontramos ninguna asociación entre las variantes estudiadas y predisposición a la enfermedad. Estos resultados coinciden con los encontrados en otras EAI en donde tampoco han conseguido encontrar una asociación con estos SNPs (288, 296, 297).

A la vista de los resultados obtenidos, parece ser que las variantes genéticas estudiadas de los genes de la *IL12B* y del *IL12RB1* no parecen jugar un papel relevante en predisposición y/o severidad al LES en nuestra población. Aunque no podemos descartar que otros genes implicados en la ruta de señalización a través de IL-12 puedan contribuir al desarrollo del LES.

5.1.4.1.2. IL23 e IL23R

La IL-23 es una citoquina relacionada con la IL-12, que está compuesta por dos subunidades: una subunidad p40 que es homologa a la subunidad p40 de la IL-12 (*IL12B*) y una subunidad p19 específica de la IL-23 (codificada por el gen *IL123A*) (298). La IL-23 comparte la subunidad β1 del receptor y las rutas de señalización de la IL-12. La IL-23 afecta a la producción de IFN-γ por las células NK, activa las células T memoria y estimula la respuesta celular Th1. Esta citoquina es capaz de aumentar la inflamación estimulando la producción de citoquinas pro-inflamatorias (274, 299). Por tanto, la IL-23 quizás tenga importantes implicaciones en la patogénesis de enfermedades inflamatorias crónicas como el LES. Por su parte el receptor de la IL-23 esta

compuesto por dos subunidades, una de ellas, la IL12Rβ1, compartida con el IL-12R; y una específica conocida como IL-23R. Este receptor está ausente durante los primeros estadíos de diferenciación de las células T, sugiriendo que la IL-23 solo ejerce su función en células más maduras. Aunque la IL-12 y la IL-23 comparten la subunidad p40, se ha demostrado que es la IL-23 y no la IL-12, la que conduce a la patogénesis en diversos modelos experimentales de enfermedades autoinmmunes (300-302). Además, al contrario que la IL-12, la IL-23 da lugar a la activación de subpoblaciones de células T caracterizadas por la producción de citoquinas IL-17, llamadas células Th17 (303-305) (Figura 5.2). Todo esto sugiere que la ruta IL-23/IL-23R quizás tenga implicaciones importantes en la patogénesis del LES.

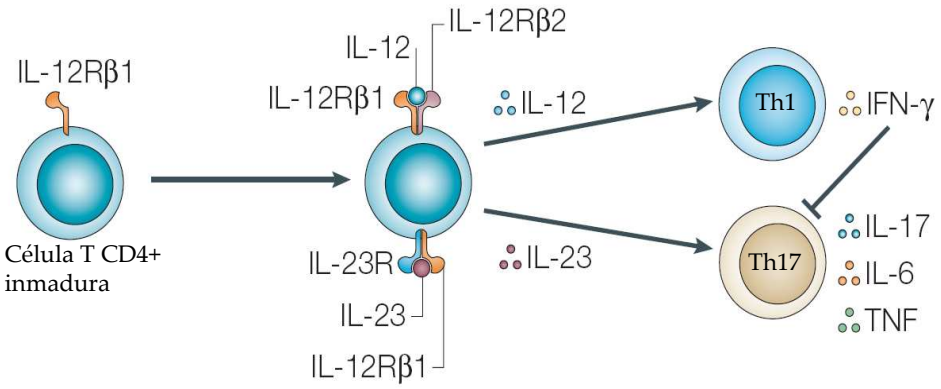


Figura 5.2. Diferenciación de las células T en base a la activación de la IL-12 y la IL-23.

Recientemente, se ha encontrado mediante un GWAS que el gen del *IL23R*, el cual se localiza en la región cromosómica 1p31, está asociado con predisposición a EII en dos cohortes independientes (306). En concreto, una variante rara que da lugar a un cambio de aminoácido (rs11209026, Arg381Gln) confiere protección frente a EII, además otras variantes no codificantes de este gen se encuentran independientemente asociadas

(rs1004819, rs7517847, rs10489629, rs1343151, rs10889677, rs11209032 y rs1495965). Poco después, y de manera independiente, en dos estudios a gran escala se observó que el mismo gen está asociado a psoriasis (294) y a espondilitis anquilosante (EA) (307). Numerosos estudios replicativos en diversas poblaciones han demostrado que *IL23R* es un gen de riesgo a EII (307-316). Por el contrario no se ha descrito hasta la fecha ninguna variante en el gen de la *IL23* asociado a EAI.

Para ver el posible efecto de estos genes en el LES, analizamos ocho SNPs de *IL23R*, que eran los que previamente se habían encontrado asociados a EII. Sin embargo, ninguno de ellos se encontró asociado a LES en nuestra población (317). Así mismo tampoco se ha encontrado asociación con ninguno de estos SNPs y diversas EAI como la AR (318), la DT1 (319), la esclerodermia (ES) (320) o la EM (291, 321). Esta falta de asociación encontrada en LES puede ser debida por una parte a que la IL-23 parece tener un papel crítico en la inflamación tisular local, tal como se ha demostrado en modelos murinos de enfermedades órgano específicas como la EAE (300), CIA (302) y EII (305), pero no se ha relacionado con el desarrollo de lupus en modelos de ratón (322). Estos indicios junto al hecho de que, el *IL23R* se asocia en humanos principalmente a enfermedades autoinmunes órgano específicas como la EII (306), la psoriasis (294) y la EA (307, 323) y no a sistémicas como el LES, la AR y la ES, nos llevan a pensar, que quizás la IL-23 tenga más importancia en la regulación de la inflamación local. Por otra parte, la IL-23 induce preferentemente la producción por parte de las células T de citoquinas como la IL-17, IL-6 y TNF-α, pero no induce la producción de interferones tipo I, los cuales juegan un papel relevante en el desarrollo y mantenimiento del proceso autoinmune en el LES (324, 325).

La subunidad p40 de la *IL23*, compartida con la *IL12*, como ya se relató en el apartado anterior, no parece tener ningún efecto en susceptibilidad al LES. Para llevar a cabo un análisis más exhaustivo de dicho gen, decidimos estudiar la otra subunidad, la cual está codificada por el gen *IL23A*. Ninguna variación se había descrito previamente en este gen, por tanto decidimos buscar polimorfismos en este gen mediante secuenciación. Para ello secuenciamos un fragmento de 4.5Kb que incluía la región promotora, los cuatro exones, tres intrones y la región 3´UTR en 30 individuos. Sin embargo, este gen resultó ser muy poco polimórfico, ya que no encontramos ninguna variación en la secuencia (282).

Estos datos, junto con los comentados en el apartado anterior encontrados en otras miembros de la familia de la citoquina IL-12 (*IL12B* y *IL12RB1*), sugieren que las variantes genéticas estudiadas de estas citoquinas no parecen tener un papel relevante en la predisposición al LES. Sin embargo, no podemos descartar estudiar los genes del otro miembro de la familia, IL-27, así como la *IL12A* e *IL12RB2*, donde reciententemente se han encontrado polimorfismos asociados a otras EAI (326).

5.1.4.2. Citoquinas pro-inflamatorias

En el LES se ha demostrado que el disbalance entre la producción de citoquinas Th1 y Th2 juega un papel clave en la inducción y el desarrollo de la enfermedad (51). Las citoquinas pro-inflamatorias regulan un amplio número de procesos inflamatorios que están implicados en la patogénesis del LES, y cada vez parece estar más claro el papel de estas citoquinas en la enfermedad (56, 57). Además estas citoquinas se han propuesto como nuevas dianas terapeúticas para el tratamiento del LES (327) y otras EAI como la AR (328).

5.1.4.2.1. Factor inhibidor de la migración de macrofagos (MIF)

El factor inhibidor de la migración de macrófagos (MIF) es una citoquina inmunoreguladora que presenta actividad pro-inflamatoria, hormonal y enzimática, y juega un importante papel en la respuesta inmune innata y adaptativa (329, 330). MIF es expresada por un amplio rango de tipos celulares como macrófagos, células B y células T entre otras (331, 332). Existen numerosas líneas de evidencia que sugieren una implicación del gen *MIF* en el LES (333) (Figure 5.3). Por un lado, *MIF* juega un papel crítico la activación de las células T, y se ha demostrado que alteraciones en esta ruta de activación dan lugar al desarrollo de LES en modelos animales (330). *MIF* está implicado en la proliferación de las células B, lo cual podría influir en la hiperactividad de células B que se produce en pacientes de LES (334). Por otro lado, *MIF* quizás contribuya también al daño inflamatorio crónico que ocurre en el LES, debido a que actúa a su vez como un inductor de diversas citoquinas pro-inflamatorias, como IL-1β, TNF-α, IL-2, IL-6, IL-8 e IFN-γ, las cuales se han encontrado asociadas con severidad a LES (331), y otros mediadores inflamatorios como el oxido nítrico, COX2, prostaglandinas y metaloproteinasas. Además, *MIF* también es capaz de inhibir la expresión del gen supresor de tumores *p53* (335) que está implicado en la apoptosis. Estudios con modelos animales de LES, han relevado que *MIF* juega un importante papel en esta enfermedad. Se ha visto que ratones MRL/*lpr* deficientes en MIF presentan un menor daño renal, menos lesiones de piel y una reducción de la mortalidad (336). Finalmente, se ha observado que los niveles en suero de MIF aumentan significativamente en pacientes con LES (337), además se ha visto que existe una correlación entre los niveles de MIF y la actividad de la enfermedad, encontrándose por ejemplo un incremento renal en la expresión de MIF en pacientes de LES que presentan glomerulonefritis (338).

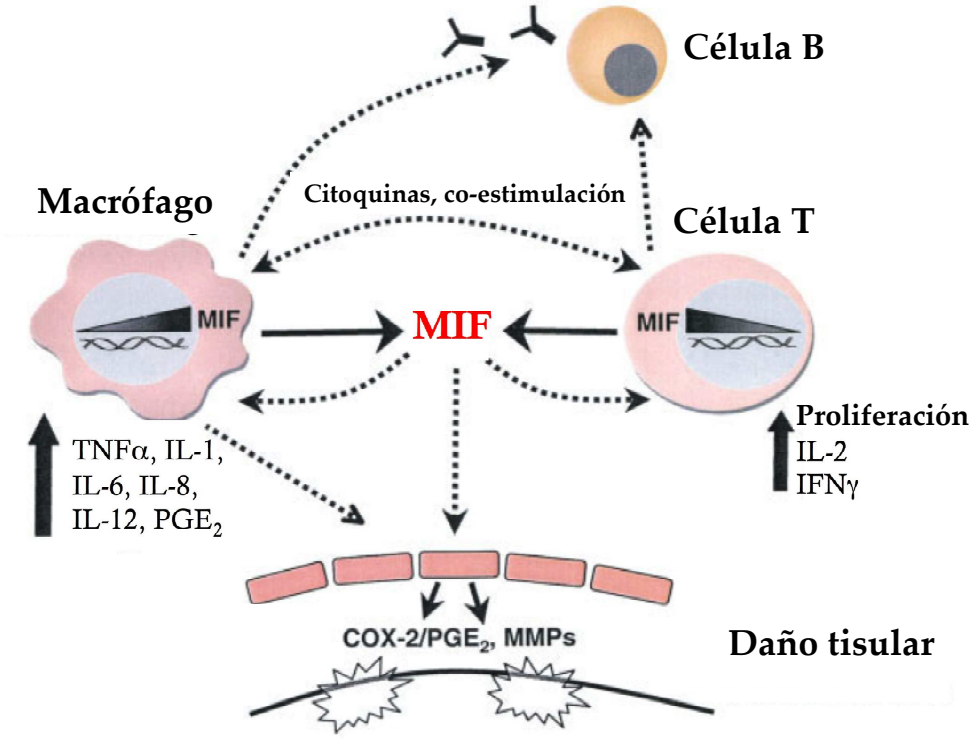


Figura 5.3. Esquema del papel inmunológico de MIF en el LES.

