Tesis doctoral

Bases genéticas de la artritis reumatoide: búsqueda de nuevos marcadores genéticos de susceptibilidad.

Memoria presentada por la licenciada Gisela Orozco Cebada para optar al grado de Doctor por la Universidad de Granada.

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Para mis padres, Francisco y Ana María
y mis hermanas, Alicia y Ana Eva.
Para Roberto.

ÍNDICE

ABREVIATURAS

ACR	Colegio Americano de Reumatología (American College of Rheumatology)
AIA	Artritis inducida por antígeno
AIJ	Artritis idiopática juvenil
AINEs o NSAIDs	Drogas antiinflamatorias no esteroideas (nonsteroideal aniinflammatory drugs)
Anti-CCP	Anticuerpos frente a péptidos cíclicos citrulinados
AR	Artritis reumatoide
ASP	Parejas de hermanos enfermos (affected sibling pairs)
BTNL2	Butyrophilin-like 2
CIA	Artritis inducida por colágeno
CIITA	Trans-activador de clase II
COX2	Ciclooxigenasa 2
CTLA-4	Antígeno de linfocitos T citotóxicos 4
CU	Colitis ulcerosa
DC	Células dendríticas
DMARDs	Fármacos antirreumáticos modificadores del curso de la enfermedad (Disease-modifying anti-rheumatic drugs)
EA	Espondilitis anquilosante
EAT	Enfermedad autoinmune tiroidea
EC	Epítopo compartido
EM	Esclerosis múltiple
FcR	Receptores Fc
FCRL	Fc receptor-like
FGFs	Factores de crecimiento de fibroblasto (fibroblast growth factors)
FOXP3	Forkhead box P3
FR	Factor reumatoide
GWAS	Estudios de asociación del genoma completo (genome-wide association studies)
HLA	Antígeno leucocitario humano (Human leukocyte antigen)
IFNγ	Interferón γ
Ig	Inmunoglobulina
IL	Interleuquina
iNOS	Óxido nítrico sintasa inducible
IRF	Factor de regulación de interferón

Jak	Kinasa Janus
LD	Desequilibrio de ligamiento (linkage disequilibrium)
LES	Lupus eritematoso sistémico
LTβ	Linfotoxina β
MHC	Complejo principal de histocompatibilidad (major histocompatibility complex)
MHC2TA	Trans-activador del complejo principal de histocompatibilidad de clase II
MIF	Factor inhibidor de la migración de macrófagos
MMPs	Metaloproteinasas de la matriz
NF-κB	Factor nuclear κB
OPG	Osteoprotegerina
OR	Odds ratio
PAMPs	Patrones moleculares asociados a patógenos (pathogen-associated molecular patterns)
PTPs	Proteín tirosín fosfatasas
PTPN22	Proteín tirosín fosfatasa no receptor 22
RANKL	Receptor activador del ligando de NF-κB
RAP	Modelo de protección de la AR (RA-protection)
RASFs	Células sinoviales tipo fibroblasto (RA synovial fibroblasts)
RUNX1	Factor de transcripción relacionado con runt 1 (Runt related transcription factor 1)
SLC22A4	Familia de transportadores de soluto 22, miembro 4
STAT	Transductor de señal y activador de la transcripción
SUMO	Modificadores pequeños similares a ubiquitina
TCR	Receptor de la célula T
TGFβ	Factor de crecimiento transformador-β (transforming growth factor β)
T_H1	Células T colaboradoras tipo 1
TLRs	Receptores tipo toll (toll like receptors)
TNF-α	Factor de necrosis tumoral alfa
T_{Reg}	Células T reguladoras
VEGF	Factor de crecimiento vascular endotelial (vascular endothelial growth factor)
WGS	Rastreo sistemático del genoma (whole genome scan)

1. RESUMEN

La artritis reumatoide es una enfermedad autoinmune, inflamatoria y crónica. Afecta principalmente a las articulaciones de todo el organismo, pero tiene un amplio rango de manifestaciones extra-articulares. Es una de las enfermedades autoinmunes más frecuentes, afectando al 1% de la población mundial aproximadamente.

Aunque no se conocen por completo las causas que la provocan, se piensa que en su aparición y desarrollo intervienen factores genéticos, ambientales y eventos al azar. La contribución de los factores genéticos supone un 60% de los factores desencadenantes de la enfermedad. La AR se caracteriza por ser una enfermedad compleja donde intervienen una gran cantidad de genes, contribuyendo cada uno de ellos de manera modesta al desarrollo de la enfermedad. Los genes más estudiados con respecto a la predisposición genética a padecer AR son los genes HLA. Concretamente, determinados alelos *HLA-DRB1*, llamados alelos del epítopo compartido son los que presentan una asociación más fuerte con AR. Hasta hace poco, no se habían identificado consistentemente otros genes que influyeran en la predisposición a AR, a pesar de que el HLA sólo es capaz de explicar el 30% del componente genético de la enfermedad. Gracias los rastreos sistemáticos del genoma, los estudios de asociación de genes candidatos y los recientes estudios de asociación de genoma completo esta situación está cambiando, con la identificación de nuevos marcadores de susceptibilidad a AR.

El objetivo general de esta tesis fue intentar contribuir al estudio de las bases genéticas de la AR, mediante estudios de asociación caso-control utilizando genes candidatos. Para ello, los genes a estudiar se seleccionaron en base a dos criterios: su localización cromosómica en regiones de ligamiento a AR, y su función dentro de rutas implicadas en los mecanismos etiopatogénicos de la AR. Se utilizaron varios grupos de pacientes de AR y controles sanos procedentes de distintas regiones de la geografía española (Granada, Sevilla, Lugo y Madrid), constituyendo una cohorte bien caracterizada y con suficiente tamaño muestral para realizar estudios de asociación con buen poder estadístico.

En un primer lugar nos interesamos en genes relacionados en procesos inflamatorios, ya que éste es un mecanismo fundamental en la iniciación de la enfermedad, que comienza con la inflamación del tejido sinovial de las articulaciones. Además, la AR se caracteriza por una situación de inflamación crónica, donde la permanente presencia de mediadores proinflamatorios provoca la destrucción de la articulación, el deterioro óseo local (erosiones) y generalizado (osteopenia), la angiogénesis que favorece la creación del pannus y la aparición de enfermedad cardiovascular, entre otros procesos patológicos.

Dentro de este grupo se seleccionaron los genes *TLR2* y *TLR4*, que son importantes receptores encargados de disparar la respuesta inflamatoria innata e iniciar la respuesta inmune adaptativa, mediante la activación de NF-κB.

También estudiamos genes relacionados con la respuesta inflamatoria mediada por NF-κB, que es un factor de transcripción fundamental, ya que coordina la expresión de numerosos genes proinflamatorios, como *NFKB1* y *SUMO4* . *NFKB1* codifica para la subunidad p50 del complejo NF-κB y *SUMO4* es un importante regulador de la actividad de éste, ya que inhibe su activación.

Los receptores de inmunoglobulinas FcγR median también en la inmunidad innata y adquirida, ya que inducen la expresión de varias moléculas efectoras que promueven un ambiente inflamatorio. Dentro de este grupo de genes estudiamos *FCRL3*, que se piensa que puede influir en el desarrollo de células B autorreactivas.

La región cromosómica 5q31 se caracteriza por contener numerosos genes implicados en rutas inflamatorias, como variadas citoquinas proinflamatorias, que regulan un amplio rango de procesos inflamatorios implicados en la patogénesis de la AR. Dentro de esta región estudiamos el gen *SLC22A4*, y un relacionado con él, *RUNX1*. Además, también investigamos el papel de varios genes de la familia de la IL-12, como *IL12B*, *IL12RB1* e *IL23R*.

Por último, dentro de la categoría de los genes implicados en inflamación, estudiamos el gen *MIF*, que codifica para una citoquina proinflamatoria que promueve la respuesta de tipo T_H1.

Además de la inflamación, la regulación de la respuesta de las células T es un mecanismo fundamental en la AR. *PTPN22* es un importante gen en autoinmunidad, ya que codifica para la PTP específica de linfocitos LYP, el cual inhibe la señalización del TCR tras su unión al complejo péptido-MHC. Tras ésta primera señal de activación, la célula T necesita de una segunda señal para activarse, la proporcionada por moléculas coestimuladoras. Ésta señal es inhibida por la molécula CTLA-4, cuyo gen fue también estudiado. Con relación a la ruta de coestimulación, otro gen que estudiamos fue *BTNL2*, que también inhibe la activación de las células T.

STAT4 es un importante mediador de la actividad de IL-12, que actúa promoviendo la proliferación de las células T_H1, e IL-23, que facilita la proliferación de las células T_H17. Ambos tipos celulares median los mecanismos efectores patológicos en AR.

Así como la regulación de la cascada de transducción de señales que se producen en la célula T tras el reconocimiento del antígeno por el TCR juega un papel fundamental en la AR, la regulación de la expresión moléculas HLA de clase II que presentarán el

antígeno a la célula T también parece ser importante. El gen *MHC2TA* es el encargado de regular esta expresión, y también fue examinado.

Por último, estudiamos el gen *FOXP3*, cuyo producto es crítico en el desarrollo de las células T_{REG}. La actividad de estas células es crucial en el mantenimiento de la tolerancia inmune y su alteración puede provocar la aparición de autoinmunidad.

Tras analizar todos estos genes, hemos encontrado que el alelo -173C del gen *MIF* está asociado a AR. Se ha visto que éste alelo produce mayor producción de MIF, lo que podría aumentar la inflamación, la apoptosis, la permeabilidad vascular y provocar una secreción anormal de otras citoquinas proinflamatorias, todo ello llevando a una situación favorable para la aparición de la enfermedad autoinmune.

Nuestros datos indicaron que el polimorfismo 1858C→T del gen *PTPN22* está asociado a AR. Esta asociación es de especial relevancia, puesto que ha sido replicada en todas las poblaciones caucásicas estudiadas. Desde hacía 30 años, con el descubrimiento de la asociación de los alelos *HLA-DRB1* del epítopo compartido, no se identificaba con tanta robustez un marcador genético de predisposición a AR.

Además, también encontramos que un polimorfismo del gen *STAT4* está asociado a AR.

2. INTRODUCCIÓN

2.1. Aspectos clínicos y epidemiológicos de la artritis reumatoide.

2.1.1. Características clínicas de la AR.

La artritis reumatoide (AR) es la artritis inflamatoria más común, y la más frecuente de las enfermedades crónicas autoimmunes sistémicas [1, 2]. Afecta fundamentalmente a las grandes y pequeñas articulaciones de todo el cuerpo de manera simétrica (Figura 2.1), donde se produce una inflamación del tejido sinovial (Figura 2.2).

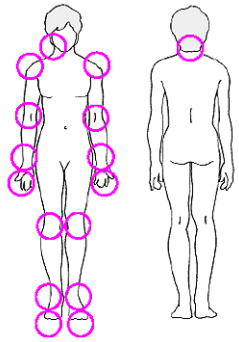


Figura 2.1. Distribución de las posibles articulaciones afectadas por la AR en el organismo.

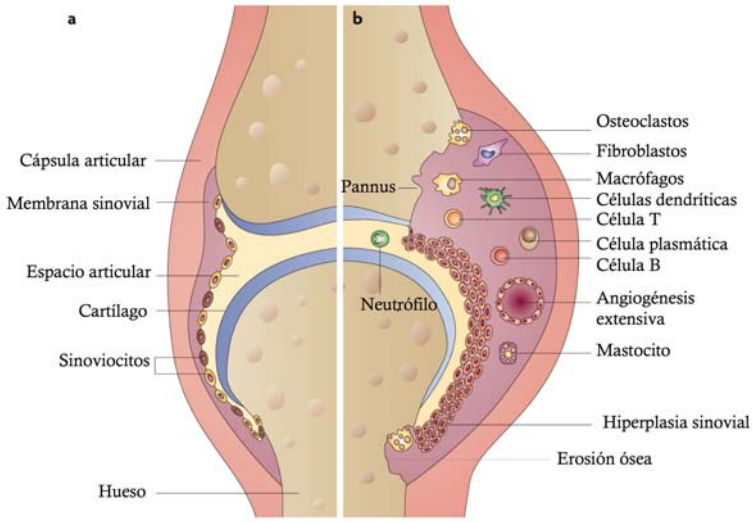


Figura 2.2. Esquema de una articulación normal (a) y una articulación afectada de AR (b). En la AR la membrana sinovial sufre una hiperplasia y se infiltra de manera crónica con células inflamatorias. Con la progresión de la enfermedad, la membrana sinovial se convierte en un tejido patológico denominado pannus, que migra sobre y dentro del cartílago articular y el hueso subyacente.

Es una enfermedad altamente heterogénea, siendo el rango de presentaciones clínicas muy amplio. Los síntomas predominantes son dolor, rigidez e inflamación de las articulaciones periféricas (Tabla 2.1) [3]. Las manifestaciones articulares pueden ser enfocadas en dos categorías: síntomas y signos reversibles relacionados con la inflamación sinovial, y daños estructurales irreversibles causados por la sinovitis crónica, que aparecen con la progresión de la enfermedad, tales como pérdida del cartílago, presencia de erosiones óseas y daño en estructuras blandas como ligamentos, tendones, músculos o meniscos (Figura 2.3).

Tabla 2.1. Características clínicas de la AR

Síntomas

- Inflamación de las articulaciones
- Dolor/rigidez
- Debilidad
- Deformidad
- Cansancio
- Malestar
- Fiebre
- Pérdida de peso
- Depresión

Características articulares

- Blandas al tacto
- Engrosamiento sinovial
- Efusión (al principio)
- Eritema (al principio)
- Dificultad de movimientos
- Anquilosis
- Subluxación

Distribución

- Simétrica
- Distal, más comúnmente que proximal
- PIF, MCF/MTP, muñeca/tobillo más comúnmente que codo/rodilla, hombro/cadera

PIF= articulación proximal interfalángica. MCF= articulación metacarpofalángica. MTP= articulación metatarsofalángica.

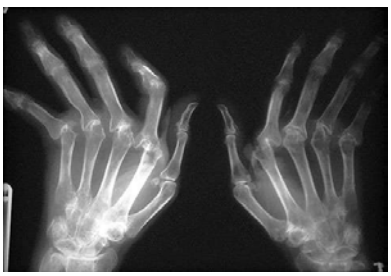


Figura 2.3. Radiografía mostrando las manos de un paciente afectado de AR avanzada, presentando deformidad y erosiones óseas.

El curso clínico de la enfermedad es extremadamente variable y se extiende desde una artritis suave auto-limitante a una inflamación multisistémica de progresión rápida. Así, existen múltiples manifestaciones extra-articulares que pueden aparecer a través del tiempo (Tabla 2.2).

Tabla 2.2. Manifestaciones extra-articulares de la AR

Sistema orgánico	**Manifestaciones**
Piel	Nódulos reumáticos, vasculitis
Ocular	Queratoconjuntivitis sicca, iritis, episcleritis
Oral	Inflamación salivar (síntomas sicca)
Respiratorio	Fibrosis pulmonar, efusión pleural, inflamación cricoaritenoide
Cardíaco	Inflamación pericardial, formación de nódulos valvulares, miocarditis
Neurológico	Mononeuritis, pinzamiento de nervios, inestabilidad cervical
Hepático	Concentración aumentada de aminotransferasa
Hematológico	Anemia, trombocitosis, leucocitosis, linfadenopatía
Vascular	Vasculitis

Se han definido factores pronósticos negativos de severidad para la destrucción articular progresiva, a través del análisis del curso clínico de los pacientes y de anormalidades de laboratorio y radiológicas (Tabla 2.3). Desafortunadamente, ninguno de ellos es lo suficientemente fiable para permitir una decisión terapéutica. Por ahora, la evaluación periódica de la actividad de la enfermedad y la respuesta a los fármacos es crucial para el tratamiento exitoso de la AR a largo plazo [3].

Tabla 2.3. Factores pronósticos de severidad

- Presencia de autoanticuerpos (FR y anti-CCP)
- Presencia de alelos del epítopo compartido
- Desarrollo temprano de erosiones articulares
- Número elevado de articulaciones afectadas
- Incapacidad temprana
- Edad de comienzo avanzada
- Presencia de manifestaciones extra-articulares

Los costes socioeconómicos de la AR son elevados. La mayoría de los pacientes requieren tratamiento continuo para retardar o parar la progresión de la enfermedad y controlar los brotes. Muchos además requieren cirugía, como reemplazo total de cadera o rodilla. Además de estos costes directos, la incapacidad para trabajar conlleva una

productividad reducida y retiro prematuro, y como resultado, sustanciales costes indirectos. El enfermo y su familia deben hacer frente al sentimiento de no contribuir a la sociedad combinado con unos roles sociales redefinidos, y los efectos del dolor, fatiga, baja autoestima y depresión.

Los pacientes de AR acusan un acortamiento medio en su esperanza de vida de entre 5 y 10 años. La severidad de la enfermedad, su actividad y la incapacidad están fuertemente asociadas a una mortalidad prematura en los pacientes de AR [4, 5].

2.1.2. Epidemiología de la AR.

La AR, como la mayoría de las enfermedades autoinmunes, se presenta con mayor frecuencia en mujeres que en hombres, en una proporción 3:1. Puede aparecer a cualquier edad, pero es más habitual entre los 40 y los 70 años. Así mismo, la incidencia aumenta con la edad [3].

La enfermedad se puede encontrar en cualquier región del mundo, afectando al 1% de la población global. Sin embargo, su prevalencia varía según el país y el área geográfica. Los países del sur de Europa tienen niveles medios de incidencia menores que los países del norte de Europa y América. Los países en vías de desarrollo tienen una incidencia aún menor, siendo la prevalencia notablemente baja en las zonas rurales de África. Por el contrario, es mucho más frecuente entre ciertas tribus nativas americanas [3, 6]. En concreto, en España cinco de cada mil adultos (0.5%) padecen AR [7].

En varios países de Europa y Norteamérica se ha percibido un descenso de la incidencia de la AR en los últimos años, pero esta tendencia es difícil de confirmar [6].

2.1.3. Diagnóstico de la AR.

Ninguno de los signos o síntomas de la AR son privativos de la enfermedad. Dado que ningún hallazgo es específico, su diagnóstico no se efectúa sólo por la presencia de datos positivos de la AR, sino por la caracterización y suma de diferentes referencias. El diagnóstico se realiza en base a los criterios definidos por el American College of Rheumatology (ACR, Colegio Americano de Reumatología) para la clasificación de la AR (Tabla 2.4) [8]. Se considera que un paciente sufre de AR cuando se cumplen al menos cuatro de estos siete criterios, los cuales aportan una sensibilidad del 91% y una especificidad del 89%.

Tabla 2.4. Criterios de la ACR para la clasificación de la AR

Criterio	Definición
1. Rigidez matutina	Rigidez matutina en y alrededor de las articulaciones, durante al menos una hora antes de la mejora máxima
2. Artritis en tres o más articulaciones	Al menos 3 articulaciones con hinchazón de tejido blando o fluido detectado por un médico. Las 14 posibles áreas son PIF, MCF, MPT, muñeca, codo, rodilla y tobillo, izquierdo o derecho
3. Artritis de manos	Inflamación en al menos un área en articulaciones de muñeca, PIF o MCF
4. Artritis simétrica	Implicación simultánea de las mismas áreas (como se ha definido en 2) en ambos lados del cuerpo (se acepta implicación bilateral de PIF, MCF o MPTs sin simetría absoluta)
5. Nódulos reumatoides	Nódulos subcutáneos, sobre prominencias óseas, en zonas de los extensores, o en regiones yuxtaarticulares
6. Factor reumatoide positivo	Niveles anormales de factor reumatoide en suero
7. Cambios radiológicos	Cambios radiológicos típicos de la AR en radiografías posteroanteriores de mano y muñeca, que deben incluir erosiones o descalcificaciones óseas inequívocas localizadas en o adyacentes a las articulaciones afectadas.

2.1.4. Tratamiento de la AR.

Debido a que la etiología de la AR no se conoce aún con suficiente profundidad, no existe un tratamiento específico para la enfermedad. Ningún tratamiento cura la AR. Por lo tanto, las metas terapéuticas consisten en la remisión de los síntomas que afectan a las articulaciones, el retorno a la funcionalidad completa y el mantenimiento de la remisión.

Los fármacos que se utilizan para tratar la AR se dividen en tres clases: drogas antiinflamatorias no esteroideas (AINEs o NSAIDs, nonsteroideal aniinflammatory drugs), corticosteroides y fármacos antirreumáticos modificadores del curso de la enfermedad (DMARDs, disease-modifying anti-rheumatic drugs) (sintéticos o biológicos) [9].

Los AINEs son un pilar importante en el tratamiento sintomático de la AR. A pesar de que no modifican la historia natural de la enfermedad, sí reducen el dolor y la inflamación articular, lo que permite mantener la capacidad funcional del paciente. Se deben usar junto a los DMARDs.

Aunque los corticosteroides son potentes supresores de la respuesta inflamatoria, su uso crea gran controversia entre los reumatólogos, debido a la toxicidad y efectos secundarios que presentan.

El tratamiento óptimo de la AR requiere una rápida y duradera supresión de la inflamación con DMARDs, que se definen como fármacos que retardan o paran la progresión de la enfermedad. Entre los DMADS sintéticos podemos encontrar metotrexate, sulfasalacina, oro intramuscular y antimaláricos, entre otros. Más recientemente se ha empezado a utilizar la terapia biológica para el tratamiento de la AR, como inhibidores del factor de necrosis tumoral alfa (TNF-α) (infliximab, etanercept y adalimumab), inhibidores de interleuquina 1 (IL-1) (anakinra), anti-CTLA4Ig y anti-CD20 (rituximab).

2.2. Fisiopatología y alteración de la respuesta inmune en la artritis reumatoide.

2.2.1 Cambios histológicos y mecanismos fisiopatológicos en la AR.

La inflamación del sinovio es central en la fisiopatología de la AR. Éste muestra una angiogénesis pronunciada, hiperplasia celular, influjo de leucocitos inflamatorios y cambios en la expresión de moléculas de adhesión a la superficie celular, proteinasas, inhibidores de proteinasas y abundantes citoquinas. La membrana sinovial se vuelve hiperplásica, con mayor cantidad de sinoviocitos tipo A (tipo macrófago) y tipo B (tipo fibroblasto). Hay una gran infiltración de células mononucleares, como células T, células B, macrófagos y células plasmáticas. La formación de este tejido sinovial localmente invasivo (pannus) es característica de la AR y está involucrado en las erosiones articulares que se encuentran en la enfermedad [3].

Angiogénesis. La angiogénesis (el proceso de formación de nuevos vasos sanguíneos) es altamente activa en la AR. Los vasos de nueva formación proporcionan oxígeno y nutrientes al sinovio hipertrófico, y proporciona el medio de reclutamiento de células inflamatorias a la articulación. Varios factores de crecimiento, citoquinas y quimioquinas influyen la angiogénesis en la AR, como los factores de crecimiento de fibroblasto (fibroblast growth factors, FGFs), TGFα y β, TNFα, angiopoyetina 1, y sobre todo, el

factor de crecimiento vascular endotelial (vascular endothelial growth factor, VEGF), que tiene un papel fundamental en el proceso angiogénico en la AR [10].

Aterosclerosis y enfermedad cardiovascular. La aterosclerosis es un proceso patológico que afecta a las paredes arteriales, y que conlleva el desarrollo de enfermedad cardiovascular. Se caracteriza por la acumulación de partículas lipídicas y células del sistema inmune en las regiones subendoteliales, lo que lleva a un estrechamiento del lumen arterial, y, tras la ruptura de la placa, a trombosis. Los pacientes de AR tienen un elevado riesgo de aterosclerosis temprana y desarrollo de enfermedad cardiovascular, debido la inflamación crónica y la desregulación del sistema inmune que caracteriza a la enfermedad [11].

Destrucción de la articulación. La AR está caracterizada por la presencia de la destrucción del cartílago de la articulación y del hueso adyacente. La destrucción de la matriz del cartílago resulta fundamentalmente de la acción de proteinasas del tejido conectivo liberadas por el tejido sinovial, condrocitos y el pannus. Además, se produce una destrucción local del hueso adyacente a la articulación, que conduce a una situación de osteoporosis y un mayor riesgo de fractura. Conjuntamente, se produce una osteopenia sistémica. Esta destrucción ósea se debe a la situación de inflamación persistente, ya que los mediadores inflamatorios aumentan la formación, actividad y supervivencia de los osteoclastos. En estos procesos parece ser fundamental el sistema receptor activador del ligando de NFκB (RANKL)/RANK/osteoprotegerina (OPG) [12-14].

2.2.2. La respuesta inmune en la AR

De manera general, la defensa inmune está mediada por dos sistemas complementarios, el sistema inmune innato y el sistema inmune adaptativo. La inmunidad innata está normalmente dirigida hacia respuestas inmediatas a amenazas que se encuentran comúnmente en el ambiente. Por el contrario, la inmunidad adaptativa se ocupa fundamentalmente del desarrollo a largo plazo de defensa y memoria hacia amenazas que nos podemos encontrar repetidamente. Ambos sistemas inmunes interactúan para formar un eficiente sistema global de defensa inmune (Figura 2.4). La patogénesis de la AR, y su base genética, debe ser entendida en este contexto [15].

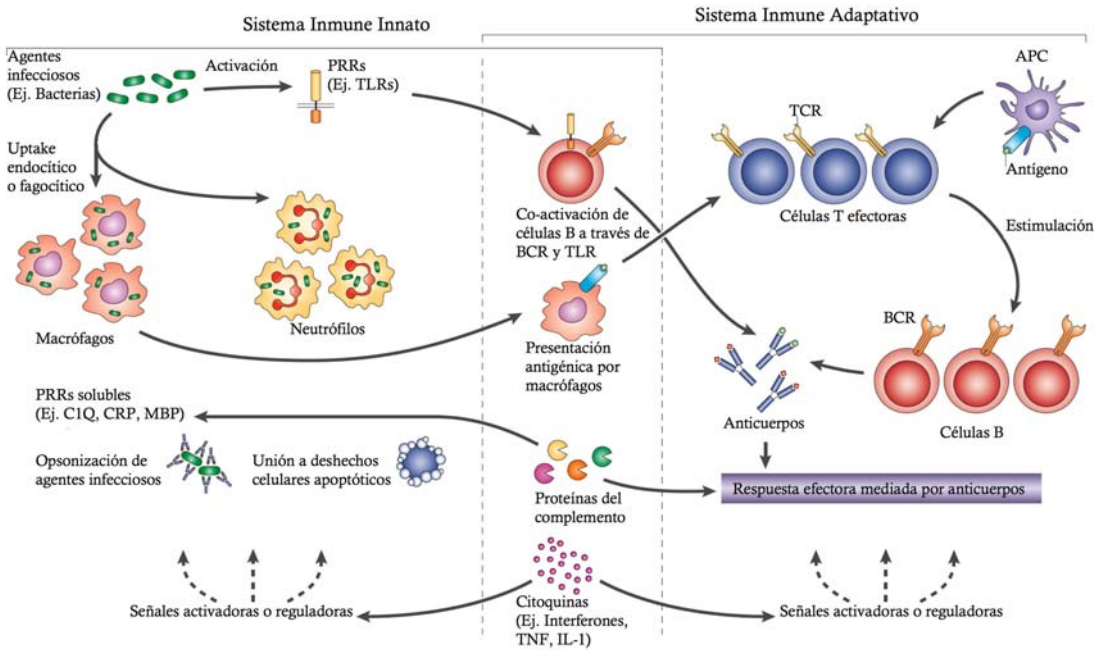


Figura 2.4. Representación esquemática de los sistemas inmunes innato y adaptativo y el solapamiento entre ambos.
Los mecanismos del sistema inmune innato implican, generalmente, respuestas inmediatas y no específicas frente a agentes infecciosos externos. Éstas incluyen funciones celulares como fagocitosis y endocitosis por parte de macrófagos y neutrófilos. Algunas de estas actividades dependen de receptores de reconocimiento de patrones (pattern-recognition receptors, PRRs), como receptores tipo toll (toll-like receptors, TLRs) y receptores tipo NOD, que reconocen patrones moleculares asociados a patógenos (pathogen-associated molecular patterns o PAMPs) presentes en gran cantidad de microorganismos. Además, varios PRRs solubles, tales como proteínas del complemento (C1Q) proteína de unión a manosa (mannose-binding protein, MBP) y reactantes de fase aguda, como la proteína C reactiva (C-reactive protein, CRP), participan en la inmunidad innata opsonizando microorganismos y uniéndose a deshechos celulares apoptóticos de manera no específica. Los mecanismos del sistema inmune adaptativo suponen la implicación de receptores que han sido seleccionados por su reactividad frente a antígenos específicos, como el receptor de la célula T (T-cell receptor, TCR) y receptores de inmunoglobulinas en las células B. El desarrollo completo de estas respuestas requiere la expansión y diferenciación de células respondedoras específicas, que establecen una memoria para la respuesta antigénica específica. Los sistemas inmunes innato y adaptativo están interrelacionados mediante mecanismos que no han sido totalmente identificados. Por ejemplo, antígenos que son fagocitados o endocitados de forma no específica por macrófagos son presentados a las células T, generando una respuesta de células T altamente específica. Además, la coestimulación de las células B a través de TLRs puede resultar en la producción de anticuerpos específicos frente a autoantígenos. Citoquinas como interferones, factor de necrosis tumoral (tumor necrosis factor, TNF) e IL-1 pueden estimular la actividad de ambas respuestas, innata y adaptativa. Las proteínas del complemento también median las repuestas efectoras inducidas por anticuerpos, y por lo tanto participan en los sistemas inmunes innato y adaptativo. APC: antigen presenting cell, célula presentadora de antígeno; BCR: B-cell receptor, receptor de la célula B.

2.2.2.1. Células T en la AR.

Las células T están implicadas en la patogénesis de la AR, como demuestran la asociación genética con alelos HLA de clase II y con el gen *PTPN22* (aspectos que serán

comentados más adelante), la detección de un elevado número de células T en el sinovio inflamado y el demostrado requerimiento de células T en varios modelos animales de artritis [16, 17]. Se ha detectado mejoría en pacientes tratados con la proteína de fusión CTLA4-immunoglobulin Fc, lo que apoya el papel de la coestimulación de las células T y la activación de células T efectoras en la AR [18].

Basándose mayoritariamente en estudios con modelos murinos, la AR ha sido clásicamente considerada una enfermedad mediada por células T colaboradoras tipo 1 (T_H1), y, por lo tanto, se pensaba que estaba dirigida por una población de células T productoras de citoquinas proinflamatorias y quemoquinas, como interferón γ (IFNγ), linfotoxina β (LTβ) y TNF [19]. Sin embargo, estudios en ratones sugieren un nuevo modelo que implica células T productoras de IL-17, una subpoblación recientemente caracterizada conocida como células T_H17, como efectores cruciales en la AR [20]. Sin embargo, no está claro si estos modelos murinos representan fielmente la enfermedad humana. Por lo tanto, se requieren más estudios para profundizar en el conocimiento del papel de las células T_H1 y T_H17 en la AR.

Además, también han sido detectadas células T reguladoras naturales (T_{Reg}) (células T reguladoras forkhead box P3 $(FOXP3)^+CD24^+CD25^+$) en el sinovio de pacientes con enfermedad activa y particularmente en fluido sinovial, que parecen tener alteraciones en su función reguladora [21, 22].

Las células T sinoviales pueden ser activadas mediante activación del TCR y rutas de coestimulación, y mediante estímulos provocados por TLRs o receptores de citoquinas (Figura 2.5). En particular, el entorno sinovial contiene IL-12, IL-23, IL-6 y factor de crecimiento transformador-β (transforming growth factor β, TGFβ), lo que promueve la diferenciación de células T_H1 y/o T_H17. Las células T activadas median funciones efectoras en la AR mediante la liberación de citoquinas proinflamatorias, que promueve la activación de leucocitos y células mesenquimales. Además, proporcionan colaboración a las células B, y, en el caso de células T efectoras $CD8^+$, tienen actividad citotóxica. También activan macrófagos, fibroblastos y células endoteliales a través de contacto celular directo [23].

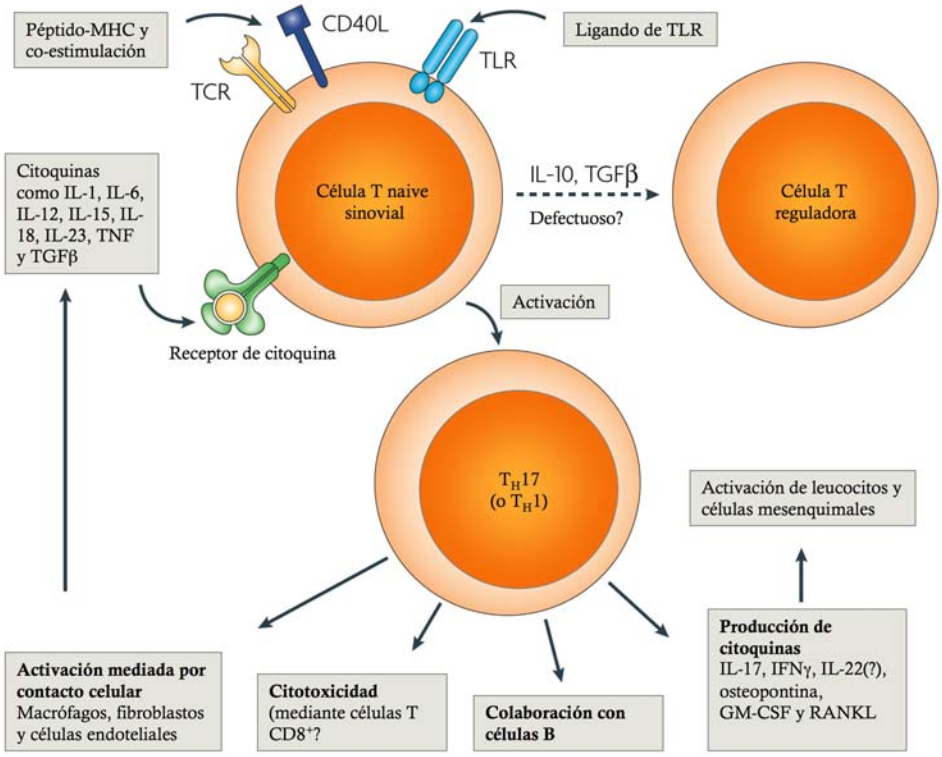


Figura 2.5. Mecanismos que provocan la activación de células T sinoviales en AR y sus mecanismos efectores. CD40L: CD40 ligando; GM-CSF: factor estimulador de colonias de granulocitos y macrófagos; RANKL: ligando del receptor activador del factor nuclear-κB.

2.2.2.2. Células B en la AR.

Se ha considerado tradicionalmente que las células B son unos de los efectores principales de la AR, ya que la anormalidad inmunológica más consistente en esta enfermedad es la presencia del factor reumatoide (FR) y los anticuerpos frente a péptidos cíclicos citrulinados (anti-CCP) , autoanticuerpos producidos por células B.

El sistema de autoanticuerpos del FR están dirigidos hacia la porción Fc de la inmunoglobulina (Ig) G y están presentes en el 70-90% de los pacientes de AR. Sin embargo, no es exclusivo de la AR y se encuentra en otras enfermedades, tales como el lupus eritematoso sistémico (LES), el síndrome de Sjögren, la tuberculosis, e incluso en personas sanas [24]. Recientemente se han descubierto los anti-CCP, que tienen mucha mayor especificidad (90-98%) para la AR [25]. Ambos autoanticuerpos (FR y anti-CCP) pueden estar presentes años antes de la aparición de los síntomas de la enfermedad [26, 27], y están asociados con la severidad de la misma [28, 29]. Aunque no se conoce por completo el papel que juegan los autoanticuerpos en los mecanismos patológicos de la AR, actualmente pueden ser considerados como biomarcadores de diagnóstico y severidad [30].

Los autoanticuerpos producidos por las células B forman complejos con los antígenos diana (Figura 2.6). Los inmunocomplejos resultantes pueden ser reconocidos por células que expresan receptores Fc (FcR) y activar el complemento. Éstos eventos provocan la implicación de leucocitos efectores que van a intervenir en procesos de inflamación crónica. También se produce un daño en distintos órganos, al depositarse los inmunocomplejos en ellos.

Además de la producción de autoanticuerpos, las células B tienen otros papeles en la patogénesis de la AR, como la presentación de autoantígenos a las células T, lo que provoca su activación, y la producción de citoquinas, como IFNγ, IL-4 o LTα/β [31, 32].

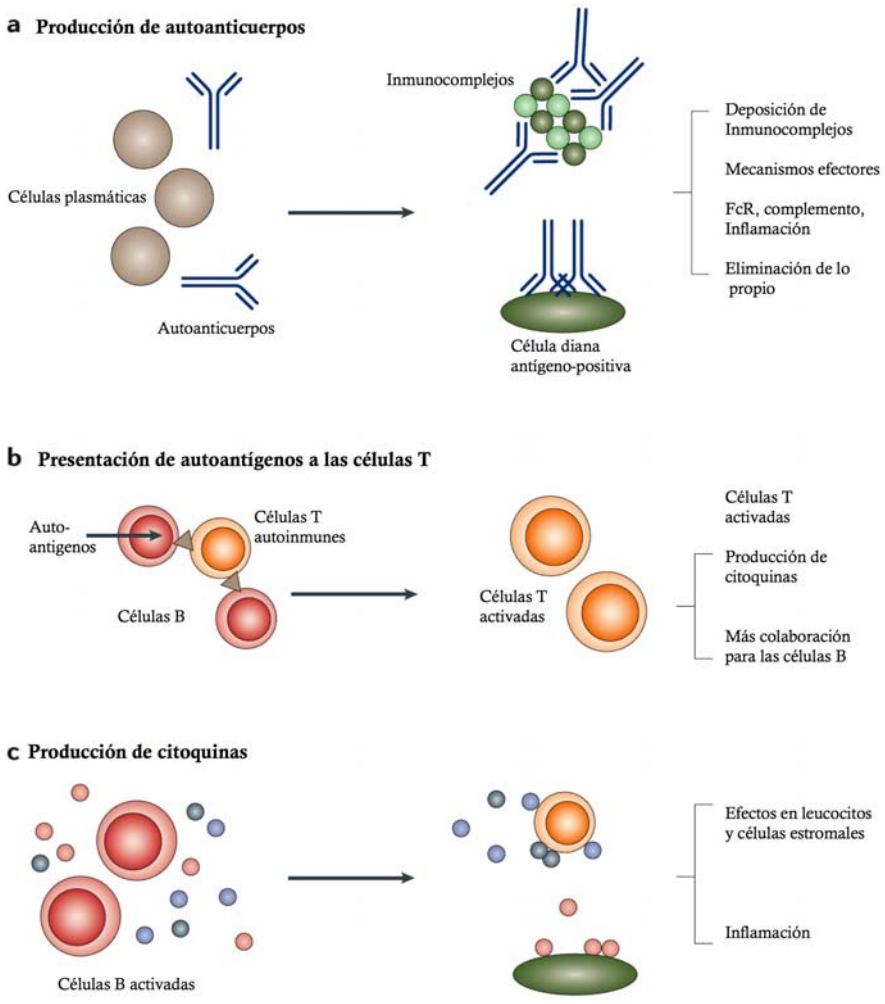


Figura 2.6. Mecanismos patológicos de la AR dirigidos por células B.