A parte de las posibles implicaciones funcionales de *MIF* en el LES, este gen también es importante en esta enfermedad desde un punto de vista posicional, ya que el gen *MIF* se localiza en el cromosoma 22q11.2, que es una región asociada a LES en estudios de ligamiento (339). Hasta la fecha se han descrito cuatro polimorfismos en el gen *MIF* (340, 341), incluyendo un microsatélite consistente en repeticiones de distinto número de CATT localizado en la región promotora (empezando en la posición -794) y tres SNPs en las posiciones -173 (rs755622), +254 (rs2096525) y +656 (rs2070766). Los SNPs +254 y +656 se localizan en intrones, mientras que el -173 se encuentra dentro del promotor del gen y se ha visto que el alelo C de este polimorfismo (-173C) se encuentra asociado con una producción elevada de la proteína MIF (340). Por otro lado, el alelo de siete repeticiones del microsatélite -794CATT ($CATT_7$) se ha asociado con altos niveles en la transcripción del gen *MIF in vitro* (340,

342). Y a su vez el haplotipo formado por los alelos -173C- $CATT_7$ produce un aumento en la expresión de los niveles de MIF (Figura 5.4). Además estas variantes se habían encontrado previamente asociadas con susceptibilidad a otras EAI como la artritis idiopática juvenil (AIJ) (340, 341, 343, 344), poliartritis inflamatoria (345), psoriasis (346) y severidad a AR (342).

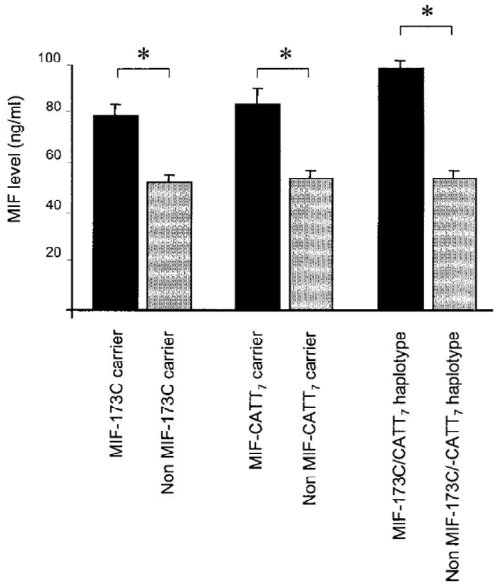


Figura 5.4. Asociación entre los niveles circulantes de MIF y los alelos -173C, $CATT_7$ y la combinación de ambos (-173C- $CATT_7$).

A la vista de todos estos datos, decidimos investigar el papel de estas dos variantes funcionales, -173G/C y -794$(CAAT)_n$ dentro del promotor del gen *MIF* con susceptibilidad y/o severidad al LES (347). Encontramos que en nuestra población, el genotipo -173CC estaba significativamente aumentado en pacientes de LES con respecto a controles sanos (*P*= 0.002, OR= 2.58, intervalo de confianza 95%(95%CI) 1.32-5.10). Esta asociación se mantenía en las frecuencias alélicas donde el alelo -173C estaba más representado en el grupo de pacientes (*P*= 0.004, OR= 1.34, 95%CI 1.05-1.27). Sin embargo, no observamos diferencias en la distribución de los distintos alelos del

microsatélite $(CAAT)_n$. Además al analizar los haplotipos formados por estas dos variantes, observamos que el haplotipo -173C-$CAAT_7$ estaba incrementado significativamente en pacientes de LES (*P*= 0.001, OR= 1.94, 95%CI 1.35-2.79).

Como *MIF* se había asociado previamente con severidad a otras enfermedades autoinmunes como la AR (342) y se había visto que los niveles de MIF aumentaban en pacientes con glomerulonefritis (338), decidimos estudiar estas variantes genéticas en relación a las distintas características clínicas que presentan los pacientes de LES. No encontramos diferencias significativas en la distribución de estos polimorfismos y pacientes de LES.

Curiosamente, las frecuencias alélicas encontradas de estos polimorfismos en nuestra población española eran similares a las encontradas en otras poblaciones de origen caucásico procedentes de Europa y Norte América (348), y significativamente diferentes de las observadas en poblaciones de origen étnico diferente (349-351). De modo que estas variaciones en la distribución de los alelos de *MIF* entre grupos raciales, sugiere la existencia de una presión selectiva actuando en el locus de *MIF*. Por tanto, quizás sea interesante investigar el papel de *MIF* en otras poblaciones étnicamente diferentes, como por ejemplo en pacientes de LES de origen africano.

Este estudio constituyó en primer dato de que variantes del gen *MIF* están implicadas en predisposición a LES, y hasta la fecha nadie lo ha estudiado en otras poblaciones. Recientemente, se ha confirmado la asociación de estas variantes con susceptibilidad a otras enfermedades autoinmunes, a parte de AIJ y poliartritis reumática, como son enfermedad celíaca (352), EII (353-355) y AR (356). Todos estos hallazgos, muestran una evidencia más que apoya la hipótesis de que diferentes EAI comparten los mismos componentes genéticos. Así mismo estos datos sugieren que terapias anti-MIF podría ser

una nueva diana terapeútica en estas enfermedades (357), ya que, se ha observado que antagonistas de MIF inhiben su función inflamatoria.

En conclusión, nuestros datos sugieren que el alelo -173C y el haplotipo -173C-CAAT$_7$ de *MIF*, que previamente se habían visto relacionadas con un aumento en los niveles de *MIF*, están asociados con riesgo a padecer LES. La mayor producción de MIF dada por la presencia de estas variantes, precipitan la respuesta autoinmune en pacientes de LES, provocando un aumento en la producción de citoquinas pro-inflamatorias, inhibición de la apoptosis y aumento de la permeabilidad tisular. Todo esto conllevaría a una mayor inflamación tisular que desencadenarían las distintas manifestaciones de la enfermedad (Figura 5.5). Sin embargo, estudios replicativos de esta asociación encontrada en LES en otras poblaciones son necesarios para establecer una asociación definitiva entre *MIF* y dicha enfermedad.

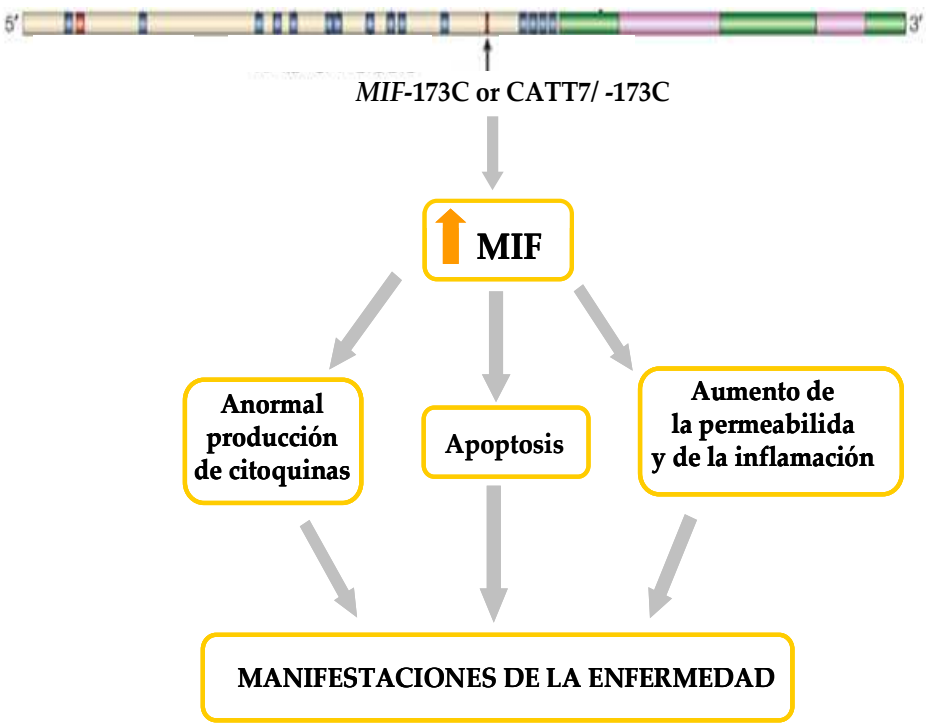


Figura 5.5. Posible papel del alelo *MIF*-173C o del haplotipo CAAT$_7$/-173C en la patogénesis del LES.

5.1.4.2.2. IL18

La IL-18, previamente conocida como factor inductor de IFN-γ (358), es una importante citoquina pro-inflamatoria, miembro de la familia de citoquinas de la IL-1. Esta citoquina esta reconocida como un importante regulador de la respuesta inmune innata y adquirida (359-361) y es capaz de mediar respuestas inmunes Th1 y Th2 (362, 363). La IL-18 es expresada por una gran variedad de células del sistema inmune (364), y se ha visto que juega múltiples papeles en inflamación crónica, enfermedades autoinmunes, en una gran variedad de canceres y de enfermedades infecciosas (360, 365-367). La IL-18 en combinación con otras citoquinas pro-inflamatorias como TNF, IL-6 o IFN-γ juega un importante papel en la propagación del proceso inflamatorio responsable de la destrucción y el daño a los tejidos (206). Por otro lado, la IL-18 en combinación con la IL-12, induce la producción de IFN-γ en células Th1, células B y células NK, promoviendo de este modo una respuesta inmune tipo Th1 (368, 369), pero esta citoquina puede también estimular la respuesta inmune Th2 en ausencia de la IL-12 (370, 371) (Figura 5.6). Además, la IL-18 actúa como un importante mediador de la enfermedad lupus-like, ya que puede acelerar está EAI espontánea en ratones MRL/*lpr*, caracterizada por presencia de glomerulonefritis, vasculitis y *rash* malar simétrico (372). En humanos, los niveles de IL-18 están más elevados en el suero de pacientes de LES comparados con controles sanos (373-375), y este aumento se ha correlacionado positivamente con el índice de actividad de la enfermedad (373). Debido a sus múltiples funciones en respuestas inflamatorias e inmunológicas, parece que la IL-18 juega un potencial papel patológico en el desarrollo de EAI como el LES.

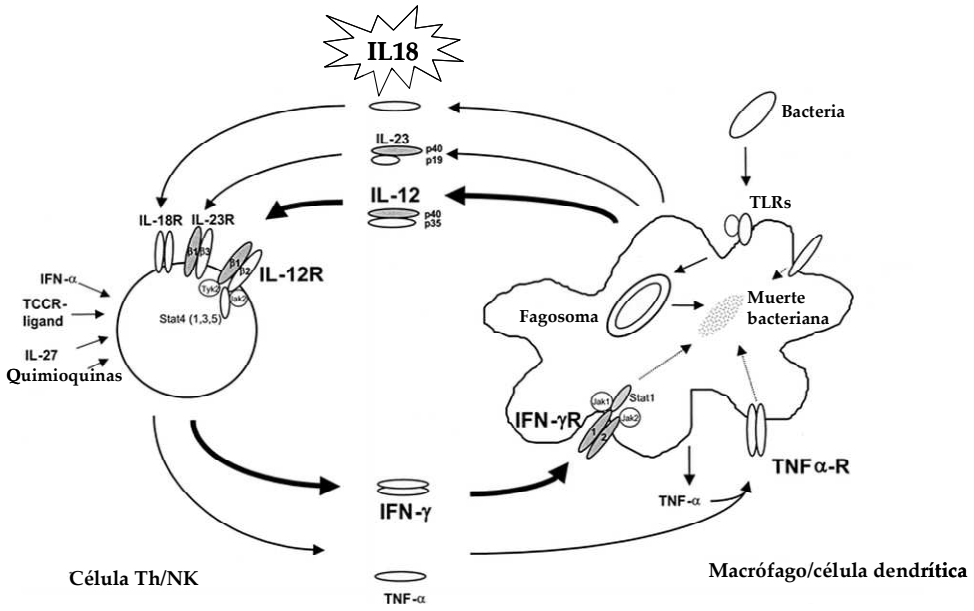


Figura 5.6. Producción de IFNγ a través de la IL-18

La expresión de la IL-18 está regulada por el promotor del gen (376-378). El gen de la *IL18* está localizado cerca de una región de asociación a LES (11q22.2-22.3) (187, 339). Además varios polimorfismos dentro de la región promotora del gen *IL18* se han visto asociados con una gran variedad de enfermedades inflamatorias y autoinmunes (367). Todo estos hallazgos, muestran a la región promotora del gen *IL18* como una región candidata atractiva para realizar estudios genéticos en LES.