2.2.2.3 Otras células que intervienen en la patogénesis de la AR.

Macrófagos. Estas células parecen jugar un papel importante en la AR, ya que se encuentran en abundancia en la membrana sinovial y en la unión cartílago-pannus. Los macrófagos, además de actuar como células presentadoras del antígeno e iniciar una respuesta inmune, contribuyen notablemente a la inflamación y a la destrucción de la articulación en la AR, ya que secretan altas cantidades de citoquinas proinflamatorias y reguladoras, así como factores de crecimiento (IL-1, IL-6, IL-10, IL-15, IL-18, TNFα, GM-CSF), quemoquinas, quimioatractantes y metaloproteinasas de la matriz (MMPs), entre otras moléculas [33].

Neutrófilos. Recientes estudios han demostrado la importancia de los neutrófilos como mediadores de la inflamación tisular. Proteasas incluidas en gránulos y compuestos intermedios de oxígeno reactivo, que son importantes para la digestión intracelular durante la fagocitosis, son liberados por los neutrófilos durante la inflamación. En el ambiente extracelular, estas proteasas derivadas de neutrófilos pueden provocar daño tisular local, pero también regular la actividad de citoquinas, receptores de citoquinas y quemoquinas. Los mismos neutrófilos son capaces de producir mediadores inflamatorios como citoquinas, quimioquinas y complemento. También expresan receptores Fc, que pueden unirse a inmunocomplejos y probablemente transportarlos al compartimento extravascular [34].

Mastocitos. Estos tipos celulares parecen estar implicados en la interrelación entre el sistema inmune inntato y el sistema inmune adaptativo. Se ha demostrado que existe una acumulación de mastocitos en el tejido sinovial, los cuales expresan varias proteasas y citoquinas proinflamatorias [35, 36]

Células dendríticas. Las células dendríticas (DC) intervienen en la patogénesis de la AR fundamentalmente por su función como células presentadoras del antígeno. Son capaces de provocar respuestas autoinmunes MHC-restringidas y participar en la producción de autoanticuerpos. Además, pueden producir mediadores inmunes proinflamatorios y contribuir en complicaciones de la AR, como la aterosclerosis [37].

Fibroblastos sinoviales. En los últimos años se ha sugerido que células sinoviales tipo fibroblasto (RA synovial fibroblasts, RASFs) contribuyen de manera significativa a la perpetuación de la enfermedad, e incluso a su iniciación. Estos RASFs constituyen un tipo celular específico que distingue a la AR de cualquier otro tipo de artritis. Una vez

activadas, estas células producen una serie de citoquinas, quemoquinas y enzimas degradadoras de la matriz que median la interacción con células inflamatorias y endoteliales vecinas, y son responsables de la destrucción progresiva del cartílago articular y el hueso [38].

En la Figura 2.7 se resumen los procesos que regulan las interacciones sinoviales entre las distintas células del sistema inmune, así como sus principales funciones efectoras.

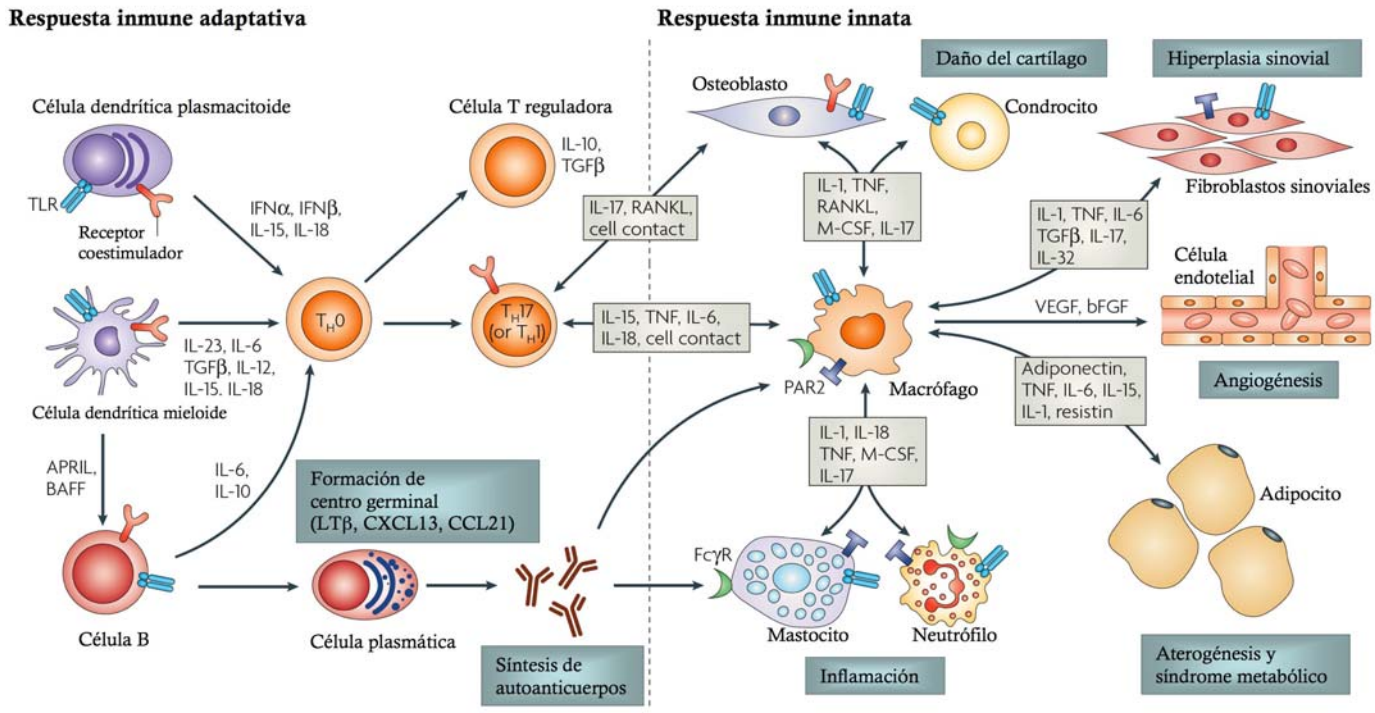


Figura 2.7. Esquema de los mecanismos patológicos que contribuyen al desarrollo de la AR.

2.3. Etiología de la artritis reumatoide.

La causa fundamental de la AR, dado su carácter autoinmune, es la pérdida de la tolerancia frente a lo propio. Los mecanismos mediante los que ocurre esta pérdida de tolerancia no se conocen, pero se cree que la AR es una enfermedad multifactorial compleja que surge como resultado de la combinación de causas genéticas, factores ambientales y eventos estocásticos, los cuales provocan una alteración de la respuesta inmune (Figura 2.8) [39].

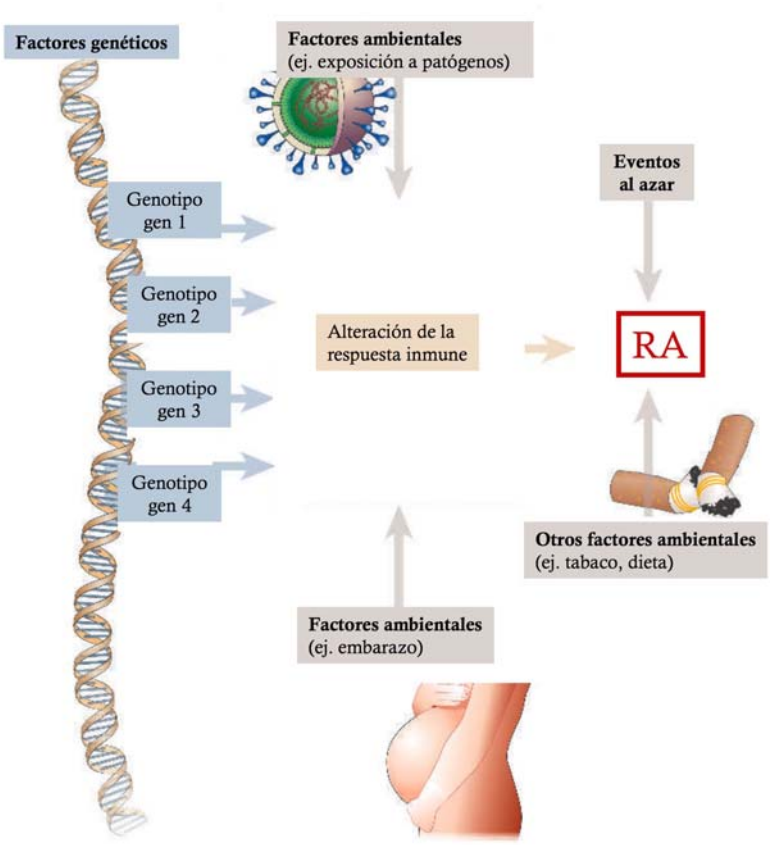


Figura 2.8. Factores que contribuyen al desencadenamiento de la AR.

Aún no se conocen con precisión los factores ambientales que contribuyen al desencadenamiento de la AR. Sin embargo, se ha sugerido que podrían influir las infecciones, el tabaco, el polvo de sílice, el aceite mineral, la dieta y factores psicológicos y hormonales, entre otros [40]. Curiosamente, de manera contraria a la creencia popular, no hay evidencias de que el clima influya en la AR [41].

2.3.1. Genética de la artritis reumatoide.

Aunque la etiología de la AR no se conoce aún en profundidad, se sabe que subyace un fuerte componente genético. Estudios realizados en familias [42] y en gemelos [43] así lo demuestran. Se ha estimado que el componente genético de la AR supone aproximadamente un 60% de los factores desencadenantes de la enfermedad [43, 44].

La presencia de agregación familiar fue la primera evidencia de susceptibilidad heredada a la AR. La prevalencia de la AR puede aumentar hasta un 12% en familiares directos de los pacientes, mientras que en la población general es del 1% aproximadamente [45]. La agregación familiar se cuantifica mediante el coeficiente λ_s o riesgo relativo en hermanos, definido como el cociente de la prevalencia de la enfermedad en hermanos de

pacientes de AR entre la prevalencia en la población general. El coeficiente λ_s en la AR varía, según el estudio, entre 3 y 15 [46]. Este riesgo relativo también se puede calcular para gemelos monocigóticos. En la AR este valor λ_{mz} es de aproximadamente 60 [47].

La AR es una enfermedad genética compleja, esto es, numerosos alelos contribuyen a la susceptibilidad a la enfermedad, cada uno de ellos con un riesgo modesto-bajo. La identificación de los factores genéticos que contribuyen al desarrollo de la AR es de gran relevancia, ya que podría establecer el marco para entender los mecanismos patogénicos de la enfermedad, en gran parte aún desconocidos. De ésta forma, se podrían establecer marcadores para el diagnóstico y el pronóstico de la AR, pudiendo proporcionar al paciente un tratamiento temprano que quizá induciría una evolución de la enfermedad más favorable y menos severa. Además, se podrían diseñar dianas terapéuticas mejores y más específicas para la AR, ya que hasta el momento, no existe un tratamiento específico para la enfermedad.

2.3.1.1. Papel de los genes HLA en la AR.

Las moléculas del complejo principal de histocompatibilidad (major histocompatibility complex, MHC) de clase II se expresan en la superficie de las células presentadoras del antígeno y constan de dos cadenas polipeptídicas unidas no covalentemente: las cadenas α y β (Figura 2.9).

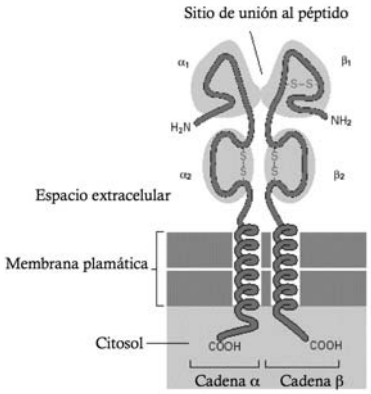


Figura 2.9. Estructura de la molécula HLA de clase II.

Estas moléculas se encargan de presentar péptidos a las células T $CD4^+$, con lo que intervienen en el mantenimiento de la tolerancia frente a lo propio y la inducción y regulación de la respuesta inmune adaptativa contra patógenos (Figura 2.10).

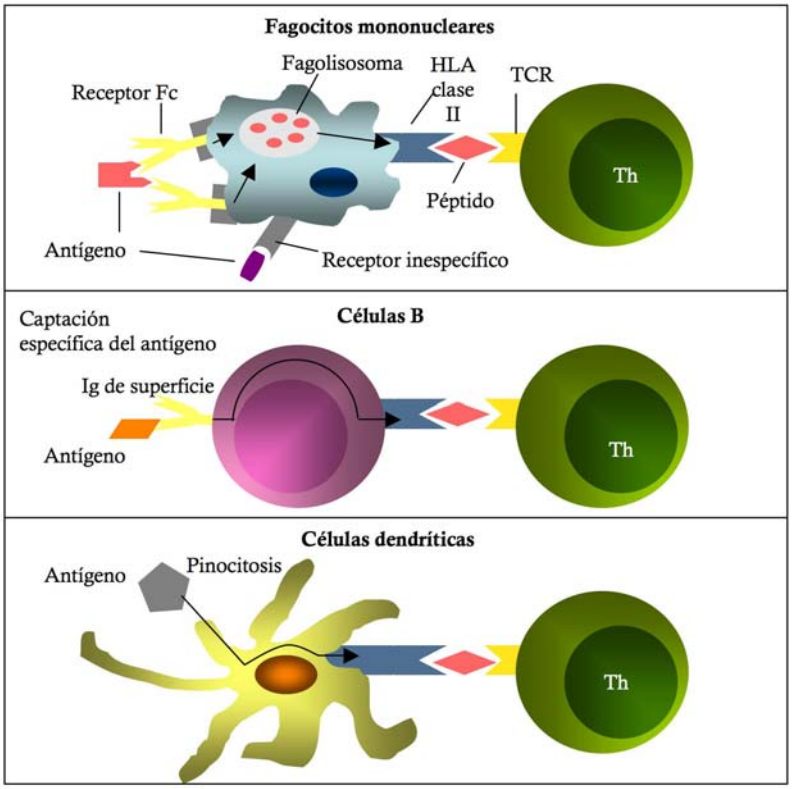


Figura 2.10. Mecanismos de presentación antigénica.

Las cadenas α y β de las moléculas MHC de clase II son codificadas por los genes *HLA* (human leukocyte antigen) de clase II: *HLA-DR*, *-DP* y *-DQ*. Estos genes se localizan en el locus MHC, situado en el cromosoma 6 (6p21.3), que constituye una región con una elevada densidad génica, conteniendo unos 220 genes, muchos de los cuales tienen funciones inmunoreguladoras (Figura 2.11).

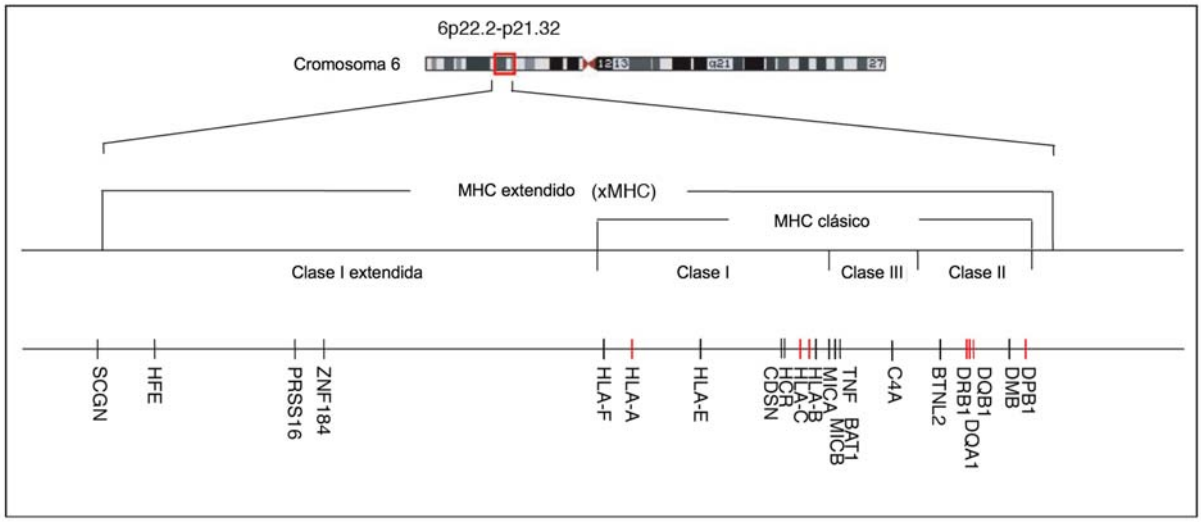


Figura 2.11. Mapa cromosómico del locus del complejo principal de histocompatibilidad.

Hace más de 30 años, Peter Stastny reparó en la alta frecuencia con que se presentaba el alelo *HLA-DRB1*04* en pacientes con AR [48]. Desde entonces, los genes HLA han sido los más estudiados y mejor caracterizados con respecto a la predisposición a AR, siendo determinados alelos *HLA-DRB1* los que presentan una asociación más fuerte con la enfermedad. Además, la asociación de los alelos *HLA-DRB1* con la AR es hasta la fecha la única sólidamente replicada en todos los grupos étnicos estudiados [49].

La hipótesis del epítopo compartido

Diversos estudios han demostrado que varios alelos *HLA-DRB1* (*0101, *0102, *0401, *0404, *0405, *0408, *0409, *0410 y *1001) están asociados a AR. El producto de estos alelos comparte una secuencia de 5 aminoácidos en la tercera región hipervariable de la molécula DRB1 (Q/R^{70} K/R RAA^{74}) (Tabla 2.5). Estos residuos son esenciales para uno de los bolsillos de unión al péptido (el bolsillo p4). Basándose en esta observación, Peter Gregersen y colaboradores formularon la hipótesis del epítopo compartido (EC o shared epitope, SE), que predice que estas moléculas DRB1 se unirían al el/los mismo/s péptido/s, provocando una respuesta inmune que resultaría en AR [50]. Sin embargo, tras 20 de investigación, aun no se conoce este péptido inductor de AR.

Tabla 2.5. Aminoácidos de la cadena β de las moléculas HLA de clase II codificadas por los alelos *HLA-DRB1* asociados a AR

HLA-DRB1	70	71	72	73	74
**0101*	Q	R	R	A	A
**0102*	Q	R	R	A	A
**0401*	Q	K	R	A	A
**0404*	Q	R	R	A	A
**0405*	Q	R	R	A	A
**0408*	Q	R	R	A	A
**0409*	Q	K	R	A	A
**0410*	Q	R	R	A	A
**1001*	R	R	R	A	A

Q: glutamina; R: arginina; K: lisina; A: alanina

El modelo RAP

Más recientemente, se ha sugerido otro modelo donde los locus *DQB1* y *DQA1* determinan la susceptibilidad a AR, mientras que determinados alelos *DRB1* protectores modularían este efecto [51]. En este modelo de protección de la AR (RA-protection, RAP), los alelos que predisponen a AR son *DQB1*0301*, **0302*, **0303*, **0304*, **0401* y **0402* combinados con *DQA1*0301* (o **0302*, pero no **0501*) y *DQB1*0501* combinado con *DQA1*0101* (o **0104*). Los alelos *DRB1* protectores *(*0103*, **0402*, **1102*, **1103*, **1301* y **1302*) se denominan DERAA positivos por presentar ese dominio aminoacídico común en la región HV3.

La hipótesis del epítopo compartido "citrulinado"

Recientemente se ha propuesto que los alelos *HLA-DRB1* EC positivos se asocian exclusivamente con un subgrupo de pacientes de AR anti-CCP seropositivos [52]. Basándose en este y otros estudios, se ha formulado un nuevo modelo en dos pasos para la patogénesis de la AR, que incorpora la hipótesis del epítopo compartido "citrulinado"[53]. En este modelo, el primer paso sería la inducción de los anti-CCP, donde la citrulinación de proteínas como resultado de la inflamación o de factores ambientales, iniciaría una respuesta de células T restringida a HLA de clase II sólo en individuos EC positivos. El segundo paso sería la expresión de antígenos citrulinados en la articulación inflamada que serían reconocidos por los anti-CCP [54].

A pesar de la relevancia de la región HLA en la predisposición genética a la AR, se ha estimado que este loci supone no más de un 1/3 de la contribución genética total [55, 56]. Por lo tanto, deben existir numerosos genes que influyen en la AR fuera de la región HLA.

Existen dos grandes aproximaciones para la identificación de los genes que confieren susceptibilidad a la AR: los estudios de ligamiento y los estudios de asociación.

2.3.1.2. Estudios de ligamiento

Los estudios de ligamiento tienen como objetivo identificar regiones cromosómicas que contienen genes que predisponen a la enfermedad, mediante la observación de polimorfismos en individuos relacionados (familias múltiples). La aproximación más extendida es la de estudiar parejas de hermanos enfermos (ASP, affected sibling pairs). Se espera que los parientes afectados muestren un exceso de haplotipos compartidos en las regiones donde se encuentre una variante causante de la enfermedad. Por lo tanto, es probable que en las regiones cromosómicas que se compartan con una frecuencia alta se encuentren genes de susceptibilidad.

Tras la identificación de regiones cromosómicas putativas de ligamiento, son necesarios estudios de mapeo fino y de asociación para poder identificar la/las variantes genéticas etiológicas y la identidad del gen responsable de la señal. Idealmente, se deberían realizar estudios replicativos en otras cohortes étnicamente distintas y estudios funcionales para poder llegar a dilucidar el papel real de estos locus en la patogénesis de la AR.

Varios estudios de ligamiento de rastreo sistemático del genoma (conocidos como whole genome scans, WGSs) se han llevado a cabo en AR, identificando numerosas

regiones putativas de ligamiento [57-65]. Los resultados de estos estudios se encuentran resumidos en la tabla 2.6 [66]. En la figura 2.12 se muestra un esquema de la localización cromosómica de los distintos locus de ligamiento para AR, donde se puede ver cómo algunas de éstas regiones se solapan entre los distintos estudios, lo que confirma su posible implicación en la AR. Sin embargo, con respecto a otras regiones existen discrepancias.

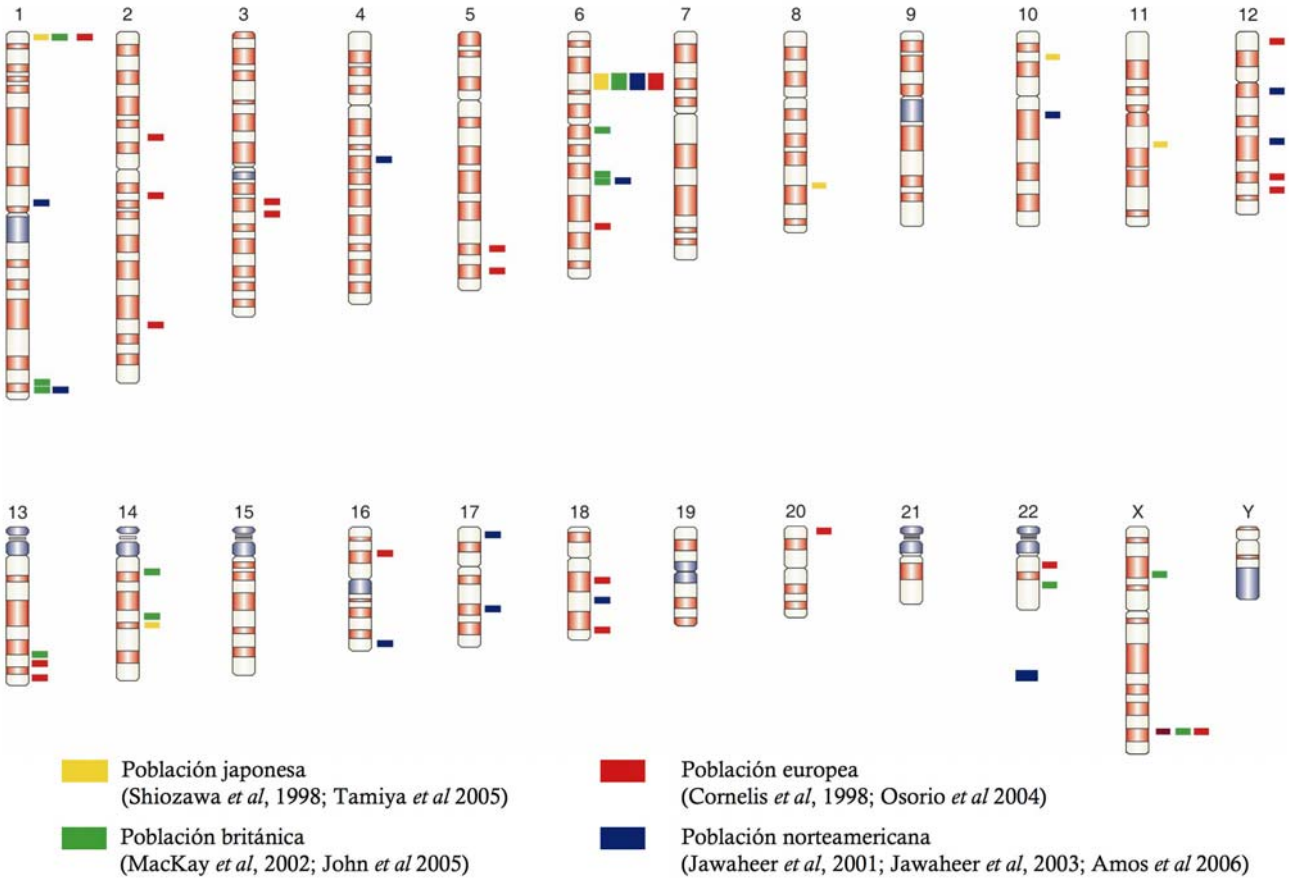


Figura 2.12. Localización cromosómica de las regiones de ligamiento a AR identificadas por los estudios de WGS.

En todos los estudios de este tipo, la señal más fuerte de ligamiento con AR se localiza en la ya conocida región HLA. Además, los WGSs sugieren otros locus de susceptibilidad a AR. Sin embargo, estas regiones putativas de ligamiento fuera de la región HLA no han sido replicadas sólidamente. La inconsistencia de estos resultados es debida en gran parte a que los estudios de WGS realizados hasta la fecha incluyen sólo unos pocos cientos de familias, por lo que el poder estadístico alcanzado no es suficiente para detectar los locus de susceptibilidad a AR, ya que individualmente contribuyen con un efecto modesto. Esta falta de poder estadístico, junto a la posibilidad de errores de tipo 1 (falsos positivos) y la heterogeneidad fenotípica podrían explicar la evidencia relativamente débil de ligamiento y la discrepancia en los resultados obtenidos en distintos estudios [67].

Tabla 2.6. Estudios de rastreo sistemático del genoma realizados en AR										
Autor, año	**Cornelis et al, 1998 [57]**	**Shiozawa et al, 1998[58]**	**Jawaheer et al, 2001 [59]**	**MacKay et al, 2002 [60]**	**Jawaheer et al, 2003 [61]**	**Eyre et al, 2004 [62]**	**Osorio et al, 2004 [63]**	**John et al, 2004 [64]**	**Tamiya et al, 2005 [68]**	**Amos et al, 2006 [65]**
Población de estudio	Europea	Japonesa	Norte-Americana	Británica	Norte-Americana	Británica	Europea	Británica	Japonesa	Norte-Americana
Nº familias	90	41	257	182	512	377	88	157	940 casos y controles	642
Nª ASP	98	45	301	208	333	425	105	217		1371
Tipo de marcador	Micro-satélites	Micro-satélites	Micro-satélites	Micro satélites	Micro-satélites	Micro-satélites	Micro-satélites	SNPs	Microsatélites + SNPs	SNPs
Nº marcadores totales	309 no-HLA c/12cM. 17 HLA c/47 cM	358 c/10.8 cM	379 c/10 cM	365 c/10cM	379 c/10cM	91*	1088 c/3.3 cM	11245 c/0.8 cM	27039 mic. c/100kb, 165 SNPs	5850
Ligamiento significativo LOD>3.6 $P<2\cdot10^{-5}$	6p21 (HLA)	1p36.31	6p21 (HLA)	6p21.3 (HLA-DRB1)	6p21.3 (HLA)	-	6p12.3-22.1 (HLA-DRB1)	6p21.31-22.1 (HLA-DRB1)	6p21.3	6p21
Ligamiento sugestivo LOD>2.2 $P<0.001$	3q13, 18q22-23	Xq27.1	-	6q16-21	-	-	20p13, 13q34	-	11q13.4, 10p13, 14q23.1	2q33, 11p12, 4q25, 5p12, 10q21
Evidencia nominal $P<0.05$	1p36, 2p13, 2q33-37, 5q32-33, 6q21-23, 12p13-q24, 13q32-qter, 16p12, 18q12, 22q11, Xq27	8q22.3-23.1	($P<0.005$) 1q43-44, 4q22, 12q21.2, 16q24.1, 17q22	1q42-44, 14q11-12, 14q22	($P<0.005$) 1p13, 1q43, 6q21, 10q21, 12q12, 17p13, 18q21	-	($P<0.01$) 18q21.32 1p36.21, 2q13, 5q34, 12q23.1, 3q21.1	6q11.1-12.3, 6q16.3-21, 13q22.3-31.1, 14q24.3-31.1, 21q22.1, Xp21.1	-	1q41-42, 7q31, 12p12, 16q12, 18q21, 20p13

* En regiones previamente identificadas por MacKay et al.
ASP: Affected sibling pairs, pares de hermanos afectados.

2.3.1.3. Estudios de asociación

Los estudios de asociación tienen como objetivo detectar si existe relación entre uno o varios polimorfismos genéticos y la enfermedad. Estos estudios pueden estar basados en poblaciones caso-control, en las que se analizan las posibles diferencias en la distribución de alelos entre el grupo de enfermos y el de individuos sanos. Además, aunque menos frecuentemente, se pueden emplear núcleos familiares, donde se incluyen individuos afectados por la enfermedad y se analiza si un alelo es transmitido preferentemente a los hijos afectos.

Los tests estadísticos basados en frecuencias alélicas en estudios caso-control tienen mayor poder para identificar alelos comunes que confieren un riesgo modesto a la enfermedad, que los basados en la segregación cromosómica en familias (estudios de ligamiento) [69]. Sin embargo, en los estudios caso-control se corre el riesgo de cometer errores de tipo 1 (falsos positivos) debido a la estructuración poblacional, sesgo que no existe en los estudios familiares.

Genes candidatos

El primer paso en el diseño de un estudio de asociación es la elección del gen candidato, para posteriormente analizar sus variantes genéticas con respecto a la asociación con la enfermedad. La selección de los genes candidatos puede tener una justificación posicional, así como funcional. La primera asume que los genes de susceptibilidad con mayor efecto están ubicadas en regiones de ligamiento previamente identificadas mediante WGSs. También se pueden tener en cuenta genes que se localicen en regiones genéticas humanas homólogas a aquellas asociadas a susceptibilidad en modelos animales de AR. En la aproximación funcional, genes involucrados en vías patofisiológicas importantes de la enfermedad (inflamación, activación de células T y B, angiogénesis, destrucción del cartílago y el hueso, apoptosis y muchas otras) serán los seleccionados para su estudio, así como genes implicados en enfermedades inflamatorias o autoinmunes relacionadas.

Desde el punto de vista de la elección de los marcadores genéticos a estudiar, podemos seleccionar polimorfismos que tienen un papel causal en la enfermedad (asociación directa) o polimorfismos que se encuentren en desequilibrio de ligamiento (linkage disequilibrium, LD) con la variante causal (asociación indirecta). En el último caso es posible seleccionar un conjunto de polimorfismos (tagSNPs) que sea capaz de proporcionar toda la información haplotípica del gen en cuestión.

Numerosos estudios genéticos de asociación se han llevado a cabo en AR hasta la fecha, los cuales han producido resultados alentadores pero aparentemente inconsistentes

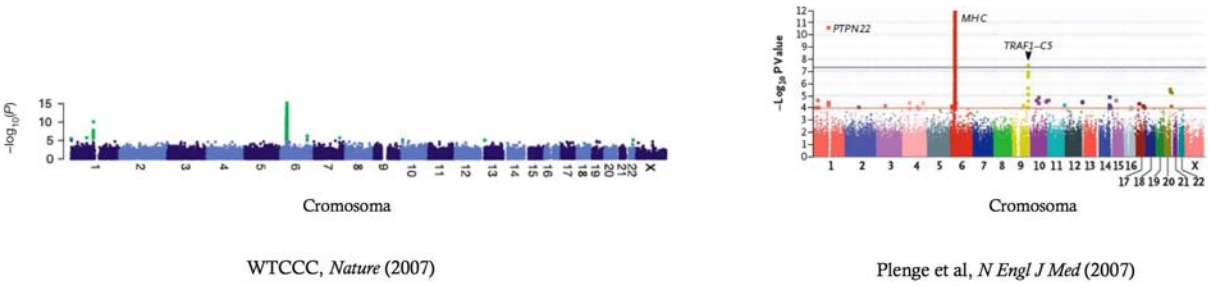
[70]. La mayoría de las asociaciones publicadas no han sido replicadas en poblaciones diferentes a las que se reportan. Estas discrepancias podrían deberse, al menos en parte, a la heterogeneidad genética. Diferentes alelos, incluso diferentes genes, pueden producir susceptibilidad a la enfermedad en 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. Por lo tanto, los estudios replicativos con elevado tamaño muestral son imprescindibles para poder determinar de manera robusta qué genes influyen en la susceptibilidad a la AR.

A pesar de las limitaciones de los estudios de asociación de genes candidatos, gracias a éstos ha sido posible en parte la identificación de la asociación con la AR más robusta y reproducible aparte de los genes HLA hasta le fecha, la que viene dada por el polimorfismo 1858C/T del gen *PTPN22* [71-73], y otros genes como *MIF* que serán comentados extensamente en la discusión de esta tesis.

Estudios de asociación del genoma completo

Hasta ahora, los estudios de asociación han estado limitados a pequeñas regiones del genoma que contienen genes candidatos o que han sido identificadas en estudios familiares de ligamiento. Gracias a tres recientes avances, ha sido posible el desarrollo de los estudios de asociación del genoma completo (genome-wide association studies, GWAS): primero, el impulso del conocimiento de los patrones de la variación génica humana con el proyecto internacional HapMap [74, 75]; segundo, la disponibilidad de nuevas técnicas de genotipado, con densa cobertura del genoma y costes más baratos; y tercero, la recolección de más amplias y mejor caracterizadas cohortes de pacientes y controles.

Hasta la fecha, dos GWAS han sido publicados en AR, los cuales han mostrado asociación con las ya conocidas regiones de susceptibilidad *HLA-DRB1* en 6p21 y *PTPN22* en 1p13 [76, 77] (Figura 2.13). Además, se han identificado prometedores nuevos locus potenciales de susceptibilidad a AR. Estudios replicativos y de mapeo fino serán necesarios para establecer el papel de esos nuevos locus en la susceptibilidad a AR.



WTCCC, *Nature* (2007)

Plenge et al, *N Engl J Med* (2007)

Figura 2.13. Regiones de asociación encontradas en dos GWASs realizados en AR.

Teniendo en cuenta las dos estrategias de selección de genes candidatos, la funcional y la posicional, hemos seleccionado una serie de genes, los cuales han sido estudiados con relación a la susceptibilidad a AR mediante estudios de asociación caso-control:

- ***Genes implicados en procesos inflamatorios:***
  - ***TLR 2 y 4: toll like receptors 2 y 4***
  - ***NFKB1: nuclear factor κB 1***
  - ***SUMO4: small ubiquitin-related modifier 4***
  - ***FCRL3: Fc receptor like 3***
  - ***SLC22A4: solute carrier family22, member 4***
  - ***RUNX1: runt related transcription factor 1***
  - ***IL12B e IL12RB1: interleukin 12B e interleukin 12 receptor B1***
  - ***IL23R: interleukin 23 receptor***
  - ***MIF: macrophage migration inhibitory factor***

- ***Genes implicados en la activación de las células T:***
  - ***PTPN22: protein tyrosine phosphatase non receptor 22***
  - ***CTLA4: cytotoxic T-lymphocyte antigen 4***
  - ***BTNL2: butyrophilin-like 2***
  - ***STAT4: signal transducer and activator of transcription 4***
  - ***MHC2TA: major histocompatibility complex II trans-activator***
  - ***FOXP3: forkhead box P3***

3. JUSTIFICACIÓN Y OBJETIVOS

La artritis reumatoide es una enfermedad crónica que ocasiona un grave sufrimiento a la persona que la padece, con discapacidad y pérdida de calidad de vida. Afecta a millones de personas en todo el mundo acortando su esperanza de vida. A pesar del alto impacto que provoca, las causas que desencadenan la AR y los mecanismos fisiopatológicos que la controlan aún no se conocen con profundidad, por lo que no existe un tratamiento específico para estos pacientes. Además, el diagnóstico es bastante complicado, al ser la AR clínicamente heterogénea. Los altos costes directos e indirectos asociados a la AR, junto a la sustancial morbilidad y mortalidad que afecta a los pacientes, justifica los potenciales beneficios que los estudios genéticos podrían aportar.