A la vista de estos interesantes datos decidimos estudiar la relación entre tres polimorfismos del promotor de la *IL18*, dos de ellos previamente asociados a LES en población asiática, -137 (rs187238) y -607 (rs1946518), y una nueva variante (-1297 o rs360719) la cual podría tener un papel clave en la expresión de la IL-18, y susceptibilidad a LES en tres poblaciones diferentes.

Aunque se encontró una tendencia de asociación entre los SNPs -137 y -607 al hacer un meta-análisis de las tres poblaciones analizadas, la asociación no resultó ser significativa después de corregir los valores de *P* por el número

de SNPs analizados en el estudio (*Pc*= 0.3 para el SNP -137 y *Pc*= 0.1 para el SNP -607). Aunque estas variaciones se habían encontrado asociadas a LES en dos estudios previos en poblaciones asiáticas (379, 380), nosotros no fuimos capaces de replicar esta asociación en tres poblaciones caucásicas (Sánchez y col. datos no publicados). Estos datos contradictorios pueden ser explicados por dos motivos principales: una razón podría ser el pequeño tamaño muestral que presentan estos estudios iniciales (379, 380), ya que presentan un poder estadístico demasiado bajo (<60%) para detectar una asociación. Otra posibilidad es la heterogeneidad genética ó ambiental, la cual está claramente presente, ya que las frecuencias alélicas y genotípicas son significativamente diferentes entre ambas poblaciones. Aunque estos polimorfismos de la región promotora del gen de la *IL18* se han sugerido que podrían estar afectando a la expresión del gen (381), estos datos deben ser tomados con precaución ya que las diferencias que encuentran en la expresión génica no llegan a ser significativas (*P*= 0.17).

En vista de que parece no estar clara la relevancia funcional de estos polimorfismos, decidimos buscar otras variantes en la región promotora que pudieran estar afectando a la expresión. En base a esto, identificamos una nueva variante funcional (*IL18* -1297) determinante de la expresión del gen de la *IL18*, y que podría ser la variante genética clave en el papel del gen de la *IL18* en enfermedades autoinmunes. Encontramos que esta variante en la población española estaba asociada significativamente con susceptibilidad a LES, ya que la frecuencia del alelo -1297C estaba aumentada en pacientes respecto a controles (*P*= 0.00003, OR= 1.45, 95%CI 1.22-1.73), y estas diferencias seguían siendo significativas después de corregir por el número de comparaciones (*Pc*= 0.00009). Resultados similares fueron encontrados en las otras dos poblaciones analizadas, aunque en estas las diferencias entre las

frecuencias alélicas no llegaban a ser significativas presentaban una clara tendencia de asociación, estando el alelo -1297C aumentado en pacientes de LES respecto a controles en ambas poblaciones (italiana y argentina). Esto quedó demostrado al realizar un meta-análisis de las tres poblaciones, el cual incluía 1342 pacientes de LES y 1138 controles sanos (Pooled OR= 1.28, 95%CI 1.13-1.46, *P*= 0.00002).

Un tema importante es saber como esta nueva variante puede afectar a la expresión del gen. Kalina y col (382), encuentras varios factores de transcripción involucrados en la regulación de la *IL18*. Se ha visto que el SNP *IL18* -1297 da lugar a la pérdida de un sitio de unión del factor de transcripción Oct-1. El Oct-1, es un factor de transcripción involucrado en la regulación de muchos genes y que también puede reprimir la expresión de algunos, incluyendo algunas ILs (382-387). En nuestro estudio demostramos mediante un ensayo de mobilidad electroforética (EMSA) y western blot que la presencia del alelo -1297T es crítica en la supresión transcripcional del gen de la *IL18*, y esta supresión está mediada a través de la unión de Oct-1. Esta supresión podría resultar en una menor producción de IL-18 y potenciar la protección frente a la sobreexpresión en desordenes como el LES, donde una respuesta inflamatoria persistente parece ser esencial en el proceso patogénico.

En conclusión, podemos decir que hemos encontrado una nueva variante funcional dentro de la región promotora del gen de la *IL18* (-1297), que parece tener un papel muy importante en la expresión de la IL-18, asociado con susceptibilidad a LES. Todos estos resultados ayudan a esclarecer el papel de los polimorfismos dentro del promotor de la *IL18* en la regulación de dicho gen y su relevancia en el LES, así como en otras EAI.

5.2. Genes en la región MHC

5.2.1. *MICA*

En el exón 5 del gen *MICA*, que codifica la región transmembrana de la molécula, se describió un microsatélite polimórfico consistente en una serie de repeticiones $(GCT)_n$ cuyos alelos determinan la presencia de un número variable de residuos de alanina. Una variante alélica del microsatélite consiste en la inserción nucleotídica de una G (GCT→GGCT) entre el segundo y el tercer triplete de repeticiones, el alelo *MICA* A5.1, que causa la alteración del marco de lectura generando un codon de parada prematuro (388). Se cree que este alelo podría dar lugar a una forma soluble de la molécula MICA o alterar su localización en el enterocito de la zona basolateral a la apical (388, 389).

La localización de los genes *MICA* en la región HLA y su asociación con otras EAI sugiere que el gen *MICA* es un posible gen candidato para el desarrollo del LES. De manera que decidimos estudiar si polimorfismos en la región TM de *MICA*, así como en los genes *HLA* de clase I (locus B) y clase II (locus *DRB1**), influyen en la predisposición genética a padecer LES en una amplia cohorte de pacientes españoles.

Aunque se encontró un ligero aumento en la frecuencia del alelo *MICA* A5.1 en pacientes de LES comparado con controles sanos, estas diferencias no llegaron a ser estadísticamente significativas (390). Con respecto al tipaje de los otros genes *HLA* clásicos, encontramos que el alelo *HLA-B*08* estaba aumentado significativamente en pacientes con LES ($P<10^{-7}$, OR= 3.17, 95%CI 2.02-5.00). Resultados similares encontramos para los alelos *HLA-DRB1*0301* ($P<10^{-7}$, OR= 2.07, 95%CI 1.59-2.68) y *HLA-DRB1*1501* (P= 0.06) de susceptibilidad a LES. Observamos que existia un fuerte LD entre los alelos *B*08*, *MICA* 5.1 y *DRB1*0301* (P= 0.00018 y $P<10^{-6}$ respectivamente). Análisis

posteriores para detectar independencia de asociación sugieren que la asociación descrita entre *MICA* A5.1 y LES es secundaria al LD que existe con el alelo *B*08*.

Estos resultados encontrados en nuestra población española contradicen un estudio previo en el cual describen una asociación positiva con los alelos A5.1 y A5 y una asociación negativa con *MICA* A9 en una población italiana con LES independiente de DR3 y DQ2 (391). Sin embargo ellos encuentran que el alelo asociado con el LES HLA-B8 presentaba un fuerte grado de LD con el alelo MICA A5.1, indicando que el alelo HLA-B8 no estaba asociado de forma independiente con el LES. Sin embargo, ellos no realizaron un análisis para detectar cual de esos genes muestra el mayor grado de asociación. Todos estos indicios nos hacen pensar que un diferente grado de LD entre HLA y *MICA* en italianos y españoles explique las discrepancias observadas. También se puede sugerir que el relativo bajo número de individuos que usan en el primer estudio (48 pacientes y 158 controles) pueda haber dado lugar a un error tipo I (falso positivo) en el estudio de asociación caso-control.

La región MHC ha sido asociada a LES en muchos estudios. Sin embargo, esta asociación HLA-LES es frecuentemente contradictoria con respecto a que genes están involucrados en la susceptibilidad a la enfermedad (392, 393). Esto puede deberse a resultados confusos causados por el fuerte LD que existe en la región.

Los alelos del gen *MICA* se han asociado con predisposición a diferentes EAI como la DT1 (394-396), la AR (397), AIJ (398), enfermedad celíaca (399, 400), ACG (401), enfermedad de Grave (402) ó CU (403). Sin embargo, su independencia con otros alelos *HLA* es contradictoria dependiendo de la enfermedad o de la población estudiada, debido principalmente al distinto grado de LD existente en esta región entre las distintas poblaciones.

En conclusión, nuestros resultados confirman los datos previos de asociación de los alelos *HLA-B08** y *DRB1*0301* con susceptibilidad a LES, pero no apoyan una asociación independiente entre *MICA* y LES.

5.2.2. *BTNL2*

En un estudio realizado por Valentonyte y col. en el cual realizaron un mapeo fino de la región 6p21, encontraron una fuerte asociación con un polimorfismo del gen *BTNL2* (rs2076530) y sarcoidosis (165). Este SNP da lugar a un cambio de una G por una A en la posición -1 del sitio donante de splicing, dando lugar a un codón prematuro de parada que impide la localización en la membrana de la proteína. A pesar de que el gen *BTNL2* se localiza cerca de los genes clásicos *HLA* de clase II, estos autores encontraron que la asociación de dicho gen con sarcoidosis era independiente del LD con *HLA-DRB1*, y que además esa asociación era incluso más fuerte. Este hecho junto con el posible papel de *BTNL2* en la ruta coestimuladora de las células T, nos llevó a estudiar el SNP en nuestra población de LES (404).

Cuando estudiamos el polimorfismo rs2076530 del gen *BTNL2* en nuestra población de LES, observamos un aumento del alelo A en pacientes respecto a controles (P= 0.00002, OR= 1.61, 95%CI 1.29-202) (404). Mientras que este alelo era protectivo para otras dos EAI comunes estudiadas en el mismo trabajo (DT1 y AR). Estas diferencias que encontramos en LES respecto a DT1 y AR probablemente sean un reflejo del efecto de los genes *HLA*, debido a la proximidad de *BTNL2* con *HLA-DRB1* y *HLA-DQB1*, cuyos diferentes alelos están involucrados en la susceptibilidad a dichas enfermedades y al fuerte LD que existe en esta región. Observamos que el alelo A estaba en LD con DR3 mientras que el alelo contrario estaba en LD con DR4. Al estratificar por la presencia de estos alelos *HLA* de clase II, observamos que la asociación

encontrada con el gen *BTNL2* en las tres enfermedades era debida al fuerte LD con estos alelos. Aunque estudios previos habian encontrado una asociación entre este polimorfismo de *BTNL2* y sarcoidosis de forma independiente a los alelos HLA-DR/DQ (165, 405), está independencia con la región MHC no ha podido ser replicada en otras poblaciones con sarcoidosis (406) e incluso en otras enfermedades autoinmunes como EM (167) y CU (407).

De manera que podemos concluir que *BTNL2* no contribuye significativamente a la susceptibilidad del LES, así como tampoco parece afectar a la DT1 y a la AR.

5.3. Genes implicados en la regulación de las célulasT y B

5.3.1. *PTPN22*

Bottini y col. a partir de un estudio basado en la estrategia de genes candidatos, encontraron una variante en la región codificante del gen *PTPN22* que da lugar a una sustitución de una arginina por un triptófano en el aminoácido 620 (R620W, 1858C/T, rs2476601), fuertemente asociada con susceptibilidad a DT1 en dos poblaciones caucásicas independientes (168). Teniendo en cuenta el importante papel que juega el gen *PTPN22* en la regulación de la respuesta de las células T y la relevancia que parece tener el SNP R620W en susceptibilidad a DT1; decidimos estudiar el papel de dicha variante en predisposición y expresión clínica del LES en nuestra población.

Al analizar esta variante genética del gen *PTPN22* observamos que al igual que el alelo T (620W) estaba aumentado significativamente en pacientes de LES (*P*= 0.03, OR= 1.45, 95%CI 1.01-2.09) (408). Simultáneamente a nuestro estudio, un grupo norteamericano reportó la misma asociación en LES (409),

de manera que esto apoya nuestros datos en los que se sugiere que el alelo 620W potencia la predisposición a LES en población caucásica.

Posteriormente a estos estudios iniciales, numerosos grupos han replicado dicha asociación en distintas poblaciones caucásicas de origen europeo con LES, tanto en estudios caso-control como en estudios familiares (410-414). Un reciente meta-análisis incluyendo gran parte de los estudios realizados hasta la fecha en caucásicos ha revelado una fuerte asociación global con el LES ($P<10^{-5}$, OR= 1.49, 95%CI 1.28-1.75) (415). Además en un reciente GWAS se ha confirmado la asociación de *PTPN22* con el LES (111)(Figura 5.7).

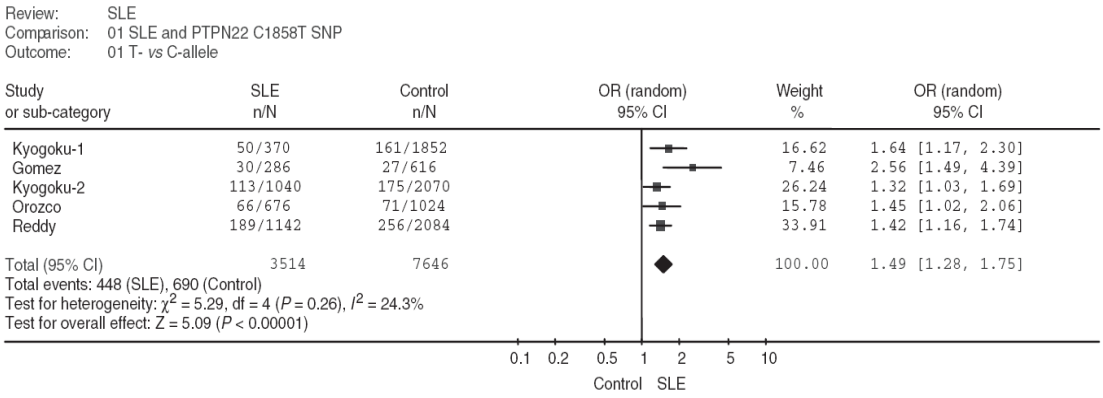


Figura 5.7. Meta-análisis del polimorfismo R620W del gen *PTPN22* incluyendo cinco poblaciones independientes, realizado por Lee y col.

Interesantemente este polimorfismo se ha encontrado asociado a multitud de EAI además de la DT1 y el LES, como son la AR (408, 416), enfermedad de Grave (417), AIJ (418), tiroiditis de Hashimoto (410), miastenia gravis (419) y ES (420) en poblaciones de descendencia europea. Todos estos fenotipos autoinmunes tienen en común un componente humoral, con autoanticuerpos específicos de la enfermedad que frecuentemente aparecen antes del comienzo clínico de la enfermedad. Sin embargo, llama la atención

que en EAI en las cuales la presencia de autoanticuerpos no es una característica predominante, como la EM (421, 422), EII (255, 423), enfermedad celíaca (421, 424) y EA (425), no se encuentra asociación con la variante R620W de *PTPN22*. En vista a estos resultados se ha sugerido que esta variante parece estar asociada solo a EAI en las que existe un gran componente humoral. En base a la importancia que parece haber entre esta variante y la presencia de anticuerpos decidimos estudiar la relación entre este polimorfismo y la presencia de autoanticuerpos en nuestros pacientes de LES. No encontramos asociación entre la presencia de algunos de los autoanticuerpos más revelantes en el LES como son: ANA, anti-ADNdc, anti-Ro, anti-La, anti-Sm, anti-Smith y anti-CL, y los distintos genotipos de dicho SNP. Igualmente tampoco encontramos asociación entre esta variantes y las demás características clínicas estudiadas (408).

El alelo de riesgo de *PTPN22* da lugar a un cambio de aminoácido en el residuo 620 el cual se localiza dentro de la región P1 de LYP la cual interacciona con el dominio rico en prolinas SH3 de CSK. Este polimorfismo tiene dos consecuencias funcionales: la unión entre PTPN22 y CSK se rompe (168, 416) y la actividad enzimática de PTPN22 se incrementa (426, 427). El efecto de esos cambios bioquímicos parece ser un aumento del nivel umbral de estimulación que es requerido para la señalización del TCR. Estos resultados fueron inesperados y paradójicos, ya que la autoinmunidad en general está ligada a una sobreactividad del sistema inmune. Además, ratones deficientes en *ptpn22* muestran una reducción en el umbral de señalización del TCR, dando lugar a una expansión de células T memoria y un aumento en la producción de inmunoglobulinas (175). Actualmente existen dos hipótesis sobre como explicar estos datos contradictorios. Una de estas posibilidades sugiere que se producen cambios durante la selección tímica de manera que se

produciría una selección positiva de timocitos que en situación normal serian eliminados, dando lugar a un aumento de células T maduras incrementándose así la autoreactividad (428). Una segunda posibilidad es que exista una menor activación de las células T reguladoras, lo que provocaría una deficiente regulación de células T autorreactivas (429) (Figura 5.8).

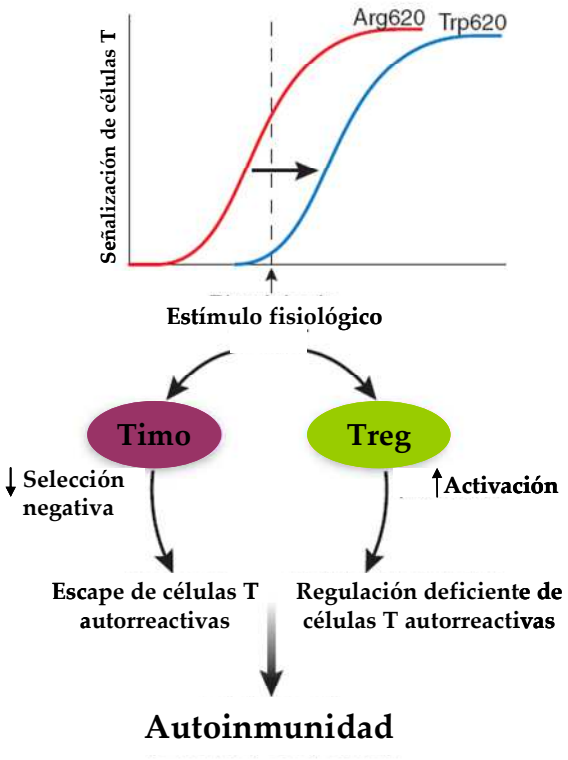


Figura 5.8. Hipótesis de la ganancia de función del polimorfismo R620W del gen *PTPN22*.

De manera interesante se ha observado que el alelo 620W de este SNP genera una mayor producción de ciertas citoquinas (426, 427), lo cual puede ser un mecanismo patogénico potencial por el cual dicho alelo contribuye al desarrollo de autoinmunidad. Por otra parte también se ha observado que esta mutación puede alterar a otros tipos celulares como es el caso de las células B, donde se ha descrito que la variante 620W da lugar a una menor producción de células B memoria y esa células van a responder menos a la estimulación

vía BCR (427). Este hecho podría finalmente resultar en una mayor producción de autoanticuerpos, lo cual apoya la teoría de que el polimorfismo R620W de *PTPN22* esta asociado principalmente con enfermedades seropositivas, como el LES.

Una característica interesante del polimorfismo *PTPN22* R620W es la gran variación que existe en las frecuencias alélicas entre las distintas poblaciones. En primer lugar existe un marcado gradiente norte-sur, siendo las frecuencias más bajas en el sur de Europa y las más altas en los países escandinavos (430) (Figura 5.9). En segundo lugar el alelo 620W está prácticamente ausente en poblaciones asiáticas y africanas (431), este hecho ha ocasionado que los intentos de replicación de esta asociación en EAI en esas poblaciones hayan resultado mayormente negativas. El claro papel que tiene este polimorfismo en poblaciones caucásicas y la falta del mismo en otras poblaciones genera la posibilidad de que existan alelos de riesgo adicionales en esas poblaciones, y que quizás puedan estar también presentes en caucásicos. Además es posible que la asociación entre la variante *PTPN22* 620W y la enfermedad sea reflejo de un LD con otra variante genética que sea la verdadera variante causal. Carlton y col, resecuenciaron el gen *PTPN22* en pacientes de AR encontrando 15 nuevos SNPs (432). Después de realizar un análisis haplotípico comprobaron que el efecto de los haplotipos se debía a la variante R620W, de manera que es bastante probable que esta sea la verdadera variante causal.

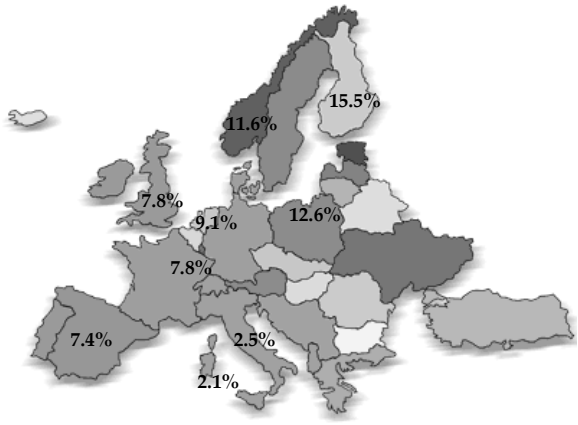


Figura 5.9. Mapa europeo que resume la diferente distribución del alelo *PTPN22* 620W.

Diversos estudios proponen a los inhibidores de PTPs como tratamiento de autoinmunidad. Dentro de ellas, *PTPN22* es hasta ahora la única posible diana terapútica (433), de manera que pequeñas moléculas inhibidoras podrían reducir la actividad catalítica del alelo 620W, de manera que se conseguiría una señalización del TCR normal neutralizándose así el proceso autoinmune inducido por dicha variante.

En resumen podemos decir, que el polimorfismo *PTPN22* R620W está fuertemente asociado con susceptibilidad a LES, y además ha sido propuesto como marcador común de predisposición a diversas EAI en poblaciones caucásicas.

5.3.2. *CD24*

Un estudio reciente identificó un polimorfismo en la región codificante del gen *CD24* (226C→T, rs8734), asociado con susceptibilidad y progresión a la EM (434). Dicha variante da lugar a un cambio de aminoácido en la posición 57 de una alanina por una valina (A57V) (434), en la posición que precede al sitio de segmentación del anclaje de la proteína GPI (Figura 5.10),

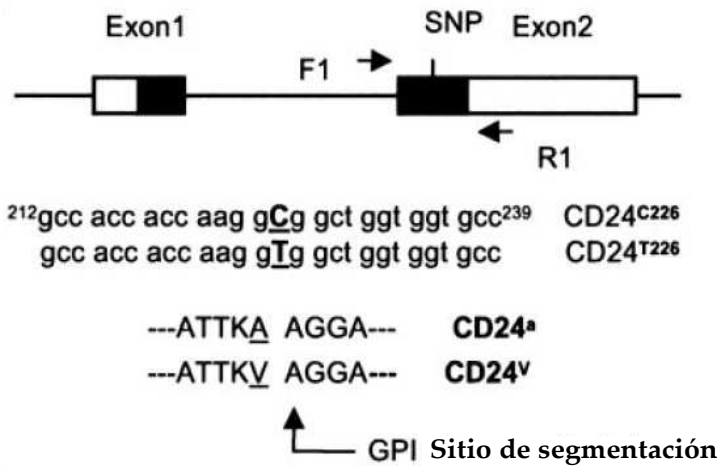


Figura 5.10. Localización del polimorfismo A57V dentro del gen *CD24*.