Los estudios genéticos podrían ayudar a dilucidar los mecanismos que intervienen en la enfermedad y por tanto, sugerir nuevas dianas terapéuticas más específicas. Los estudios genéticos también pueden ser aplicados en el campo de la farmacogenética, que podría permitir la administración tratamientos personalizados. Finalmente, en combinación con otros datos clínicos, la identificación de marcadores genéticos se podría utilizar en el diagnóstico de la enfermedad, o en la predicción de las características clínicas que presentará el paciente, con la intención de realizar una acción terapéutica temprana e incluso la prevención.

El objetivo general de esta tesis fue la identificación de nuevos marcadores genéticos de predisposición a AR, mediante una estrategia basada en estudios de asociación caso-control con genes candidatos. En concreto, nos planteamos los siguientes objetivos:

1. Estudiar el posible papel de los SNPs Arg677Trp y Arg753Gln del gen *TLR2*, y Asp299Gly y Thr399Ile del gen *TLR4* en la predisposición genética a sufrir AR.
2. Evaluar si los polimorfismos -94isn/delATTG y $(CA)_n$ de *NFKB* están asociados a AR.
3. Determinar la posible influencia del SNP 163A→G de SUMO4 en la susceptibilidad a AR.
4. Investigar la asociación de la variante genética -169T→C del gen *FCRL3* con AR.
5. Analizar los polimorfismos de los genes *SLC22A4* y *RUNX1* con relación a la susceptibilidad a padecer AR.
6. Establecer si los polimorfismos más estudiados de los genes de la familia de la IL-12 *IL12B*, *IL12RB1* e *IL23R* están implicados en la AR.
7. Comprobar si la predisposición a sufrir AR está influenciada por los polimorfismos funcionales de *MIF*, -173G→C y $(CATT)_n$.

8. Estudiar el papel del polimorfismo 1858C→T del gen *PTPN22* en la susceptibilidad a AR, y evaluar su valor como marcador de susceptibilidad y pronóstico en conjunto con los alelos *HLA* del epítopo compartido y la presencia de autoanticuerpos como FR y anti-CCP.
9. Analizar los polimorfismos funcionales de los genes *CTLA4* y *BTNL2*, que intervienen en la ruta de coestimulación de la célula T, como marcadores de susceptibilidad a AR.
10. Determinar la influencia del SNP rs7574865 de *STAT4* en la predisposición a AR.
11. Investigar si la variante *MHC2TA* -168A→G está asociada a AR.
12. Establecer si el microsatélite $(GT)_n$ del gen *FOXP3* está implicado en la susceptibilidad a AR.

4. ANEXO I: ARTÍCULOS PUBLICADOS

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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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* The authors have contributed equally to this study

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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Toll-like receptor 4 Asp299Gly polymorphism and rheumatoid arthritis: a replicative study in three different populations

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POLIMORFISMO ASP299GLY DEL GEN DEL RECEPTOR TIPO TOLL 4 Y ARTRITIS REUMATOIDE: UN ESTUDIO REPLICATIVO EN TRES POBLACIONES DIFERENTES

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RESUMEN

Los polimorfismos de los receptores tipo toll (TLRs) han sido extensamente estudiados con respecto a la predisposición genética a varias enfermedades complejas humanas. En este contexto, el papel del polimorfismo Asp299Gly de *TLR4* en la patogénesis de la artritis reumatoide (AR) no está claro. El objetivo del presente estudio fue comprobar la posible implicación de este polimorfismo en la susceptibilidad a la AR. Genotipamos el polimorfismo mediante reacción en cadena de la polimerasa y posterior análisis de la longitud de fragmentos generados por endonucleasa de restricción específica (PCR-RFLP) en tres poblaciones diferentes de Granada (sur de España), Lugo (norte de España) y Colombia. No encontramos diferencias estadísticamente significativas en la distribución de alelos y genotipos en ninguna de las cohortes a estudio. Nuestros datos, junto a los de otros grupos de investigación, no apoyan un papel relevante del polimorfismo Asp299Gly de *TLR4* en la susceptibilidad a la AR.

PALABRAS CLAVE: Artritis reumatoide/ Polimorfismo/ Gen del receptor tipo toll 4.

ABSTRACT

Toll-like receptors (TLRs) polymorphisms have been extensively studied with regard to genetic predisposition to several human complex diseases. In this context, the role of *TLR4* Asp299Gly in the pathogenesis of rheumatoid arthritis (RA) is not clear. The aim of this study was to test the possible implication of this polymorphism in the susceptibility to RA. We genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in three different populations from Granada (Southern Spain), Lugo (Northern Spain), and Colombia. We did not find statistically significant differences in the distribution of alleles and genotypes in any of the cohorts under study. Our data, together with those from other groups, do not support a relevant role of *TLR4* Asp299Gly polymorphism in the susceptibility to RA.

KEY WORDS: Rheumatoid arthritis/ Polymorphism/ Toll-like receptor 4 gene.

TABLE I. Toll-like receptor 4 allele and genotype frequencies in patients with RA and healthy controls in three independent cohorts

TLR4 Asp299Gly Frequencies	Granada		Lugo		Colombia	
	RA patients n= 337 (%)	Healthy controls n= 275 (%)	RA patients n= 211 (%)	Healthy controls n= 100 (%)	RA patients n= 141 (%)	Healthy controls n= 130 (%)
Alleles						
Asp	641 (95.1)	510 (92.7)	395 (93.6)	189 (94.5)	273 (96.8)	244 (93.8)
Gly	33 (4.9)	40 (7.3)	27 (6.3)	11 (5.5)	9 (3.2)	16 (6.1)
Genotypes						
Asp/Asp	304 (90.2)	237 (86.2)	185 (87.7)	89 (89)	132 (93.6)	114 (87.7)
Asp/Gly	33 (9.8)	36 (13.1)	25 (11.9)	11 (11)	9 (6.4)	16 (12.3)
Gly/Gly	0	2 (0.7)	1 (0.4)	0 (0)	0 (0)	0 (0)

Rheumatoid arthritis (RA) is among the most common autoimmune inflammatory diseases[1]. Although the etiology of RA is still poorly understood, family studies, twin studies and segregation analyses have provided evidence of a strong genetic component[2]. However, the genetic background of this disorder is complex and likely involves multiple genes encoding proteins with significant function in the modulation of the immune system.

Toll-like receptors (TLRs) are phylogenetically conserved receptors that are involved in the recognition of pathogen-associated molecular patterns (PAMPs) and endogenous ligands, and play an important role in regulating inflammatory response and signalling the activation of adaptive immunity[3]. Therefore, genetic variation of *TLR* genes may play a role in determining susceptibility to chronic human diseases which have an inflammatory component, such as rheumatoid arthritis (RA)[4].

Radstake and colleagues[5] investigated the influence of a functional *TLR4* gene polymorphism, at amino acid 299 (Asp299Gly)[6,7], on the susceptibility and severity/outcome of rheumatoid arthritis (RA). The authors presented data showing a lower frequency of the *TLR4* heterozygous condition for the polymorphism in 282 patients with RA (10.6%) than in 314 control individuals (17.2%), suggesting that the hypofunctional 299Gly allele may protect against RA. The authors also reported no association between *TLR4* genotypes and disease severity and/or outcome. As noted by the authors, validation of these findings in independent cohorts is needed to establish firmly a role of *TLR4* polymorphism in susceptibility to RA.

We have reported a similar study with different conclusions[8]. Our cohort was composed of 224 RA cases and 199 unrelated healthy control subjects, all Caucasian subjects from the South of Spain. Although we observed a difference in the distribution of *TLR4* Asp299Gly heterozygous individuals between RA patients (9%) and control (13%), it did not reach statistical significance. In order to replicate Radstake et al. data, we have analysed the *TLR4* Asp299Gly polymorphism in an extra group of RA patients and controls from Southern Spain, being the total individuals analysed of 337 RA patients and 275 healthy controls (Table I). We determined *TLR4* genotypes by a PCR-based method, as previously described[9]. Thirty-three (9.8%) of the RA patients and thirty-six (13.1%) of the healthy controls were heterozygous for the Asp299Gly polymorphism. No statistically significant differences in genotype or allele distribution of the *TLR4* polymorphism were observed between RA patients and control individuals. To further test the effect of *TLR4* Asp299Gly polymorphism on RA susceptibility, we replicated the study in two additional cohorts of patients with RA and healthy subjects from Lugo (Northern Spain) and Colombia (Table 1). Among the population from Lugo, 11.9% of the patients with RA and 11% of the controls were heterozygous for the allelic variant. Regarding the Colombian cohort, 6.4% of the patients with RA and 12.3% of the healthy controls were heterozygous for the SNP. We did not observe statistically significant differences when we compared allele and genotype frequencies between RA patients and healthy subjects in both cohorts.

In agreement with our data, a study conducted in a British population showed no evidence of association of the *TLR4* Asp299Gly polymorphism with RA, in a cohort of 212 RA patients and 879 control subjects[10].

Since the number of patients with RA and control subjects included in the present study is higher than that in the Radstake et al study, the lack of statistically significant results is unlikely to have resulted from low power. Based on the previous study, one would expect that a power of 99.0% would be achieved by including 305 and 270 individuals in the patient and control groups, respectively (p=0.05 and

TT genotype frequency in the control group 43.7%). Lack of replication of a previous association is a common event in the search for genetic determinants of complex human traits. Among the most common causes of irreproducibility are population stratification, publication bias, time-lag bias and methodology bias.

The effects of genetic, population and clinical heterogeneity must be considered when attempting to detect susceptibility genes for RA in different populations. In European Caucasian healthy populations the frequency of *TLR4* Asp299Gly heterozygous range from 8% to 13%[8,10-13]. A possible bias due to the genotyping rate may explain the contradictory results obtained in relation to *TLR4* polymorphism and RA[5,8,10]. It is worth noting that the frequency of *TLR4* Asp299Gly heterozygous in the group of healthy controls is of 17% in Radstake's manuscript, which is considerable higher that the reported in most studies[8,10-13]. In this regard, it is interesting to mention that a study of *TLR4* polymorphisms in familial hypercholesterolaemia has found that only 11% of healthy controls were heterozygous for the Asp299Gly polymorphism[11]. This study was carried out in a Dutch population from the same geographical area as Radstake's study, suggesting that the association between *TLR4* and RA susceptibility observed in Radstake's study may be due to genotyping uncertainty.

In support of ours and Kilding et al. results, whole-genome scan linkage studies have revealed no evidence of linkage between RA susceptibility and the 9q23 region, where *TLR4* gene maps, although the lack of linkage does not exclude the possibility of a disease gene mapping to a region.

In our opinion, the above considerations question the role of the *TLR4* Asp299Gly polymorphism in RA disease susceptibility.

CONCLUSIONS

In the light of our findings, replicated in three different cohorts, it seems that the *TLR4* Asp299Gly polymorphism does not play a relevant role in the pathogenesis of RA, which is in agreement with other recent reports.

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Brief communication

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

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

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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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SLC22A4, RUNX1, and *SUMO4* Polymorphisms Are Not Associated with Rheumatoid Arthritis: A Case-Control Study in a Spanish Population

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ABSTRACT. ***Objective***. To replicate the association reported in Japanese individuals of functional *SLC22A4* and *RUNX1* polymorphisms with rheumatoid arthritis (RA), and to test the possible role in this trait of a functional variant of the *SUMO4* gene that was shown to be associated with another related autoimmune disease, type 1 diabetes (T1D).

Methods. Our study population consisted of 886 patients with RA and 987 healthy controls. All subjects were of Spanish Caucasian origin. We conducted a case-control association study with 6 single-nucleotide polymorphisms (SNP) spanning the *SLC22A4* gene. SNP mapping in the *RUNX1* gene associated with RA in a Japanese population and a *SUMO4* polymorphism associated with T1D were also studied.

Results. No statistically significant differences between patients with RA and healthy controls were observed when comparing the distribution of the genotypes or alleles of any of the *SLC22A4* polymorphisms tested. Similarly, no evidence of association between RA and the *SLC22A4* haplotype previously reported to be associated in a Japanese population was found. With regard to the *RUNX1* and *SUMO4* SNP, we did not observe statistically significant differences in the distribution of genotypes or alleles between patients with RA and healthy controls.

Conclusion. These results suggest that the *SLC22A4*, *RUNX1*, and *SUMO4* polymorphisms analyzed do not confer a relevant role in susceptibility to RA in the Spanish population. (J Rheumatol 2006;33:1235–9)

Key Indexing Terms:
RHEUMATOID ARTHRITIS | SUSCEPTIBILITY | POLYMORPHISM
SLC22A4 | *RUNX1* | *SUMO4*

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Rheumatoid arthritis (RA) is a chronic complex inflammatory disease thought to have an autoimmune origin. Although the precise etiology of RA is unknown, a strong genetic component is well established[1]. The genetic background of systemic autoimmune diseases such as RA is complex and probably involves multiple genes encoding proteins with significant functions in the regulation of the immune system. A genetic approach to identify genes associated with autoimmune disorders is proposed as one of the promising methodologies to elucidate the cause of these diseases.

The chromosomal region 5q31 is particularly interesting with regard to RA genetic predisposition because it contains many genes involved in immune and inflammatory pathways[2]. This region has been reported to be associated with Crohn's disease, which, like RA, has an inflammatory and autoimmune pathogenesis[3]. A recent study in a Japanese population reported an association between RA and a functional variant of the *SLC22A4* gene (solute carrier family 22, member 4), which maps in the 5q31 region and encodes the organic cation transporter 1[4]. This polymorphism disrupts a

RUNX1 binding site and affects the expression of *SLC22A4*. Further, in the same study, an association between RA and a single nucleotide polymorphism (SNP) located in the *RUNX1* gene was also found. RUNX1 is an essential hematopoietic transcription factor, whose abnormality is frequently found in leukemia[5]. Recently, regulatory polymorphisms mapping in *RUNX1* binding sites have been independently reported to be associated with systemic lupus erythematosus and psoriasis[6,7]. These findings support the hypothesis that autoimmune diseases may share a common pathogenesis and susceptibility genes[8].

Besides replication studies, considering the possible role of a gene previously associated with a related trait is a useful tool to clarify the genetic component of RA. We have therefore chosen *SUMO4* as a candidate gene for susceptibility to RA. Members of the *SUMO* (small ubiquitin-related modifiers) gene family encode a family of proteins involved in post-translational modification[9]. A new member of this gene family, *SUMO4*, located on 6q25, has recently been identified[10,11]. SUMO4 protein conjugates to IκB and negatively regulates nuclear factor-κB (NF-κB) transcriptional activity[10]. NF-κB activates transcription of different genes encoding proteins involved in the immune response. Therefore, impaired control of NF-κB function may lead to the development of autoimmune inflammatory disorders. Recently, evidence was reported for an association of *SUMO4* common nonsynonymous SNP 163 A→G, resulting in the amino-acid substitution M55V, with susceptibility to type I diabetes[10,11]. Further, the *SUMO4* M55V substitution was shown to result in an increased NF-κB transcriptional activity and a higher expression of *IL12B* gene[10].

The aim of our study was to: (1) replicate the reported association of functional SNP of *SLC22A4* and *RUNX1* with RA in a Caucasian population, and (2) test the possible role of the *SUMO4* polymorphism in RA.

MATERIALS AND METHODS

Subjects. A total of 886 patients with RA meeting the American College of Rheumatology (ACR) 1987 revised classification criteria for RA[12] were recruited from 5 Spanish hospitals: Hospital Virgen de las Nieves (Granada), Hospital Universitario Virgen del Rocio (Seville), Hospital Xeral-Calde (Lugo), Hospital 12 de Octubre (Madrid), and Hospital Universitario La Paz (Madrid). RA patients had been genotyped for HLA-DRB1. Among the RA patients 75.3% were women; the mean age at disease onset was 50.3 ± 14 years; 55.7% carried the shared epitope; 75.8% were rheumatoid factor-positive; 27% presented extraarticular manifestations; and 20% presented nodular disease. A total of 987 blood bank and bone marrow donors from corresponding cities were included as healthy controls. Patients and controls were all of Spanish Caucasian origin and were included after giving written informed consent. We obtained approval for the study from all participating hospital ethical committees.

Genotyping. DNA from patients and controls was obtained from peripheral blood using standard methods. SNP were selected according to previous studies in autoimmune diseases, including SNP studied in Japanese patients with RA spanning the *SLC22A4* region (rs3763112 [slc2-E1], rs1007602 [slc2-1], rs3792876 [slc2-F2], rs2073838 [slc2-F1], and rs2269822 [slc2-3])[4], and the *SLC22A4* SNP associated with Crohn's disease in a Caucasian population (rs1050152 [SLC22A4*L503F])[13] (Figure 1). We also tested the *RUNX1* rs2268277 variant, which has been reported to be associated with RA[4], and the *SUMO4* 163 A→G polymorphism previously shown to be associated with type 1 diabetes (T1D)[10].

Samples were genotyped for *SLC22A4*, *RUNX1*, and *SUMO4* polymorphisms using a TaqMan 5' allelic discrimination Custom TaqMan® SNP Genotyping Assay method (Applied Biosystems, Foster City, CA, USA). Allele-specific probes were labeled with the fluorescent dyes VIC and FAM, respectively. PCR reaction was carried out in a total reaction volume of 8 μl with the following amplification protocol: denaturation at 95ºC for 10 min, followed by 40 cycles of denaturation at 95ºC for 15 s, and to finish, annealing and extension at 60ºC for 1 min. Following PCR, the genotype of each sample was attributed automatically by measuring the allelic specific fluorescence on the ABI Prism 7000 Sequence Detection System using SDS 1.1 software for allelic discrimination (Applied Biosystems).

Statistical analysis. Allelic and genotypic frequencies of all the genetic variants were obtained by direct counting. Statistical analysis to compare allelic and genotypic distributions was performed by the chi-square test. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated according to Woolf's method. The software used was the Statcalc program (EpiInfo 2002; Centers for Disease Control and Prevention, Atlanta, GA, USA). p values < 0.05 were considered statistically significant. In all tables, uncorrected p values are presented. 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 linkage disequilibrium measures were investigated and haplotypes constructed by the expectation-maximization algorithm implemented using Unphased software[14]. Sample sizes were estimated *a priori* by Quanto 0.5 software (Department of Preventive Medicine, University of Southern California, Los Angeles, CA, USA) according to previously reported allele frequencies[4,3,10], so that each association study had at least 80% power to detect an association with the same OR as detected in previous studies (OR 1.5–2.0) at the 5% significance level assuming a dominant inheritance model.

RESULTS

SLC22A4 genotypes were in Hardy-Weinberg equilibrium in patients and controls. We observed that the *SLC22A4* rs3792876 and rs2073838 SNP were in complete linkage disequilibrium, as described in a Japanese population. No statistically significant differences in allele and genotype frequencies of different SNP tested in the *SLC22A4* region were found between RA patients and controls (Table 1). Of note, the frequencies of these *SLC22A4* polymorphisms in our population differed significantly from those found in the Japanese population[4].

Additionally, we carried out a haplotype analysis of 5 SNP common to the Japanese study, which define the *SLC22A4* haplotype associated with RA in the Japanese population (rs3763112, rs1007602, rs3792876, rs2073838, and rs2269822; Table 2). Four haplotypes with frequency > 5% were found in the Spanish population. We did not observe statistically significant differences in the distribution of these haplotypes when comparing RA patients with the control group. The RA-associated *SLC22A4* haplotype in the Japanese study was present at an extremely low frequency in our population.

With regard to the rs2268277 *RUNX1* polymorphism, genotypes were in Hardy-Weinberg equilibrium in patients and controls. Similarly, no statistically significant differences

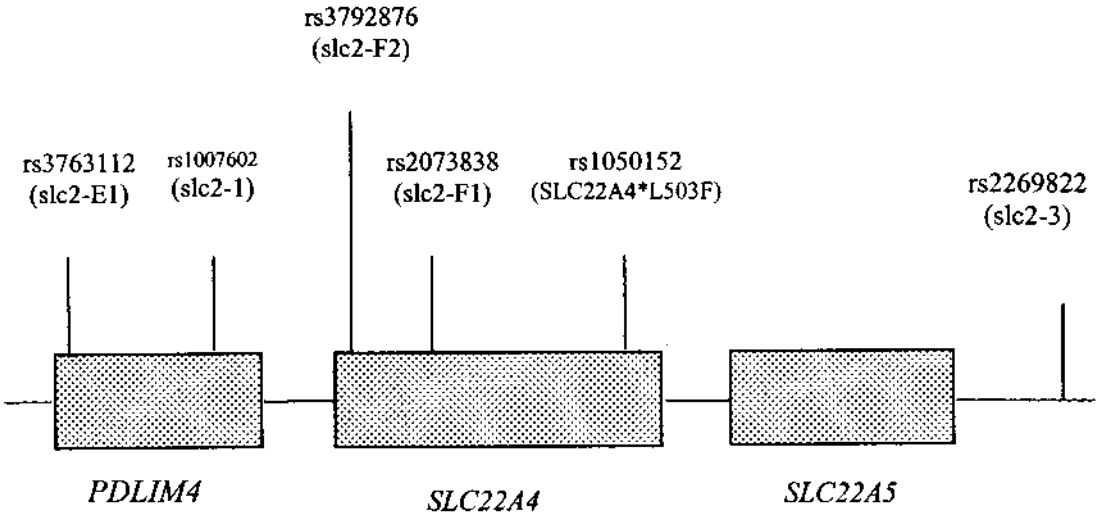

Figure 1. Location of the SNP tested in the 5q31.1 region.

Table 1. Genotype and allele frequencies of different *SLC22A4*, *RUNX1*, and *SUMO4* SNP among RA patients and healthy controls.

	Genotypes and Alleles	RA Patients, n = 886 (%)	Healthy Controls, n = 987 (%)	p	OR (95% CI)
SLC22A4 rs3763112 (slc2-E1)	G	992 (57.4)	1055 (53.4)	0.11	1.11 (0.97–1.26)
	A	780 (42.6)	919 (46.6)		
SLC22A4 rs1007602 (slc2-1)	C	1127 (63.6)	1235 (62.6)	0.51	1.05 (0.91–1.20)
	T	645 (36.4)	739 (37.4)		
SLC22A4 rs3792876 (slc2-F2)	C	1643 (92.7)	1844 (93.4)	0.40	0.9 (0.69–1.16)
	T	129 (7.3)	130 (6.7)		
SLC22A4 rs2073838 (slc2-F1)	G	1643 (92.7)	1844 (93.4)	0.40	0.9 (0.69–1.16)
	A	129 (7.3)	130 (6.7)		
SLC22A4 rs1050152 (SLC22A4*L503F)	C	997 (56.3)	1130 (57.2)	0.54	0.96 (0.84–1.10)
	T	775 (43.7)	844 (42.8)		
SLC22A4 rs2269822 (slc2–3)	C	1527 (86.2)	1701 (86.2)	0.98	1.00 (0.83–1–21)
	T	245 (13.8)	273 (13.8)		
RUNX1 rs2268277	C	711 (40.1)	788 (39.9)	0.89	1.01 (0.88–1.15)
	G	1061 (59.9)	1186 (60.1)		
SUMO4 163A→G	A	856 (48.3)	915 (46.4)	0.23	1.08 (0.95–1.23)
	G	916 (51.7)	1059 (53.6)		

between RA patients and controls were observed when the distribution of the genotypes or alleles of this *RUNX1* SNP were compared (Table 1). We did not observe the epistatic effect reported by Tokuhiro, *et al*[4] concerning the susceptible alleles of both *SLC22A4* and *RUNX1* genes. The number of individuals bearing the combination of these genotypes was much lower in our population than in the Japanese population, due to the marked difference between allelic and genotypic frequencies.

Regarding *SUMO4*, genotype and allele frequencies of the 163A→G SNP in patients with RA and controls are shown in Table 1. The genotype frequencies were not found to be significantly different from those predicted by Hardy-Weinberg equilibrium testing in controls. The observed allele frequencies in our control population were in concordance with those found in other Caucasian populations[10,11,15]. However, they differ significantly from those described in Asian populations (Spanish vs Taiwanese, $p < 10^{-7}$; Spanish vs Chinese, $p = 6 \cdot 10^{-6}$;

Table 2. SLC22A4 haplotypes with frequency > 5% in Spanish patients with RA and healthy controls. Haplotypes were constructed taking into account the following SNP: rs3763112 (slc2-E1), rs1007602 (slc2-1), rs3792876 (slc2-F2), rs2073838 (slc2-F1), and rs2269822 (slc2-3).

Haplotype	RA Patients, 2n = 1772 (%)	Healthy Controls, 2n = 1974 (%)	p*	OR (95% CI)
GCCGC	780 (44)	830 (42)	0.22	1.08 (0.95–1.24)
ATCGC	638 (36)	730 (37)	0.53	0.96 (0.84–1.10)
ACCGC	106 (6)	138 (7)	0.21	0.85 (0.65–1.11)
GCCGT	88 (5)	118 (6)	0.18	0.82 (0.61–1.10)

* Overall p = 0.24.

Spanish vs Korean, $p = 12 \cdot 10^{-6}$)[10]. No statistically significant differences in the distribution of the alleles or genotypes of the *SUMO4* 163A→G polymorphism were found when we compared RA patients with the control group (Table 1).

Next, we analyzed demographic and clinical characteristics of RA patients according to their *SLC22A4*, *RUNX1*, and *SUMO4* genotypes (gender, age at disease onset, presence of shared epitope, rheumatoid factor, rheumatic nodules, and extraarticular disease); however, no significant differences were observed (data not shown).

DISCUSSION

In our study, no evidence of an association with RA of the reported *SLC22A4*, *RUNX1*, and *SUMO4* susceptibility SNP was observed. With regard to *SLC22A4* and *RUNX1*, failure to replicate reported associations is a common event in the search for genetic determinants of complex diseases, due either to genuine population heterogeneity or a different sort of bias, such as publication bias or time-lag bias[16]. The first published report usually suggests a stronger genetic effect, and subsequent studies often fail to confirm the original findings[16]. The lack of replication in our study may have arisen due to a type 2 error (false negative). According to the *a priori* calculation, our sample size had at least 80% power to detect the relative risk for the individual SNP reported in the Japanese study at the 5% significance level. Nevertheless, we found a very low minor allele frequency of the RA-associated polymorphism (slc2-F1) in our population (6.7%) compared with that found in the Japanese population (31%). Because of our low minor allele frequency, our sample size was underpowered to detect the homozygous slc2-F1, a risk genotype found in the Japanese population. Indeed, according to the frequency of homozygous AA reported in our population and other Caucasian populations, more than 5000 patients and 5000 controls would have to be tested to find an association with similar OR to that described in the Japanese population. Regarding rs3792876 (slc2-F2), this SNP was in complete linkage disequilibrium with rs2073838 (slc2-F1), and considerations about the *posteriori* power were the same. Regarding the rest of the comparisons, we had more than 80% power to detect a relative risk similar to the Japanese study at the 5% significance level in every case.

The genetic heterogeneity between populations is clearly present in this case, since *SLC22A4* allele and genotype frequencies are significantly different between the Spanish and the Japanese populations, which may also account for the failure to replicate the *SLC22A4* association with RA. In this sense, there are several reported RA genetic associations in the Japanese population, such as peptidyl-arginine deiminase 4 (*PADI4*)[17] or inhibitor of κB-like[18] gene variants, which were not replicated in Caucasian populations[19,20]. Although an association of the *SLC22A4* gene with Crohn's disease has been reported in both Japanese[21] and Caucasian populations[13], in Japanese the associated disease polymorphism was the rs3792876, while in the Europeans it was rs1050152. It is possible that disease-relevant genes or alleles may be specific for certain populations, and vary among different ethnic groups.

During the course of this work, and in agreement with our results, 2 studies showing lack of association of *SLC22A4* with RA in other Caucasian populations have been reported[22,23]. All 3 studies in Caucasian populations have the same power calculation problems. Our study and data reported in the Canadian population show a trend similar to the Japanese study. In these 3 studies slc2-F1 AA homozygotes are overrepresented among patients, whereas in the UK population they are underrepresented. It seems inadequate to draw conclusions using SNP with a very low frequency of the minor allele, taking into account the moderate OR found in Japanese. Nevertheless, for the rest of the SNP studied in the region having a higher minor allele frequency, no association was detected.

With regard to the *RUNX1* rs2268277 polymorphism, the lack of replication of the association with RA was due neither to a lack of power nor genetic heterogeneity, because the minor allele frequency found in the Spanish population (35%) was very similar to that found in the Japanese population (37%); thus our RA sample size (886 patients) was large enough to reach a 98% statistical power to detect a relative risk similar to the Japanese study at the 5% significance level. In addition, the association of *RUNX1* polymorphism with RA has not been replicated in another Caucasian population[24].

Another possibility to explain discrepancies among studies is environmental heterogeneity. Some genes may play a role in susceptibility to RA only in the presence of specific envi-

ronmental factors to which Japanese, but not the Spanish population, are exposed. Therefore, investigation of possible gene-environmental interaction would be very useful to determine this effect.

Regarding *SUMO4*, our study attempted to assess the potential implication of the functional variant 163 A→G of the gene, which has been associated with T1D, in susceptibility to a related systemic autoimmune disorder such as RA. No evidence of an association of *SUMO4* 163 A→G SNP with RA susceptibility was found, which is in accordance with a recent study in a British population[25]. This lack of association is not attributable to the sample size, because the power of our study to detect a difference with OR = 1.5 at $\alpha = 0.05$ was > 99%. The allele and genotype frequencies observed in our study were similar to those described in other Caucasian populations[10,11,15].

The reported association of the *SUMO4* gene to T1D is now under debate[26,27]. Of note, Guo, *et al* did not find an association of the *SUMO4* polymorphism and T1D in a case-control study carried out in a Spanish population[10]. Therefore, it seems that *SUMO4* does not play a relevant role in the genetic predisposition to susceptibility to autoimmune disorders such as RA and T1D in the Spanish population, although a small effect cannot be excluded; this can be verified only in an extremely large data set.

We have been unable to replicate the association of functional variants of *SLC22A4* and *RUNX1* with RA as previously described in a Japanese population. In addition we did not find an association between RA and a functional polymorphism of *SUMO4*, which has been associated with T1D.

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EXTENDED REPORT

Epistatic interaction between *FCRL3* and *NFκB1* genes in Spanish patients with rheumatoid arthritis

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Background: A Japanese study has described a strong association between rheumatoid arthritis and several polymorphisms located in the Fc receptor-like 3 (*FCRL3*) gene, a member of a family of genes related to Fc receptors located on chromosome 1q21–23.
Objectives: To evaluate the association between rheumatoid arthritis and *FCLR3* polymorphisms in a large cohort of Caucasian patients with rheumatoid arthritis and healthy controls of Spanish origin. Owing to the described functional link between the *FCRL3* polymorphisms and the transcription factor nuclear factor κB (NFκB), a functional polymorphism located in the *NFκB1* gene was included.
Methods: 734 patients with rheumatoid arthritis from Madrid and Granada, Spain, were included in the study, along with 736 healthy controls. Polymorphisms in the *FCRL3* gene were studied by TaqMan technology. The −94ins/delATTG *NFκB1* promoter polymorphism was analysed by fragment analysis after polymerase chain reaction with labelled primers. Genotypes were compared using 3×2 contingency tables and χ^2 values.
Results: No overall differences were found in any of the *FCRL3* polymorphisms and in the *NFκB1* promoter polymorphism when patients were compared with controls. However, when stratified according to *NFκB1* genotypes, a susceptibility effect of *FCRL3* polymorphisms was observed in patients who were heterozygotes for *NFκB1* ($p_c = 0.003$).
Conclusions: The *FCRL3* polymorphisms associated with rheumatoid arthritis in a Japanese population are not associated per se with rheumatoid arthritis in a Spanish population. A genetic interaction was found between *NFκB1* and *FCRL3* in Spanish patients with rheumatoid arthritis. These findings may provide a general rationale for divergent genetic association results in different populations.

Rheumatoid arthritis is one of the most common autoimmune diseases in Western countries, with an estimated prevalence of 1% in the Spanish population. Recent advances in its genetic basis have been fuelled by an ongoing Japanese genomewide study, which has described several causative polymorphisms in the past few years.[1–3] However, some of these outstanding findings have been difficult to replicate in European populations, as has recently been shown by our group and by others with regard to the gene peptidylarginine deiminase 4 (*PADI4*),[4–6] the haematopoietic isoform of the citrullinating enzyme. *SLC22A4*, an organic cation transporter gene, is another example of lack of association in Caucasian populations of a gene previously shown by the Japanese group[7 8] to be associated with the disease. The causes underlying such discrepancies may vary, but arguably they include both distinct environmental inputs and different genetic structure. The purely genetic reasons for a failure to replicate the association of a polymorphism described as aetiological are many. Firstly, the polymorphism studied may be found in another population in such a low frequency that a significant difference may be difficult to achieve. Secondly, the supposedly aetiological polymorphism may not be really aetiological, but instead it may act as a marker of a nearby causative polymorphism. The associations between different polymorphisms in the same or nearby genes (linkage disequilibrium) may vary widely among populations. Finally, a true aetiological polymorphism may act only on a defined genetic background, and therefore discloses itself only in specific populations. A similar situation has been reported repeatedly in mice, where a defined inactivating mutation may yield different phenotypic outcomes, depending on genetic background.[9]

A recent genetic report from the Yamamoto group[3] has described the association of four single-nucleotide polymorphisms (SNPs) located in the Fc receptor-like 3 (*FCRL3*) gene with an increased susceptibility to rheumatoid arthritis and several other autoimmune diseases, thereby raising the possibility of *FCRL3* being a general autoimmune susceptibility factor. *FCRL3*, also termed *FCRh3*, is one of a family of six related genes located on chromosome 1q21–23, sharing homologies with other classic Fc receptor genes located in the same cluster. These previously known classic genes code for receptors for the constant portion (Fc) of the immunoglobulin molecules. The Fc receptors have relevant roles in diverse

Table 1 Clinical characteristics of patients with rheumatoid arthritis

Mean (SD) age at onset (years)	50 (14)
Female:male ratio	3.2
Erosive disease (%)	81
Shared epitope positivity (%)	60
Rheumatoid factor positivity (%)	75

Abbreviations: *FCRL3*, Fc receptor-like 3; HLA, human leucocyte antigen; NFκB, nuclear factor κB; SNP, single-nucleotide polymorphism

Table 2 Genotypic and allelic distribution of *FCRL3* −169 SNP alleles

	n	AA	AG	GG	Alleles	A	G
Controls from Madrid	489	153 (31)	233 (48)	103 (21)	978	539 (55)	439 (45)
Controls from Granada	229	75 (33)	113 (49)	41 (18)	458	263 (57)	195 (43)
Patients from Madrid	448	117 (26)	229 (51)	102 (23)	896	463 (52)	433 (48)
Patients from Granada	221	61 (28)	122 (55)	38 (17)	442	244 (55)	198 (45)

FCRL3, Fc receptor-like 3; SNP, single-nucleotide polymorphism.
Values in parentheses are percentages.
Overall genotype comparison (Madrid and Granada), p = 0.10.
Minimum detectable allelic RR = 1.24.

aspects of immunobiology.[10] This fact, along with their polymorphic nature, led long ago to their consideration as natural candidates to be susceptibility genes in several autoimmune inflammatory conditions. The region 1q21–23 has been reported to be linked to systemic lupus erythematosus in familial linkage studies with several affected members, but it has not been detected in a recent study on rheumatoid arthritis (interestingly, in the Japanese population) using microsatellites,[11] or in previous genomewide scans in populations of European ancestry. Kochi *et al*[3] examined the region with high-density SNP mapping and found a peak of association not in the classic genes, but on *FCRL3*, a family member expressed on some subsets of B cells. Although they were cautious enough not to discard fully other genes located in the vicinity, the main candidate they propose is clearly *FCRL3* itself. They suggest that the −169C susceptibility allele binds the transcription factor nuclear factor κB (NFκB) more strongly than the non-susceptible −169T allele, thereby enhancing the transcription rate of the *FCRL3* gene.

Although some recent research in the Spanish population has been undertaken to ascertain the role of the classic Fc receptor genes in rheumatoid arthritis,[12] we have tried to replicate directly the Japanese findings, as their functional studies convincingly showed that the polymorphism may be mechanistically relevant to the pathological process. We also looked for possible genetic interactions between the polymorphisms in the *FCRL3* promoter and the functional polymorphism in the promoter of *NFKB1*, coding for the p105 subunit of NFκB. This p105 subunit is proteolytically activated on cell stimulation, yielding the p50 subunit, a component (in combination with c-Rel) of the active transcription factor binding to the −169 *FCRL3* promoter polymorphism.[3]

METHODS

Our study included 734 white Spanish patients with rheumatoid arthritis, consecutively recruited in two hospitals in Madrid (n = 489) and one hospital in Granada (n = 245). The diagnosis was established on the basis of the 1987 American College of Rheumatologists (formerly ARA) criteria.[13] Table 1 shows their main clinical characteristics. Most of these patients have been included in previous studies from our groups. The written consent of the participants was obtained according to the Declaration of Helsinki. The control group included 736 people, 489 from Madrid and 247 from Granada: white Spanish blood donors and healthy laboratory staff and students. Although the blood donors were not specifically asked about the presence of rheumatoid arthritis, the 1% frequency of this disease in the Spanish population precludes this fact from being a major concern. The ethics committees of the participating hospitals (Clinico San Carlos, La Paz, Virgen de las Nieves, Spain) approved this study.

The *FCRL3* polymorphisms were analysed using the C_1741825_10 (*FCRL3* −169, rs7528684, in Madrid only), C_1741826_10 (*FCRL3* −110, rs11264799) and C_2741972_10 (*FCRL3* intron 3, rs1537947, in Granada only) Assays-on-Demand of Applied Biosystems (Foster City, California, USA), according to the conditions recommended by the manufacturer. In all, 94 samples were genotyped for both the −169 SNP and the polymorphism in the intron 3 of the *FCRL3* gene. As described previously (see supplementary table in the Japanese study[3]), the concordance was almost total (>97%; *FCRL3* −169T is equivalent to *FCRL3_6*G, and *FCRL3* −169C is equivalent to *FCRL3_6*A), and it allowed us to consider them as quasi-equivalent markers. They are both referred to as −169SNP hereafter, for simplicity. Alleles were discriminated in an ABI 7900 Sequence Detector (Applied Biosystems). The −94ins/delATTG *NFκB1* promoter polymorphism was analysed as explained previously.[14]

The presence of the shared epitope (defined as DRB1*0101, DRB1*0102, DRB1*0401, DRB1*0404, DRB1*0405, DRB1*0408 or DRB1*1001 alleles) was ascertained as described elsewhere.[15 16] The shared epitope is a common stretch of amino acids present in human leucocyte antigen (HLA)-DRB1 alleles associated with the disease, and it is the strongest rheumatoid arthritis susceptibility factor identified.