Teniendo en cuenta estos hallazgos, decidimos investigar la posible implicación de dicho polimorfismo de *CD24* en susceptibilidad a LES en tres poblaciones europeas independientes.

En la cohorte española, encontramos que la frecuencia del alelo *CD24V* estaba aumentada significativamente en el grupo de pacientes de LES comparado con controles (*P*= 0.0001, OR= 3.6, 95%CI 2.13-6.16) (435). Además demostramos que las frecuencias del genotipo *CD24V/V* en pacientes de LES es más del doble que en el grupo control (10.2% vs 4.3%, *P*<0.00001, OR= 3.7, 95%CI 2.16-6.34). Estos resultados fueron corroborados al analizar una segunda cohorte procedente de Alemania; donde encontramos que la frecuencia del alelo *CD24V* era mayor en el grupo de pacientes, aunque las diferencias no llegaban a ser significativas. Esto puede ser explicado por el tamaño de muestra, el cual era mucho más bajo en alemanes (n= 257) que en españoles (n= 696), resultando en una perdida de poder estadístico para demostrar una asociación de susceptibilidad. Sin embargo, no encontramos ninguna evidencia de asociación entre esta variante y los pacientes de LES en la cohorte sueca. Al realizar un test de homogeneidad entre cohortes

observamos que las tres cohortes eran combinables a nivel alélico. De manera que decidimos realizar un meta-análisis juntando los datos de estas tres poblaciones, obteniendo como resultado un aumento del alelo *CD24V* en el grupo de pacientes (Pooled OR= 1.20, 95%CI 1.05-1.36, *P*= 0.007).

A pesar de que nuestros datos concuerdan con resultados previos encontrados en otras poblaciones caucásicas, en las cuales se ha encontrado este SNP asociado con susceptibilidad a EM (434, 436), ACG (437) y AR (438), un reciente estudio en dos poblaciones caucásicas de pacientes de EM procedentes de Bélgica y Reino Unido, no fueron capaces de replicar dicha asociación (439). Estos resultados controvertidos, pueden ser explicados por la hererogeneidad de la población, ya que suecos, belgas e ingleses muestras diferencias en las frecuencias alélicas comparados con españoles, norteamericano y alemanes. Parece que las poblaciones del Norte de Europa tienen una mayor frecuencia del alelo *CD24V*, exhibiendose un claro gradiente norte-sur. Estas diferencias parecen estar fundamentalmente en los controles, mostrándose una frecuencia similar del alelo *CD24V* entre nuestros controles del Sur de España (23.8%) y los controles del norte de España (25.3%), sin embargo las frecuencias en alemanes eran ligeramente diferentes (27.4%), y estas diferencias eran aún mayores en controles de Bélgica (30%), Suecia (30.8%) y Reino Unido (34%) donde la frecuencia de dicho alelo era mayor. Sin embargo estas diferencias parecen no existir entre los grupos de pacientes tanto de LES como de EM. Sugiriendo la existencia de "clinas" entre controles, con una asociación del alelo *CD24V* a LES. Estos gradientes en las frecuencias de un polimorfismo presentes en controles y no en pacientes ocurre con otro gen asociado a LES y del que ha habido gran controversia. Este gen es el *PDCD1* (440) el cual muestra un claro gradiente de distribución del alelo PD1.3A en individuos sanos. Este gradiente ha ocasionado resultados

divergentes en las distintas poblaciones estudiadas. Este patrón de variación ha sido caracterizado en un gran número de SNPs, definiéndose dos grandes grupos de subpoblaciones europeas: una procedente del sur de Europa y otra del norte de Europa (441).

Todos estos datos, sugieren la existencia de otra u otras variantes de *CD24* que puedan tener un papel importante en el riesgo a enfermedades en otras poblaciones diferentes a la española. Un reciente estudio, encuentra una inserción/deleción de dos pares de bases (TG/del), donde encuentran que la deleción de este dinucleótido confiere protección frente a LES y EM; y que esta deleción reduce la estabilidad del ARNm (442). Posteriormente a la publicación de nuestro trabajo, analizamos esta nueva variante funcional en nuestra cohorte de pacientes de LES, no encontrando diferencias significativas en la distribución de este polimorfismo y la enfermedad (datos no publicados).

En conclusión, podemos decir que la variante del gen *CD24* A57V juega un papel importante en susceptibilidad a LES en la población española, y que se ha observado la misma tendencia de asociación en una cohorte alemana. Sin embargo, parece que existen resultados contrarios en distintas poblaciones, sugiriéndose un gradiente en la frecuencia de dicho polimorfismo entre las distintas poblaciones. De manera que sería interesante investigar en el papel de esta variante del gen *CD24* en otros grupos étnicamente distintos para confirmar esta hipótesis.

5.4. Genes seleccionados mediante GWAS

Como ya se mencionó anteriormente, en los últimos años los GWAS han tenido gran relevancia en los estudios genéticos de enfermedades complejas. Gracias a ellos diferentes genes previamente asociados a LES mediante estudios de ligamiento o mediante la estrategia de los genes candidatos han

sido confirmados, como son el caso de los genes *HLA*, *PTPN22*, *IRF5*, *FCGR3A*, *FCGR2A* ó *PDCD1* (Figura 5.11). Igualmente estos estudios han conseguido encontrar nuevos genes, algunos de ellos de función aún desconocida, asociados con susceptibilidad a LES y que rápidamente han empezado a replicarse en multitud de poblaciones diferentes. Dentro de estos nuevos genes de predisposición a la enfermedad hemos estudiado los siguientes: *BANK1*, *STAT4* y *TRAF1/C5*.

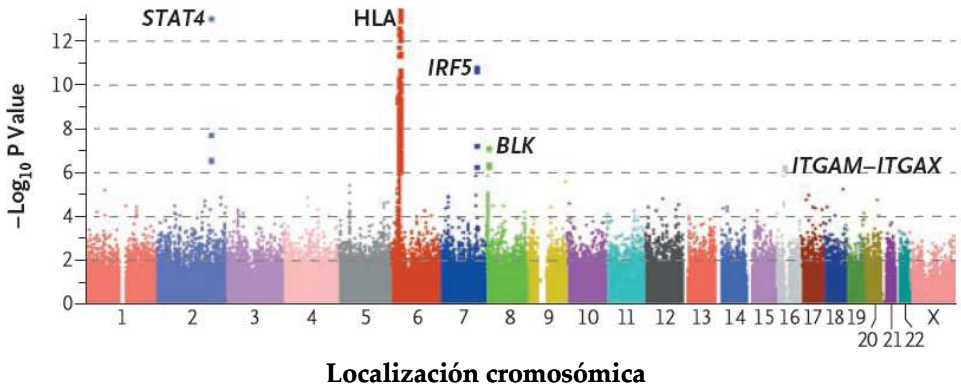


Figura 5.11. Representación de algunos de los loci más asociados a LES mediante los últimos GWAS.

5.4.1. *BANK1*

Hace solo unos meses los primeros GWAS en LES salieron a la luz. El grupo de la Doctora Marta Alarcón-Riquelme de la universidad de Uppsala (Suecia) ha sido uno de los primeros en lanzar resultados sobre ellos. Este grupo realizó un GWAS que incluia 85.042 SNPs en pacientes de LES y controles sanos de Suecia, encontrando diversos SNPs asociados a la enfermedad (189). Entre todos estos polimorfismos asociados, uno de ellos es una mutación no-sinónima (rs10516487) que da lugar a una sustitución de una arginina por una histidina en el aminoácido en posición 61 (R61H) en la proteína BANK1. Además otros cuatro polimorfismos en *BANK1* estaban

también asociados. Debido a que *BANK1* se expresa específicamente en células B y su potencial papel en la regulación de la activación de dichas células, decidimos estudiar en colaboración con el grupo de la Dra. Alarcón-Riquelme el gen *BANK1* en nuestra población de LES española y otras 4 poblaciones independientes procedentes de Suecia, Alemania, Italia y Argentina.

Para realizar una mejor cobertura de los SNPs de *BANK1* y asi poder refinar la señal de asociación, llevaron acabo el genotipado de 30 SNPs a lo largo de una región de 248-kb del gen (incluyendo el polimorfismo rs10516487 previamente asociado) en la población sueca (189). De todos ellos encontraron 9 polimorfismos asociados y todos ellos se encontraban situados entre los intrones 1 y 7. Posteriormente decidieron llevar a cabo un análisis exhaustivo de la expresión y estrutura de *BANK1*. En estos estudios observaron que *BANK1* se expresa principalmente en células B CD19+, y al clonar *BANK1* vieron que aparecian dos bandas principales. Se sabe que *BANK1* tiene dos isoformas completas (FL) alternativas, una de ellas contiene el exón 1A y otra el exón 1B (192). Después de secuenciar esta segunda banda, observaron una nueva isoforma desconocida hasta el momento, la cual se caracteriza por una deleción completa del exón 2 (isoforma Δ2). Tras analizar un gran número de pacientes y controles observaron que esta nueva isoforma aparecia siempre y que se presentaba también en chimpancé y bazo de ratones, sugiriendo por tanto que se trata de una forma consitutiva de splicing y que está altamente conservada en las distintas especies.

Posteriormente examinaron la región proxima al exón 2 en busqueda de un posible sitio que pudiera estar afectando a la señal de splicing, identificando un SNP, rs17266594, el cual se localiza en un sitio conocido como "branch point" o "punto de bifurcación", este punto está involucrado en el primer paso del splicing y parece que también es potencialmente importante

en el segundo paso (443). Descubrieron que exisitian diferencias en la expresión de las distintas isoformas de *BANK1* entre los distintos genotipos de este polimorfismo (189). De manera que los individuos homocigotos para el alelo T, que son los que presentan el sitio "branch point" clásico (YNYTG**A**YYN), presentan una expresión mayor de la isoforma completa, mientras que esta expresión era hasta un 40% menor en individuos homocigotos para el alelo menor C (Figura 5.12).

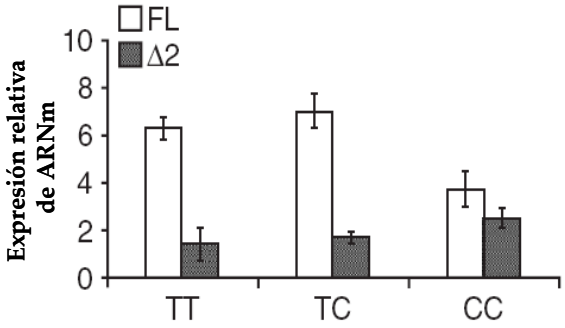


Figura 5.12. Expresión relativa de ARNm de las isoformas FL y Δ2, en función de los distintos genotipos del polimorfismo rs17266594.

Para determinar si otras variantes genéticas pueden estar contribuyendo a esta forma alternativa de splicing, secuenciaron la región promotora, el exón 1A, el exón 1B y el exón 2, encontrando una nueva variante no-sinónima (rs3733197) que da lugar a un cambio de una alanina por una treonina (A383) en el exón 7 y que codifica para un motivo rico en repeticiones de ankirina.

Cuando analizamos estos tres polimorfismos con un posible papel funcional del gen *BANK1* en nuestra población, observamos que el alelo G del SNP rs10516487 estaba aumentado significativamente en pacientes de LES (*P*= 0.0065, OR= 1.30, 95%CI 1.07-1.59) (189). Resultados similares fueron encontrados con el polimorfismo rs17266594, donde el alelo T estaba

aumentado en pacientes de LES comparado con controles sanos (P= 0.01, OR= 1.29, 95%CI 1.06-1.56). Posteriormente al realizar el análisis haplotípico observamos que estas dos variantes presentaban un alto grado de LD (D´=0.95, r^2= 0.90). Sin embargo, no encontramos asociación en la distribución alélica y genotípica del polimorfismo rs3733197 entre pacientes de LES y controles sanos. Al ampliar el estudio en otras 4 poblaciones caucásicas observamos que en todas ellas se replicaba la asociación encontrada para los SNPs rs10516487 y rs17266594, y que la variante rs3733197 estaba asociada a LES en argentinos, alemanes e italianos (189). De manera que se decidió llevar a cabo un meta-análisis incluyendo estas cinco poblaciones de LES, comprendiendo un total de 3971 individuos. Para ello realizamos un test de Mantel-Haenszel, encontrando una fuerte asociación en las tres variantes estudiadas (pool OR= 1.38, *P*= 3.74 x 10^{-10} para rs10516487; pool OR= 1.42, *P*= 4.74 x 10^{-11} para rs17266594 y pool OR= 1.23, *P*= 4.67 x 10^{-5} para rs3733197). El análisis haplotípico, demuestra que el haplotipo formado por los tres alelos de susceptibilidad (TGG) es de riesgo, mientras que existen dos haplotipos de protección (CAA y CAG), lo cual desmuestra que esta tercera variante rs3733197 no parece estar ejerciendo un efecto importante en la predisposición a la enfermedad. Además observamos que ninguno de los tres polimorfismos es independiente de los otros, como demuestra el grado de LD entre ellos.

Como se comentó previamente en la introducción, BANK1 es una proteína adaptadora de células B (190, 191), que presenta dos isoformas completas de 785 y 755 aminoácidos, de manera que solo difieren en 30 aminoácidos en la región N-terminal. El canal de calcio IP3R se asocia al dominio N-terminal de BANK1 (codificado por el exon 2), mientras que la PTK Lyn interacciona con la porción C-terminal (192) (Figura 5.13). El polimorfismo rs10516487 se situa en el exón 2, dentro de la región esencial de unión al IP3R,

especulándose que la variante R61, la cual está altamente protonada bajo condiciones de pH fisiológico, podría alterar potencialmente la afinidad de BANK1 por IP3R, favoreciéndose así la unión entre ambos. Por otro lado el polimorfismo rs17266594 quizás este afectando a la eficiencia relativa del splicing, de las isoformas FL y Δ2 de *BANK1*. Mutaciones que afectan la timidina de la secuencia consenso del "branch point" y alteran la eficiencia de splicing, ya han sido previamente descritas (444). Esta mayor eficiencia de splicing junto con una mayor unión al IP3R dada por el alelo R61, podría dar lugar a una proteína más activa en los individuos que llevan este haplotipo de riesgo. Por el contrario, ya que la isoforma Δ2 pierde el exón 2 que está codificado por el dominio de unión a IP3R, es posible que esta isoforma funcione como un dominante negativo atenuando la señalización mediada por BANK1. El alelo de protección C de rs17266594 afecta a la isoforma Δ2, de manera que cuando esta presente dicho alelo protectivo, BANK1 pierde afinidad de unión a IP3R y disminuye la señalización mediada por BANK1. Este efecto negativo de BANK1 dado por la isoforma Δ2, puede explicar en parte el resultado negativo de inhibición por parte de BANK1 de la activación de células B que se encontró en los modelos knockout de ratón (194)

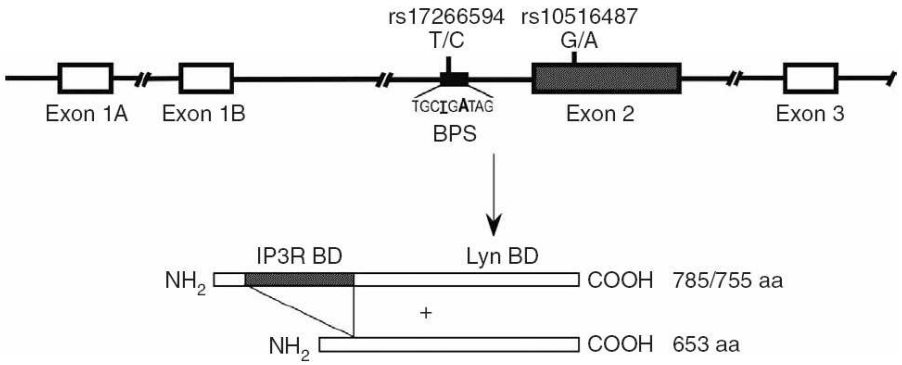


Figura 5.13. Estructura esquemática del extremo 5´de *BANK1*.

Debido a que todos estos datos han sido publicados muy recientemente, hasta el momento esta poco estudiado el papel de estos polimorfismos de *BANK1*, en otras EAI. Sin embargo, en nuestro laboratorio ya hemos estudiado dichas variantes en AR encontrando solo una débil asociación de dichos polimorfismos con la AR (Orozco y col. datos en prensa).

En conclusión podemos decir que estas tres variantes genéticas del gen *BANK1*, individualmente o en haplotipos, confieren susceptibilidad a LES en poblaciones caucásicas. Sin embargo, futuros estudios en otras poblaciones de razas diferentes principalmente, son necesarios para poder establecer el papel de *BANK1* como marcador general de LES.

5.4.2. *STAT4*

Recientemente, se ha encontrado una fuerte asociación genética entre el SNP rs7574865 del gen *STAT4* con AR y LES (197). Dicha asociación ha sido replicada en distintas poblaciones y EAI (445-451), y en dos estudios independientes de GWAS se ha encontrado a dicho gen *STAT4* asociado a LES (452, 453). Además se ha encontrado que la variante que confiere mayor riesgo a la enfermedad (rs7574865) está más fuertemente asociada en individuos con LES caracterizados por la presencia de autoanticuerpos anti-ADNdc, nefritis y una edad de diagnóstico menor de 30 años (450).

Estudios replicativos y de un denso genotipado de genes en diferentes poblaciones es requerido para definir si hay más de una señal independiente que pudiera ser importante en susceptibilidad y si la asociación genética es además aplicable a todas la etnias y barreras geográficas. Por ello, el objeto de nuestro estudio fue revisar la asociación genética de *STAT4* usando poblaciones independientes y un gran número de SNPs diana para definir si

solo la variante rs7574865 contribuye a la susceptibilidad en *STAT4* (Abelson y col. en prensa). Para ello realizamos un mapeo fino de la región que abarca los genes *STAT1* y *STAT4* en nuestra cohorte de paciente de LES y controles españoles. En este estudio analizamos 29 SNPs diana seleccionados de la base de datos HapMap y donde se incluyó el SNPs previamente asociado con AR y LES, rs7574865 (197) (Figura 5.14). Tras el análisis, observamos que las asociaciones más fuertes con LES eran con las variantes génicas rs3821236 (P= $7.07x10^{-8}$), rs7574865 (P= $9.37x10^{-6}$), rs3024866 (P= $3.83x10^{-7}$) y rs1467199 (P= $7x10^{-5}$). Con objeto de ampliar el estudio de asociación y de incrementar el poder estadístico de nuestro análisis, el grupo de la Dra. Alarcón-Riquelme realizó el genotipado de estos SNPs en 5 poblaciones independientes procedentes de Italia, Alemania, Argentina y México (una cohorte adulta y otra infantil). Mientras que el polimorfismo rs7574865 aparecía asociado en todas las poblaciones estudiadas, salvo en alemanes donde la asociación estaba en el borde de la significancia estadística (P= 0.05); todos los demás SNPs estaban asociados en las demás poblaciones a excepción de la variante rs1467199 cuya asociación solo se replicó en la población argentina. El porque de estas diferencias entre poblaciones no parece deberse a estratificaciones en la población o a una falta de poder estadístico debido a un pequeño tamaño de muestra. La razón de estas diferencias podría ser la presencia de varios haplotipos de riesgo independientes involucrados en la susceptibilidad a la enfermedad, donde algunos tengan un efecto mayor en algunas poblaciones comparadas con otras, como es el caso de los genes *HLA-DRB1*. Al realizar un análisis de independencia observamos que los polimorfismos rs3821236 y rs7574865 tenían un efecto genético independiente dentro del gen *STAT4*, proveyendo cada uno de estos SNPs el mismo riesgo predictivo para LES.

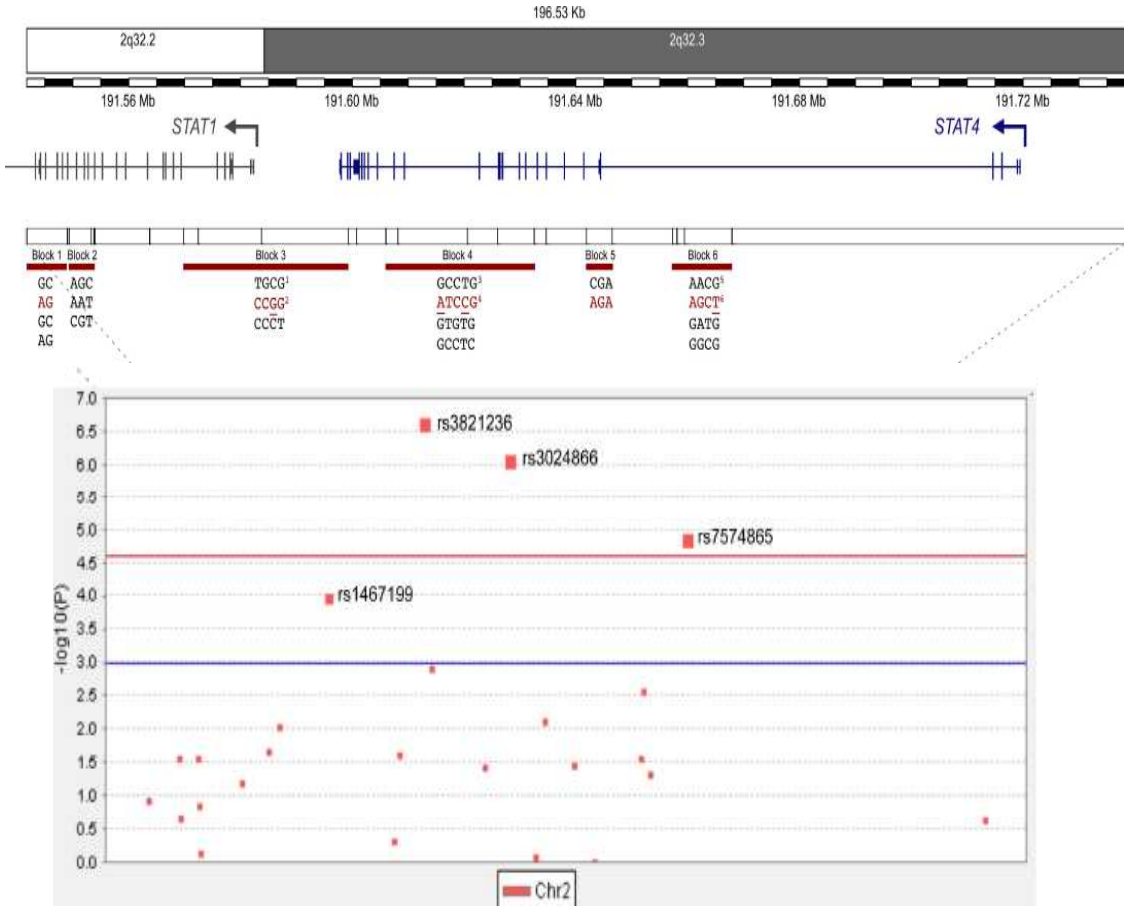


Figura 5.14. Análisis de 29 SNPs diana de la región 2q33.2 en AR.