Genotype frequencies (3×2 contingency tables), carriage rates (2×2 tables) and allelic frequencies (2×2 tables) were compared with the χ^2 statistics and $p<0.05$ was considered to be significant. The p values were calculated with a standard free software package (Epi Info V.2000, CDC, Atlanta, Georgia, USA). Statistical power (indicated below each table

Table 3 Genotypic and allelic distribution of *FCRL3* −110 SNP

	n	GG	AG	AA	Alleles	G	A
Controls from Madrid	479	235 (49)	196 (41)	48 (10)	958	666 (70)	292 (30)
Controls from Granada	225	115 (51)	89 (40)	21 (9)	450	319 (71)	131 (29)
Patients from Madrid	420	196 (47)	184 (44)	40 (10)	840	576 (69)	264 (31)
Patients from Granada	206	103 (50)	94 (46)	9 (4)	412	300 (73)	112 (27)

FCRL3, Fc receptor-like 3; SNP, single-nucleotide polymorphism.
Values in parentheses are percentages.
Overall genotype comparison (Madrid and Granada), p = 0.23.
Minimum detectable allelic RR = 1.26.

Table 4 Genotypic and allelic distribution of *NFκB1* insertion or deletion in patients with rheumatoid arthritis and in controls

	n	Del/del	Del/ins	Ins/ins	Alleles	Del	Ins
Controls from Madrid	458	74 (16)	214 (47)	168 (37)	916	362 (40)	554 (60)
Controls from Granada	247	35 (14)	107 (43)	105 (43)	494	177 (36)	317 (64)
Patients from Madrid	376	61 (16)	168 (45)	147 (39)	752	290 (39)	462 (61)
Patients from Granada	245	28 (11)	124 (51)	93 (38)	490	180 (37)	310 (63)

del, deletions; ins, insertions; *NFκB1*, nuclear factor κB1; RA, rheumatoid arthritis.
Values in parentheses are percentages.
Overall genotype comparison (Madrid and Granada), p = 0.80.
Minimum detectable allelic RR = 1.25.

as the minimum detectable relative risk (RR) value for p = 0.05 and a statistical power of 80%) was calculated with the online calculator at http://calculators.stat.ucla.edu/powercalc/binomial/case-control/index.php.

RESULTS

SNPs located in the *FCRL3* region (the ones identified as primary sources of the association signal described in the Japanese study[3]) were examined in the Spanish population. The Madrid and Granada populations were very similar in terms of their genotypic distributions. We found no evidence of phenotypic, genotypic or allelic association with rheumatoid arthritis (tables 2 and 3), even when results from both populations were pooled. Shared-epitope stratification did not improve the significance. We could not confirm the association described in the Japanese population.

Table 4 shows the allelic distribution of the −94ins/delATTG *NFKB1* promoter polymorphism found in the promoter region of the *NFKB1* gene. Data from Granada have been published previously[14] and they are included here for completeness, with only minor modifications. No differences were found when patients and controls from each separate location were compared. When controls from Madrid were compared with controls from Granada, we found no differences, showing that the two populations are homogeneous. Similarly, when patients with rheumatoid arthritis from Madrid were compared with those from Granada, we found no differences. This prompted us to pool both populations, to improve statistical power, but again no differences were found between patients with rheumatoid arthritis and controls. No differences were apparent when patients were stratified according to the number of shared epitope alleles carried (data not shown).

As the functional interaction between *NFκB1* and *FCRL3* is proved, we decided to investigate whether any genotype combination showed an altered pattern when patients were compared with controls. Table 5 shows the compound genotypes observed in patients with rheumatoid arthritis and controls. Among patients heterozygotic for the −94ins/delATTG *NFκB1* promoter polymorphism, there is a skewed distribution of the −169 polymorphism (p = 0.001, corrected by three different *NFκB1* genotypes, $p_c = 0.003$). Similarly to the Japanese population, the less common allele was over-represented in patients with rheumatoid arthritis when compared with controls.

DISCUSSION

We report that the association of the promoter polymorphism at position −169 on the *FCRL3* gene depends on the *NFκB1* genotype, thereby suggesting that a genetic and functional link between both genes is relevant in onset of rheumatoid arthritis. This is to our knowledge the first attempt to replicate the *FCRL3* association reported by the Yamamoto group.[3] In contrast with that report, our results show no association of *FCRL3* polymorphisms when considered alone.

The previous Japanese report did not include the polymorphism on the *NFκB1* gene in its analysis. However, their results were highly relevant per se. Our finding of an interaction between both genes seems to reconcile their results with the negative results we obtained when *FCRL3* alone is considered. If the polymorphisms are indeed aetiological this lack of association is otherwise problematic to explain, as the allelic frequencies in our population are by no means low enough to cause problems related to statistical power.

A genetic cooperation between distinct loci is not new in the genetics of rheumatoid arthritis. The relevance of rheumatoid arthritis susceptibility of mutations affecting an intronic *RUNX1* binding site on the *SLC22A4* gene (an organic cation transporter) and of mutations in the *RUNX1* gene itself has been recently described.[1 17] A few years ago, we also described the protection against rheumatoid arthritis afforded by major histocompatibility complex class I chain-related gene A alleles in the presence of the shared epitope at the *HLA-DRB1* locus.[15]

A functional hypothesis explaining our results is not straightforward. The ins/del polymorphism in the *NFκB1* promoter has been related to transcriptional activity of the gene encoding this transcription factor,[18] fundamental in orchestrating immune and inflammatory reactions. But this differential activity is probably not the sole cause behind the results we have presented, because a specific association of *FCRL3* genotypes in *NFκB1* heterozygotes (as opposed to homozygotes or carriers of any allele) is difficult to interpret. There have been a few reports in the literature of the effect of heterozygous genotypes on susceptibility to autoimmune

Table 5 Distribution of patients with rheumatoid arthritis (n = 592) and controls (n = 646) according to the compound *NFκB1* insertion or deletion and *FCRL3* −169SNP genotype

NFκB1	del/del	ins/del	ins/ins
Patients (Madrid and Granada)			
FCRL3			
AA	24 (29)	68 (24)	68 (30)
AG	45 (54)	145 (52)	123 (53)
GG	14 (17)	66 (24)	39 (17)
Controls (Madrid and Granada)			
FCRL3			
A	30 (31)	112 (38)	67 (27)
AG	46 (47)	137 (46)	127 (51)
GG	22 (22)	48 (16)	57 (23)

del, deletions; *FCRL3*, Fc receptor-like 3; ins, insertions; *NFκB1*, nuclear factor κB1; RA, rheumatoid arthritis; SNP, single-nucleotide polymorphism.
Percentages are calculated across columns.
Overall *FCRL3* genotype comparisons for each *NFκB1* genotype, p = 0.54, p = 0.001 and p = 0.28, for del/del, ins/del and ins/ins patients, respectively.

diseases. An *IL4* polymorphism has been described as negatively associated with multiple sclerosis in heterozygosis.[19] The authors suggest that perhaps the association could result from linkage disequilibrium with distant markers. This situation is reminiscent of the one found at the *HLA-DR* locus in coeliac disease. The association of *DR7-DR5* heterozygotes with the disease stems from the fact that *DR7* is strongly linked in our population to *DQB1*02*, and *DR5* is almost invariably linked to *DQA1*05*. These two alleles, located in distinct but nearby genes, are the primary susceptibility factors in coeliac disease, and *DR* is acting only as a linkage disequilibrium marker. A speculative scenario would be that a specific genetic configuration at the *NFκB1* locus confers the ability to become a susceptibility factor to variations found in *FCRL3*. And perhaps what is different between our population and the Japanese population is the distribution of genetic configurations in the *NFκB1* locus.

In summary, our results show complex genetic interactions between different genes to determine the final outcome: susceptibility to rheumatoid arthritis. Further studies are necessary to expand this knowledge and to delineate the genetic susceptibility pathways operating in distinct populations.

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ELSEVIER

Interleukin 12 (*IL12B*) and Interleukin 12 Receptor (*IL12RB1*) Gene Polymorphisms in Rheumatoid Arthritis

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ABSTRACT: The aim of this study was to assess the possible association between the *IL12B* and the *IL12RB1* gene polymorphisms and the systemic autoimmune disease rheumatoid arthritis (RA). Our study population consisted of 545 patients with RA and 393 healthy subjects. All the individuals were of white Spanish origin. Genotyping of the *IL12B (IL12Bpro* and *IL12B* 3′ untranslated region) and *IL12RB1* (641A→G, 1094T→C, and 1132G→C) polymorphisms was performed by polymerase chain reaction–restriction fragment length polymorphism and polymerase chain reaction–fluorescent methods. No statistically significant differences in the distribution of the *IL12B* and the *IL12RB1* genotypes and alleles between patients with RA and control subjects were observed. In addition, no association was found between the above-mentioned polymorphisms with any of the demographic and clinical parameters tested in patients with RA. These results suggest that *IL12B* and *IL12RB1* genes may not play a relevant role in the susceptibility or severity of RA in the Spanish population. *Human Immunology 66, 711–715 (2005).* © American Society for Histocompatibility and Immunogenetics, 2005. Published by Elsevier Inc.

KEYWORDS: interleukin 12; interleukin 12 receptor; rheumatoid arthritis; polymorphism; susceptibility

ABBREVIATIONS

IL-12	interleukin 12
IL-12R	interleukin 12 receptor
RA	rheumatoid arthritis
SNP	single nucleotide polymorphism
Th1	helper T cell type 1
UTR	untranslated region

INTRODUCTION

Rheumatoid arthritis (RA) is the most common autoimmune inflammatory disorder, affecting 1% of the adult population worldwide, and is usually associated with progressive joint destruction [1]. Although the pathogenesis of RA is unknown, the role of genetic factors in the pathogenesis of this disorder is well established [2, 3]. Cytokine genes play an important role in RA pathogenesis, and hence cytokine polymorphism could influence the development or severity of the disease [1].

Interleukin-12 (IL-12) is a heterodimeric proinflammatory cytokine formed by a 35-kDa chain (p35 or IL-12α) and a 40-kDa chain (p40 or IL-12β) [4]. IL-12β is encoded by the *IL12B* gene on chromosome 5q31–33, a region that has been demonstrated to be in linkage to RA [5]. Given its pivotal role in helper T cell type 1 (Th1) differentiation, the *IL12B* gene might be an important functional candidate gene for Th1-mediated diseases such as RA. In addition, excessive IL-12 production has been found in RA [6]. Two polymorphisms have been described mapping to *IL12B* gene, which may have a biologic significance: a 4-bp insertion-deletion within the promoter region, referred to here as *IL12Bpro,* and a A→C single nucleotide polymorphism (SNP) located in the 3′ untranslated region (UTR) at position 1188,

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referred to hereafter as *IL12B* 3′ UTR [7]. It has recently been described that *IL12Bpro* is not a single insertion/deletion, but a compound polymorphism involving a GC/TT transition combined with an AGAG microinsertion [8]. The biological activities of IL-12 are mediated through high-affinity binding to IL-12 receptor (IL-12R), which is composed of two subunits, IL-12Rβ1, encoded by *IL12RB1,* and IL-12Rβ2, encoded by *IL12RB2* [4]. Three SNPs have been described in *IL12RB1* (641A→G, 1094T→C, and 1132G→C), causing three missense variants (Q214R, M365T, G378R) [9], which may influence IL-12–induced signaling [10].

In light of these findings, the aim of this study was to examine the possible influence of the *IL12B* and *IL12RB1* gene polymorphisms in the susceptibility or severity of RA.

MATERIALS AND METHODS

A total of 545 patients with RA meeting the American College of Rheumatology 1987 revised criteria for RA [11] were recruited from three hospitals in Spain: Hospital Virgen de las Nieves (Granada), Hospital Xeral-Calde (Lugo), and Hospital La Paz (Madrid). A total of 393 blood bank donors and bone marrow donors from the corresponding cities, matched for sex and age, were included as healthy controls. All the subjects, patients, and controls were of white Spanish origin and were included in this study after providing written informed consent. We obtained approval for the study from all local ethical committees of the corresponding cities.

Genomic DNA was isolated from anticoagulant treated peripheral blood mononuclear cells by means of standard methods. We determined the *IL12B* promoter 4-bp insertion/deletion, *IL12B* 3′ UTR 1188 SNP, and polymorphisms within the *IL12RB1* gene genotypes, as previously described [7, 12, 13].

Statistical analysis to compare allele and genotype distributions was performed by χ^2 test. Odds ratios and 95% confidence intervals were calculated according to Woolf's method. The software used was Statcalc (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. For the haplotype analysis, pairwise linkage disequilibrium measures were investigated and haplotypes constructed by the expectation-maximization (EM) algorithm implemented in UNPHASED software [14]. The power of the study to detect an effect of a polymorphism in disease susceptibility was estimated by Quanto 0.5 software (Department of Preventive Medicine University of Southern California, Los Angeles, CA).

RESULTS

The genotype frequencies of all the polymorphisms tested were not found to be significantly different from those predicted by the Hardy-Weinberg equilibrium in healthy subjects. *IL12Bpro, IL12B* 3′ UTR, and *IL12RB1* genotype and allele frequencies in patients with RA and their healthy controls are listed in Table 1. In our Spanish population, the *IL12RB1* polymorphisms were in perfect linkage disequilibrium, forming the two common haplotypes, as previously described in a Japanese population [9]. No statistically significant differences were observed when the genotypes and allele distribution of all the analyzed polymorphisms were compared between patients with RA and healthy controls (Table 1).

Next, we analyzed the demographic and clinical characteristics of patients with RA according to their genotypes (Table 2). No significant differences were observed for most of the clinical features except for the presence of extraarticular disease and sex in the distribution of *IL12B* 3′ UTR genotypes. However, these differences turned out to be nonsignificant after correction for the

TABLE 1 Allele and genotype frequencies of *IL12B* promoter polymorphism in RA patients and healthy controls

Frequency	RA patients (%) (*n* = 545)	Healthy controls (%) (*n* = 393)
IL12B promoter alleles		
1	518 (47.5)	395 (50.3)
2	572 (52.5)	391 (49.7)
IL12B promoter genotypes		
1/1	113 (20.7)	87 (22.1)
1/2	292 (53.6)	222 (56.5)
2/2	140 (25.7)	84 (21.4)
IL12B 3′ UTR alleles		
A	889 (81.6)	623 (79.3)
C	201 (18.4)	163 (20.7)
IL12B 3′ UTR genotypes		
A/A	364 (66.8)	249 (63.4)
A/C	161 (29.5)	125 (31.8)
C/C	20 (6.7)	19 (4.8)
IL12RB1 alleles[a]		
1	745 (68.3)	527 (67)
2	345 (31.7)	259 (33)
IL12RB1 genotypes		
1/1	244 (44.7)	171 (43.5)
1/2	257 (47.2)	185 (47.1)
2/2	44 (8.1)	37 (9.4)

Abbreviations: IL12 = interleukin 12; IL12R = interleukin 12 receptor; RA = rheumatoid arthritis; UTR = untranslated region.

[a] Allele 1, Q214-M365-G378; allele 2, R214-T365-R378.

TABLE 2 Distribution of *IL12B* and *IL12RB1* genotypes by clinical and demographic features of RA patients

Genotype	Shared epitope (%)	Rheumatoid factor (%)	Rheumatoid nodules (%)	Extraarticular disease (%)	Female sex (%)	Age at onset (mean ± SD years)
IL12Bpro						
1/1	51	73	14	25	83	51.7 ± 12.1
1/2	59	74	16	27	84	48.3 ± 12.9
2/2	63	80	13	25	83	53.1 ± 13.3
IL12B 3′ UTR						
A/A	59	77	15	26	74	51.6 ± 13
A/C	56	72	14	19	77	49.2 ± 12.6
C/C	59	80	20	53[a]	75	58 ± 15.9
IL12RB1						
1/1	59	72	17	22	79	49.2 ± 12
1/2	58	77	18	28	72[b]	50.9 ± 14
2/2	50	67	28	36	90	52.9 ± 13.5

Abbreviations: IL12 = interleukin 12; IL12R = interleukin 12 receptor; Pro = promoter region; RA = rheumatoid arthritis; UTR = untranslated region.

[a] For genotype C/C vs A/A + A/C, $p = 0.01$, odds ratio (OR) 3.59 (95% confidence interval [CI] 1.13–11.44), and corrected $p = 0.06$.

[b] For genotype 1/2 vs 1/1 + 2/2, $p = 0.02$, OR 0.60 (95% CI 0.38–0.94), and $p_c = 0.12$.

number of stratifications performed by the Bonferroni inequality correction (Table 2).

We found that the *IL12Bpro* and *IL12B* 3′ UTR polymorphisms revealed linkage disequilibrium (Table 3), although the most commonly occurring haplotypes did not appear to be associated with RA.

DISCUSSION

The present study analyzed the *IL12B* (*IL12pro* and *IL12B* 3′ UTR) and *IL12RB1* (641A→G, 1094T→C, and 1132G→C) polymorphisms in RA, but provided no evidence for association of the SNPs investigated with predisposition or severity of RA. This is the fist attempt to test the influence of *IL12pro* and *IL12RB1* polymorphisms in RA. The fact that we have not found an association of the polymorphisms tested with RA cannot be the result of a lack of power because our sample size had 80% power to detect the effect of a polymorphism, conferring an odds ratio of 1.5 at the 5% significance level (assuming an allele frequency of 50% in the control population). The frequency distribution of the *IL12B* promoter and *IL12B* 3′ UTR polymorphism was comparable to that described in European and Australian populations [15–17]. Our findings are in accordance with those of a previous study, where no association with *IL12B* 3′ UTR polymorphism was found in a group of patients with RA from the United Kingdom and Greece [16]. On the basis of the results of these two studies involving three European populations, the role of this polymorphism in susceptibility to RA can be excluded.

TABLE 3 Distribution of the most common occurring *IL12B* haplotypes in RA patients and healthy controls

IL12B haplotypes	RA patients (%) ($n = 393$)	Healthy controls (%) ($n = 545$)
IL12pro1-IL12B 3′ UTR A	118 (30)	164 (30)
IL12pro1-IL12B 3′ UTR C	71 (18)	104 (19)
IL12pro2-IL12B 3′ UTR A	197 (50)	262 (48)
Global D′	0.77	0.71

Abbreviations: IL12 = interleukin 12; Pro = promoter region; RA = rheumatoid arthritis; UTR = untranslated region.

A primary role of the *IL12B* 3′ UTR gene polymorphism in type 1 diabetes was proposed [18]. However, several large case-control and familial studies have failed to replicate the association between the *IL12B* 3′ UTR SNP and type 1 diabetes [19–21]. Similarly, a lack of association with the *IL12B* gene has been observed in a range of autoimmune diseases, such as celiac disease [22], multiple sclerosis [23], and Crohn's disease [17], 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 *IL12B* 3′ UTR may play in the susceptibility to autoimmune diseases, the biologic significance of this polymorphism has been also been called into question. Although several studies observed differences in the *IL12B* gene expression regarding the *IL12B* 3′ UTR genotypes [17, 18], other authors have not observed any correlation between *IL12B* genotypes and IL-12 expression [20, 24].

Our findings are in agreement with recent reports where no association with *IL12RB1*polymorphisms was found in type 1 diabetes [13, 25]. The frequency distribution of the *IL12RB1* polymorphism was similar to that described in a white population [13]. Further analysis to observe functional differences between *IL12RB1* haplotypes is important to speculate on their significance in Th1-mediated immune diseases. The fact that other *IL12RB1* polymorphisms not in linkage disequilibrium

with the alleles tested, or other genes in the IL-12 pathway, may contribute to development of RA cannot be excluded. In addition, cytokines that are involved in Th1 differentiation, such as IL-23, also become candidate genes for involvement in RA.

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Investigation of the *IL23R* gene in a Spanish rheumatoid arthritis cohort

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Summary Recently, a genome-wide association study identified the interleukin-23 receptor gene (*IL23R*) as an inflammatory bowel disease (IBD) associated gene. Given the involvement of *IL23R* in T-cell regulation, we decided to test whether this gene is associated with rheumatoid arthritis (RA). Eight *IL23R* gene polymorphisms (rs1004819, rs7517847, rs10489629, rs11209026, rs1343151, rs10889677, rs11209032, and rs1495965) were selected among the 10 most associated SNPs from the IBD study. A total of 322 RA patients and 342 healthy controls were genotyped for the selected SNPs using a Taqman 5′ allelic discrimination assay. We did not find statistically significant differences when we compared allele and genotype frequencies between RA patients and controls for none of the *IL23R* gene polymorphisms under study. We did not observe significant differences when RA patients were stratified according to their clinical and demographic features. We conclude that the *IL23R* gene does not seem to be associated with RA predisposition in a Spanish population.

Introduction

Rheumatoid arthritis (RA), one of the most common systemic autoimmune diseases, is characterized by chronic joint inflammation and subsequent joint destruction [1]. Although RA etiology is not fully understood, a strong genetic component has been established [2]. The best-characterized genetic factor contributing to RA is the association of different *HLA-DRB1* alleles with the disease. Estimates suggest that the HLA locus accounts for approximately one third of the total genetic component of RA susceptibility [3]. Therefore, it is suggested that other non-HLA genes may play a relevant role in RA susceptibility. To date, the most replicated and best studied association with RA besides the HLA locus is that found for the *PTPN22* gene [4–6]. However, a great number of genes contributing to RA susceptibility remain to be discovered.

Recently, a genome-wide association study identified several SNPs in the interleukin-23 receptor (*IL23R*) gene associated with inflammatory bowel disease (IBD) [7]. Shortly afterward, *IL23R* was found to be associated with psoriasis in an independent large-scale association study [8]. IL-23R is the receptor for interleukin-23 (IL-23), a pro-inflammatory

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ABBREVIATIONS

IBD	inflammatory bowel disease
IL	interleukin
IL23R	interleukin 23 receptor
MAF	minor allele frequency
OR	odds ratio
RA	rheumatoid arthritis
SNP	single nucleotide polymorphism

cytokine composed of a common p40 subunit (which is shared with IL-12) and a unique p19 subunit, and is involved in memory T-cell regulation [9]. Recent findings have shown an important role of IL-23 in RA, as IL-23–deficient mice are resistant to collagen-induced arthritis [10]. In addition, IL-23p19 is over-expressed in RA synovial fibroblasts [11]. Furthermore, expression of p19 induces inflammation in transgenic mice [12]. It is worth noting that *IL23R* is located on the chromosomal region 1p, which has been shown to have linkage to RA [13].

The co-localisation of susceptibility loci in genome-wide scan studies have led to the hypothesis that common genes may contribute to the susceptibility to autoimmune diseases [14]. In addition, case-control– and family-based association studies support this hypothesis [15]. Therefore, investigation of genes associated with related autoimmune diseases seems to be a good way of studying the genetic basis of RA.

Because of its functional relevance in RA, its position in the genome, and its association with other autoimmune diseases, we sought to test the possible role of *IL23R* polymorphisms in the susceptibility to RA in a Spanish population.

Subjects and methods

Subjects

A total of 322 RA patients meeting the American College of Rheumatology (ACR) 1982 revised criteria for RA [16] were recruited from Hospital Virgen de las Nieves in Granada, southern Spain. A total of 342 blood donors from the same city were included as healthy controls. The RA patients had been genotyped for *HLA-DRB1*. 75.3% of the RA patients were women; the mean age of onset was 50.3 ± 14 years. Of the patients, 55.7% carried the shared epitope; 75.8% were positive for the rheumatoid factor; 63% were positive for anti-cyclic citrullinated peptide autoantibodies; 20% presented with nodular disease; and 27% showed additional extra-articular manifestations.

IL23R SNP selection and genotyping

Selection of the SNPs was done on the basis of their previously reported association with IBD susceptibility [7]. Ten polymorphisms (rs1004819, rs7517847, rs10489629, rs2201841, rs11465804, rs11209026, rs1343151, rs10889677, rs11209032, and rs1495965) spanning the *IL23R* gene located in intronic, coding and 3′ untranslated regions were reported to be strongly associated with IBD, although, using the tagger algorithm, we determined that two of the SNPs (rs2201841 and rs11465804) were in complete linkage disequilibrium with rs10889677 and rs11209026, respectively ($r^2 = 1$, Hapmap CEU dataset) and were therefore not included in the study. Thus, eight of the previously studied *IL23R* genetic variants were analyzed in our population and genotyped using a Taqman 5′ allelic discrimination assay, by Taqman Pre-designed SNP Genotyping Assays (Applied Biosystems, Foster City, CA).

Statistical analysis

Both allelic and genotypic frequencies were calculated and compared by χ^2 tests using the Statcalc software (Epi Info 2002; Centers for Disease Control and Prevention, Atlanta, GA). Significance was calculated by 2 × 2 contingency table and Fisher's exact test. The Armitage trend test was applied to counts of genotypes when deviations from Hardy-Weinberg equilibrium were observed as recommended [17,18] and was implemented in the FINETTI software (Wienker TF and Strom TM, unpublished data http://ihg.gsf.de/cgi-bin/hw/hwa2.pl).

Statistical significance was considered at values of $p < 0.05$. The Haploview software was used to obtain linkage disequilibrium pairwise values, haplotype construction and to implement the tagger algorithm. Power calculations were performed with Quanto software (M. J. Gauderman, University of Southern California, 2005; http://hydra.usc.edu/GxE/).

Table 1 Case-control association study results for *IL23R* SNPs in a Spanish cohort.

			RA patients (n = 322)				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	167	131	24	0.28	170	137	35	0.30	0.32	1.12 (0.89−1.43)
rs7517847	A	C	109	159	54	0.41	121	153	68	0.42	0.77	1.03 (0.83−1.28)
rs10489629	A	G	91	160	71	0.47	78	192	72	0.49	0.40*	0.91
rs11209026	G	A	283	36	3	0.07	302	34	6	0.07	0.89*	0.93
rs1343151	G	A	119	139	64	0.41	136	157	49	0.37	0.12	0.84 (0.67−1.05)
rs10889677	C	A	143	151	28	0.32	167	134	41	0.32	0.82	0.97 (0.77−1.23)
rs11209032	G	A	170	125	27	0.28	161	137	44	0.33	0.04	1.27 (1.00−1.61)
rs1495965	A	G	109	155	58	0.42	135	139	68	0.40	0.51*	1.05

CI = confidence interval; MAF = minor allele frequency; OR = odds ratio; RA = rheumatoid arthritis.
* Armitage trend test.

Results

Allele and genotype frequencies for the eight *IL23R* SNPs tested in RA patients and healthy controls are shown in Table 1. Minor allele frequency (MAFs) in controls were similar to those found for a North American population in a previously published IBD study [7].

Genotypes at all loci were in Hardy-Weinberg equilibrium in our RA patients and healthy controls, except for rs10489629, rs11209026, and rs1495965 in the control cohort.

We did not find statistically significant differences when allele and genotype frequencies between RA patients and controls were compared for any of the polymorphisms under study. We did observe a trend for association with RA when comparing *IL23R* rs11209032 allele frequency between RA patients and healthy controls ($p = 0.04$), although it did not reach statistical significance when the Bonferroni correction was applied for multiple testing correction. Furthermore, the odds ratio (OR) showed a not relevant association (OR = 1.27, 95% confidence interval = 1.00–1.61). No statistically significant differences were found when we compared genotypic frequencies for all SNPs under study. In addition, haplotype analysis did not reveal significant association with RA (data not shown).

Next, we analyzed demographic and clinical characteristics of RA patients according to their *IL23R* genotypes (gender, age at disease onset, presence of shared epitope, rheumatoid factor, rheumatic nodules, and extra-articular disease). However, no significant differences were observed (data not shown).

Discussion

In this work, we have tried for the first time to assess the possible role of *IL23R* gene polymorphisms in RA predisposition. We selected *IL23R* as a candidate gene mainly for its recently reported association with a related autoimmune inflammatory disease, IBD [7,19,20]. This association has been replicated in an ethnically different Spanish population [21]. In addition, the SNP that showed the strongest association with IBD in the work by Duerr *et al*. has been associated with childhood-onset IBD in a Scottish population [22].

Although it seems that *IL23R* plays an important role in IBD susceptibility, we did not find an association with RA or with systemic lupus erythematosus in our cohort [23]. Although different autoimmune diseases share a common genetic basis, it seems that *IL23R* is not a common susceptibility gene for autoimmunity, as it does not play a relevant role in RA susceptibility. This lack of association was not caused by a type II error (false negative), as our sample size had ≥70% power to detect an association with an OR between 1.5 and 2 at the 5% significance level, assuming a dominant model. We found no association of the *IL23R* polymorphisms with RA clinical features. Despite this, we cannot discard a possible role of *IL23R* polymorphism in RA clinical subgroups, because of the insufficient power of this analysis, given the high degree of stratification performed.

The fact that *IL23R* is associated with organ-specific autoimmune diseases, such as IBD [7] and psoriasis [8], and not with systemic autoimmune diseases such as RA and systemic lupus erythematosus, suggest that *IL23R* may play a more important role in regulating local inflammation rather than systemic inflammation. This hypothesis is strengthened by recent findings that suggest that IL23 is essential for local tissue inflammation but not for systemic inflammation in mice [24].

Interestingly, in a previous work, we failed to find an association between *IL12B* and *IL12RB1* polymorphisms and RA [25] or systemic lupus erythematosus susceptibility [26]. Furthermore, our group searched for polymorphism in the *IL23A* gene by sequencing the entire gene, including 5′and 3′ regions, in 30 individuals; however, no sequence variations were found [26]. Our data, together with those from other groups, possibly rule out the IL-12 cytokine family members as key regulators of RA genetic predisposition. Nevertheless, little is known about IL-27, the other member of the IL-12 family, and genetic susceptibility to autoimmunity. Thus further investigation is needed in this regard.

It has been shown that IL-23 drives the development of a novel T-cell subset characterized by the production of the proinflammatory cytokine IL-17 (Th17), which plays a central role in mediating chronic inflammatory responses [27,28]. IL-17 has been proposed as a key molecule in the etiopathologic pathways that drive to the development of RA. IL-17 is present in the sera, synovial fluids, and synovial biopsy samples from most RA patients, whereas it is absent in osteoarthritis [11,29–32]. Therefore, it would be of great interest to analyze whether *IL17* gene polymorphisms play a role in RA genetic susceptibility.

Functional studies regarding the phenotypic effect of the SNPs associated with IBD in the previous work by Duerr *et al*. are lacking [7]. Given that the most associated SNP, Arg381Gln, is located near the first tyrosine phosphorilation site, it is temping to speculate that it may have functional consequences in the transducing pathway triggered by the binding of IL-23 to its receptor. Studies revealing the functional relevance of *IL23R* SNPs are needed to clarify the way by which the polymorphisms exert their influence in IBD and psoriasis susceptibility.

In conclusion, our data suggest that the *IL23R* gene polymorphisms do not play a relevant role in the susceptibility to RA in a Spanish Caucasian population, which is in contrast with the results previously obtained for IBD. Additional studies in other ethnically different cohorts will be necessary before completely discarding *IL23R* polymorphisms as RA susceptibility markers.

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Macrophage migration inhibitory factor gene: Influence on rheumatoid arthritis susceptibility

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KEYWORDS
MIF gene;
Rheumatoid arthritis susceptibility;
Early-onset

Summary The macrophage inhibitory factor (MIF) is a cytokine that has been implicated in several inflammatory and autoimmune diseases, including rheumatoid arthritis, systemic lupus, glomerulonephritis, and multiple sclerosis. In rheumatoid arthritis (RA), results ranging from lack of association of *MIF* polymorphisms with RA, to involvement in either severity or susceptibility to the disease have been reported in the past. We aimed at investigating the role of this gene in RA in the Spanish population. Two well-known *MIF* promoter polymorphisms were tested in 606 adult RA patients and 886 healthy controls: a single nucleotide polymorphism at −173G/C and a tetranucleotide repeat $(CATT)_{5-8}$ located at −794. We found a significant association of the allele −173C with RA ($p = 0.01$; odds ratio [OR] = 1.31; 95% confidence interval [CI] = 1.06–1.62). The −173C risk allele, previously reported to be transmitted in excess in patients with juvenile idiopathic arthritis, was significantly more frequent in early-onset adult RA patients than in healthy controls ($p = 0.003$; OR = 1.57; 95% CI = 1.14–2.15), whereas late-onset patients were not significantly different to controls ($p = 0.6$; OR = 1.09; 95% CI = 0.77–1.55). In conclusion, the −173C allele in the *MIF* promoter region is associated with increased RA predisposition, mainly in early-onset patients.

Introduction

Rheumatoid arthritis is an autoimmune disease characterized by synovial inflammation of multiple joints, affecting approximately 1% of the population worldwide. RA susceptibility is complex, with both genetic and environmental factors influencing predisposition. Twin and family studies suggest that approximately 60% of susceptibility is caused by genetic factors, the HLA locus being the main contributor [1]. The shared epitope hypothesis pointed to a short amino acid sequence common to all HLA-DRB1 alleles found to be associated with RA [2]. The identification of additional ge-

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ABBREVIATIONS

MIF macrophage inhibitory factor
RA rheumatoid arthritis

netic risk factors for RA is an ongoing process that will aid in the understanding of its etiology. One of these risk determinants that has captured attention during past years is the macrophage migration inhibitory factor (MIF). This cytokine has been shown to be a key regulator of innate and adaptive immune responses (for a review see [3]). MIF enhances the expression of proinflammatory cytokines and adhesion molecules and inhibits p53-dependent apoptosis [4–6]. MIF also increases the expression of metalloproteinases, exacerbating the destructive process in the joints [7]. MIF protein levels are elevated in serum and synovial fluid of RA patients as compared with healthy controls [8], and *in vitro* addition of MIF antagonists to synoviocytes inhibit inflammatory pathways [9]. Moreover, treatment with neutralizing anti-MIF antibodies led to delayed onset and lower frequency of collagen-induced arthritis in mice [10] and administration of endotoxin to *MIF*-deficient mice results in decreased macrophage viability and proinflammatory function, and in increased apoptosis compared with wild-type controls [11]. In summary, MIF takes part in several features involved in RA disease and its therapy [12,13].

A short gene located on chromosome 22q11.2 encodes the 12.5-kDa MIF protein [14,15]. Given the proinflammatory activities of MIF *in vivo* and *in vitro*, two polymorphisms in the promoter region of this gene have been analyzed in the past. One is a G/C change at −173 and the other a $(CAAT)_{5-8}$ tetranucleotide repeat at −794, being the −173C allele in strong linkage disequilibrium with the $(CAAT)_7$ repeat allele. This −173C allele creates a putative binding site for the transcription factor AP4, and it has been shown to affect promoter activity in a cell-type–dependent manner [16]. Several association studies with RA in different populations have been published to date. Two of them found a role of the *MIF* polymorphisms with RA severity in adults from the United States and the Netherlands [17,18], whereas two other studies reported changes in disease susceptibility [19,20]. Moreover, in two studies, a lack of association with RA has been reported, although in one of them the power of the study could be compromised, as they tested both *MIF* polymorphisms in a small cohort (from Germany) [21,22]. Considering all these apparently contradictory results, we endeavored to replicate the association of these MIF polymorphisms in a well-powered adult RA cohort from an independent population.

Subjects and methods

Patients and control subjects

The study group consisted of 606 adult, unrelated, white, Spanish patients with RA (62% women) consecutively recruited from three centers (Hospital Clínico, Madrid; Hospital La Paz, Madrid; and Hospital Virgen de las Nieves, Granada). The RA diagnosis was established based on the American College of Rheumatology (ACR) criteria for diagnosis [23] and the samples were previously genotyped for HLA-DRB1. Phenotypic details were obtained with the clinical history and personal interviews with patients. The mean age at onset was 54 ± 14 years. Of the patients, 61% carried the shared epitope (SE); 66% and 50% were positive for rheumatoid factor and for cyclic citrullinated peptide, respectively; and 32% presented nodular disease. A group of 886 healthy unrelated subjects (61% women and 39% men, mainly hospital employees and blood donors) from both Madrid and Granada were selected as controls. Cases and controls were all white Spanish subjects and were included in this study after written informed consent. The Ethics Committees from the three hospitals approved the study.

MIF polymorphisms

The *MIF* −173G/C polymorphism was genotyped by an Assay on Demand (C___2213785_10; Applied Biosystems, Foster City, CA) following the manufacturer's recommendations and analyzed in an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). The tetranucleotide repeat $(CATT)_{5-8}$, located −794 base pairs (bp) upstream of the transcription start site, was amplified with an annealing temperature of 56°C using the following set of primers: Forward: 5′ FAM-TTG CAC CTA TCA GAG ACC-3′; Reverse: 5′-TCC ACT AAT GGT AAA CTC G-3′.

Samples were subsequently denatured and run on an ABI Prism 3100 automatic sequencer (Applied Biosystems). Each sample included an internal size standard to achieve a highly consistent measure, and the results were analyzed using the GeneMapper software (Applied Biosystems).

Statistical analysis

Allele and genotype frequencies in patients and controls were compared by χ^2 test or Fisher exact test; p values were considered significant at a level of <0.05. Odds ratios (OR) and p values were calculated using a standard software package (Epi Info v. 6.02; Centers for Disease Control and Prevention, Atlanta, GA).

Results

The allelic frequencies of the two *MIF* promoter markers in healthy controls of our Spanish population (−173G/C: 87.3/12.7; $(CAAT)_{5-8}$: 25.5, 66.2, 8.2, and 0.1) resemble those previously described control subjects in a British study [19] (−173G/C: 88/12; $(CAAT)_{5-8}$: 25.3, 65.6, 8.8, and 0.3), but differ from those found in Germany [20] (−173G/C: 79/21; $(CAAT)_{5-8}$: 11, 63, 26, and 0) or Japan [24] (−173G/C: 77.7/22.3; $(CAAT)_{5-8}$: 39.4, 42.6, 17.4, and 0.6), evidencing genetic heterogeneity. Both polymorphisms conformed to the Hardy-Weinberg equilibrium in the Spanish cohorts. Linkage disequilibrium between the two alleles −173C and $(CAAT)_7$ has been described, and we corroborated it in our population ($D' = 1$; $r^2 = 0.6$).

Table 1 details the frequencies of the two MIF promoter markers in our adult RA patients and healthy controls. As shown for the −173G/C polymorphism, a significant difference in the overall distribution of genotype frequencies was observed between both cohorts ($p = 0.042$; $\chi^2 = 6.33$) and the −173C allele was associated with increased predisposition to RA ($p = 0.01$; OR = 1.3; 95% CI = 1.06–1.62). Because the MIF gene has been previously found associated with juvenile idiopathic arthritis, we classified our RA patients in tertiles by age at onset (early onset: 19–48 years, middle-age onset: 48–59 years; and late onset: 59–89 years; $n = 352$

each, as age at onset was not available for 78 patients). When early-onset patients were compared with controls (Table 2), the susceptibility allele −173C already reported in juvenile idiopathic arthritis was found associated (p = 0.003; OR = 1.57; 95% CI = 1.14–2.15), whereas late-onset patients were not significantly different from controls (p = 0.6; OR = 1.09; 95% CI = 0.77–1.55).

Conditioning for the presence/absence of either shared epitope, DAS28 calculated at onset, rheumatoid factor, or cyclic citrullinated peptide and gender stratification revealed no significant differences in the allelic distribution of the −173G/C polymorphism or the tetranucleotide repeat (data not shown).

Table 2 Analysis of −173G/C macrophage inhibitory factor *(MIF)* polymorphism in rheumatoid arthritis (RA) patients stratified by age at onset.

	Early-onset RA (n = 352)	Late-onset RA (n = 352)	Controls (n = 886)
GG	118 (67%)	132 (75%)	681 (77%)
GC	51 (29%)	40 (23%)	188 (21%)
CC	7 (4%)	4 (2%)	18 (2%)
G	287 (81%)	304 (86%)	1550 (87%)
C	65 (19%)	48 (14%)	224 (13%)

Early onset vs controls: −173C vs. G: p = 0.003; OR = 1.57; 95% CI = 1.14–2.15.

Discussion

MIF is a unique protein involved in inflammation and immune response. Data ranging from lack of influence of the *MIF* gene in adult RA predisposition [21,22] to a role of this gene either in severity [17,18] or in susceptibility [19,20] to the disease have been published, and impelled us to study two polymorphic markers in the promoter region of the gene. The data point toward a susceptibility effect of the *MIF* gene in our population, given that the −173C allele yielded a significant difference between RA patients and healthy controls. The effect of the gene seems to be stronger in early-onset adult RA patients. One possible explanation for the seemingly opposing previous reports could be population heterogeneity, which can be demonstrated even among European populations, as recently noted by Daly and Rioux [25]. However, an additional factor that may explain some of the discrepancy in association between *MIF* and RA is the impact that age has on disease susceptibility. Heterogeneity in the distribution by age among cohorts could explain the differences observed in previous studies with different populations. The effect detected in the youngest adult RA subgroup could be easily overlooked in a RA cohort enriched with elderly patients and, on the contrary, an overall increase in RA susceptibility caused by *MIF* polymorphisms would be only evidenced when patients with an early debut were globally more abundant.

Table 1 Frequencies of macrophage inhibitory factor (*MIF*) polymorphisms in Spanish rheumatoid arthritis (RA) patients and control subjects.

Polymorphism	RA patients (n = 606)	Controls (n = 886)
−173G/C *MIF*		
GG	431 (71%)	681 (77%)
GC	157 (26%)	188 (21%)
CC	18 (3%)	18 (2%)
G	1019 (84%)	1550 (87%)
C	193 (16%)	224 (13%)
$(CAAT)_{5-8}$ *MIF*		
5	342 (28%)	472 (27%)
6	758 (63%)	1160 (65%)
7	111 (9%)	141 (8%)
8	1	1

−173C vs G allele: p = 0.01; OR = 1.31; 95% CI = 1.06–1.62.

Both polymorphisms are putative functional genetic markers; the $(CAAT)_5$ allele showed reduced basal and serum forskolin-stimulated transcriptional activity, lower than any other $(CAAT)_{6-8}$ allele *in vitro* [26]. Although the association of the various $(CAAT)_n$ and −173G/C alleles with transcriptional activity seemed to be cell-type dependent [27], significantly higher levels of human circulating MIF have been found in carriers of the −173C or of the $(CAAT)_7$ alleles [18,28]. The significant association of the −173C allele with increased RA risk would underlie the proinflammatory effect of elevated circulating/synovium MIF protein. The −173C risk allele, previously observed to be transmitted in excess in patients with juvenile idiopathic arthritis [27], was found associated after age stratification in the younger subgroup.

Our data and those from others support the etiologic origin of the increased MIF synovial/circulating levels found in RA patients as compared with healthy controls, disregarding the possibility that the observed increased expression of this cytokine would be a consequence of initial events in this autoimmune disease. An interesting emerging hypothesis is the common role of MIF in several diseases with an autoimmune multifactorial etiology and inhibition of its proinflammatory action is likely to be a therapeutic strategy, given the promising results obtained in the preclinical models [29]. Neutralization of MIF either with anti-MIF antibody or with chemically derived inhibitors of MIF activity could be a new tool for treatment of inflammatory disorders, including arthritis. Given the ability of MIF to antagonize the effects of glucocorticoids [30–32], the therapeutic inhibition of MIF would contribute to RA treatment. The description of the *MIF* gene as risk factor for RA offers important insights into the disease pathogenesis through the knowledge of the mechanistic pathways implicated in MIF proinflammatory effect.

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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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Autoantibodies, *HLA* and *PTPN22*: Susceptibility markers for rheumatoid arthritis.

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ABSTRACT

Objective: To analyze the relationship between the presence of autoantibodies (RF and anti-CCP), *HLA-DRB1* alleles and *PTPN22* 1858 C/T polymorphism and test the value of their combination as susceptibility markers for RA.

Methods: Patients with early arthritis were included. At entry in the cohort or during follow up, 191 patients fulfilled criteria for RA and 184 individuals suffered from other arthropathies. RF was measured by nephelometry and anti-CCP antibody by ELISA. HLA class II alleles were determined by PCR. Samples were genotyped for *PTPN22* 1858C/T variants using a TaqMan 5'-allele discrimination assay.

Results: The presence of SE alleles was strongly associated with anti-CCP and RF positive RA (P= $7.05 \cdot 10^{-10}$; OR 4.57, 95% CI 2.76-7.57, and P= $1.68 \cdot 10^{-6}$; OR 2.99, 95% CI 1.89-4.74, respectively). The combination of the *PTPN22* 1858T variant and anti-CCP antibodies gave a high specificity for the disease, and was significantly associated with RA (P= $8.86 \cdot 10^{-5}$; OR 10.05, 95% CI 1.88-53.73).

Conclusion: The combination of the T variant of the 1858 polymorphism of the *PTPN22* gene in combination with the presence of anti-CCP antibodies, preferentially in a SE-positive individual, is associated with the development of RA.

Keywords: Rheumatoid arthritis, autoantibodies, rheumatoid factor, cyclic citrullinated peptides, *HLA-DRB1, PTPN22* gene, polymorphism.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease, affecting up to 1% of the adult population worldwide. Although the precise aetiology of RA is unknown, a strong genetic component is well established [1].

The strongest and best-known genetic association with RA is that found for particular alleles of *HLA-DRB1* [2]. To date, the association of the 1858C/T polymorphism of the *PTPN22* gene with RA is the most robust and reproducible one outside the HLA region [3,4]. Recently it has been discovered that shared epitope (SE) *HLA-DRB1* alleles are exclusively associated with a subgroup of RA patients that test positive for auto-antibodies against CCP [5]. This has led to the establishment of a "citrullinated" SE hypothesis [6,7]. Furthermore, autoantibodies have also been related with *PTPN22* 1858C/T polymorphism. Subsequent studies have confirmed this suggestion, since both RF and anti-CCP autoantibodies have been associated with *PTPN22* 1858T allele [8-11]. And what is more, the combination of the 1858T variant and anti-CCP antibodies gave a 100% specificity for the disease and strongly predicted the future onset of RA [9].

In the light of these evidences, we tested the value of the combination of *PTPN22* 1858C/T polymorphism, *HLA-DRB1* alleles and the presence of RF and anti-CCP as susceptibility markers for the onset of RA using in an inception cohort of patients with inflammatory arthritis.

MATERIALS AND METHODS

Patients

Our cohort comprised of 375 subjects (70% women, age 52,9 ± 16,4 years and disease duration before entry 14,9 ± 8,1 weeks). Patients were referred to the early arthritis clinic (EAC) of La Paz University Hospital, and were included when a rheumatologist confirmed arthritis at least in one joint and if the symptoms have been present <1 year. All patients were taking nonsteroidal antiinflammatory drugs (NSAIDs) and/or low dosis of oral corticosteroids and none received disease-modifying antirheumatic drugs (DMARD). Patients were excluded if they had crystal-induced, septic arthritis or inflammatory flares of osteoarthritis.

All patients were followed up every 6 months in the EAC for at least 2 years, unless they went into clinical and analytical remission and diagnosis was different from RA. At entry in the cohort or during follow up, 191 patients fulfilled 4 or more of the 1987 revised ACR criteria for RA [12], and the remaining 184 individuals suffered from other arthropathies (mainly undifferentiated arthritis, psoriatic arthritis, reactive arthritis or undifferentiated spondyloarthropathy).

At baseline, blood tests were carried out to determine acute phase reactants and rheumatoid factor (RF) and serum samples were stored at –40ºC for posterior determinations. In all patients autoantibodies were measured every 12 months and no change from negative to positive was seen in the first 2 years of the disease.

All the subjects were of Spanish Caucasian origin and were included in this study after informed consent. We obtained approval for the study from local ethical committee of the hospital.

Autoantibodies measurement

Rheumatoid factor was measured by nephelometry (Behring, Nephelometer Analyzer II), with a detection limit of 15 u/ml. Anti-CCP antibody was detected by a second-generation enzyme-linked immunosorbent assay (ELISA) in patient sera (Immunoscan RA Mark2; Eurodiagnostica, Arnhem, The Netherlands). The cut-off level for anti-CCP antibody positivity was set at 25 arbitrary units, according to the manufacturer´s instructions.

Genotyping methods

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), as previously described [4].

HLA genotyping

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

Statistical analysis

Statistical analysis to compare allelic and genotypic distributions was performed by χ^2 test calculated on 2x3 or 2x2 contingency tables. Statistical significance was $p<0.05$. For non-parametric data analysis, Mann-Whitney *U*-test was used for ordinal variables and Fisher's exact test for dichotomous variables. Multivariate stepwise logistic regression analysis was performed using the Statistical Package for the Social Sciences, version 10.0 (SPSS, Chicago, IL).

RESULTS

Seropositivity for anti-CCP and RF was strongly associated with RA ($P= 7.68\cdot10^{-28}$; OR 18.91, 95% CI 10.12-35.34 and $P= 2.83\cdot10^{-26}$; OR 11.92, 95% CI 7.26-19.61, respectively). However, the association for the RF was weaker than that found for anti-CCP. In addition, there was a strong association between RF positivity and anti-CCP positivity ($P= 3.64\cdot10^{-21}$; OR 38.82, 95% CI 14.54-103.66; Kappa= 0.667, standard error= 0.06, 95% CI 0.56-0.78).

As shown in Table 1, the presence of SE alleles was strongly associated with anti-CCP positive RA ($P= 7.05\cdot10^{-10}$; OR 4.57, 95% CI 2.76-7.57). Furthermore, the presence of DR3 allele (DRB1*0301) showed a high trend of protection against anti-CCP positive RA ($P=$ 0.01; OR 0.48, 95% 0.26-0.89). Similarly, DERAA alleles carriage seems to be protective for anti-CCP positive RA ($P=$ 0.01; OR 0.49, 96% CI 0.28-0.89). Interestingly, no association was observed with the SE in the anti-CCP negative RA patients, indicating that the SE does not associate with RA as such, but rather with a defined anti-CCP phenotype. In addition, we did not observe any association with DERAA and DR3 alleles in the anti-CCP negative RA patients group.

With regard to RF autoantibodies, the carriage of SE alleles was also associated with RA in the RF positive patients group, due to the strong correlation between RF positivity and anti-CCP positivity ($P= 1.68\cdot10^{-6}$; OR 2.99, 95% CI 1.89-4.74). Moreover, no association was observed with the SE in the RF negative RA patients. Furthermore, we found an association between the presence of the DERAA alleles and RF positivity, in a

protective way (P= 0.03; OR 0.57, 95% CI 0.34-0.98). However, we did not observe any association with the carriage of the DR3 allele in the RF positive RA group.

These data indicate that the SE alleles primarily predispose to the presence of anti-CCP antibodies, and are not an independent risk factor for the development of RA.

Regarding the *PTPN22* 1858C/T polymorphism, the genotype and allele distribution of the SNP were in agreement with the Hardy-Weinberg equilibrium among both the RA patients and the non-RA patients. Allele and genotype frequencies did not differ significantly from those described in a Spanish population [4]. However, when allele or genotype frequencies were compared, no statistically significant differences were found. Thus, it seems that *PTPN22* itself does not predispose to the onset of RA in our inception cohort of patients with inflammatory arthritis from Spain, which is in contrast with the previously reported association of *PTPN22* with RA compared to the general Spanish population [4].

Next, we analyzed the relationships between the *PTPN22* 1858C/T polymorphism and antibodies to CCP and the RF (Table 2). The combination of the 1858T variant and anti-CCP antibodies gave a high specificity for the disease (83.2%, calculated as number of patients with a true negative test divided by the total number of patients without the disease), since only one of the controls presented this combination. This patient suffer from palindromic rheumatism which has been described to be an abortive for of RA and without treatment can progress to RA [13]. Carriage of the *PTPN22* 1858T variant and presence of anti-CCP antibodies were significantly associated with the development of RA, compared with the rest of combinations (P= $8.86 \cdot 10^{-5}$; OR 10.06, 95% CI 1.88-53.73), whereas the combination of the lack of the T variant of the SNP and anti-CCP negativity was strongly protective (P= $8.2 \cdot 10^{-21}$, OR 0.12, 95% CI 0.07-0.19). We also observed an association with RA for the combination of the T variant and the presence of RF antibodies (P= 0.002, OR 4.18 95% CI 1.47-11.91), however, this association was much weaker than that found for the combination of the *PTPN22* T allele and anti-CCP autoantibodies.

The distribution in RA patients and non-RA patients in the combination of the presence of SE, anti-CCP and T allele of the *PTPN22* polymorphism did not reach statistically significant differences, probably due to the relatively low number of individuals included in the study and the high degree of stratification performed (data not shown).

Using multivariate stepwise logistic regression analysis with all variables included and RA diagnosis as a dependent variable, only anti-CCP and RF autoantibodies entered in the forward and backward method (OR 22.4 95% CI 11.2-44.6 and 9.8 95% CI4.3-22.0, respectively).

DISCUSSION

The most interesting findings of this work are the following: (1) The presence of the SE allele is strongly associated with anti-CCP positivity, although the SE does not associate with RA as such, but rather with a defined anti-CCP phenotype, and (2) The combination of the 1858T variant and anti-CCP antibodies gave a high specificity for the disease. Therefore, we have replicated

previously described remarkable findings in an ethnically different Spanish cohort [5,9,14-16]. Despite of the high specificity for RA given by the 1858T variant of the *PTPN22* gene and seropositivity for anti-CCP, this test had low sensitivity for RA diagnostic. Therefore, in order to establish a combination of variables as a diagnostic tool, it would be necessary to include other markers of severity/outcome, such as radiological changes or bone erosions. Unfortunately, these data were not available for our cohort. However, our data suggest that *PTPN22* polymorphism and anti-CCP might be helpful as RA prognostic markers.

Our data, together with data from other groups [14,17-20] suggest that the SE alleles predispose for anti-CCP positivity rather than for RA. Thus, it has been proposed the hypothesis that HLA SE containing molecules play a role in the activation of CD4+ T cells through preferential presentation of citrullinated antigens. These citrulline specific T cells may provide the help required for the IgG antibodies response to citrullinated antigens. On the basis of these and other data, van Gaalen et al formulated a two-hit model for the pathogenesis of RA, which incorporates a novel "citrullinated" SE hypothesis [7].

In addition, we have found that the absence of *HLA-DRB1*0301* is associated with anti-CCP positive RA, which is slightly different from the finding by Verpoort et al and Irigoyen et al [21,22] who stated that *HLA-DR3* is associated with anti-CCP negative RA. They proposed that this association is not due to the *HLA-DR3* gene itself, but to other genes in linkage disequilibrium with it. In this sense, a microsatellite marker (MIB*350) that is part of an ancestral haplotype associated with DRB1*0301 was described as a risk factor for RA in Dutch and Spanish populations independently of DR3 [23,24]. Nevertheless, it remains unclear which locus inside this cluster is responsible for the association. A fine mapping of this locus could help to shed light on the different associations between DR3 and anti-CCP positivity in North American, Dutch and Spanish populations.

We found an association between the absence of the protective DERAA alleles and anti-CCP and RF positive RA. However, DERAA alleles were not associated with anti-CCP and RF negative RA. This is in contrast with previous findings, which show that the protective effect of DERAA is independent of the patient's anti-CCP status [21,25].

We have replicated the finding that carriage of the T allele of this polymorphism, in combination with seropositivity for anti-CCP autoantibodies predisposes for RA and has a high specificity for the disease [9], suggesting that the T variant could influence the outcome of RA once autoantibodies, such as anti-CCP autoantibodies, have developed. In fact, the only non-RA patient in this cohort with anti-CCP antibodies and the T variant, has palindromic rheumatism which has been described as an abortive form of RA [13]. Nevertheless, a relatively low number of patients were included in the study. Therefore, further investigation using bigger cohorts is needed in order to confirm the possible clinical application of these markers.

We did not observe any association of the T allele with RA in combination with the presence of the RF in accordance with previous works [4,26,27], which strengthens

the arising hypothesis that anti-CCP, rather than RF, are the autoantibodies that characterize RA. This also could explain why *PTPN22* 1858C/T is not associated with several autoimmune diseases in which autoantibodies are present, such as celiac disease [28], since the polymorphism was proposed as a common genetic marker for autoantibody-mediated autoimmune disease [29]. In the light of these evidences, it seems that the SNP could be involved in the regulation of some autoantibodies, such as anti-CCP, but not others, such as RF. Thus, the implication of *PTPN22* in autoimmunity seems to be conditioned to the autoantibody milieu of each disease.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

KEY MESSAGE

The combination of the T variant of the 1858 polymorphism of the *PTPN22* gene with the presence of anti-CCP antibodies, preferentially in a SE-positive individual, is associated with the development of RA.

Table 1.- Distribution of SE, DERAA and DR3 alleles in RA patients, stratified by the presence of anti-CCP and RF, and non-RA patients.

		RA patients (n= 191)											
		Anti-CCP positive (n= 116)			Anti-CCP negative (n= 75)			RF positive (n= 135)			RF negative (n= 56)		
	Non RA (n= 184), n (%)	n (%)	*P* value	OR (95% CI)	n (%)	*P* value	OR (95% CI)	n (%)	*P* value	OR (95% CI)	n (%)	*P* value	OR (95% CI)
SE +	66 (35.9)	84 (72.4)	$7.05 \cdot 10^{-10}$	4.57 (2.76-7.57)	22 (29.3)	0.31	0.76 (0.42-1.34)	85 (63)	$1.68 \cdot 10^{-6}$	2.99 (1.89-4.74)	21 (37.5)	0.82	1.09 (0.59-2.01)
SE -	118 (64.1)	32 (27.6)			53 (70.7)			50 (37)			35 (62.5)		
DERAA +	53 (28.8)	19 (16.4)	0.01	0.49 (0.28-0.89)	23 (30.7)	0.77	1.10 (0.61-1.97)	25 (18.5)	0.03	0.57 (0.34-0.98)	16 (28.6)	0.97	1.01 (0.52-1.95)
DERAA -	131 (71.2)	97 (83.6)			52 (69.3)			110 (81.5)			40 (71.4)		
DR3 +	47 (25.5)	16 (13.8)	0.01	0.48 (0.26-0.89)	16 (21.3)	0.47	0.81 (0.43-1.54)	23 (17)	0.07	0.61 (0.35-1.06)	9 (16.1)	1.14	0.59 (0.28-1.29)
DR3 -	137 (74.5)	100 (86.2)			59 (78.7)			112 (83)			47 (83.9)		

The following alleles were classified as SE positive: DRB1*0101, *0102, *0401, *0404, *0405, *0408, *0410 and *1001; and as DERAA positive: DRB1*0103, *0402, *1102, *1103, *1301, *1302 and *1416. ORs were calculated for each RA subgroup compared with non-RA individuals.

Table 2.- Distribution of the *PTPN22* 1858C/T SNP stratified by the presence of anti-CCP and RF antibodies.

Combination of variables	RA patients n= 191 (%)	Non-RA n= 184 (%)
PTPN22 CC + anti-CCP$^-$	68 (35.6)	153 (83.2)
PTPN22 CT+TT + anti-CCP$^-$	7 (3.7)	18 (9.8)
PTPN22 CC + anti-CCP$^+$	98 (51.3)	12 (6.5)
PTPN22 CT+TT + anti-CCP$^+$	18 (9.4)	1 (0.5)
PTPN22 CC + RF$^-$	50 (26.2)	139 (75.5)
PTPN22 CT+TT + RF$^-$	6 (3.1)	15 (8.2)
PTPN22 CC + RF$^+$	116 (60.7)	26 (14.1)
PTPN22 CT+TT + RF$^+$	19 (9.9)	4 (2.2)

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Cytotoxic T-lymphocyte antigen-4-CT60 polymorphism in rheumatoid arthritis

Key words:
CTLA-4; CT-60 SNP; rheumatoid arthritis; susceptibility

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Abstract: Cytotoxic T-lymphocyte antigen-4 (*CTLA-4*) is a functional candidate gene with susceptibility to rheumatoid arthritis (RA). The aim of this work was to investigate the possible influence of the recently described CT60A/G dimorphism of the *CTLA-4* gene in the susceptibility to RA in Spanish patients. A total of 433 RA patients and 398 control subjects were included in the study. Genotyping of CTLA-4 CT60 was performed using two different methods: polymerase chain reaction restriction fragment length polymorphism system using an amplification-created restriction site and a TaqMan 5′-allelic discrimination assay. In order to validate results obtained by different methods, a quality-control exercise was performed. No significant deviation in the distribution of the alleles or genotypes of the CT60 was found when we compared RA patient and control groups. In addition, no differences in CTLA-4 CT60 genotypic distribution was found when RA patients and controls were stratified by the presence or absence of the shared epitope. In conclusion, our results do not support an association between CT60A/G polymorphism and susceptibility to RA in the Spanish population, although the contribution of other positions located within the 3′ region of the *CTLA-4* gene to RA susceptibility cannot be discarded.

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Rheumatoid arthritis (RA) is a common autoimmune inflammatory disorder that is usually associated with progressive joint destruction. Although the pathogenesis of RA is unknown, the higher concordance of RA in monozygotic twins and the familial clustering provide evidence for the role of genetic factors in the pathogenesis of this disorder (1). Recent genome-wide linkage analyses in RA has confirmed that the major histocompatibility complex, which has been found to be associated with RA in many case–control studies, is a major susceptibility locus for RA (2–4). However, evidence for non-human leukocyte antigen (HLA) susceptibility loci has also been reported which confirms that RA is a complex multigenic trait.

T cells play a major role in the pathogenesis of RA, therefore, regulators of T-cell activity are candidates for influencing disease

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susceptibility. The cytotoxic T-lymphocyte antigen-4 (CTLA-4) molecule is a homolog for CD28, and both molecules and their common ligands (B7-1 and B7-2) constitute the B7/CD28-CTLA-4 co-stimulatory pathway for T-cell activation. Whereas the CD28/ligand interaction plays a critical role in increasing and maintaining the T-cell response initiated through T-cell antigen receptor engagement, the CTLA-4/ligand interaction has an inhibitory effect on T-cell activation and might contribute to peripheral tolerance (5, 6). Thus, *CTLA-4* is a functional candidate gene to susceptibility to RA. Several polymorphisms have been described in the *CTLA-4* gene, some of them are: −1722T/C (7) and −319C/T (8), both within the promoter region; +49A/G in the exon 1 (9); and a microsatellite $(AT)_n$ 3′UTR polymorphism (10) and a recently described CT60A/G dimorphism (SNP 3087243), which has recently been associated with a variety of autoimmune diseases, with the CT60 A allele being protective and the G allele increasing susceptibility (11, 12). The CT60 G allele was shown to be associated with lower mRNA levels of soluble CTLA-4 isoform, that could increase T-cell activation and might have an important role in determining susceptibility to autoimmune diseases (11).

It is likely that RA shares a common genetic background with other autoimmune diseases. We, therefore, investigated the possible influence of the recently described CT60A/G dimorphism of the *CTLA-4* gene in the susceptibility to RA in Spanish patients.

Materials and methods

Subjects

A total of 433 RA patients meeting the American College of Rheumatology (ACR) 1982 revised criteria for RA (13) and 398 control subjects were included in the study. RA patients and control individuals were recruited from two South of Spain hospitals: Hospital Universitario Virgen del Rocio (Seville) and Hospital Virgen de las Nieves (Granada). All the subjects, cases and controls, were of Spanish Caucasian origin and were included in this study after written informed consent. The mean age of controls at analysis was 45 ± 12, 75% were female and 25% were male. Patients and controls had been previously genotyped in HLA-DRB1.

CTLA-4 CT60 genotyping

DNA from patients and controls was obtained from peripheral blood using standard methods. Genotyping of CT60 was performed using two different methods. Samples from Seville were genotyped by polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) system using an amplification-created restriction site method, as previously described, was performed (12). Briefly, a fragment of 216 bp of *CTLA4* gene was amplified using the following primers: sense 5′CACCACTATTTGGGATATACC3′ (the nucleotide deliberately changed to introduce a restriction site is shown underlined) and antisense 5′AGCTCTATATTTCAGGAAGGC3′. The PCR product was digested with the restriction enzyme *Nco*I (Boehringer Mannhein, Germany). Samples demonstrating only a 216 bp band were assigned as GG, samples demonstrating only a 196 bp band were typed as AA, and samples demonstrating two bands of 216 and 196 bp were assigned as AG. Samples from Granada were genotyped for CTLA-4 CT60 variants using a TaqMan 5′-allelic discrimination assay; the primers, probes, and reaction conditions were similar as previously described (11). In order to validate results obtained by different methods, a quality control consisting of CT60 genotyping in exchanged samples and sequencing of selected samples was also performed.

Statistical analyses

Allelic and genotypic frequencies of CT60A/G were obtained by direct counting. Statistical analysis to compare allelic and genotypic distributions was performed by Chi-square test calculated on 2×3 or 2×2 contingency tables. The software used was STATCALC program (Epi Info 2002; Centers for Disease Control and Prevention, Atlanta, GA, USA). The *P*-values were corrected (P_c) by multiplying by the number of comparisons. Statistical significance was $P < 0.05$.

Results and discussion

To check the quality control, no discrepancies between results obtained by the two different methods used were found. The allele and genotype distribution of CTLA-4 CT60 marker in the case–control cohorts from Granada and Seville showed no significant differences between these two case–control cohorts, hence we combined the two cohorts to form a RA case–control group which was used in the further analysis. The genotypic frequencies were not found to be significantly different from those predicted by the Hardy–Weinberg equilibrium in healthy controls. No significant deviation in the distribution of the alleles or genotypes of the CT60 was found when we compared RA patient and control groups (Table 1). In addition, no differences in CTLA-4 CT60 genotypic distribution was found when RA patients and controls were stratified by the presence or absence of the shared epitope (Table 2).

In order to address clinical heterogeneity, subgroup analysis of more phenotypically homogeneous subgroups of patients may be undertaken. Therefore, we analysed demographic and clinical characteristics of RA patients (rheumatoid factor, rheumatic nodules, extra-articular disease, sex, and mean age at disease onset) according

Distribution of the CTLA4 genotypes and alleles in RA Spanish patients and healthy controls

	RA (%)	HC (%)
Genotypes	$n=433$	$n=398$
AA	118 (27.3)	98 (24.6)
AG	198 (45.7)	199 (50.0)
GG	117 (27.0)	101 (25.4)
Alleles	$2n=866$	$2n=796$
A	434 (50.1)	395 (49.6)
G	432 (49.9)	401 (50.4)

RA, rheumatoid arthritis; HC, healthy controls.
No significant differences were found.

Table 1

Distribution of the CTLA-4 genotypes and alleles in RA Spanish patients and healthy controls stratified according to the presence or absence of the human leukocyte antigen (HLA)-shared epitope

	Presence of HLA-shared epitope		Absence of HLA-shared epitope	
	RA (%)	HC (%)	RA (%)	HC (%)
Genotypes	$n=207$	$n=143$	$n=151$	$n=252$
AA	52 (25.1)	29 (20.3)	49 (32.5)	68 (27.0)
AG	100 (48.3)	76 (53.1)	60 (39.7)	123 (48.8)
GG	55 (26.6)	38 (26.6)	42 (27.8)	61 (24.2)
Alleles	$2n=414$	$2n=286$	$2n=302$	$2n=504$
A	204 (49.3)	134 (46.9)	158 (52.3)	259 (51.4)
G	210 (50.7)	152 (53.1)	144 (47.7)	245 (48.6)

RA, rheumatoid arthritis; HC, healthy controls.

Table 2

to their CTLA-4 CT60 genotypes and alleles, and no association was found with any of the parameters tested (data not shown).

Recently, genotyping data in multiple SNPs obtained in a study of other autoimmune diseases (Grave's disease, autoimmune hypothyroidism, and type 1 diabetes) suggest the presence of a common locus of susceptibility in the 6.1-kb 3′ region of *CTLA-4* gene (11). The strongest association in the region corresponds to the marker CT60 (SNP3087243) which is located more than 800 nucleotides after the $(AT)_n$ microsatellite region, whereas association with the promoter region and the exon 1 was ruled out. The CT60 allelic variation was reported to be correlated with lower mRNA levels of the soluble form of CTLA-4 (sCTLA), suggesting that differential expression of alternatively spliced forms of CTLA-4 might have an important role in determining susceptibility to autoimmune diseases. In addition, a clinical trial demonstrated a significant improvement in patients with RA after treatment with CTLA-4Ig (24), thus, in the same way, genotypes producing higher levels of sCTLA-4 could protect against the development of RA.

This is the first study looking for an association between the recently described CT60A/G polymorphism in the *CTLA-4* gene and the susceptibility to RA, although during the preparation of this manuscript an article was published (14). Barton et al. (14) investigated the association of seven CTLA-4 polymorphism with RA and, in concordance with our data, the authors failed to find an association with none of the CTLA-4 polymorphisms analysed, including the CT60 variation and RA in a British population. The sample size used in our study had 85% power to detect the relative risk for the polymorphism (considering an odds ratio 1.66–2.00) at the 5% significance level. These results would discard any association of the CTLA-4 CT60 with RA. However, association with other polymorphic positions located within the 3′ region of the gene and involved in the alternative splicing could be possible.

CTLA-4 has been proposed as a general susceptibility locus in autoimmune diseases. Nevertheless, in the case of the RA, the majority of the studies that have focused on the polymorphism located at exon 1 (15–23), have not been conclusive and sometimes contradictory. Practically all these studies failed to find a direct association between CTLA-4 polymorphism and RA, although several groups have reported a possible interaction between HLA-DRB1 specificities and CTLA-4 exon 1 polymorphism. Nevertheless, these results could not be replicated in other studies. We explored the possibility that CTLA-4 CT60 variation can only act in certain HLA-DRB1 backgrounds. However, no evidence of association was observed when the RA patients and control were stratified by the presence of shared epitope alleles, which is in agreement with the data in Barton et al. (14).

In summary, our results do not support an association between CT60A/G polymorphism and susceptibility to RA in the Spanish population, although the contribution of other positions located within the 3′ region of the *CTLA-4* gene to susceptibility to the disease cannot be discarded.

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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 *STAT4* gene with rheumatoid arthritis: A replication study in three European populations

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ABSTRACT

Objective: To study the previously reported association of the *STAT4* polymorphism rs7574865 with rheumatoid arthritis (RA) in three different European populations from Spain, Sweden and the Netherlands, comprising 2,072 patients and 2,474 controls in total.

Methods: Three different collections were included in the study: 923 RA patients and 1296 healthy controls from Spain, 273 RA patients and 285 healthy controls from Sweden, and 876 RA patients and 893 healthy controls from the Netherlands. DNA from patients and controls was obtained from peripheral blood. Samples were genotyped for *STAT4* SNP rs7574865 using a TaqMan 5′-allele discrimination assay. χ^2 test was performed to compare the allelic and genotypic distributions.

Results: We observed a significantly increased frequency of the minor T allele in RA patients compared with healthy controls (24.8% vs 20.8%, *P*= 0.001, OR 1.26; 95% CI 1.09-1.45) in the Spanish population. This association was confirmed in both the Swedish population (*P*= 0.03, OR 1.35; 95% CI 1.03-1.77) and the Dutch population (*P*= 0.03, OR 1.45; 95% CI 1.21-1.73). The overall *P* value for all three populations was 9.79×10^{-6}, OR (95% CI) 1.25 (1.13-1.37). No association between rs7574865 and the presence of rheumatoid factor or anti-CCP was observed. A meta-analysis of all published *STAT4* associations revealed an OR of 1.25 (95%CI 1.19-1.33, $P=10^{-5}$).

Conclusion: We found an association of the rs7574865 *STAT4* polymorphism with RA in three different populations from Spain, Sweden and the Netherlands, thereby confirming previous data.

INTRODUCTION

Rheumatoid arthritis (RA) is the most common chronic autoimmune disease; it affects approximately 0.5-1% of the adult population worldwide. It is characterized by the chronic inflammation and destruction of the synovial joints, leading to progressive joint damage, and it is associated with significant disability and early mortality (1). RA etiology is complex, in common with other autoimmune disorders, and is not completely understood. However, it is known that RA risk is probably influenced by an interaction between environmental and genetic factors.

Data obtained in familial and twin studies suggest that up to 60% of disease susceptibility is due to genetic factors (2). The strongest and best-known genetic association with RA is that found for particular alleles of *HLA-DRB1* (3). Estimates suggest that the HLA locus probably accounts for no more than one-third of the total genetic component of susceptibility (4), so that many genes contributing to RA susceptibility remain to be discovered. To date, the association of the 1858C/T (rs2476601) polymorphism of the *PTPN22* gene with RA is the most robust and reproducible one outside the HLA region in European populations (5-7).

Despite past efforts to discover the genetic basis of RA, only a few studies have yielded significant results. Fortunately, this situation may be about to change due to recent genome-wide association studies (GWAS) that have detected a relatively large number of new, potential susceptibility loci (8, 9). The next challenge is to sift through these new putative susceptibility loci and determine which are indeed general risk

factors for RA, whether they are specific for certain populations, or even false-positives.

Recently, an RA linkage peak in chromosome 2q was detected in families of European ancestry (10). A follow-up study identified several polymorphisms in the third intron of the *STAT4* (signal transducer and activator of transcription 4) gene as the markers responsible for the signal in 2q. Four polymorphisms in tight linkage disequilibrium (LD) (rs11889341, rs7574865, rs8179673 and rs10181656, r^2>0.97 in Caucasians) form a susceptible haplotype tagged by the T allele of rs7574865, which showed the most significant association with RA and systemic lupus erythematosus (SLE) (11). The association of *STAT4* with RA has recently been replicated in a Korean population (12).

We aimed to determine whether the association of the *STAT4* polymorphism rs7574865 with RA could be extended to three different RA cohorts from Spain, Sweden and the Netherlands.

METHODS

Patients

Spanish cohort

A total of 923 RA patients meeting the American College of Rheumatology (ACR) 1982 revised classification criteria for RA (13) were recruited from Spanish hospitals. Their characteristics have been described elsewhere (7). A total of 1296 blood bank and bone marrow donors were used as healthy controls. All the subjects were of Spanish Caucasian origin, and they gave written informed consent for the study. The study was approved by the local ethics committees of the corresponding hospitals.

Swedish cohort

A total of 273 Swedish patients were recruited from the BARFOT (a Swedish abbreviation for Better AntiRheumatic PharmacOTherapy) registry, which includes patients participating in a structured program for follow-up of newly diagnosed RA patients in southern Sweden, from 1992–2005. This registry includes most of the adult patients with new onset of inflammatory polyarthritis and fulfilling the 1987 ACR classification criteria for RA (13) within the catchment areas of the six participating rheumatology centers of the BARFOT program (total population: approximately 1.5 million inhabitants) (14). All the subjects gave their written informed consent and the study was approved by all the regional ethics committees. We recruited 285 Swedish control subjects from the Uppsala Academic Hospital Blood Bank. All individuals were of Swedish ancestry and had four grandparents born in Sweden.

Dutch cohort

The Dutch RA group comprised 876 patients from Nijmegen (n= 599) and Groningen (n= 277). The patients from Nijmegen attend the outpatient clinic of the Department of Rheumatology at the Radboud University Nijmegen Medical Center or the outpatient clinic of the centres participating in the Dutch Rheumatoid Arthritis Monitoring (DREAM) register. The patients were diagnosed according to the ACR criteria (13) and belong to two prospective inception cohorts which have been described elsewhere (15). The RA patients from Groningen were recruited from the outpatient clinic of the Department of Rheumatology, University Medical Center Groningen (UMCG), the Netherlands; these

patients were diagnosed according to the ACR criteria (13) and all were RF-positive and/or erosive. A total of 893 unrelated Dutch individuals were selected for being born in the Netherlands and had at least three out of four grandparents also born in the Netherlands (16). All the patients and controls gave their informed consent and the medical ethics committees of the participating centres approved the respective original studies.

Genotyping methods

DNA from patients and controls was obtained from peripheral blood, using standard methods. Samples were genotyped for *STAT4* rs7574865 variants using a TaqMan 5′ -allele discrimination assay (Applied Biosystems, Foster City, CA, USA). Allele-specific probes were labeled with the fluorescent dyes VIC and FAM, respectively. PCR reaction was carried out in a total reaction volume of 4 μl with the following amplification protocol: denaturation at 95ºC for 10 min, followed by 40 cycles of denaturation at 92ºC for 15 sec and finished with annealing and extension at 60ºC for 1 min. Post-PCR, the genotype of each sample was attributed automatically by measuring the allelic-specific fluorescence on the ABI PRIM 7900 Sequence Detection Systems using SDS 2.3 software for allelic discrimination (Applied Biosystems, Foster City, CA, USA). Duplicate samples and negative controls were included to check the accuracy of genotyping.