Posteriormente se analizó la posible interacción génica (epiptasis) entre los polimorfismos del gen *IRF5* asociados con LES y estas variantes de *STAT4*, observándose que no existe una interacción significativa entre ellos. Si bien ambos genes tienen efectos independientes en el riesgo al LES, existe también un efecto aditivo entre los SNPs de ambos genes, particularmente con el rs7574865 y rs2070197, para incrementar el riesgo de padecer LES. El próximo paso sería determinar si alguna de estas variantes de *STAT4* asociadas a la enfermedad tiene algún efecto funcional. Para ello analizamos los niveles de expresión de *STAT4* en células mononucleares de sangre periférica (PBMCs),

encontrando una correlación entre dichos niveles y los tres polimorfismos más asociados, rs7574865, rs3024866 y rs3821236.

Aunque aún queda mucho por hacer para discernir el papel de *STAT4* en LES, nuestros resultados apuntan a que el gen *STAT4* tiene un papel importante en susceptibilidad a la enfermedad, un papel que parece variar entre las diferentes poblaciones derivando en dos variantes de riesgo diferentes e independientes, cuya significancia funcional aún necesita ser estudiada.

5.4.3. *TRAF1/C5*

Recientemente mediante un GWAS se ha identificado una nueva región de asociación a AR conocida como *TRAF1/C5* (454), la cual se encuentra situada en el cromosoma 9 en la región 9q33.2. En este estudio después de analizar un gran número de SNPs en dos amplias poblaciones independientes de AR positivas para anticuerpos antipéptido citrulinados (CCP), encontraron que el SNP del cromosoma 9 más asociado con susceptibilidad a la AR (rs3761847) estaba en LD con dos genes relevantes en inflamación crónica como son el *TRAF1* y el *C5*. Esta asociación con AR se ha confirmado más recientemente en un segundo GWAS de poblaciones independientes (455), así como en un estudio de mapeo fino de genes candidatos de susceptibilidad a la enfermedad (456) y en estudios replicativos en distintas poblaciones (451, 457). Esta región también se ha encontrado asociada con susceptibilidad a AIJ y los distintos subtipos de la misma, sugiriéndose así que estas variantes son un factor de riesgo de artritis inflamatoria en general. Sin embargo, esta asociación no se ha replicado ni en pacientes de AR y ni de LES en una población Colombiana (447) Teniendo en cuenta la importancia de estos dos genes *TRAF1* y *C5* en inmunidad y en respuesta inflamatoria, así como el

hecho de que diversas EAI poseen un componente genético común; decidimos estudiar en colaboración con el grupo del Dr. Rene E.M. Toes del centro médico universitario de Leiden (Holanda), el posible papel de dichos genes en diversas EAI y dos diferentes poblaciones caucásicas.

En un estudio previo, el grupo del Dr. Toes observó una fuerte asociación entre dichos genes y susceptibilidad a AR en tres poblaciones independientes procedentes de Holanda, Suecia y América (456); lo cual confirmaba los resultados del GWAS comentado anteriormente (454). La variante más fuertemente asociada en este estudio rs3761847, presentaba un alto grado de LD con la variante rs10818488, la cual es la variante más asociada en el estudio del Dr. Toes junto con el SNP rs2416808.

En nuestro estudio analizamos el SNP más informativo, rs10818488, en muestras de pacientes con DT1, enfermedad celíaca, ES y LES en dos poblaciones independientes procedentes de Holanda y España. Al realizar el análisis encontramos que el alelo A de dicho polimorfismo estaba aumentado en pacientes de DT1 (P= 0.027, OR= 1.14 95%CI 1.02-1.28) y de LES (P= 0.016, OR= 1.16 95%CI 1.03-1.31). Dicha asociación se replicó en una población independiente de Creta tanto en DT1 como en LES. Sin embargo, no se encontró ninguna señal de asociación con ES y enfermedad celíaca en ninguna de las poblaciones estudiadas.

A pesar de que el polimorfismo rs10818488 o alguno de sus *proxy* aún no se ha probado que sean una variante causal dentro del locus *TRAF1*/*C5*, este presenta la asociación mas fuerte en esta región con la AR. Además en nuestro trabajo se deja clara la evidencia de que esta asociación es reproducible en otras EAI como la DT1 y el LES en poblaciones caucásicas. Contribuyendo estos resultados a apoyar la hipótesis de que esta región quizás sea una vía compartida en estas tres enfermedades. Sin embargo no se puede descartar la

posibilidad de que otros alelos adicionales en este locus estén también involucrados. Interesantemente esta asociación encontrada en la región *TRAF1*/*C5*, ha sido recientemente publicada en dos GWAS en LES (111, 452), así como en AR y DT1 (458).

En resumen podemos concluir que en nuestro trabajo confirmamos la asociación del locus *TRAF1*/*C5* con DT1 y LES, lo cual en combinación con el mayor riesgo previamente encontrado que confiere dicha región a AR y AIJ, indica que esta región está involucrada probablemente en un mecanismo común esencial en varias EAI. Sin embargo, a pesar de la evidencia del papel de este locus en EAI, son necesarias nuevas secuenciaciones, mapeo fino y estudios funcionales en dicha región para identificar el/los alelo/s causales y como este alelo podría afectar a la función de *TRAF1* y/o *C5* dando lugar a la enfermedad.

6. CONCLUSIONS

1. The inflammatory pathway candidate genes: *TLR2*, *TLR4*, *TLR5*, *TLR7*, *NFKB* and *FCRL3*, showed no evidence of association with SLE, suggesting a minor role of these genes in SLE susceptibility in our population.

2. The genetic variants studied in the *IL12* gene family: *IL12B*, *IL12RB1*, *IL23A* and *IL23R* seem not play a relevant role in the genetic of SLE in our population.

3. The functional variants *MIF*-173C and the *MIF*-173-$CATT_7$ haplotype, which have been associated with a higher production of MIF protein, confer susceptibility to SLE in our population. These findings together with the data found in other autoimmune diseases, suggest that *MIF* gene is a common genetic marker in autoimmunity.

4. We have found a new functional variant within the *IL18* gene promoter region that seems to have a very important role in IL-18 expression, in addition to be associated with susceptibility to SLE in the Spanish population.

5. The *MICA* and *BTNL2* genes, localized near to the *HLA* genes in the chromosomic region 6p21, are not independent associated with susceptibility to SLE in our population.

6. The *PTPN22* R620W gene polymorphism is strongly associated with SLE in our population. Our work has contributed to the establishment of the *PTPN22* gene as a common predisposition genetic marker to autoimmune diseases in Caucasian populations.

7. The no-synonymous *CD24* A57V polymorphism has been found associated for the first time with susceptibility to SLE in the Spanish population. However, further studies in other populations are needed to confirm this association.

8. Our work has contributed to the establishment of *BANK1* gene as a new susceptibility gene to SLE.

9. We have replicated the previously association found between the *STAT4* gene and SLE in several populations. In addition, we performed a fine mapping of this gene, contributing to a better understanding of the contribution of *STAT4* to SLE predisposition.

10. We report for the first time a role of the *TRAF1/C5* locus in SLE predisposition in two independent cohorts, indicating, together with previous results by different groups, that *TRAF1/C5* may be considered as a candidate locus for autoimmunity.

7. PERSPECTIVAS

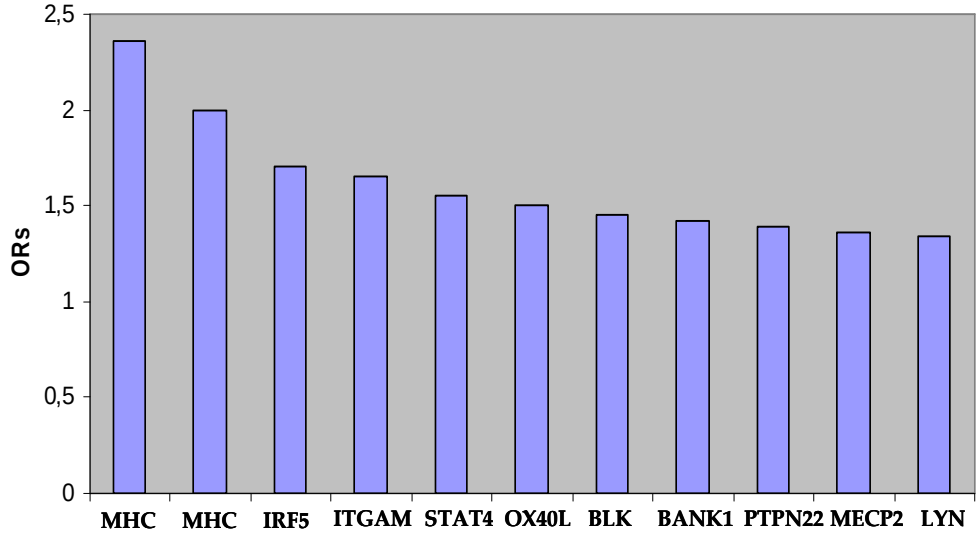
En menos de 20 años hemos sido testigos de grandes avances en el campo de la genética humana, lo cual nos ha llevado a un mayor conocimiento de las bases genéticas que predisponen al LES. Sin embargo, la gran parte de los genes que contribuyen a la enfermedad están aún por descubrir. Algunos de estos genes han sido estudiados en este trabajo y como resultado se ha podido establecer que los genes *MIF*, *IL18*, *PTPN22*, *CD24*, *BANK1*, *STAT4* y *TRAF1/C5* son marcadores genéticos de susceptibilidad al LES. Mientras que otros genes como los *TLR2*, *TLR4*, *TLR5*, *TLR7*, *NFκB*, *FCRL3*, *IL12B*, *IL12RB1*, *IL23A*, *IL23R MICA* y *BTNL2* no parecen estar contribuyendo al riesgo genético de la enfermedad. A diferencia de otras áreas de investigación, en el campo de la epidemiología genética los resultados "negativos" de no asociación poseen un alto valor divulgativo, ya que ayudan a descartar posibles genes que *a priori* parecen tener un papel importante en la enfermedad.

Las aproximaciones basadas en estudios de asociación para identificar las variaciones genéticas causales son más directas que los análisis de ligamiento y además son más útiles para detectar factores de riesgo individuales que confieren solo un riesgo moderado. Inicialmente, los estudios genéticos de asociación han estado limitados ya que analizaban cohortes relativamente pequeñas y un bajo número de genes. Desafortunadamente, este tipo de aproximaciones han dado lugar a un gran número de falsos positivos.

La progesión revolucionaria que ha experimentado el campo de la genética de enfermedades complejas en los últimos años, ha sido posible gracias a los recientes avances tecnológicos que permiten analizar de forma rápida y efectiva miles de SNPs. Además, los avances informáticos han permitido el análisis del gran volumen de datos generado en estos estudios de asociación del genoma completo (GWAS). Estas técnicas de genotipado son cada vez más comunes, y los resultados obtenidos con ellas han dado alguna

indicación de que variaciones a lo largo del genoma contribuyen al desarrollo de las enfermedades complejas como el LES. En contraste con los típicos estudios de rastreamiento del genoma (WGS) que analizaban un número pequeño (300-5.000) de marcadores genéticos, los GWAS permiten analizar más de 500.000 SNPs. Para catalogar los distintos SNPs y la correlación entre ellos (conocido como haplotipos) con objeto de facilitar los estudios genéticos en enfermedades humanas, el proyecto Internacional HapMap ha catalogado los patrones haplotípicos a través del genoma humano analizando más de tres millones de SNPs y ha creado una base de datos pública con dicha información (www.hapmap.org).