Data analysis

Statistical analysis to compare allelic and genotypic distributions was performed using the χ^2 test. Odds ratios (OR) and 95% confidence intervals (95% CIs) were calculated according to Woolf´s method using the Statcalc modules implemented in Epi Info, version 2002 software for Windows, Centers for Disease Control and Prevention, Atlanta, GA, USA). *P* values less than 0.05 were considered statistically significant.

The meta-analysis of all published *STAT4* associations was conducted using Mantel-Haenszel test to calculate pooled OR. To accommodate the effect of different ethnic backgrounds on the association between *STAT4* and RA, heterogeneity between studies was tested using the χ^2 test, and the confidence interval for the odds ratio was estimated using a random effect model. Meta analysis was conducted using the Cochrane Review Manager software, version 4.1.2.

RESULTS

Table 1 shows the *STAT4* rs7574865 genotype and allele frequencies in patients and controls for our three independent collections of RA patients and controls of Spanish, Swedish and Dutch origin. Genotype frequencies were in Hardy-Weinberg equilibrium in patients and controls in all three populations.

In our Spanish cohort, rs7574865 GT and TT genotypes were present in RA patients at a significantly (*P* trend= 0.002) higher frequency than in controls [*P*= 0.01, OR 1.27 (95% CI 1.06-1.51), and *P*= 0.02, OR 1.56 (95% CI 1.07-2.30), respectively]. Accordingly, we observed a statistically significant increase of the minor T allele in RA patients compared to healthy controls (24.8% vs 20.8%, *P*= 0.001, OR 1.26; 95% CI 1.09-1.45; Table 1).

A similar effect was found in our Swedish cohort (*P* trend= 0.026). Carriers of rs7574865*T allele were more frequently RA

patients than controls (P= 0.02, OR 1.51, (95% CI 1.07-2.30) and P= 0.05, OR 2.01, (95% CI 1.00-4.08), for the GT and TT genotypes, respectively), and the overall frequency of the rs7574865*T allele was significantly increased in patients versus controls (28.9% versus 23.2%, P= 0.03, OR 1.35; 95% CI 1.03-1.77; Table 1).

This trend was further confirmed in our Dutch cohort, with similar increases in rs7574865 GT and TT genotypes and rs7574865*T allele (P trend= 0.03), although only TT showed a border line significance (P= 0.13, OR (95% CI) 1.16 (0.95-1.42) and P= 0.08, OR (95% CI) 1.43 (0.95-2.14), for the GT and TT genotypes respectively, and P= 0.03, OR 1.45; 95% CI 1.21-1.73 for the rs7574865*T allele; Table 1). Given these similar results between our three population cohorts, we combined them for an overall analysis, which showed strong evidence for association both with genotypes (P= 6.05×10^{-5}) and allele frequencies (P= 9.79×10^{-6}, OR 1.25; 95% CI 1.13-1.37 for the rs7574865*T allele). Interestingly, *STAT4* rs7574865 minor allele frequency (MAF) were similar to those found for other Caucasian populations in all three populations included in our study (11).

A meta-analysis of all the Caucasians populations studied to date showed an overall association between the *STAT4* polymorphism and RA (P= 1×10^{-5}, OR 1.25; 95% CI 1.18-1.33; Figure 1, the analysis included Spanish, Swedish and Dutch populations from our study, North American (NARAC and replication cohorts) populations, and the EIRA Swedish cohort) (11). This association remained unchanged when we included the data from the study in the Korean population (P=1×10^{-5}, OR 1.25 95%CI 1.19-1.33). Of note, the magnitude of the risk from the *STAT4**rs7574865 T allele did not differ significantly among the study populations, implying that the *STAT4* rs7574865 T allele may predispose to RA with a similar effect across different populations.

In addition, we tested whether the *STAT4* polymorphism was associated with the presence of serum autoantibody, such as rheumatoid factor (RF) and anti-cyclic citrullinated peptide autoantibodies (anti-CCP). For this we used the Spanish cohort, since autoantibody data was available for this population. We observed a statistically significant association of the T allele of the *STAT4* SNP in RA groups, both positive and negative for RF, compared with healthy controls [P= 0.0003, OR 1.36 (95% CI 1.15-1.60); P= 0.01, OR 1.36 (95% CI 1.07-1.74), respectively] (Table 2). In the same way, we found a statistically significant association of the T allele in RA groups both positive and negative for anti-CCP autoantibodies compared with controls [P= 0.001, OR 1.41 (95% CI 1.15-1.73); P= 0.03, OR 1.33 (95% CI 1.03-1.70), respectively] (Table 2). To further investigate the effect of the serum autoantibody on the *STAT4* association with RA, we analyzed the distribution of the rs7574865 genotypes and alleles among RA patients stratified according to their autoantibody status. We found a similar trend of distribution between RF+ and RF-, and anti-CCP+ and anti-CCP- groups, respectively. This finding suggests that the susceptibility to RA endowed by the rs7574865 T allele is not differential across RF+ or anti-CCP+ seropositivity in patients with RA.

DISCUSSION

Here we confirm the reported association of the rs7574865 *STAT4* polymorphism with RA in three independent collections of Spanish, Swedish and Dutch origin (11). Moreover, the overall association including 2072 RA patients and 2474 healthy controls was highly significant [$P=1\times10^{-4}$, OR 1.24 (95% CI 1.12-1.37)]. The association of rs7574865 was initially reported in individuals of European ancestry from the USA and Sweden and it was replicated in a Korean population (12). These findings, together with the data presented here, suggest that *STAT4* is a common RA susceptibility marker for European and Asian populations. Further replication in ethnically different cohorts will be required to definitively establish *STAT4* as a RA susceptibility marker. The hypothesis that *STAT4* is also a risk factor for other common autoimmune diseases, such as SLE, remains to be tested (11). Additional studies are needed to clarify the role of *STAT4* as a marker for novel common pathways involved in autoimmune diseases.

The Janus kinase and signal transducer and activator of the transcription (Jak-STAT) pathway is the signaling target of a multitude of cytokines that are thought to have biologically significant roles in rheumatoid synovial inflammation (17). In particular, STAT4 transmits signals induced by IL-12, IL-23 and type 1 interferons (18). A major action of IL-12 through STAT4 signaling is to promote the differentiation of naive $CD4^+$ T cells into T-helper (Th) 1 cells, which produce IFNγ. These Th1 cells are thought to drive the chronic autoimmune response (19). STAT4 is also important for the development of the recently identified IL-17 secreting Th cells in response to IL-23 (20). These Th17 cells play critical roles in autoimmune diseases, such as RA, through IL-17 production (21-23). Furthermore, IL-17 expression is increased in sera, synovial fluids and synovial biopsies of RA patients (24-28). Therefore, *STAT4* plays a key role in the regulation of Th1 and Th17 cells response. As both lineages are master regulators of RA ethiopathology in human, *STAT4* may exert its influence in RA through a defective signaling in these pathways. *STAT4* is also highly expressed in RA synovium compared with normal tissue (29-31).

Studies using animal models of autoimmunity have provided further evidence that *STAT4* is involved in these pathologies. Interestingly, $Stat4^{-/-}$ mice are resistant to proteoglycan-induced arthritis (32) and develop significantly less severe collagen-induced arthritis (CIA) than wild-type control mice (33). Moreover, the specific targeting of *STAT4* expression by antisense phosphorothioate oligonucleotide suppresses CIA (33). This suggests a possible therapeutic targeting directed to *STAT4*.

Although it seems clear that *STAT4* plays a key role in several pathways involved in RA pathogenesis, the functional role(s) of the associated polymorphism(s) remains to be elucidated.

Finally, a unique mature dendritic cell subset, apparently specific to seropositive RA patients that strongly express *STAT4* (31), has been identified. In view of the fact that these cells are correlated with the presence of serum rheumatoid factor, we investigated whether the association of *STAT4* with RA was dependent on RF and anti-CCP seropositivity in the Spanish cohort. Our

results suggest that *STAT4* is a risk factor for RA, independently of the presence of serum autoantibody. A previous report found similar results for anti-CCP in a Korean population (12). However, verification of this finding in larger populations is needed, since the autoantibody study in the Korean population and in the present study lack sufficient power to be able to assess the effect of RF and anti-CCP in *STAT4*'s contribution to RA risk convincingly.

In conclusion, we found an association of the rs7574865 *STAT4* polymorphism with RA in three different European cohorts from Spain, Sweden and the Netherlands, thereby confirming previous data.

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Table 1. Genotype and allele frequencies for *STAT4* rs7574865 polymorphism in RA patients and healthy controls in three populations.

	RA patients	Healthy controls	*P**	OR (95% CI)
Spanish[1]				
GG	521 (56.4)	813 (62.7)	-	1.0 (Ref.)
GT	347 (37.6)	428 (33.0)	0.01	1.27 (1.06-1.51)
TT	55 (6.0)	55 (4.2)	0.02	1.56 (1.07-2.30)
G	1389 (75.2)	2054 (79.2)	-	1.0 (Ref.)
T	457 (24.8)	538 (20.8)	0.001	1.26 (1.09-1.45)
Swedish[2]				
GG	134 (49)	169 (59.3)	-	1.0 (Ref.)
GT	120 (44)	100 (35.1)	0.02	1.51 (1.07-2.15)
TT	19 (7)	16 (5.6)	0.05	2.01 (1.00-4.08)
G	388 (71.1)	438 (76.8)	-	1.0 (Ref.)
T	158 (28.9)	132 (23.2)	0.03	1.35 (1.03-1.77)
Dutch[3]				
GG	503 (57.4)	552 (61.80)	-	1.0 (Ref.)
GT	313 (35.7)	295 (33.0)	0.13	1.16 (0.95-1.42)
TT	60 (6.8)	46 (5.2)	0.08	1.43 (0.95-2.14)
G	1319 (75.3)	1399 (78.3)	-	1.0 (Ref.)
T	433 (24.7)	387 (21.7)	0.03	1.45 (1.21-1.73)
Overall genotypic associations: [1]*P*=0.007, [2]*P*=0.28, [3]*P*=0.1.				

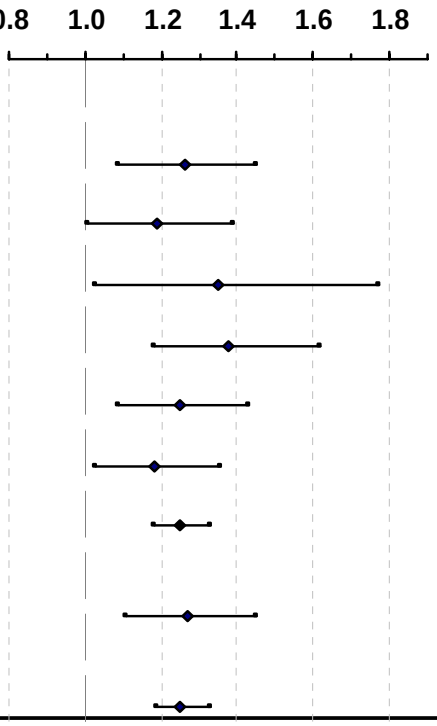
Table 2. Genotype and allele frequencies for *STAT4* rs7574865 polymorphism in Spanish RA patients, stratified by presence or absence of serum autoantibodies and healthy controls.

STAT4 rs7574865	Healthy controls n= 1296 (%)	RF+ n= 549	*P* value [1]	OR (95% CI)	RF- n= 202	*P* value [2]	OR (95% CI)	AntiCCP + n= 288	*P* value [3]	OR (95% CI)	AntiCCP- n= 187	*P* value [4]	OR (95% CI)
GG	813 (62.7)	299 (54.5)	-	1.00 (Ref)	109 (54)	-	1.00 (Ref)	152 (52.8)	-	1.00 (Ref)	106 (56.7)	-	1.00 (Ref)
GT	428 (33.0)	212 (38.6)	0.005	1.35 (1.08-1.67)	80 (39.6)	0.03	1.39 (1.01-1.93)	117 (40.6)	0.005	1.46 (1.11-93.)	66 (35.3)	0.32	1.18 (0.84-1.66)
TT	55 (4.2)	38 (6.9)	0.003	1.88 (1.19-2.96)	13 (6.4)	0.07	1.76 (0.89-3.45)	19 (6.6)	0.03	1.85 (1.03-3.30)	15 (8.0)	0.01	2.09 (1.09-3.97)
G	2054 (79.2)	810 (73.8)	-	1.00 (Ref)	298 (73.8)	-	1.00 (Ref)	421 (73.1)	-	1.00 (Ref)	278 (74.3)	-	1.00 (Ref)
T	538 (20.8)	288 (26.2)	0.0003	1.36 (1.15-1.60)	106 (26.2)	0.01	1.36 (1.07-1.74)	155 (26.9)	0.001	1.41 (1.15-1.73)	96 (25.7)	0.03	1.33 (1.03-1.70)

Overall genotypic associations: [1]*P*= 0.001, [2]*P*= 0.04, [3]*P*= 0.005, [4]*P*= 0.04. *P* values and ORs were obtained by comparing each RA group with controls.

Figure 1. Meta-analysis testing the association of the STAT4 rs7574865*T allele with RA in Caucasians and Asian populations. Data on the NARAC USA, the Replication USA, and the ERA studies are extracted from ref. 11, and from reference 12 for the Korean study. Abbreviations: n. Number of RA chromosomes, N. number of control chromosomes; #. The 95% confidence interval estimated using random effect model; *. The Mantel-Haenszel test for overall association of T allele to RA (P value)=0.00001, and test for heterogeneity P =0.8 (Chi^2 =3.01, df =6) and P =0.70 (Chi^2 =2.98, df =5) in overall analysis of all studies and total analysis of Caucasians, respectively. Vertical line represents no effect i.e. disease risk =1.0.

The study	RA patients n/N	Controls n/N	Odds Ratio (95% CI)#
Caucasians			
This study			
Spanish cohort	457/1846	538/2592	
Dutch cohort	433/1752	387/1786	
Swedish cohort	158/546	132/570	
NARAC study -USA	340/1212	576/2618	
Replication study -USA	527/2026	583/2652	
ERA study -Sweden	765/3058	388/1762	
Total*	2680/10440	2604/11980	
Asians			
Korean	795/2064	601/1816	
Overall*	3475/12504	3205/13796	



Letters to the Editor

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Study of the role of a functional polymorphism of *MHC2TA* in rheumatoid arthritis in three ethnically different populations

Sir, Rheumatoid arthritis (RA) is a chronic complex inflammatory disease that is thought to have an autoimmune origin. Although the precise aetiology of RA is unknown, a strong genetic component is well-established. The strongest genetic association with RA has been found for particular alleles of HLA-DRB1. Furthermore, it has been reported that the differential expression of HLA class II genes is associated with both susceptibility and progression of RA [1]. Also, it has been recently reported about an association between the more severe forms of RA (extra-articular RA) and the presence of the HLA-DRB1*04 allele as well as between the HLA C3 allele and vasculitis in RA [2, 3]. Therefore, it seems that regulatory factors of the expression of major histocompatibility complex (MHC) class II molecules could play an important role in the pathogenesis of RA, and polymorphisms in these genes might confer susceptibility to the disease.

The MHC class II transactivator (CIITA), encoded by the MHC II transactivator (*MHC2TA*) gene, is a major physiological regulator of the expression of MHC class II genes, and it maps to chromosome 16p13, a region which has shown linkage with RA [4]. Therefore, it is an attractive functional and positional candidate gene for RA. It has been recently reported that the –168A→G polymorphism (rs3087456) in *MHC2TA* is associated with RA, multiple sclerosis (MS) and myocardial infarction in a Nordic population [5]. In addition, the single nucleotide polymorphism (SNP) leads to reduced *MHC2TA* expression, and hence reduced production of MHC II molecules. Our aim was to replicate the reported association of the *MHC2TA* –168A→G SNP with RA in three ethnically different populations from Spain, Argentina and Sweden.

MHC2TA genotypes were in Hardy–Weinberg equilibrium in cases and controls for the three populations under study. An association was found when we compared allele frequencies between Spanish RA patients and healthy controls, with a higher frequency of the *MHC2TA*–168A allele in the RA group [$P = 0.01$, odds ratio (OR) 1.23 (95% confidence intervals (CI) 1.04–1.47)] (Table 1). In addition, the *MHC2TA*–168 AA genotype was more frequent among RA patients than in healthy controls [$P = 0.01$, OR 1.31 (95% CI 1.06–1.63)]. Next, we analysed the demographic and clinical characteristics of Spanish RA patients (gender, age at disease-onset, presence of shared epitope, rheumatoid factor, rheumatic nodules and extra-articular disease) according to the *MHC2TA*–168A→G genotype; however, no statistically differences were observed (data not shown). Additionally, we sought to replicate the association of the *MHC2TA*–168A→G SNP with RA in two ethnically different population from Argentina and Sweden. No evidence of association was observed in the RA Argentinean and Swedish sets (Table 1). Finally, the three independent cohorts were pooled in order to carry out a meta-analysis, showing that *MHC2TA*–168A→G SNP is not associated with RA (pooled OR 1.08, 95% CI 0.96–1.21, $P = 0.206$).

In an attempt to replicate the reported association of the *MHC2TA*–168A→G polymorphism with RA in three different cohorts, we found that, in contrast to the original study by Swanberg *et al.* [5], the A allele is the susceptibility allele in the Spanish population, not the G allele, while a lack of association was observed in the Argentinean and Swedish cohort. Notably, Akkad *et al.* [6] were not able to replicate the association of *MHC2TA*–168A→G polymorphism and RA in a German population.

Lack of replication of a previous association is a common event in the search for genetic determinant of complex human traits that may be due to a number of factors. The effects of genetic, population and clinical heterogeneity must be considered when attempting to detect susceptibility genes for RA in different populations. In this regard, we have found striking differences in allele frequencies for healthy controls between the Spanish and Argentinean populations, and those found in the Nordic population ($P = 5\times10^{-5}$ and $P < 10^{-7}$, respectively) [5]. Furthermore, these allele frequencies are statistically different between Spanish and Argentinean subjects ($P = 2\times10^{-6}$). Interestingly, Spanish allele frequencies were similar to those found in Germans [6]. However, allele frequencies for the Argentinean population were different from the German population ($P = 3\times10^{-6}$). It is worth mentioning that we have found genetic heterogeneity between the Swedish population from the original study by Swanberg *et al.* [5] and the Swedish population tested in the present study. Although the frequency of the RA patients was similar for the two Nordic populations, they differ in the allele frequency of the SNP in healthy controls ($P = 0.006$). Surprisingly, the frequencies in RA patients and controls for the Swedish population that we studied are comparable with those found in the Spanish population.

In light of this evidence, it seems possible that a cause of discrepancy could be genetic heterogeneity, which can be explained by differences in the haplotype structure of the *MHC2TA* locus across populations. However, the association found in the Spanish population between RA and *MHC2TA* –168A allele is possibly due to a type I error (false positive). The lack of replication of the original finding in two cohorts from Argentina and Sweden, and the results from the meta-analysis carried out pooling the three RA cohorts, also points to this hypothesis.

Other groups have evaluated the possible role of *MHC2TA* SNPs in autoimmune disease, but none of them have reached robust conclusions. *MHC2TA*–168A→G polymorphism is weakly associated with MS in an English population [7]. In a Japanese study a trend of association was found only between 485(A→A/G) *MHC2TA* and systemic lupus erythematosus [8].

In conclusion, it appears that this genetic variation on *MHC2TA* does not play a role in the susceptibility to RA. Inherent genetic differences in the allele frequencies and haplotype structure in the *MHC2TA* region in the populations studied can explain the lack of confirmation of the initial positive finding. This could be avoided using a gene-based replication approach, not allele-based, in which all genetic variants in the *MHC2TA* gene are re-examined for association with autoimmune diseases.

Table 1. Genotype and allele frequencies for *MHC2TA* rs3087456 polymorphism in RA patients and healthy controls

	Spanish cohort				Argentinean cohort				Swedish cohort			
MHC2TA –168A→G	RA patients $n=748$ (%)	Healthy controls $n=676$ (%)	P*	OR (95% CI)	RA patients $n=287$ (%)	Healthy controls $n=287$(%)	P**	OR (95% CI)	RA patients $n=278$ (%)	Healthy controls $n=478$ (%)	P***	OR (95% CI)
AA	444 (59.4)	356 (52.7)	0.01	1.31 (1.06–1.63)	114 (39.7)	109 (38%)	0.66	1.08 (0.76–1.53)	160 (57.6)	265 (55.4)	0.57	1.09 (0.80–1.49)
AG	262 (35)	274 (40.5)	0.03	0.79 (0.63–0.99)	117 (40.8%)	139 (48.4%)	0.06	0.73 (0.52–1.03)	94 (33.8)	184 (38.5)	0.19	0.82 (0.59–1.13)
GG	42 (5.6)	46 (6.8)	0.35	0.81 (0.52–1.28)	56 (19.5%)	39 (13.6%)	0.05	1.54 (0.96–2.47)	24 (8.6)	29 (6.1)	0.18	1.46 (0.80–2.66)
A	1150 (76.9)	986 (72.9)	0.01	1.23 (1.04–1.47)	345 (60.1%)	357 (62.2%)	0.46	0.92 (0.72–1.17)	414 (74.5)	714 (74.7)	0.92	0.99 (0.77–1.27)
G	346 (23.1)	366 (27.1)	0.01	0.81 (0.68–0.96)	229 (39.9%)	217 (37.8%)	0.46	1.09 (0.86–1.39)	142 (25.5)	242 (25.3)	0.92	1.01 (0.79–1.3)

*3×2 overall P-value 0.03.
**3×2 overall P-value 0.08.
***3×2 overall P-value 0.24.
Meta-analysis: pooled OR 1.08, 95% CI 0.96–1.21, $P=0.206$

For the Spanish cohort, a total of 748 RA patients meeting the American College of Rheumatology (ACR) 1982 revised classification criteria for RA [9] were recruited from three Spanish hospitals: Hospital Virgen de las Nieves (Granada), Hospital Xeral-Calde (Lugo), and Hospital Universitario La Paz (Madrid). A total of 676 blood bank and bone marrow donors of the corresponding cities were included as healthy controls. For the Argentinean cohort, 287 RA patients meeting the ACR 1982 revised classification criteria for RA [9] were recruited in a multicentre collaboration covering 22 Health Centra in Argentina, although only cases and controls from 3 centres were available for analysis in this study, and 287 blood donors were included as healthy controls. The Swedish patients were recruited from the BARFOT (a Swedish abbreviation for Better AntiRheumatic PharmacOTherapy) registry, which includes patients participating in a structured program for follow-up of newly diagnosed RA in southern Sweden, including 3100 patients from 1992–2005. This registry includes most adult patients with new onset of inflammatory polyarthritis within the catchment area of the six participating rheumatology centres of the BARFOT program (total population: approximately 1.5 million inhabitants) [10], including patients fulfilling the 1987 ACR classification criteria for RA [9]. All the subjects were included in this study after written informed consent. We obtained approval for the study from all local ethical committees of the corresponding hospitals. Samples were genotyped for *MHC2TA* polymorphism using a TaqMan 5′ allelic discrimination Custom TaqMan® SNP Genotyping Assays method (Applied Biosystems, Foster City, CA, USA). Duplicate samples and negative controls were included to ensure accuracy of genotyping. Statistical analysis to compare allelic and genotypic distributions was performed by the χ^2 test. Odds ratios (OR) 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 <0.05 were considered statistically significant. Pooled OR in the meta-analysis was performed by Mantel–Haenszel statistic, using StatsDirect software.

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Severe cutaneous manifestations in a child with refractory Kawasaki disease

Sir, We report a child with refractory Kawasaki disease (KD), who presented severe scattered crusting skin lesions as predominant manifestation of the disease.

Case report

A 17-month-old boy presented to our department with a 3 day history of fever reaching 39°C, resistant to amoxicillin, along with a maculopapular rash on the face, trunk and limbs. Past medical history was unremarkable. Routine laboratory work-up revealed erythrocyte sedimentation rate (ESR) 72 mm/h, C-reactive protein (CRP) 13.67 mg/dl (nv < 0.35), haemoglobin 10 g/dl, white blood cell count $26.50 \times 10^3/mm^3$, fibrinogen 837 mg/dl and sodium 129 mEq/l (Table 1). Microbiological evaluation for bacterial and viral infections, including adenovirus, cytomegalovirus, parvovirus, herpes and Epstein–Barr virus, Staphylococcus and Group A Streptococcus, were negative. Throat, nasopharyngeal and cutaneous swabs for culture were also negative. Chest X-ray and abdominal ultrasound were unremarkable. On the day after admission, he developed non-exudative conjunctivitis, cervical lymphoadenopathy and mucositis, and KD was suspected. Echocardiogram revealed normal systolic and diastolic left ventricular dimensions (29/19 mm), with normal fractional shortnening (32%) and ejection fraction (56%). The diameter of the left coronary artery was increased: 3.6 mm (*z*-score size for age: 2.5 mm). Intravenous immunoglobulin (IVIG) and aspirin were promptly administered on day 5 from the fever onset. Notwithstanding, fever rose up to 40°C and a significant worsening of the skin lesion occurred: itching and burning tender rash all over the body, scalp included. Cracked and scabbed lips appeared. A further IVIG cycle resulted unsuccessful in subsiding fever and cutaneous manifestations that later also presented blisters at the ankles and ear lobes. The ESR and CRP were still raised and platelet count rose up to $771 \times 10^3/mm^3$. On day 8, an echocardiogram confirmed the previously reported coronary lesion. Despite a third dose of IVIG on day 10, fever persisted and diffuse scabs progressively involving cheeks, forehead, eyelids and legs occurred (Fig. 1). Intravenous methylprednisolone (MP), 30 mg/kg, was then given on day 11, but two additional steroid pulses were required over the following days, due to the persistent spiking fever along with elevated inflammatory parameters. On day 16, fever dropped and skin alterations significantly improved. Peeling at fingers and toes was then noted. No changes were detected on echocardiogram and the boy was discharged on aspirin (3 mg/kg). At 1 month follow-up, he had complete resolution of skin lesions and echocardiogram showed normal coronary artery diameter. Artery peripheral involvement was excluded by systemic echo Doppler evaluation.

Table 1. Laboratory values

Variable	Day 3	Day 16
Erythrocyte sedimentation rate (mm/h)	72	56
C-reactive protein (mg/dl)	13.67	0.31
White cells (per mm^3)	5100	12.540
Neutrophils (%)	56	55
Platelets (per mm^3)	300.000	1.057.000
Haemoglobin (g/dl)	10	8.5
Sodium (mEq/l)	129	138
Albumin (%)	48.6	34.6
Fibrinogen (mg/dl)	837	732
C_3 (90–180)	112	
C_4 (10–40)	15	
IgG (mg/dl)	762	
IgA (UI/ml)	60	
IgM (mg/dl)	112	

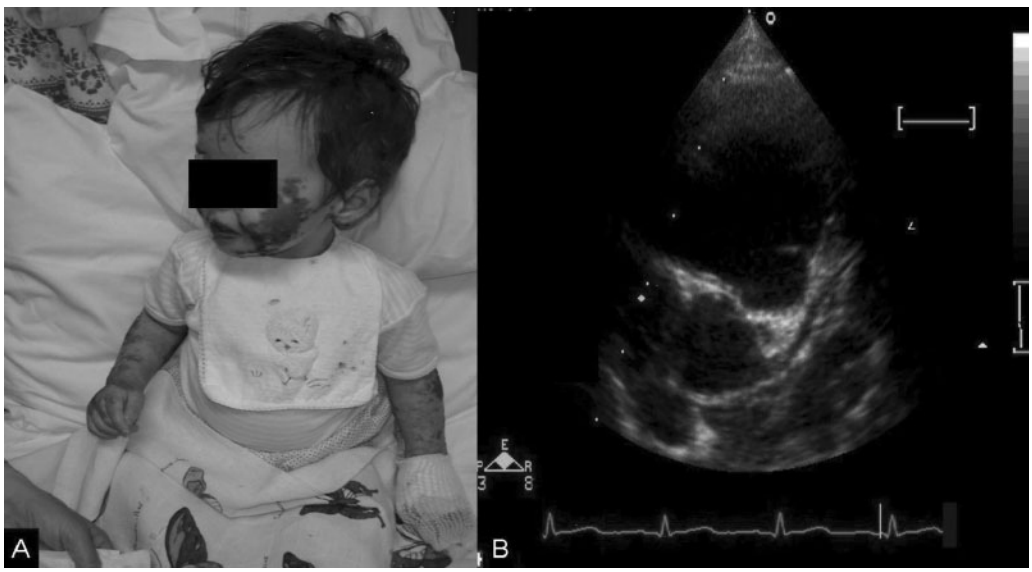

Fig. 1. **(A)** A 17-month-old boy with the Kawasaki disease: extensive cutaneous crusts all over the body. (**B**) Echocardiographic finding of dilatation of the left coronary artery.

ELSEVIER

Analysis of a GT Microsatellite in the Promoter of the *foxp3/scurfin* Gene in Autoimmune Diseases

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ABSTRACT: The aim of this study was to assess the possible association of the functional $(GT)_n$ microsatellite polymorphism in the *F0XP3* gene with predisposition to several autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), ulcerative colitis (UC), Crohn's disease, and celiac disease. We analyzed a case-control cohort composed of 231 SLE patients, 293 RA patients, 528 inflammatory bowel disease (354 Crohn's disease patients and 260 UC patients) patients, 103 celiac disease patients, and 274 healthy controls ethnically matched. Genotyping of $(GT)_n$ microsatellite was performed by polymerase chain reaction (PCR)-based method combined with fluorescent technology. We found no evidence for association of this polymorphism between controls and these autoimmune disease patients. Additionally, no differences in the genotype and allele distribution were found when patients were stratified according to clinical manifestation. The $(GT)_n$ microsatellite of the *F0XP3* gene may not play a relevant role in the susceptibility to SLE, RA, inflammatory bowel disease, and celiac disease in our population. *Human Immunology 66, 869–873 (2005).* © American Society for Histocompatibility and Immunogenetics, 2005. Published by Elsevier Inc.

KEYWORDS: *F0XP3* gene; systemic lupus erythematosus (SLE); rheumatoid arthritis (RA); ulcerative colitis (UC); Crohn's disease; celiac disease; autoimmune diseases (AID); microsatellite

ABBREVIATIONS

AID	autoimmune diseases
CI	confidence interval
HLA	human leukocyte antigen
IBD	inflammatory bowel disease
OR	odds ratio
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
RA	rheumatoid arthritis
SLE	systemic lupus erythematosus
T1D	type 1 diabetes
Tregs	regulatory T cells
UC	ulcerative colitis

INTRODUCTION

Autoimmune diseases (AID) affect approximately 5% of the population and are characterized by loss of self-tolerance causing immune-mediated tissue destruction [1]. AID share a number of characteristics that suggest common etiologic pathways or mechanisms, including reactivity to self-antigens, inflammatory manifestation, as well as genetic associations with human leukocyte antigens (HLA) [2]. Although the etiology of AID is

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unknown, it is widely accepted that both environmental and genetic factors are involved in the pathogenesis of these disorders [3]. The genetic background of AID is complex and likely involves multiple genes encoding proteins with significant functions in the regulation of the immune system.

Regulatory T cells (Tregs) are important components of the homeostasis of the immune system, because impaired regulatory T-cell activity can cause AID [4, 5]. There is compelling evidence that the role of Treg is not limited to the prevention of autoimmunity, but is important in controlling virtually all forms of immune response, including inflammation. *FoxP3/Scurfin* gene encodes a protein that is a member of the forkhead/winged-helix family of transcriptional regulators, and is specifically expressed in naturally occurring $CD25^+CD4^+$ regulatory T cells [6, 7]. Furthermore, retroviral gene transfer of Foxp3 converts naive T cells toward a regulatory T-cell phenotype similar to naturally occurring CD4+ regulatory T cells [7]. Thus, *Foxp3* is a master regulatory gene for the development of regulatory T cells.

A rare recessive monogenetic disorder called IPEX (immune dysregulation, polyendocrinopathy, including type 1 diabetes, enteropathy, and X-linked syndrome), is caused by a mutation in the *FOXP3* gene on human chromosome Xp11.23 [8]. Because of the pathologic role in the maintenance of the immune system and the variation in the balance between pro-inflammatory and anti-inflammatory cytokines, dysregulation of *FOXP3/Scurfin* gene expression may lead to the development of AID. Of interest, Bassuny *et al.* reported an association of a functional microsatellite polymorphism $(GT)_n$ of *FOXP3* gene with susceptibility to type 1 diabetes (T1D) in a Japanese population [9].

There is increasing evidence that AID share a common genetic risk factor, which is suggested by the familial aggregation of autoimmunity and also that the chromosomal region linkage to AID tends to overlap. Taking into account these findings, the aim of our study was to examine the possible influence of the functional $(GT)_n$ polymorphism in the *FOXP3* gene on genetic predisposition of a panel of autoimmune diseases, namely rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD), and celiac disease.

PATIENTS AND METHODS

Patients

A total of 231 SLE patients, 293 RA patients, 528 IBD patients (354 Crohn's disease patients and 260 UC patients), 103 celiac disease patients, and 274 healthy controls included in this study were recruited from Hospital Virgen de las Nieves (Granada) and Hospital Clínico San Cecilio (Granada), Hospital Puerta del Mar (Cadiz), and Hospital Materno-Infantil (Granada). SLE and RA patients fulfilled the classification criteria of the American College of Rheumatology [10, 11]. Celiac disease patients were diagnosed following the European Society for Pediatric Gastroenterology and Nutrition criteria for celiac disease [12]. IBD patients were diagnosed according to standard clinical, endoscopic, radiologic, and histopathologic criteria [13]. All the subjects, cases and controls, were Caucasian Spanish, living in the same geographic area and were matched for age and sex. All study subjects were included in this study after written informed consent. We obtained approval for the study from all local ethical committees of the corresponding hospitals.

Genotyping

Genomic DNA was isolated from anticoagulant-treated peripheral blood mononuclear cells (PBMCs) using standard methods. Genotyping of the $(GT)_n$ microsatellite marker located between exon −1 and exon 1 of the *FOXP3* gene was performed by a polymerase chain reaction (PCR)-based method as previously described [9], using the following primers: forward, 5′-CAACCATTGCCCTCATAGAGG-3′, and reverse, 5′-GGCGGTATGAGATACTCGACCA-3′. The forward primer was 5′ labeled with the fluorescent dye 6-FAM and the lengths of the fragments were analyzed in an ABI PRISM 3100 Genetic Analyzer and using a Genescan 672 software (Applied Biosystems, Foster City, CA). To verify the repeat numbers of each allele we used direct sequencing using ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Statistical Analyses

Allelic and genotypic frequencies of *FOXP3* polymorphism were obtained by direct counting. Statistical analysis to compare allelic and genotypic distributions was performed by chi-square test. Odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated according to Wolf's method. The software used was Statcalc program (Epi Info 2002; Centers for Disease Control and Prevention, Atlanta, GA). p Values were corrected for the number of alleles determined using the Bonferroni test, and p values below 0.05 was considered statistically significant.

RESULTS

The genotype and allele distribution frequencies of the $(GT)_n$ *FOXP3* microsatellite in AID patients and healthy controls is shown in Tables 1 and 2. In both patient and control groups, genotype and allele frequencies did not deviate significantly from those expected from Hardy-Weinberg equilibrium. We found seven different alleles comprising repeat and bp ranges $(GT)_{12}$–$(GT)_{18}$ and 255–267, respectively. The most frequent alleles were $(GT)_{15}$ (54.1%) and $(GT)_{16}$ (36.6%) in the control

TABLE 1 Genotype distribution of *F0XP3/Scurfin* $(GT)_n$ in female and male controls and SLE, RA, CD, UC, and celiac disease patients

Female	SLE (%) (n = 206)	RA (%) (n = 232)	CD (%) (n = 177)	UC (%) (n = 130)	Celiac disease (%) (n = 65)	Controls (%) (n = 147)
$(GT)_{14}/(GT)_{15}$	0	1 (0.4)	8 (4.6)[a]	2 (1.5)	0	1 (0.7)
$(GT)_{14}/(GT)_{16}$	0	0	2 (1.1)	0 (0)	0	0
$(GT)_{15}/(GT)_{15}$	62 (30.1)	72 (31)	45 (25.4)	34 (26.2)	18 (27.7)	44 (30)
$(GT)_{15}/(GT)_{16}$	94 (45.7)	108 (46.6)	74 (41.8)	50 (38.5)	29 (44.6)	62 (42.2)
$(GT)_{15}/(GT)_{17}$	9 (4.3)	8 (3.5)	16 (9)	6 (4.6)	5 (7.7)	8 (5.4)
$(GT)_{16}/(GT)_{16}$	29 (14)	33 (14.2)	14 (7.9)	30 (23.1)[b]	8 (12.3)	18 (12.2)
$(GT)_{16}/(GT)_{17}$	8 (3.9)	8 (3.5)	12 (6.8)	5 (3.8)	2 (3)	5 (3.4)
Others	4 (2)	2 (0.8)	6 (3.4)	3 (2.3)	3 (4.7)	9 (6.1)
Males	**SLE (%) (n = 25)**	**RA (%) (n = 61)**	**CD (%) (n = 107)**	**UC (%) (n = 114)**	**Celiac disease (%) (n = 38)**	**Controls (%) (n = 127)**
$(GT)_{14}$	0	0	2 (1.9)	2 (1.8)	0	0
$(GT)_{15}$	16 (64)	32 (52.5)	59 (55.1)	57 (50)	25 (65.7)	69 (54.3)
$(GT)_{16}$	7 (28)	25 (41)	42 (39.2)	50 (43.8)	11 (29)	47 (37)
$(GT)_{17}$	1 (4)	4 (6.5)	3 (2.8)	5 (4.4)	2 (5.3)	11 (8.7)
Others	1 (4)	0	1 (0.9)	0	0	0

Abbreviations: SLE = systemic lupus erythematosus; RA = rheumatoid arthritis; CD = Crohns disease; UC = ulcerative colitis.
[a] $p = 0.03$, p corrected = NS.
[b] $p = 0.01$, p corrected = NS.

group. *F0XP3*genotypes and alleles with a frequency of ≤2% in patients or controls were jointly considered. The allele frequencies observed in our control population were in good agreement with allele frequencies found in other Caucasian South-European population [14]. However, these allele frequencies contrast significantly with those detected in the Japanese population (Table 3).

No statistically significant differences were observed between allele frequencies of SLE, RA, or celiac patients and controls. In addition, we found no association of this polymorphism and genotype frequencies in female and male patients with these AID. With regard to the IBD patients, the $(GT)_{16}/(GT)_{16}$ genotype was slightly increased in UC patients (23.1% versus 12.2% in the control group, $p = 0.01$, p_c = not significant, OR = 2.15, 95% CI = 1.08–4.29). We observed a statistically significant deviation in the distribution of the $(GT)_{14}$ allele among the female Crohn's patients (5.1% versus

TABLE 2 Distribution of allele frequencies of *F0XP3/Scurfin* $(GT)_n$ in female and male SLE, RA, CD, CU, celiac disease, and healthy controls

Alleles Female	Size (bp)	SLE (%) (n = 412)	RA (%) (n = 462)	CD (%) (n = 354)	UC (%) (n = 260)	Celiac Disease (%) (n = 130)	Controls (%) (n = 294)
$(GT)_{14}$	259	0	1 (0.2)	18 (5.1)[a]	2 (0.8)	0	4 (1.3)
$(GT)_{15}$	261	228 (55.3)	263 (57)	189 (53.4)	127 (48.8)	72 (55.4)	159 (54.1)
$(GT)_{16}$	263	162 (39.3)	182 (39.4)	116 (32.7)	115 (44.2)	47 (36.2)	107 (36.4)
$(GT)_{17}$	265	18 (4.4)	16 (3.4)	30 (8.5)	15 (5.8)	9 (6.9)	19 (6.5)
Others	Others	4 (1)	0	1 (0.3)	1 (0.4)	2 (1.5)	5 (1.7)
Males	**Size (bp)**	**SLE (%) (n = 25)**	**RA (%) (n = 61)**	**CD (%) (n = 107)**	**UC (%) (n = 114)**	**Celiac disease (%) (n = 38)**	**Controls (%) (n = 127)**
$(GT)_{14}$	259	0	0	2 (1.9)	2 (1.8)	0	0
$(GT)_{15}$	261	16 (64)	32 (52.5)	59 (55.1)	57 (50)	25 (65.7)	69 (54.3)
$(GT)_{16}$	263	7 (28)	25 (41)	42 (39.2)	50 (43.8)	11 (29)	47 (37)
$(GT)_{17}$	265	1 (4)	4 (6.5)	3 (2.8)	5 (4.4)	2 (5.3)	11 (8.7)
Others	Others	1 (4)	0	1 (0.9)	0	0	0

Abbreviations: SLE = systemic lupus erythematosus; RA = rheumatoid arthritis; CD = Crohns disease; UC = ulcerative colitis.
[a] $p = 0.009$, p corrected = 0.05; OR = 3.88 (1.22–13.72).

TABLE 3 Comparative allele frequencies between Japanese and Spanish populations

Alleles	Japanese controls (%) (n = 472)	Spanish controls (%) (n = 421)	p Value	p_c
$(GT)_{12}$	0	3 (0.7)	0.03	ns
$(GT)_{13}$	0	2 (0.5)	ns	ns
$(GT)_{14}$	1 (0.2)	4 (1)	ns	ns
$(GT)_{15}$	154 (32.6)	228 (54.1)	>0.000001	>0.000001
$(GT)_{16}$	296 (62.8)	154 (36.6)	>0.000001	>0.000001
$(GT)_{17}$	11 (2.3)	30 (7.1)	0.002	0.01
$(GT)_{18}$	10 (2.1)	0	0.001	0.006

GT = genotyping; ns = not significant.

1.3% in the control group; $p = 0.009$, OR 3.88, 95% CI = 1.22–13.72) that turned out to be nonsignificant after applying the Bonferroni test ($p_{corr} = 0.05$).

DISCUSSION

There is increasing evidence that common genes may underlie autoimmunity [3, 15]. However, evidence that specific risk alleles are associated with multiple AID is relatively sparse. Genes within the MHC complex, and not only the HLA alleles, are associated with multiple autoimmunity diseases [16]. Recently there have also been examples showing that non-HLA complex genes play a role in the development of more than one autoimmune disease. Thus, *CTLA4* gene variants are associated with T1D, Graves' disease, and SLE [17, 18]. In addition, the *PTPN22* R620W polymorphism has been associated with a number of AID [19, 20]. This leads to the hypothesis of common predisposing genes to autoimmunity and consequently when a potential risk factor for an autoimmune disease is discovered, such as the *FOXP3* gene, investigating its involvement in different AID is of interest.

In the current case-control study, the possible association of RA, SLE, IBD, and celiac disease with variants in the *FOXP3* gene was investigated. The data reveal no association between the AID investigated with the *FOXP3* variant that was found to be associated with T1D in the Japanese population.

The possible reason for the lack of association for this polymorphism in our population may result from a *FOXP3*/*Scurfin* gene located on one of the T1D susceptible loci, Xp11.23, which is not a RA, SLE, IBD, or celiac disease susceptibility loci. However, the absence of linkage evidence in a particular chromosomal region does not mean that genes in that region do not contribute to disease risk. Our negative findings could be due to lack of power to detect a true association in these diseases, specially in Crohn's disease, in which we observed a trend of association for the GT14 allele. Therefore, genotyping in larger number of materials is warranted before drawing any definitive conclusions.

In accordance with our findings, no association was detected between T1D and *FOXP3* polymorphisms in an Italian population [14]. Zavattari *et al.* extensively characterized the *FOXP3* region, searching for genetic variants, and no evidence of association between T1D and the 12 *FOXP3* polymorphisms analyzed was observed. Differences in the ethnicities of the study population may account for the failure to replicate the T1D association with *FOXP3* in a Japanese population [9]. This possibility is consistent with the marked differences in *FOXP3* allele frequencies in the Japanese versus the white population. Alternatively, it is possible that the specific disease-relevant alleles for at least some susceptibility genes vary among different ethnic groups. Thus, our findings together with those of Zavattari *et al.* suggest that the *FOXP3* microsatellite is not a susceptibility allele for most common AID, at least in the Caucasian population. Further studies are warranted to assess the *FOXP3* variants' relevance to AID in the Japanese population.

With regard to the functional relevance of the *FOXP3* gene microsatellite polymorphism, the different microsatellite alleles appear to affect promoter activity of the *FOXP3* gene [9]. Nevertheless, caution should be exercised in extrapolating the results of previous functional assays, because the presence of other polymorphisms within intron zero or other distant genetic variants in strong linkage disequilibrium with *FOXP3* gene microsatellite might be those that are really affecting *FOXP3* gene expression.

In summary, we found no evidence of association between this genetic variant in *FOXP3* gene with AID patients in our Spanish population. Obviously, the participation of FOXP3 protein in autoimmunity is not brought into question by these data, and the existence of other *FOXP3* gene regulatory polymorphisms that might affect *FOXP3* expression and influence AID risk cannot be excluded.

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5. DISCUSIÓN

5.1. Genes implicados en la inflamación.

5.1.1. La inmunidad innata: *TLR2* y *TLR4*

Los receptores tipo toll (toll-like receptors, TLRs) son receptores filogenéticamente conservados que están involucrados en el reconocimiento de patrones moleculares asociados a patógenos (pathogen-associated molecular patterns, PAMPs) y ligandos endógenos. Los TLRs juegan un importante papel en la regulación de la respuesta inflamatoria innata y la iniciación de la respuesta adaptativa, lo que les posiciona en la cima de la pirámide de la respuesta inmune [78].

Aunque se han identificado hasta 10 miembros de esta familia en humanos, TLR-2 y TLR-4 son los receptores tipo toll más estudiados en AR. Estos receptores se unen fundamentalmente a PAMPs basados en lípidos, unión que provoca su activación y la iniciación de una cascada de transducción de señales que termina en la activación de NF-κB, con la consiguiente inducción de la expresión de mediadores inflamatorios y moléculas coestimuladoras. En la figura 5.1 se muestra un esquema de esta vía. Sin embargo, existe una ruta de activación independiente de la proteína adaptadora MyD88, que también lleva a la activación de NF-κB [79, 80].

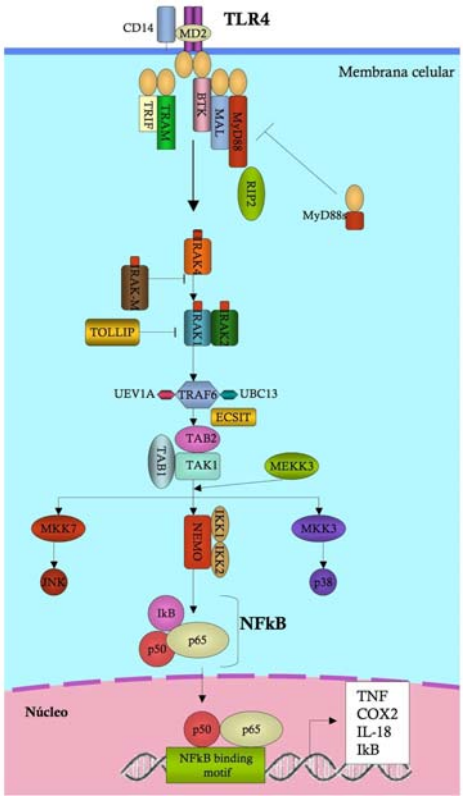

Figura 5.1. Señalización a través de TLR

Estudios en modelos murinos sugieren que la disponibilidad de ligandos de TLR puede ser suficiente para iniciar una artritis en individuos susceptibles [81-83]. Se han encontrado ligandos de TLR de origen microbiano en articulaciones de pacientes con AR [84]. Además, los TLRs podrían ser activados por ligandos endógenos que se encuentran en articulaciones inflamadas, como proteínas de choque térmico, fibrinógeno y ácido hialurónico. El reconocimiento de estos ligandos por parte de fibroblastos y macrófagos sinoviales a través de TLRs provocaría su activación y la expresión de citoquinas proinflamatorias, quimioquinas y enzimas destructoras de tejido. En un hipotético loop de retroalimentación, ligandos endógenos generados a través de estos procesos inflamatorios resultaría en la estimulación crónica de las células sinoviales (Figura 5.2) [85, 86].

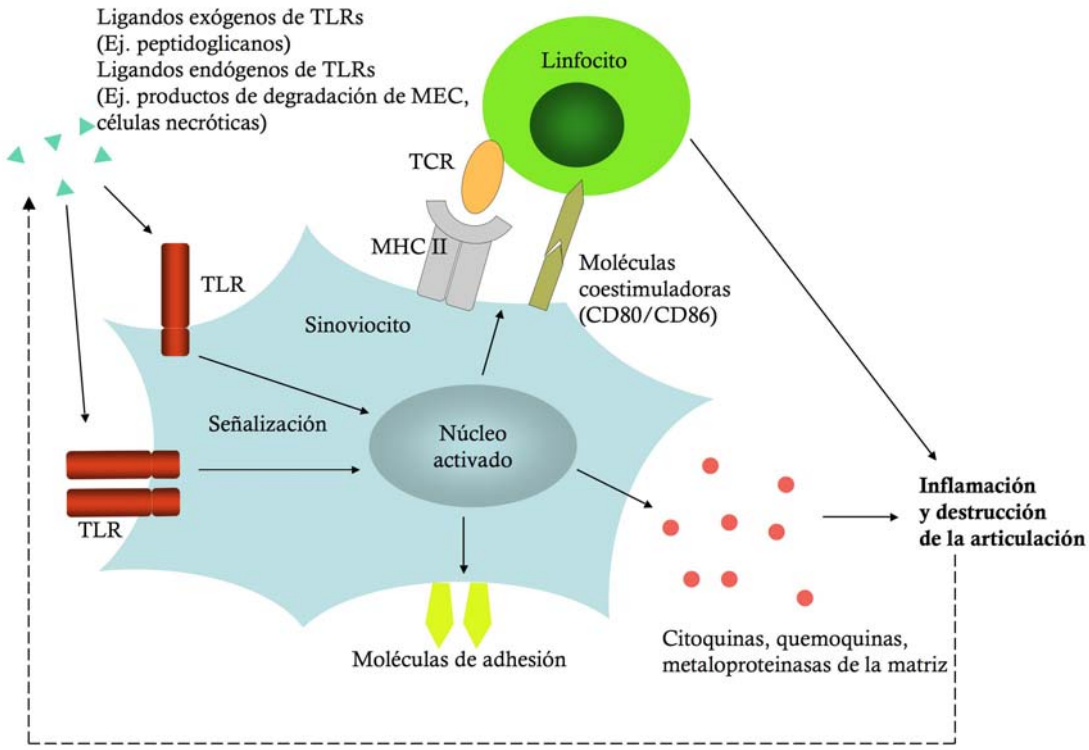

Figura 5.2. Papel de los TLRs en AR.

La expresión de TLR-2 y TLR-4 está aumentada en las articulaciones inflamadas de pacientes de AR [87]. Además, diversos estudios recientes han demostrado que la activación de TLR-2 y TLR-4 en sinoviocitos conduce a la expresión de diversas moléculas efectoras involucradas en la patogénesis de la AR, como RANKL que promueve la destrucción ósea [87], citoquinas proinflamatorias como IL-15 [88] y MIF [89], y promotores de la angiogénesis como VEGF [90].

En vista del importante papel que TLR-2 y TLR-4 parecen tener en la AR, decidimos estudiar varios de sus polimorfismos genéticos. Dos interesantes SNPs no sinónimos habían sido descritos en el gen *TLR4*: una sustitución de una adenina (A) por una guanina (G) en el nucleótido en posición 896 desde el codón de iniciación, que resulta en el cambio de un de un ácido aspártico a una glicina en el aminoácido 299 de la proteína (Asp299Gly) y una segunda mutación que origina una sustitución de una treonina por isoleucina en posición 399 (Thr399Ile). Estos SNPs parecen tener una influencia en la función de TLR-4, ya que se correlacionan con una menor respuesta a endotoxinas en humanos [91]. Además, están asociados a susceptibilidad shock séptico por gram-negativas [92] y a aterosclerosis [93]. 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 gram-positivas [94]. Además, otro polimorfismo en *TLR2* (Arg677Trp) se ha visto asociado con lepra [95].

En vista del importante papel que *TLR2* y *TLR4* parecen tener en la AR, decidimos estudiar estos cuatro polimorfismos genéticos por primera vez en una cohorte de AR [96]. Aunque ninguno de los dos genes mapea en regiones de ligamiento a AR (4q32 y 9q32, respectivamente), su importante función en la inmunidad innata y adquirida, hace de *TLR2* y *TLR4* unos interesantes genes candidatos.

Con respecto a los SNPs de *TLR2*, no encontramos ningún individuo en nuestra población española que portara el polimorfismo Arg677Trp. Además, vimos que el otro polimorfismo de *TLR2*, Arg753Gln, se encontraba con muy baja frecuencia. Esta baja frecuencia de los polimorfismos de *TLR2* ha sido observada en otras poblaciones caucásicas [94, 97]. Parece ser que los SNPs sólo son frecuentes en poblaciones asiáticas, debido a la heterogeneidad genética entre distintos grupos étnicos.

En cuanto a *TLR4*, pudimos observar que en nuestra población los SNPs Asp299Gly y Thr399Ile se encuentran en alto desequilibrio de ligamiento, y sus frecuencias alélicas son similares a las previamente descritas para otras poblaciones europeas y norteamericanas [91, 92, 98, 99]. Por el contrario, estos polimorfismos son muy raros en población japonesa [100]. Por lo tanto, parece que existe una clara heterogeneidad en los polimorfismos de *TLR2* y *TLR4* entre poblaciones caucásicas y asiáticas, como puede ser observado en numerosos estudios de asociación.

Cuando comparamos la frecuencia de los SNPs de *TLR4* entre pacientes de RA y controles, no encontramos diferencias significativas, por lo que parece que estos polimorfismos no están implicados en la susceptibilidad a AR. De acuerdo con nuestros resultados, un estudio independiente realizado en población británica, y publicado casi simultáneamente al nuestro, tampoco encontró asociación entre Asp299Gly y la AR [101].

Sin embargo, en un estudio publicado con posterioridad, se reportó que este SNP estaba asociado a AR en población holandesa [102]. Con objeto de intentar aclarar el papel del polimorfismo de *TLR4* en la AR, decidimos realizar un estudio replicativo incluyendo tres cohortes distintas de Granada, Lugo y Colombia [103]. De nuevo, no encontramos diferencias significativas en la distribución de Asp299Gly entre pacientes de AR y controles sanos. Debido al mayor poder estadístico de nuestro estudio (99%) en comparación con el del grupo holandés, podemos concluir que este SNP no influye en la susceptibilidad a AR. La asociación obtenida en holandeses pudo deberse a errores de genotipado, ya que la frecuencia de heterocigotos Asp/Gly que encontraron en controles era bastante superior a la que previamente se había encontrado en otras poblaciones [96, 99, 101, 104, 105]. De manera interesante, tampoco encontramos asociación entre los polimorfismos de TLR2 y TLR4 y la susceptibilidad a LES [96].

En un estudio más reciente se ha sugerido que *TLR4* Asp299Gly está asociado a una menor remisión de la AR tras el tratamiento con DMARDs y un aumento del DAS28 [106]. Sin embargo, debido al bajo número de pacientes incluidos en este estudio sería necesario realizar ensayos con mayor poder estadístico para poder establecer una relación entre el polimorfismo de *TLR4* y la respuesta al tratamiento. Además, se ha estudiado un nuevo polimorfismo de *TLR2*, un microsatélite $(GT)_n$ en el intrón 2, cuyos alelos cortos parecen estar asociados a AR en coreanos [107]. Sin embargo, los estudios funcionales proporcionaron datos contradictorios, ya que encontraron que esos alelos cortos producía una menor expresión de *TLR2*, de manera contraria a lo que cabría esperar. Además, esta asociación no ha sido replicada en otras poblaciones.

Por lo tanto, parece ser que los polimorfismos Asp299Gly y Thr399Ile de *TLR4* y Arg677Trp y Arg753Gln de *TLR2* no son relevantes en la AR, aunque no se pueden descartar todavía otros polimorfismos de estos genes, así como otros genes de la familia toll que podrían estar involucrados en la enfermedad que aun no han sido estudiados.

5.1.2. Activación de genes proinflamatorios por NF-κB

5.1.2.1. *NFKB1*

El factor nuclear κB (NF-κB) es un factor de transcripción de gran importancia en los procesos inflamatorios, ya que coordina la expresión de una amplia variedad de genes que controlan la respuesta inmune innata y adquirida [108]. Las moléculas de la familia NF-κB/REL están compuestas por heterodímeros codificados por los genes *RELA* (p65), *NFKB1* (p50; p105), *NFKB2* (p52; p100), *c-REL* y *REL*. La principal forma activa de NF-κB

es un heterodímero formado por la subunidad p65 asociada con p50 o p52, proteínas generadas a partir de la proteolisis de las precursoras p105 y p100, respectivamente.

En células no estimuladas, NF-κB se encuentra en el citoplasma de manera inactiva unido a la proteína inhibidora IκB, lo que previene su entrada en el núcleo. Tras la estimulación de la célula por distintas vías, NF-κB es capaz de activarse rápidamente. Esta activación puede ser inducida por el reconocimiento de patógenos o ligandos endógenos a través de los TLR (Figura 5.3 a), la unión de citoquinas a sus receptores, como por ejemplo TNF-α o IL-1 (Figura 5.3 b), o tras la activación del receptor de la célula T (Figura 5.3 c). Tras estos estímulos, se produce una cascada de señalización que confluye en la fosforilación y ubiquitinación de IκB, lo que la señaliza para su degradación por el proteasoma. De esta forma, NF-κB queda liberado y es capaz de translocarse al núcleo, donde activa la transcripción de numerosos genes implicados en procesos inflamatorios, tales como citoquinas proinflamatorias, quimioquinas, moléculas de adhesión, MMPs, ciclooxigenasa 2 (COX2) y óxido nítrico sintasa inducible (iNOS).

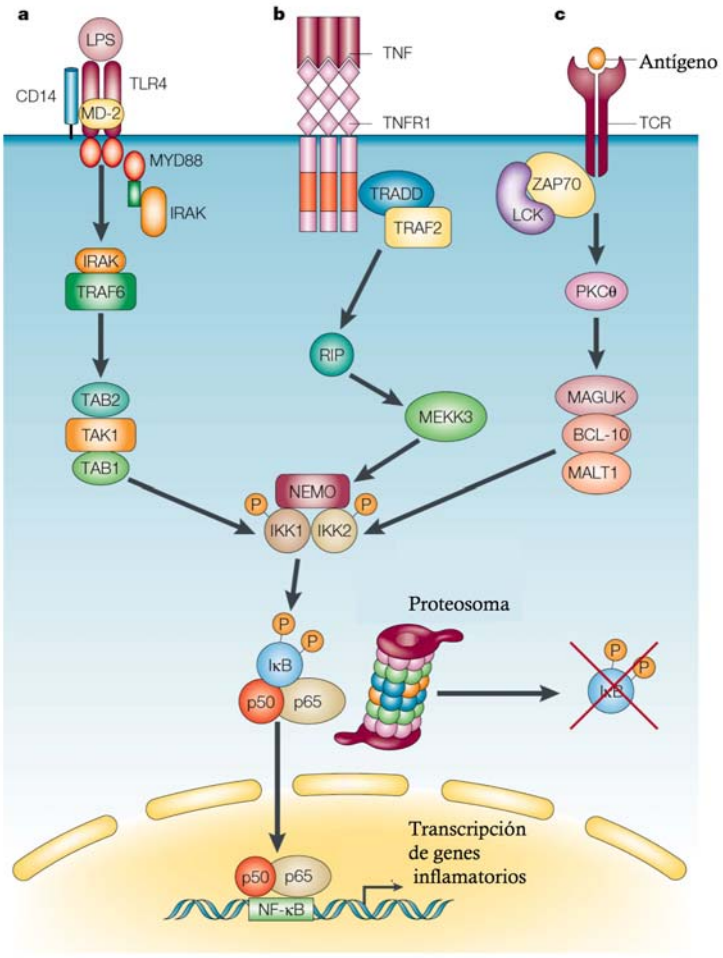

Figura 5.3. Mecanismos de activación de NF-κB

Con respecto al papel de NF-κB en la AR, numerosas evidencias sugieren que este factor de transcripción está involucrado en múltiples mecanismos etiopatológicos de la enfermedad (Figura 5.4) [109]. Se ha observado que NF-κB se encuentra sobreactivado en el sinovio de articulaciones artríticas humanas [110, 111]. Así mismo, hay una mayor expresión de *NFKB1* en tejido sinovial de pacientes de AR [112]. El bloqueo de la actividad de NF-κB por sobreexpresión de IκB inhibe la respuesta inflamatoria y la destrucción tisular en el sinovio reumatoide [113], y la administración de bloqueantes de NF-κB parece ser efectivo para mejorar la enfermedad en modelos murinos de AR [114]. A la vista de todos estos indicios, se ha propuesto que NF-κB podría ser una buena diana terapéutica para la AR [115, 116]. Sin embargo, debido al amplio espectro de acción de NF-κB en la diferenciación, activación y supervivencia de gran cantidad de tipos celulares, el tratamiento prolongado con inhibidores podría tener efectos adversos, por lo que recientemente se está apostando por la inhibición específica de NF-κB en células inflamatorias, como células dendríticas [117].

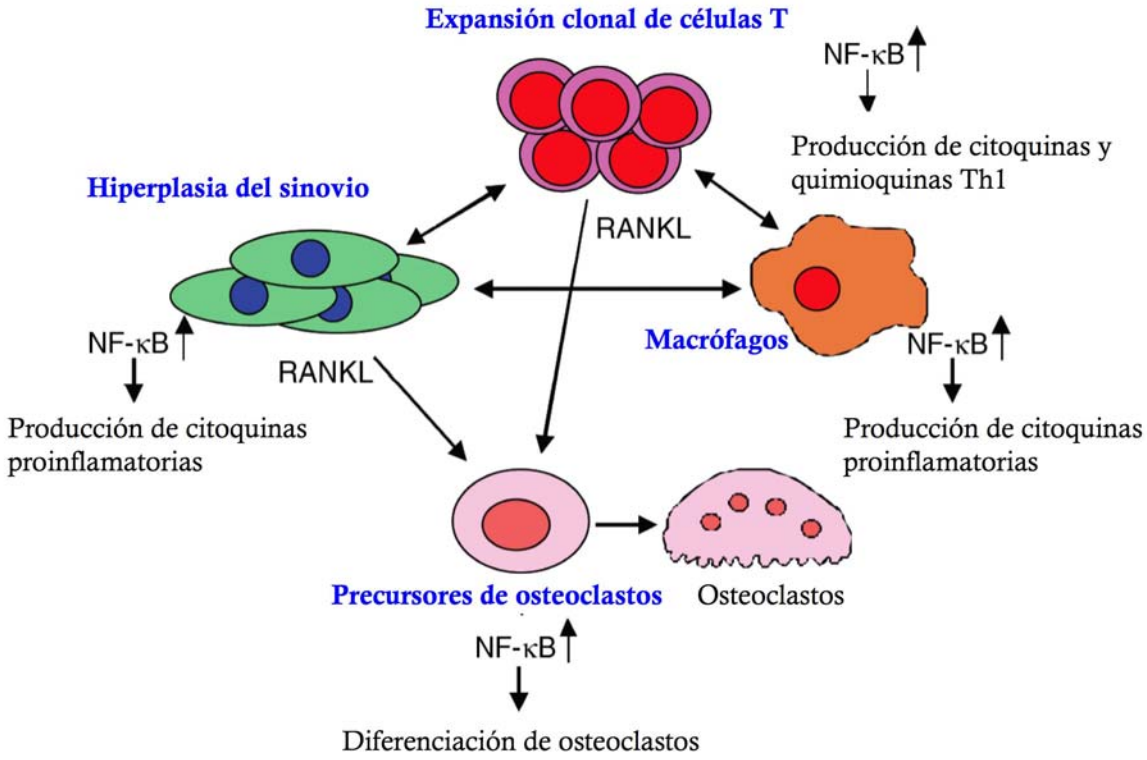

Figura 5.4. Esquema de los procesos derivados de la activación por NF-κB de distintos tipos celulares que intervienen en la patogénesis de la AR

Teniendo en cuenta la importancia de NF-κB en la patogénesis de la AR, nos propusimos analizar dos de los polimorfismos del gen *NFKB1*. Cuando realizamos el estudio, se había identificado un nuevo polimorfismo funcional en el promotor que consiste en una inserción/delección (-94isn/delATTG) [118]. La presencia de la delección resulta en una menor actividad promotora, y está asociada a colitis ulcerosa (CU) en población norteamericana [118]. Además, un potencial polimorfismo regulador de la expresión de *NFKB1* localizado en la proximidad del gen se había visto asociado a diabetes tipo 1, un microsatélite $(CA)_n$ [119]. Cuando comparamos la frecuencia con que se presentan estos polimorfismos en pacientes de AR frente a controles sanos, no encontramos diferencias significativas, lo que sugiere que estos polimorfismos no influyen en el desarrollo de la AR [120].

Nuestro estudio fue el primero en analizar la influencia de estos dos polimorfismos en la susceptibilidad a AR. Posteriormente, ningún otro estudio ha sido publicado, por lo que sería interesante saber si estos resultados se replican en poblaciones diferentes. Por el contrario, varios estudios replicativos en CU han sido publicados después del de Karban y col, generando un gran debate en torno al papel de -94isn/delATTG en la susceptibilidad a esta enfermedad. Mientras que un estudio pudo replicar la asociación [121], otros, incluyendo datos de nuestro grupo, no encontraron relación entre *NFKB1* -94isn/delATTG y UC [122-124]. Un reciente meta-análisis ha revelado que esta variante genética no parece tener especial relevancia en la predisposición a CU [125]. A todo esto hay que añadir que la disminución de la actividad promotora encontrada para el alelo -94ins asociado a CU está en contra de los datos que demuestran una mayor actividad de NF-κB en esta enfermedad [118]. En cuanto a la diabetes tipo 1, no se ha podido replicar la asociación del microsatélite $(CA)_n$ en población española [126]. En el mismo estudio, tampoco se encontró asociación con -94isn/delATTG.

Con respecto al papel de las dos variantes de *NFKB1* en otras enfermedades autoinmunes, sólo se ha encontrado asociación de -94isn/delATTG con la enfermedad de Graves [127]. Por el contrario, parece que este polimorfismo no está asociado a LES [120], espondilitis anquilosante (EA) [128], enfermedad celiaca [129], arteritis de células gigantes [130], y artritis psoriásica [131]. Observando globalmente todos los datos de los que disponemos hasta la fecha, podemos sugerir que los polimorfismos -94isn/delATTG y $(CA)_n$ de *NFKB1* no influyen en la susceptibilidad genética a un gran número de enfermedades autoinmunes.

Está claro que NF-κB es un importante regulador en la autoinmunidad y la inflamación. Sin embargo, los procesos en los que actúa son complejos, por lo que para poder establecer el papel de *NFKB1* en la predisposición genética a autoinmunidad serían

necesarios estudios más profundos. Además, aun no han sido estudiados otros miembros importantes de la familia de NF-κB, como *NFKB2*, *c-REL* o *REL* o *RELA*.

5.1.2.2. *SUMO4*

La familia de proteínas modificadores pequeños similares a ubiquitina (small ubiquitin-like modifiers, SUMO) participan en la modificación post-traduccional de proteínas de manera similar a la ubiquitina, pero a diferencia de ésta no se polimeriza y no señaliza proteínas para su degradación, sino para cambiar su localización, para modificar la actividad de factores de transcripción, regular las interacciones proteína-proteína o la unión de proteínas al DNA [132].

Una característica importante de la sumoilación es que puede actuar como antagonista de la ubiquitinación. Cuando SUMO se une a determinadas proteínas consigue estabilizarlas y favorecer la formación de complejos. Dado que SUMO se une en los mismos sitios aceptores que la ubiquitina, se impide la unión de ésta, y por lo tanto, se inhibe la degradación de la proteína. Este fenómeno ocurre en la regulación de la activación de factores de transcripción como NF-κB, la cual es muy importante en los procesos inflamatorios y autoinmunes, como hemos visto anteriormente. Para que IκB se degrade y libere a NF-κB, debe ser fosforilado y ubiquitinizado. IκB puede ser sumoilado en las mismas lisinas aceptoras de ubiquitina, con lo que se impide su degradación, inhibiendo la activación de NF-κB [133]. Por lo tanto, las proteínas SUMO podrían modular la respuesta inflamatoria a través de la inhibición de NF-κB (Figura 5.5).

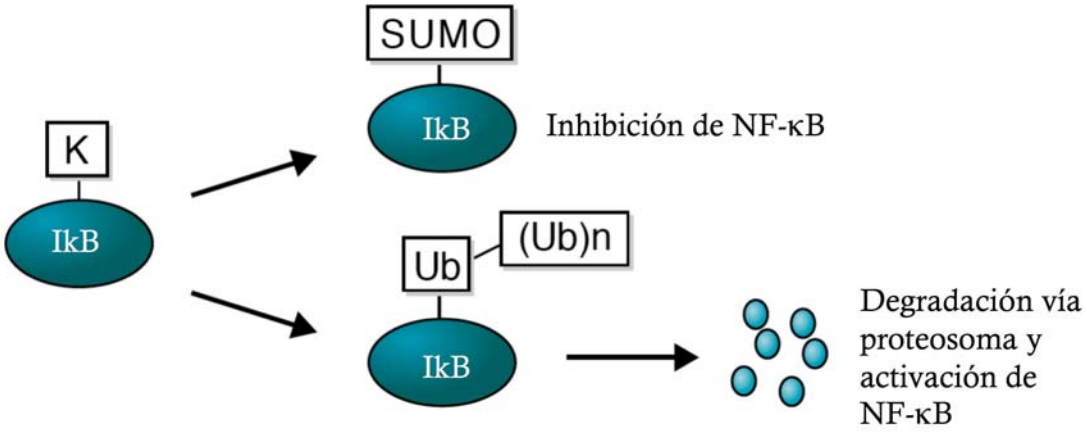

Figura 5.5. Mecanismo por el que SUMO inhibe la activación de NF-κB.

Se han identificado varios locus de ligamiento en diabetes tipo 1, entre ellos la región *IDDM5* en el cromosoma 6q25 [134]. Guo y col realizaron un mapeo fino de esta zona y observaron que la asociación más fuerte con diabetes tipo 1 en esta zona se debía a un polimorfismo en el gen *SUMO4* (Figura 5.6) [135]. El SNP (163A→G, M55V) es además relevante desde el punto de vista funcional, ya que resulta en una mayor actividad transcripcional de NF-κB y una mayor expresión del gen *IL12B*, el cual tiene gran importancia en autoinmunidad e inflamación, como más tarde veremos.

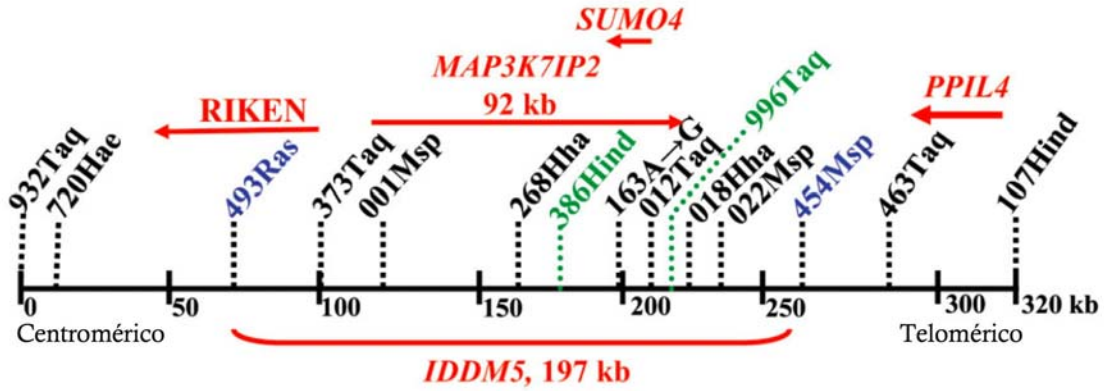

Figura 5.6. Mapa del locus *IDDM5*.

Muchas enfermedades autoinmunes comparten características clínicas similares, lo que indica que existen rutas biológicas comunes para estas dolencias. Varios indicios sugieren que esto puede ser debido a una base genética común, como por ejemplo la asociación con los genes HLA, que es común para muchas autoinmunidades, y otros genes no-HLA [136, 137]. Esta base genética común también se pone de manifiesto por el hecho de que la región 6q25, que ha mostrado ligamiento con diabetes, ha sido identificada como región de susceptibilidad a AR en numerosos estudios [57, 60, 61, 64, 76].

Teniendo en cuenta esta hipótesis de la base genética común en autoinmunidad y el potencial papel que SUMO4 podría tener en la regulación de la inflamación, decidimos estudiar el papel del polimorfismo funcional 163A→G del gen *SUMO4* en la susceptibilidad a AR en población española. En contra de lo esperado, comprobamos que este SNP no está asociado a AR en nuestra cohorte [138]. De nuevo, las frecuencias de este polimorfismo son muy diferentes en población caucásica y asiática. De acuerdo con nuestros resultados, otros estudios no han encontrado asociación entre *SUMO4* 163A→G y AR, ni artritis idiopática juvenil (AIJ), en poblaciones británica y española [139, 140].

La asociación de *SUMO4* con diabetes ha sido el tema de estudio de numerosos artículos, que han generado resultados conflictivos. Antes del estudio de Guo y col se

reportó una asociación del alelo A del polimorfismo *SUMO4* 163A→G en familias norteamericanas y británicas [141]. Por el contrario, Guo y col encontraron asociación con el alelo contrario, el G [135]. Sin embargo, los experimentos funcionales llevados a cabo por ambos estudios muestran que el alelo G podría tener un papel importante en la alteración de mecanismos implicados en autoinmunidad. Después se han realizado más estudios replicativos, unos mostrando asociación [142, 143] y otros no [144-147]. Se ha sugerido que la conflictiva asociación alélica de *SUMO4* con diabetes podría ser debida a un falso positivo, a epistasis entre *SUMO4* y otro loci que pudiera influir en la dirección de la asociación, o a interacciones complejas gen-ambiente.

En otras enfermedades autoinmunes tampoco se ha encontrado asociación con el SNP de *SUMO4*, como en LES [148] o enfermedad de Graves [149].

La falta de asociación entre este polimorfismo y AR no quiere decir que podamos descartar el locus 6q como región de asociación. Muchos otros genes que mapean en esta región podrían ser buenos candidatos. De hecho, muy recientemente se ha identificado un prometedor SNP fuertemente asociado a AR en la proximidad de esta zona [76]. Futuros estudios de mapeo fino ayudarán a dilucidar el papel de este loci cromosómico en la susceptibilidad a AR.

5.1.3. *FCRL3*

La región cromosómica 1q21-23 está implicada en la susceptibilidad a múltiples enfermedades autoinmunes, como LES [150], psoriasis [151] y esclerosis múltiple (EM) [152]. Esta región contiene los genes de la familia Fcγ receptor (FcγR) II/III, además del cluster de una nueva familia de genes, los Fc receptor-like (FCRLs, también conocidos como FcRHs, IRTASs o SPAPs). Los FCRLs tienen una gran homología estructural con los FCγRs, aunque aun no se conocen ni su función ni sus ligandos .

Los FcγR son receptores de la parte Fc de la inmunoglobulina G (IgG), siendo parte de ellos inhibidores y otros activadores. Estos receptores relacionan el sistema inmune innato y el adquirido entre sí. El balance en la señalización a través de FcγR inhibidores y activadores regula la actividad de varios tipos celulares del sistema inmune. La unión de la IgG a los receptores FcγRIII (y FcγRI) provoca la activación de una cascada de transducción de señales que termina en funciones efectoras tales como fagocitosis, producción de citoquinas y quimioquinas proinflamatorias, citotoxicidad dependiente de anticuerpos, liberación de metabolitos tóxicos derivados del oxígeno, así como la facilitación de la presentación de antígenos, todo ello promoviendo un ambiente inflamatorio (Figura 5.7) [153, 154].