El año 2007 será recordado como el año cuando los GWAS fuerón usados extensamente por primera vez en el estudio de las enfermedades complejas. Durante estos dos últimos años numerosos GWAS en diversas enfermedades autoinmunes e inflamatorias como la AR o la EC han salido a la luz. Aunque los primeros GWAS en LES se han hecho esperar un poco más, a principios de 2008 se publicaron los primeros trabajos en los cuales se confirmó que la región HLA es el factor de riesgo más importante en susceptibilidad al LES y han revelado que varios genes previamente desconocidos (*C8orf13-BLK*, *ITGAM-ITGAX*, *PXK*, *KIAA1542*, *BANK1* y *TNFAIP3*) contribuyen a la susceptibilidad del LES (111, 189, 452, 453, 459) (Figura 7.1).



Figua 7.1.

Aunque estos nuevos estudios de GWA están descubriendo nuevas regiones y genes de susceptibilidad al LES y a numerosas enfermedades autoinmunes el gran incoveniente de estos estudios es la aparición de falsos-positivos. Un método inicial para reducir el riesgo de estos errores tipo I ha sido aplicar un criterio de significancia más riguroso. La presencia de estos errores hace imprescindible la replicación en estudios caso-control independientes. La necesidad de replicación, junto con los avances técnicos, ha aumentado el desarrollo de colaboraciones internacionales entre centros académicos (SLEGEN).

Otro riesgo en este tipo de estudios es que variantes modestas no sean detectadas, tal como se observa en la asociación encontrada con *TNFSF4*, un gene identificado por estudios de asociación en familias (460), el cual no se ha encontrado en los recientes GWAS realizados en LES. Del mismo modo, el gen *BANK1* y *TNFAIP3*, identificados mediante GWAS (189, 453) no se ha encontrado en otros dos GWAS que empleaban chip de 500K SNPs (111, 452). Esto es debido a que los chips actuales de entre 300-550Kb solo cubren entre el

80-90% de las variantes, dejando al resto fuera del análisis. Un incremento en el número de observaciones (los nuevos chips de ~1.000.000 de SNPs están ya disponibles) quizás ayuden a encontrar las variantes escondidas.

La última meta en estudios de asociación genética es la identificación de rutas biológicas que ayuden a crear nuevas terapias y a dilucidar los mecanismos fisiopatológicos de las enfermedades (Figura 7.2). Para llegar a este fin se requieren tres pasos principales. Primero, las asociaciones observadas mediante GWAS necesitan ser verificadas mediantes estudios replicativos en poblaciones independientes. Segundo, los locus cercanos a las variantes genéticas identificadas necesitan ser meticulosamente genotipadas para determinar cual SNP o haplotipo revela la asociación más fuerte y que quizás represente la variante genética causal. Tercero, las consecuencias funcionales de portar la variante de riesgo tienen que ser determinada mediante estudios de genética funcional. Hasta el momento, el segundo paso es alcanzable, aunque este requiere tiempo, material, y fuentes de financiación, mientras que el tercero, determinar la funcionalidad de una variante genética es difícil, tal como se ha visto con la asociación encontrada entre LES y la variante genética encontrada en la región ITGAM (459).

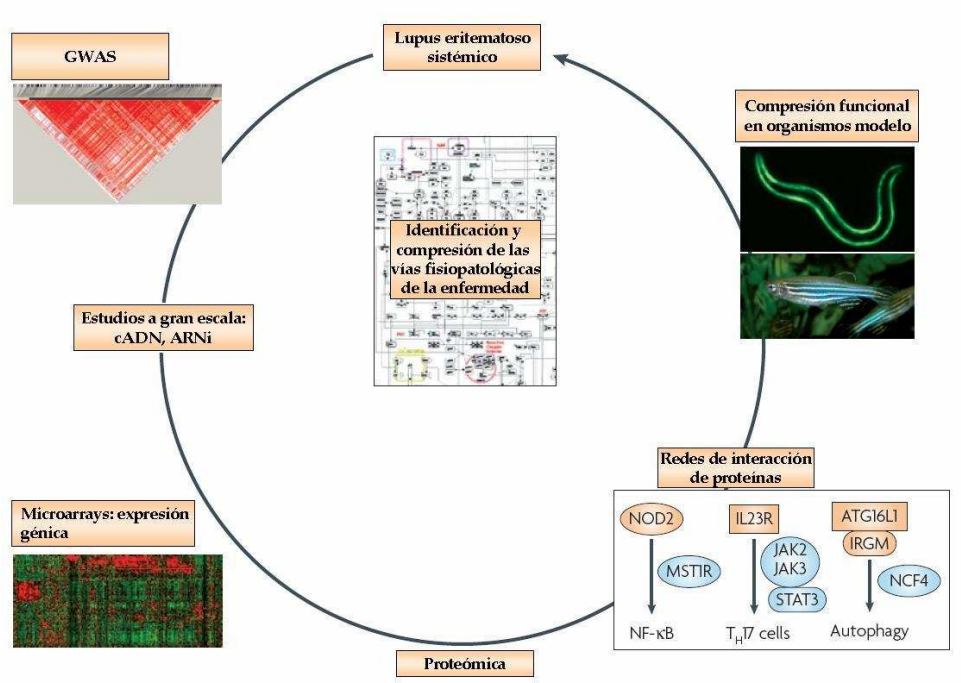


Figura 7.2. Esquema de trabajo desde los genes candidatos hasta el estudio de las vías de señalización implicadas en la enfermedad específica.

Aunque los GWAS han constituido un gran avance en la búsqueda de los genes de susceptibilidad al LES, la contribución de otras formas de variación genética aparte de los SNPs, como la variación estructural, sobretodo las variaciones en el número de copias (CNV), está aún por explorar (461). Hay regiones en el genoma que aún no están bien cubiertas en HapMap debido en parte a la poca información en algunas secuencias, y también a la presencia de CNVs y segmentos duplicados (462, 463). Esas regiones contienen segmentos de ADN, con un tamaño que oscila entre kilobases y megabases, las cuales pueden estar delecionadas en algunos individuos, pero duplicados, quizás algunas veces, en otros. Las implicaciones de estos CNV en la variación fenotípica es aún incierta, pero algunas de estas variantes están claramente asociadas con enfermedades. En el caso del LES se ha encontrado un CNV en el gen *FCGRB3*, relacionado con la expresión de la proteína, asociado con

susceptibilidad a la enfermedad (464) (Figura 7.3). Recientemente, se ha encontrado otra región de CNV asociada a LES, observándose que un variable número de copias del componente del complemento 4 (*C4A* y *C4B*) está asociado con susceptibilidad al LES (465) (Figura 7.3).

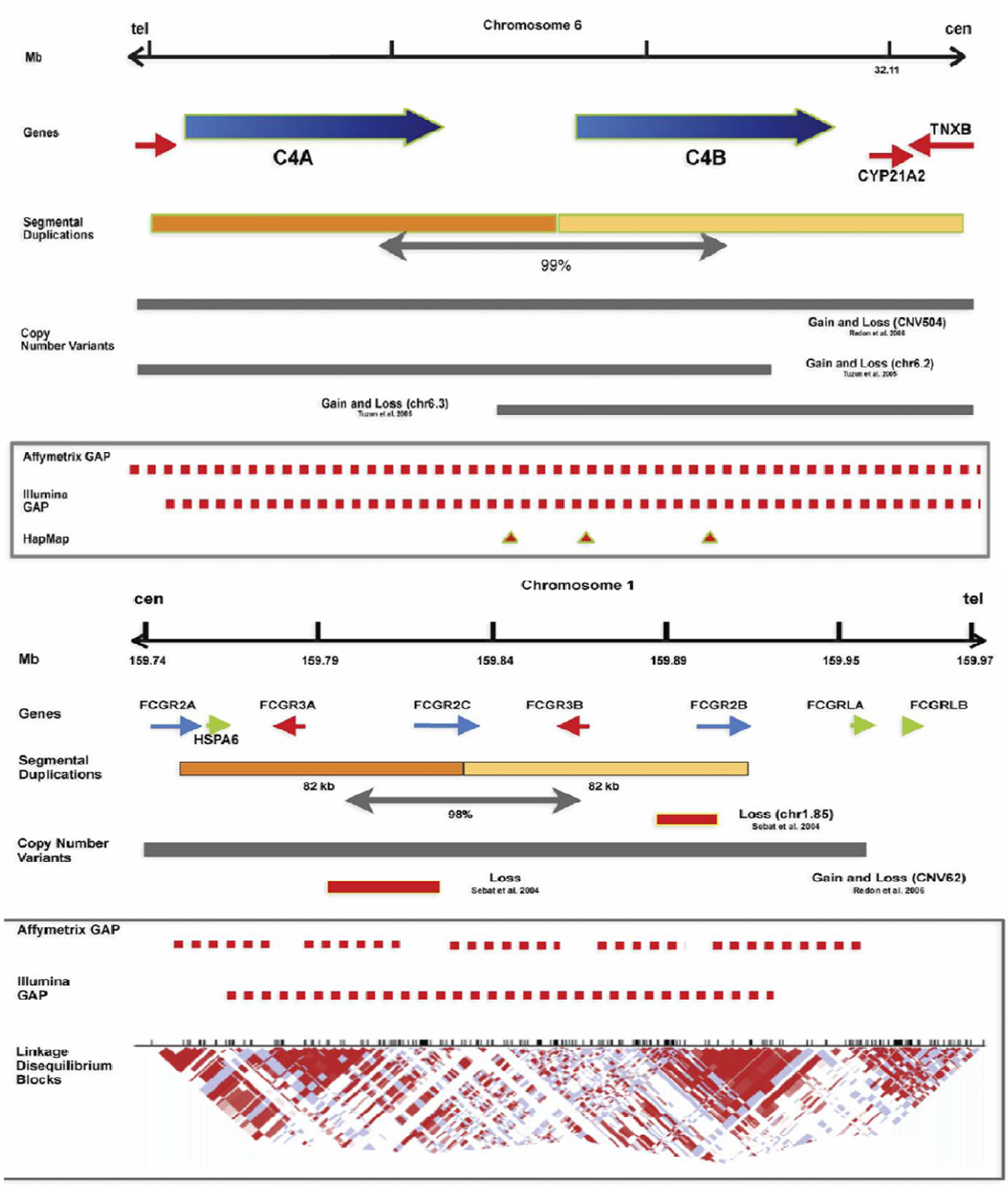


Figura 7.3. Representación esquemática de dos regiones genómicas que contienen CNVs asociadas a LES.

Otras nuevas variantes cada vez más de moda dentro del campo de la genética de enfermedades, son los microARNs (miARN). Los miARN son una nueva clase de ARNcs reguladores, que funcionan como represores endógenos en la translación de genes a proteínas en animales a través de la unión de estos a sitios diana presentes en la región 3´ de los ARNm. La presencia de polimorfismos o mutaciones en dichos miARN, sobretodo en los sitios de unión a estos miARN, pueden afectar a su regulación (466) y probablemente puedan afectar a la susceptiblidad a diversas enfermedades (467).

Otras fuentes novedosas a la espera de abrirse camino en este campo son estudios epipstático que estudien la relación entre genes y gen-ambiente, variaciones que afectan a los patrones de metilación (epigenética), así como el estudio de mutaciones raras.

Con la integración de todas estas aproximaciones se espera que en un futuro próximo se pueda determinar la arquitectura genética del LES, lo cual sería de gran ayuda para el mejor conocimiento de las bases moleculares de la enfermedad, el establecimiento de nuevas estrategias de diagnóstico y la identificación de nuevas dianas terapéuticas.

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9. TRABAJOS ADICIONALES

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