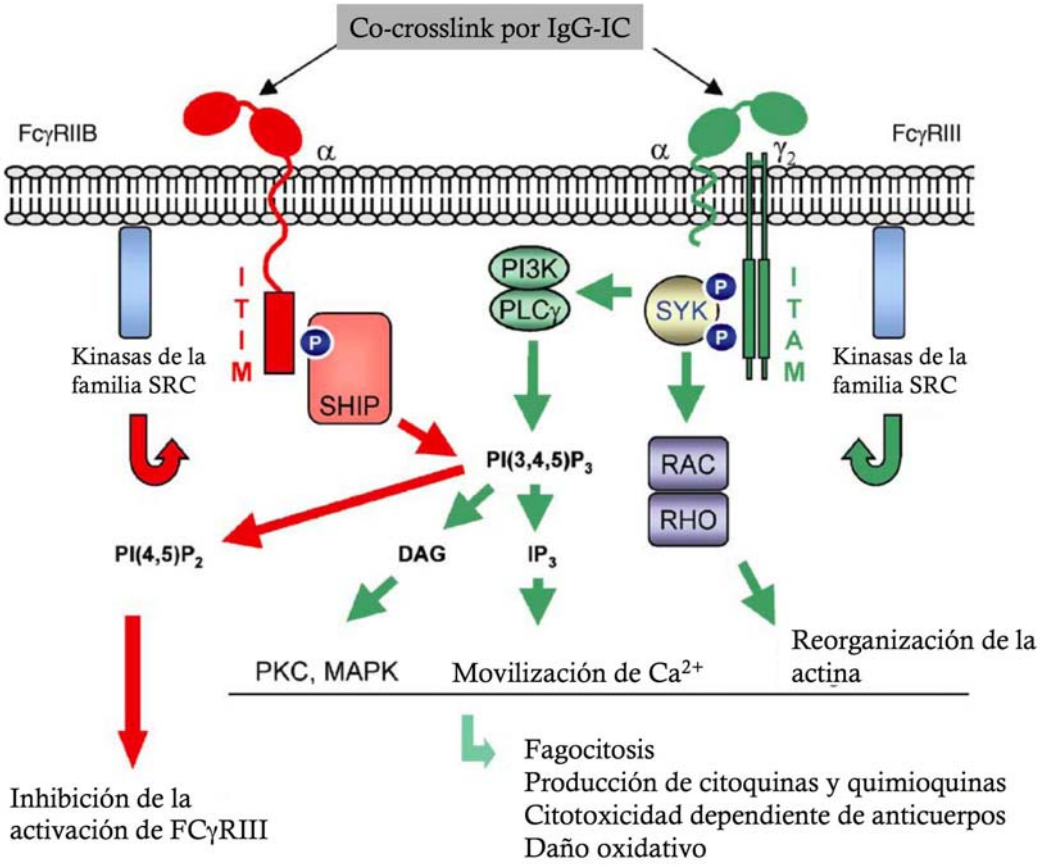

Figura 5.7. Regulación positiva y negativa de la señalización por FcR (FcγRIII vs FcγRIIIB)

Numerosos estudios realizados en ratones han puesto de manifiesto la importancia de los FcγR en la patogenia de la AR [155]. Por ejemplo, la expresión de FcγRIII es necesaria para el desarrollo de artritis inducida por colágeno (CIA); ratones FcγRIII-/- no desarrollan signos de inflamación ni destrucción del cartílago [156]. Además, la inyección de anticuerpo IgG anti-colágeno tipo II en ratones deficientes para este receptor, era suficiente para iniciar una artritis [157]. El papel de los FcγRs en la inflamación sinovial en humanos está menos claro, pero varias evidencias sugieren que estos receptores son de considerable importancia [153, 155].

Dada la importancia que los genes de la región 1q21-23 parecen tener, el grupo japonés de Kochi y col examinaron esta región mediante un mapeo de alta densidad con SNPs, y observaron un pico de asociación con AR no en los genes FcγR clásicos, sino en *FCRL3*, un gen de la familia expresado en células B (Figura 5.8) [158]. Encontraron 4 SNPs, tres de ellos en alto desequilibrio de ligamiento, fuertemente asociados a AR, enfermedad autoinmune tiroidea (EAT) y LES en población japonesa. Además, uno de esos SNPs (-169T→C) altera la afinidad de NF-κB y regula la expresión de *FCRL3*.

Aunque la función precisa de *FCRL3* no se conoce, se piensa que puede influir en el desarrollo de células B, y aumentar la presencia de células autorreactivas en los centros germinales [158].

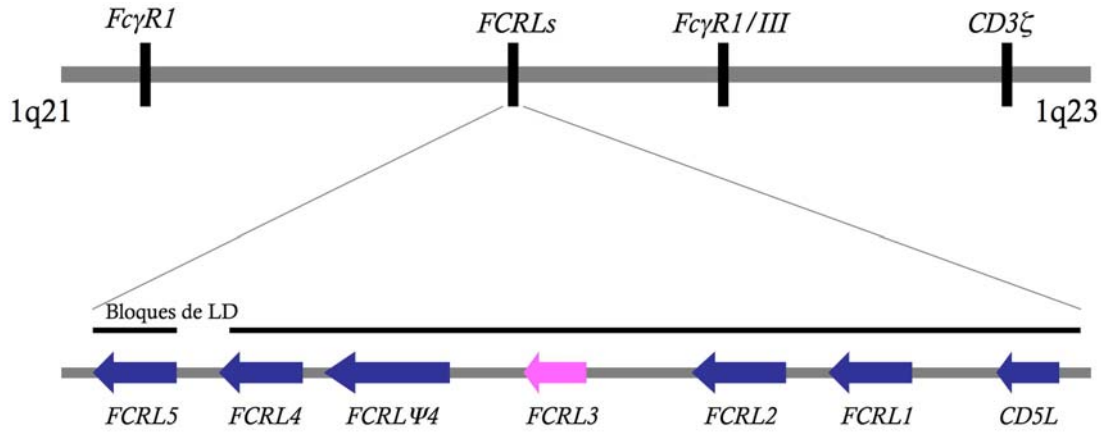

Figura 5.8. Estructura del cluster génico FCRLγ

Nuestro grupo, en colaboración con el grupo de la Dra Urcelay en Madrid, llevamos a cabo un estudio replicativo en población española, utilizando los polimorfismos -169T→C, -110A→G y +1381A→G. Sin embargo, no encontramos diferencias significativas cuando frecuencias de estos SNPs fueron comparadas entre pacientes de AR y controles sanos en nuestra población [159].

Numerosos estudios replicativos han tratado de esclarecer el papel de los polimorfismos de *FCRL3* en AR, pero se han encontrado datos conflictivos. En población asiática, un estudio japonés ha replicado la asociación [160], mientras que otro coreano no [161]. En poblaciones caucásicas, dos estudios han observado una modesta tendencia de asociación con AR [162, 163], pero en la mayoría de los estudios, incluyendo el nuestro, la asociación de *FCRL3* con AR no se replica [159, 162, 164-166]. Dos recientes meta-análisis han revelado que el SNP -169T→C de *FCRL3* parece ser importante en la susceptibilidad a AR en poblaciones asiáticas, pero no en poblaciones blancas de ascendente europeo [167, 168]. Parece ser que la asociación de este locus con AR es población-específica, como ocurre con otros genes estudiados previamente. Como por ejemplo, el polimorfismo 1858C→T del gen *PTPN22*, que se asocia a AR en poblaciones europeas pero no se encuentra presente en poblaciones asiáticas [169], el cual será ampliamente comentado más adelante en esta tesis. Sin embargo, en este caso, los polimorfismos de *FCRL3* son frecuentes en ambos grupos étnicos, por lo que la discrepancia podría deberse a diferencias en la exposición a factores de susceptibilidad ambientales. Otro ejemplo similar lo

encontramos en el gen *PADI4*, cuya asociación con la AR varía según la población, y como *FCRL3*, el haplotipo de susceptibilidad es frecuente en todas las poblaciones [170].

Por otro lado, ya que en el estudio de Kochi y col se demostraba la interacción funcional entre *FCRL3* y NFκB [158], estudiamos si alguna combinación de genotipos de *FCRL3* -169T→C y *NFKB1* -94ins/delATTG mostraba un patrón alterado al comparar pacientes de AR con controles. Pudimos observar que en los pacientes heterocigotos para el polimorfismo de *NFKB1* había un aumento significativo del alelo C del SNP de *FCRL3*. Esta asociación es difícil de interpretar, pero podría ser debida al desequilibrio de ligamiento con marcadores distantes, indicando que una configuración genética específica en el locus *NFKB1* podría hacer que variaciones de *FCRL3* actuaran como marcadores de susceptibilidad.

Con respecto al papel de *FCRL3* en otras enfermedades autoinmunes, los resultados obtenidos en estudios de asociación en distintas poblaciones también han sido controvertidos. Sin embargo, este gen sí parece estar involucrado en la enfermedad de Graves [167].

Estos hallazgos no descartan la posibilidad de que otros SNPs en el cluster 1q21-23 se asocien con AR en caucásicos. Por otro lado, resaltan la importancia de los estudios replicativos amplios y con buen poder estadístico en el estudio genético de las enfermedades complejas.

5.1.4. Citoquinas proinflamatorias

Las citoquinas regulan un amplio rango de procesos inflamatorios que están implicados en la patogénesis de la AR. Es bien conocido que en las articulaciones artríticas existe un disbalance entre las citoquinas pro- y anti-inflamatorias que favorece la inducción de autoinmunidad, inflamación crónica y por lo tanto, daño articular. Se piensa que las citoquinas podrían ser buenas dianas terapéuticas para el tratamiento de la AR, pero aún no se conoce por completo la intrincada red reguladora que controla la actividad de estas moléculas [23].

5.1.4.1. El cluster de citoquinas 5q31

5.1.4.1.1. *SLC22A4* y *RUNX1*

La región cromosómica 5q31 es de particular interés en relación a la predisposición genética a la AR, ya que contiene numerosos genes implicados en rutas inflamatorias, como IL-3, IL-4, IL-5, IL-9, IL-13, factor de regulación de interferón 1 (IRF1), factor

estimulador de colonia 2 (CSF2) y un factor de transcripción de célula T [171]. De hecho, esta región se conoce con el nombre de "cluster genético de citoquinas". Además, esta región, aunque no ha sido identificada como de susceptibilidad a AR en estudios de ligamiento, está asociada a la enfermedad de Crohn (EC) que, como la AR, tiene un componente autoinmune e inflamatorio [172]. Por estas razones, un grupo japonés decidió realizar una búsqueda de genes asociados a AR en el cluster 5q31, mediante un mapeo de LD con SNPs [173]. Encontraron que una variante del gen *SLC22A4* (familia de transportadores de solutos 22, miembro 4), la denominada slc2F1, estaba fuertemente asociada a AR (Figura 5.9). Este SNP está en alto LD con slc2F2, el cual altera el sitio de unión del factor de transcripción RUNX1 (factor de transcripción relacionado con runt 1), afectando la expresión de *SLC22A4*. (Figura 5.10). A su vez, encontraron un polimorfismo en el gen *RUNX1* también asociado a AR. De forma interesante, otros grupos han reportado de manera independiente asociación de variantes en sitios de unión a RUNX1 con LES y psoriasis [174, 175].

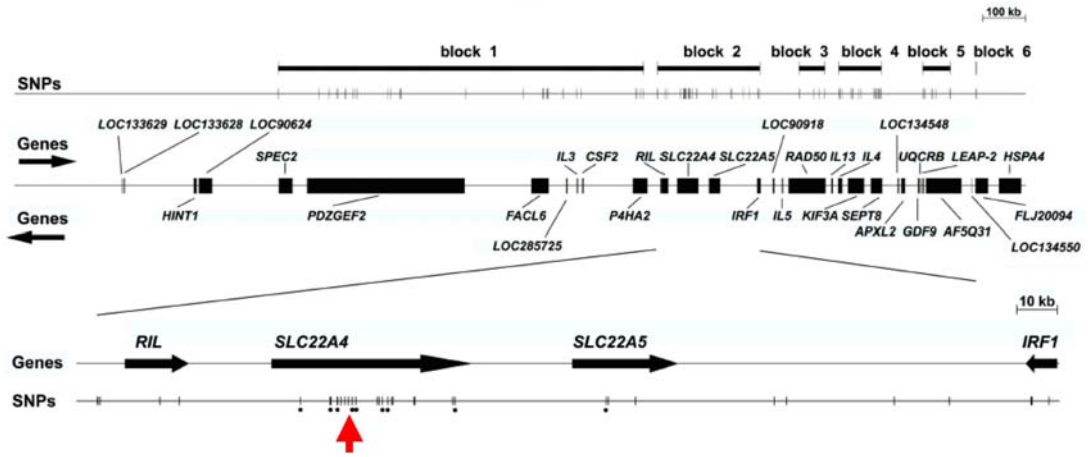

Figura 5.9. Bloques de desequilibrio de ligamiento y estructura génica de parte de la región 5q31

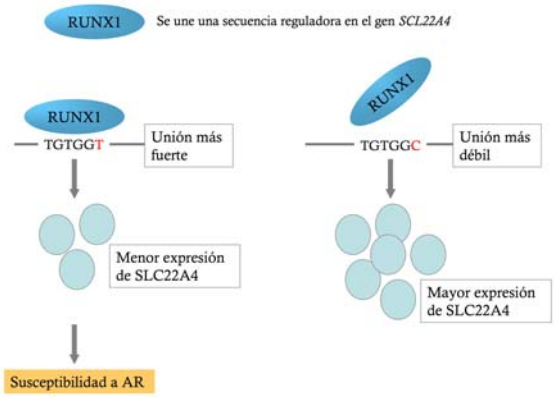

Figura 5.10. Posible papel funcional del polimorfismo de *SLC22A4*.

SLC22A4 codifica para el transportador de cationes orgánicos OCTN1, de función fisiológica desconocida, por lo que no podemos especular acerca del posible papel que pudiera jugar esta molécula en la patogénesis de la AR. RUNX1 es un factor de transcripción que regula la expresión de genes involucrados en hematopoyesis y diferenciación mieloide, cuya disfunción parece estar involucrada en leucemia [176].

A la vista de estos interesantes datos, y dada la heterogeneidad genética que frecuentemente se presenta entre asiáticos y caucásicos, decidimos intentar replicar la asociación de *SLC22A4* y *RUNX1* con AR en población española. Para ello seleccionamos los seis SNPs de la región 5q31 que a nuestro parecer eran los más interesantes: el SNP funcional asociado con AR en japoneses [173], el SNP más fuertemente asociado a EC en población caucásica [177] y los SNPs que forman un haplotipo en japoneses [173] (Figura 5.11).

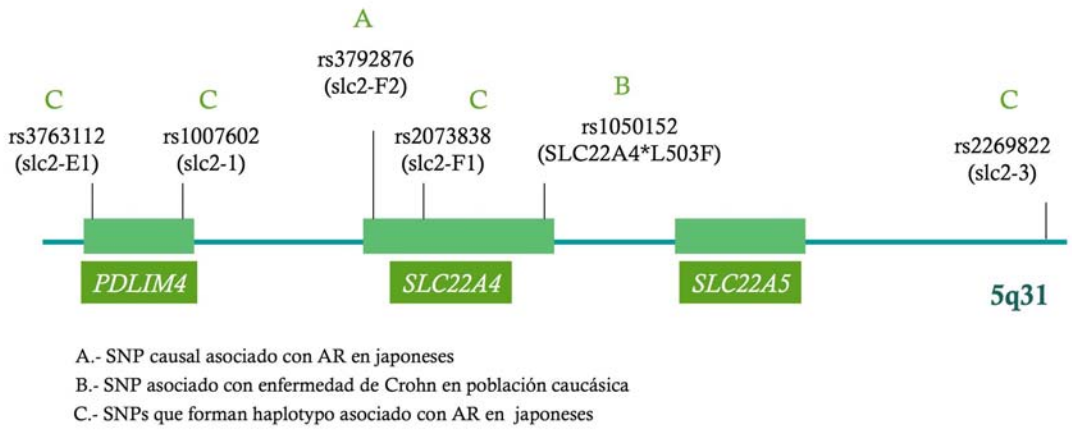

Figura 5.11. Selección de SNPs en la región de *SLC22A4*. La flecha roja indica la localización del SNP slc2F1.

Como en otras ocasiones, las frecuencias de estos SNPs en nuestra población diferían bastante de las encontradas en japoneses. En cambio, esto no ocurría para el SNP de *RUNX1*. En concreto, la variante más asociada a AR en japoneses, slc2F1, es poco frecuente en españoles, por lo que para detectar una posible asociación se necesitaría un mayor número de individuos que los incluidos en nuestro estudio, por lo que éste podría tener bajo poder estadístico. Sin embargo, para el resto de los polimorfismos, nuestro poder estadístico fue mayor del 80%. Es de destacar que el SNP más asociado a EC en caucásicos en la región 5q31 no es slc2F1, sino L503F. Esto nos hace pensar en la presencia de

heterogeneidad genética en este locus para ambas poblaciones, japonesa y caucásica. Es posible que los genes o alelos relevantes en autoinmunidad sean específicos para ciertas poblaciones, y varíen entre los distintos grupos étnicos.

Ambas enfermedades, AR y EC responden a terapia con anti-TNFα, lo que nos hace pensar que deben existir rutas inflamatorias comunes, y por lo tanto, solapamiento en el control génico de estas rutas. Sin embargo, no encontramos asociación entre el gen de susceptibilidad de EC, *SLC22A4*, y AR. Aunque los pacientes con EC tienen mayor riesgo de desarrollar artritis inflamatoria, esta suele ser seronegativa para el FR, y no existen evidencias de que la incidencia de la AR sea mayor en estos pacientes [178]. De igual forma, los polimorfismos del gen *CARD15*, clásicamente asociados a EC, no están asociados a AR [179, 180]. Por lo tanto, nuestros datos proporcionan más información acerca de las diferencias etiopatogénicas y genéticas de las dos enfermedades.

De acuerdo con nuestros resultados, varios estudios con alto poder estadístico han rechazado la posibilidad de que *SLC22A4* sea un gen de predisposición a AR en caucásicos. No se ha encontrado asociación en canadienses [181], británicos [182], y en una población española independiente de la nuestra [183]. Sorprendentemente, tampoco se ha podido replicar la asociación de *SLC22A4* en población japonesa con el mismo background genético que la población del primer artículo [173], en un amplio estudio con alto poder estadístico [184]. En este caso, ni la heterogeneidad genética, ni la exposición a un medio ambiente distinto, ni la falta de poder estadístico podría explicar la falta de replicación. Aunque serían necesarios más estudios replicativos para rechazar totalmente este gen como de susceptibilidad a AR, los datos publicados hasta la fecha sugieren que la asociación reportada por Tokuhiro y col podría ser un falso positivo. Esto pone de manifiesto la importancia que los estudios replicativos tienen en el análisis del componente genético de susceptibilidad a AR.

En otras enfermedades autoinmunes, los polimorfismos de *SLC22A4* tampoco parecen tener gran relevancia, como en LES [148], enfermedad celiaca [185] y psoriasis [186]. Sin embargo, existen resultados controvertidos en diabetes tipo 1. Mientras que un estudio realizado en españoles detectaba asociación significativa [187], otro estudio británico desechaba la asociación [188]. El elevado número de pacientes y controles empleado en el estudio británico en comparación con el español hace pensar que *SLC22A4* no está involucrado en diabetes tipo 1, aunque harían falta más estudios replicativos para poder descartarlo con toda seguridad.

El polimorfismo de *RUNX1* tampoco se ha visto asociado a AR en estudios posteriores [184, 189, 190], a pesar de que mapea en región de ligamiento a AR (21q22) [64], ni a diabetes tipo 1 [188] ni LES [148].

Todos estos indicios sugieren que ni *SLC22A4* ni *RUNX1* son relevantes en la predisposición genética a las enfermedades autoinmunes.

5.1.4.1.2. La familia de la IL-12

La IL-12 es una interleuquina proinflamatoria heterodimérica formada por una cadena p35 (o IL-12α) y una cadena p40 (o IL-12β) [191]. Es el miembro prototipo de una pequeña familia de citoquinas, de la que también forman parte IL-23 e IL-27 (Figura 5.12). IL-12 e IL-23 comparten la subunidad p40, la cual es producida con un gran exceso en comparación con los heterodímeros IL-12 e IL-23. IL12-p40 está codificada por el gen *IL12B,* localizado en el cluster de citoquinas 5q31.

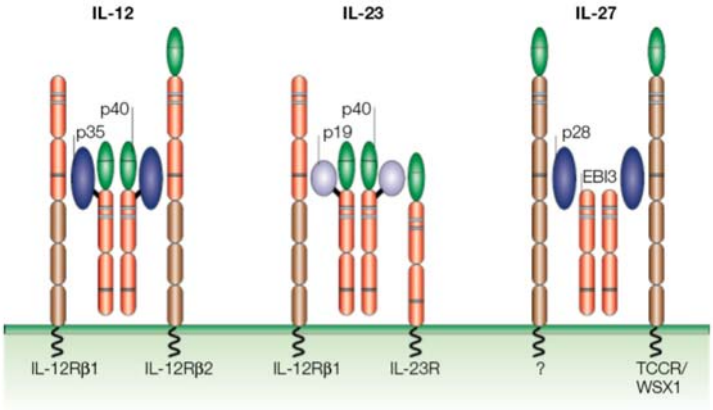

Figura 5.12. La familia de la IL-12.

Las funciones que IL-12 tiene como inductora de la producción de IFN-γ, de la diferenciación de células T hacia el subtipo T_H1 y como eslabón de unión entre el sistema inmune innato y adaptativo, hacen que esta citoquina tenga un papel crucial en la AR (Figura 5.13). Los principales productores de IL-12 son macrófagos, neutrófilos y células dendríticas en respuesta a varios estímulos activadores.

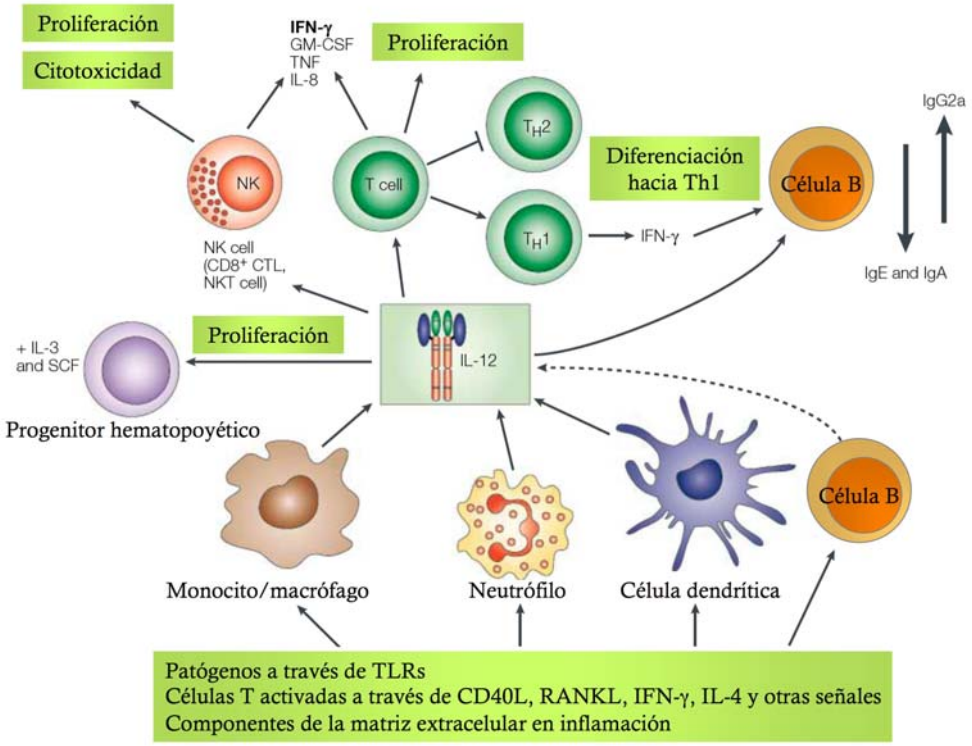

Figura 5.13. Funciones biológicas de la IL-12.

***IL12B*:** IL-12p40 es un interesante candidato para su estudio en AR, por su función y su localización cromosómica, como hemos comentado anteriormente. Además, se ha detectado una elevada expresión de *IL12B* mRNA en tejido sinovial de pacientes con AR, así como de la proteína completa IL-12p70 [192]. De los polimorfismos de *IL12B*, dos de ellos son de particular interés, ya que parecen tener relevancia biológica: un SNP 1188A→C en la región 3´ no traducida (*IL12B 3´UTR*), que se vio asociado a diabetes tipo 1, y además, altera los niveles de expresión del gen [193] y una inseción/delección en la región promotora (*IL12Bpro*), que se vio asociado a severidad del asma en niños [194] y a diferencias en la expresión de IL-12p70 [195].

Teniendo en cuenta la importancia de este gen, y la relevancia de los polimorfismos descritos en la literatura, decidimos estudiar la influencia de estas variantes genéticas en la predisposición a padecer AR en nuestra población. Cuando comparamos las frecuencias de ambos polimorfismos entre pacientes y controles, no encontramos diferencias estadísticamente significativas. Tampoco encontramos asociación con las distintas características clínicas de la enfermedad [196]. El poder estadístico alcanzado en el estudio fue suficiente para demostrar que nuestros resultados no fueron un falso negativo. Además,

las frecuencias que observamos fueron similares a las encontradas en otras poblaciones caucásicas [194, 197, 198]. Así mismo, de acuerdo con nuestros resultados, otro estudio tampoco pudo encontrar asociación entre los polimorfismos de *IL12B* y AR en dos poblaciones independientes griega y británica [197]. Esto sugiere que las variantes estudiadas de *IL12B* no influyen en la susceptibilidad a AR.

Aunque en un principio se propuso que *IL12B 3´UTR* se asociaba a diabetes tipo 1 [193], estudios posteriores no han podido replicar esta asociación [199-203], lo que cuestiona el papel de este gen en la enfermedad. La asociación de *IL12Bpro* con asma tampoco se ha replicado [204]. Además, también se ha puesto en duda la relevancia funcional de los polimorfismos *IL12Bpro* e *IL12B 3´UTR* [200, 205] y no se ha encontrado asociación de éstos con otras enfermedades autoinmunes relacionadas, como LES [206], enfermedad celiaca [201, 207, 208], esclerosis múltiple [209], y miastenia gravis [210].

El conjunto de estos indicios, nos llevan a descartar que las variantes *IL12B 3´UTR* e *IL12Bpro* estén implicadas en autoinmunidad. Sin embargo, otros hallazgos sugieren que sí podrían estar involucradas en la susceptibilidad a infecciones [211]. Finalmente, en un reciente estudio se ha identificado sólidamente a *IL12B* como gen de susceptibilidad a psoriasis [212]. Parece claro que este gen está involucrado en importantes mecanismos inmunológicos, así que estudios más profundos utilizando más marcadores podría aclarar el papel de *IL12B* en AR.

***IL12RB1*:** La actividad biológica de IL-12 está mediada mediante su unión al receptor IL-12R, el cual está compuesto de dos subunidades (Figura 5.12): IL-12Rβ1, codificado por *IL12RB1* y compartido con el receptor de IL-23, e IL-12Rβ2, codificado por *IL12RB2*. Tres SNPs no sinónimos en el gen *IL12RB1* nos parecieron interesantes: 641A→G, 1094T→C y 1132G→C, que provocaban cambios de aminoácido (Q214R, M365T y G378R). El haplotipo formado por 214R, 365T y 378R parece que provoca una menor respuesta del receptor a la unión de IL-12 e IL-23, lo que origina susceptibilidad a infecciones [213, 214]. Dado que en la AR existe una respuesta inmune exagerada, pensamos que quizá este haplotipo de susceptibilidad a infecciones pudiera proteger frente a la autoinmunidad. Por lo tanto, genotipamos estos tres polimorfismos en nuestra cohorte de pacientes de AR y controles sanos, pero no encontramos asociación con la enfermedad [196]. Del mismo modo, estos SNPs tampoco se asocian a otras enfermedades autoinmunes como LES [206] y diabetes tipo 1 [215, 216].

Varios estudios intentaron estudiar el gen que codifica para la otra molécula que forma parte del receptor de IL-12, *IL12RB2*. Sin embargo, no encontraron mutaciones potencialmente funcionales [213, 217].

***IL23R*:** Otro importante miembro de la familia de la IL-12 es IL-23, que recientemente ha recibido una mayor atención debido a su papel en la diferenciación de un nuevo subtipo de célula T $CD4^+$, las T_H17 (Figura 5.14) [218].

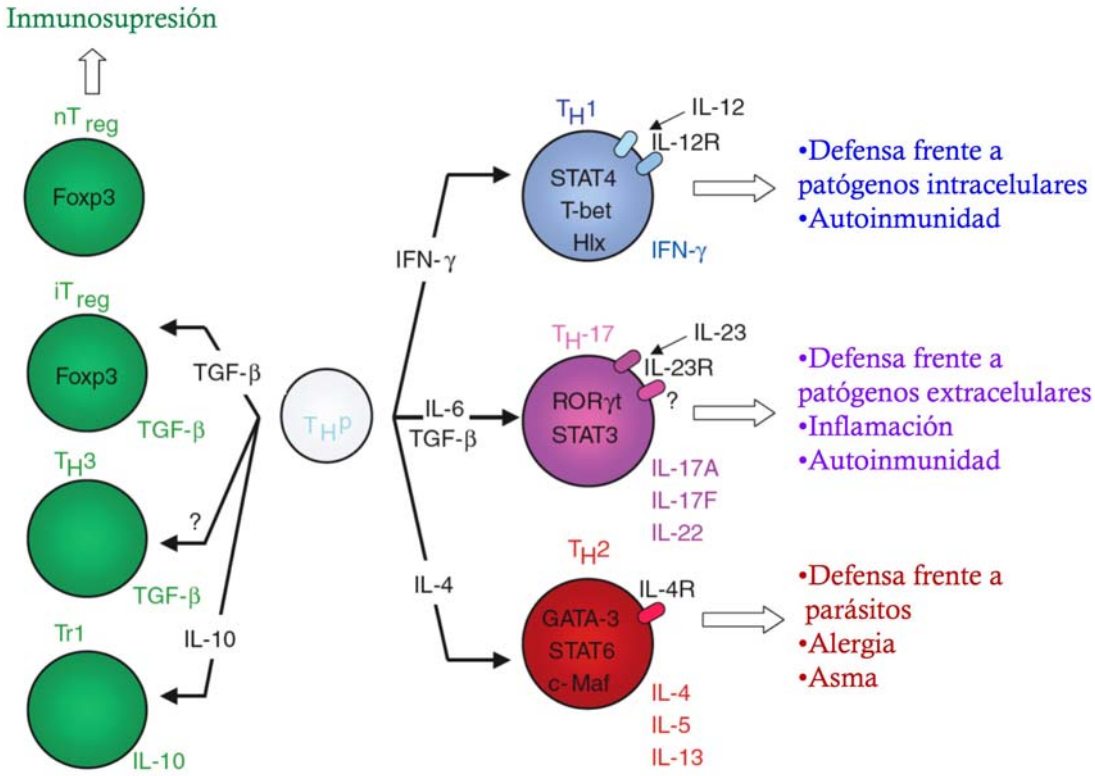

Figura 5.14. Diferenciación de las células T $CD4^+$

Para poder explicar el mecanismo mediante el cual se produce la autoinmunidad, durante años prevaleció la hipótesis Th1/Th2, mediante la cual se pensaba que en condiciones fisiológicas debía existir un equilibrio entre estos dos tipos de respuestas celulares, que se inhiben recíprocamente. Por lo tanto, una pérdida del equilibrio hacia una respuesta T_H1 produciría una autoinmunidad, mientras que una respuesta exagerada T_H2 produciría alergia o asma [219]. Más tarde, este modelo se ha ido retocando y modificando, mediante la incorporación al modelo de las células T reguladoras, que serán comentadas más adelante. Estudios más recientes han sugerido que en la patogénesis de las enfermedades autoinmunes como la AR debe estar interviniendo otro tipo celular además de las T_H1 productoras de INFγ, ya que se ha visto que ratones deficientes en INFγ o su receptor no son resistentes a autoinmunidad [220]. Posteriores estudios con ratones deficientes en IL-12 e IL-23 pusieron de manifiesto que ratones deficientes solamente en IL-23 sufrían de encefalomielitis autoinmune experimental (EAE), un modelo de esclerosis

múltiple, y de artritis inducida por colágeno (CIA), un modelo de AR [221]. Parece ser que esto es debido a que IL-23 promueve la proliferación de un nuevo subtipo de células T, las células Th17. Su diferenciación se estimula mediante IL-6 y TGF-b, y se caracterizan por la producción de IL-17A, IL-17F y en menor medida, TNF e IL-6. Así que parece ser que no sólo una alteración de la activación y la actividad de las células T_H1 promueven la autoinmunidad, sino que también hay que tener en cuenta a las células T_H17, aunque aún no se conocen en profundidad sus mecanismos efectores (Figura 5.14).

La IL-23 está formada por las subunidades p40 y p19 (Figura 5.12). Como hemos visto en el apartado anterior, los polimorfismos más relevantes de *IL12B* (el gen que codifica la subunidad p40) parecen no influir en la susceptibilidad a AR. Con respecto a la subunidad p19, el gen *IL23A* fue secuenciado por completo utilizando 30 individuos de nuestra población de Granada y no se encontró ningún polimorfismo en su secuencia, de acuerdo con lo depositado en las bases de datos [206].

Muy recientemente, en un estudio de GWA, se identificó al gen *IL23R* como uno de los mayores responsables de la susceptibilidad genética a enfermedad inflamatoria intestinal (EII) [222]. Poco después, y de manera independiente, en un estudio a gran escala se observó que el mismo gen está asociado a psoriasis [223]. Numerosos estudios replicativos en múltiples poblaciones han demostrado que *IL23R* es un gen de riesgo a EII [224-229].

Varias publicaciones recientes han probado que IL-23, a través de la unión a su receptor, también tiene un importante papel en AR. Ratones deficientes en IL-23 son resistentes a CIA [230], y la expresión de p19 induce inflamación en ratones transgénicos [231]. En humanos, se ha visto que hay una sobreexpresión de p19 en fibroblastos sinoviales [232]. Además, *IL23R* se localiza en el cromosoma 1p, en una región de ligamiento con AR [233]. Dado el importante papel que *IL23R* parece tener en la AR y teniendo en cuenta la co-localización de loci de susceptibilidad entre distintas enfermedades autoinmunes, pensamos que *IL23R* podría ser un loci común para EII y AR. Para estudiar este posible efecto, genotipamos 8 polimorfismos de *IL23R*, que eran los que muestran mayor asociación con EII en estudios anteriores. Sin embargo, ninguno de ellos está asociado a AR en nuestra población [234]. Así mismo, nuestro grupo tampoco encontró asociación con LES [235]. Un estudio realizado en ratones ha mostrado que IL-23 es esencial en la inflamación tisular local, pero no en la inflamación sistémica [236]. Estos indicios, junto al hecho de que *IL23* se asocia a enfermedades autoinmunes órgano-específicas (EII y psoriasis) y no sistémicas (AR y LES), nos lleva a pensar, que quizás IL-23 tenga más importancia en la regulación de inflamación local. Recientemente se ha visto que *IL23R* es un factor de riesgo para la espondilitis anquilosante (EA) [237], lo que ha sido

corroborado en nuestra cohorte de EA española (Rueda y col, datos no publicados). A la vista de todos estos indicios, parece ser que este gen está implicado en enfermedades autoinmunes seronegativas, tales como EII, psoriasis y EA, y no en RA y SLE, donde los autoanticuerpos juegan un papel fundamental.

Hemos estudiado gran parte de los genes de la familia de la IL-12 (*IL12B*, *IL12RB1*, *IL23A* y *IL23R*) sin encontrar asociación con AR. Sin embargo, no podemos descartar otros polimorfismos de esta familia de genes como posibles factores de riesgo. Quedan por estudiar los genes del otro miembro de la familia, IL-27, así como *IL12A* e *IL12RB2*. Sin embargo, el papel de estos genes en autoinmunidad aun no se ha estudiado en profundidad, por lo que futuras investigaciones ayudarán a conocer mejor la implicación de la familia de la IL-12 en la AR.

5.1.4.2. *MIF*

El gen factor inhibidor de la migración de macrófagos (*MIF*) ha acaparado la atención de numerosos grupos de investigación en los últimos años, dado su importante papel regulador en la inmunidad innata y adquirida. MIF es una citoquina proinflamatoria expresada por una gran cantidad de células y tejidos, que además tiene actividades hormonal y enzimática [238]. Tras la estimulación de las células del sistema inmune por distintas señales, éstas son capaces de liberar MIF rápidamente, el cual induce la activación de la cascada pro-inflamatoria mediada por ERK1/ERK2. Directa o indirectamente, MIF estimula la producción de un amplio panel de moléculas proinflamatorias tales como citoquinas (TNFα, IFNγ, IL-1β, IL-2, IL-6, IL-8 y proteína inflamatoria de macrófagos 2), óxido nítrico, COX2 y productos de la ruta del ácido araquidónico (como PGE_2) y varias MMPs. Además, MIF es capaz de inhibir la apoptosis a través de la supresión de la actividad de p53 y de promover la respuesta de tipo T_H1 [238].

Estudios con modelos animales de AR, han revelado que MIF juega un importante papel en esta enfermedad. Se ha visto que el tratamiento con anticuerpos anti-MIF protege frente a CIA [239], y ratones $MIF^{-/-}$ sufren de una artritis inducida por antígeno (AIA) menos severa [240]. En humanos, los niveles de MIF están aumentados en suero y tejido sinovial de pacientes de AR y se correlacionan con la actividad de la enfermedad [241]. En la figura 5.15 se representa un esquema del papel que MIF juega en la AR. MIF es producido por los macrófagos y células T activadas siendo capaz de auto-estimular a estas células por un efecto paracrino. MIF induce la producción de citoquinas proinflamatorias, contribuyendo a la perpetuación del ambiente inflamatorio. Además, activa la producción de prostaglandinas (fundamentalmente PGE_2) que median el dolor y el aumento de la permeabilidad vascular en el sinovio, así como MMPs, todo ello promoviendo la

destrucción de la articulación. MIF también regula la función de células endoteliales y células B, y controla la proliferación y apoptosis de sinoviocitos mediante su influencia sobre la expresión de p53 [242].

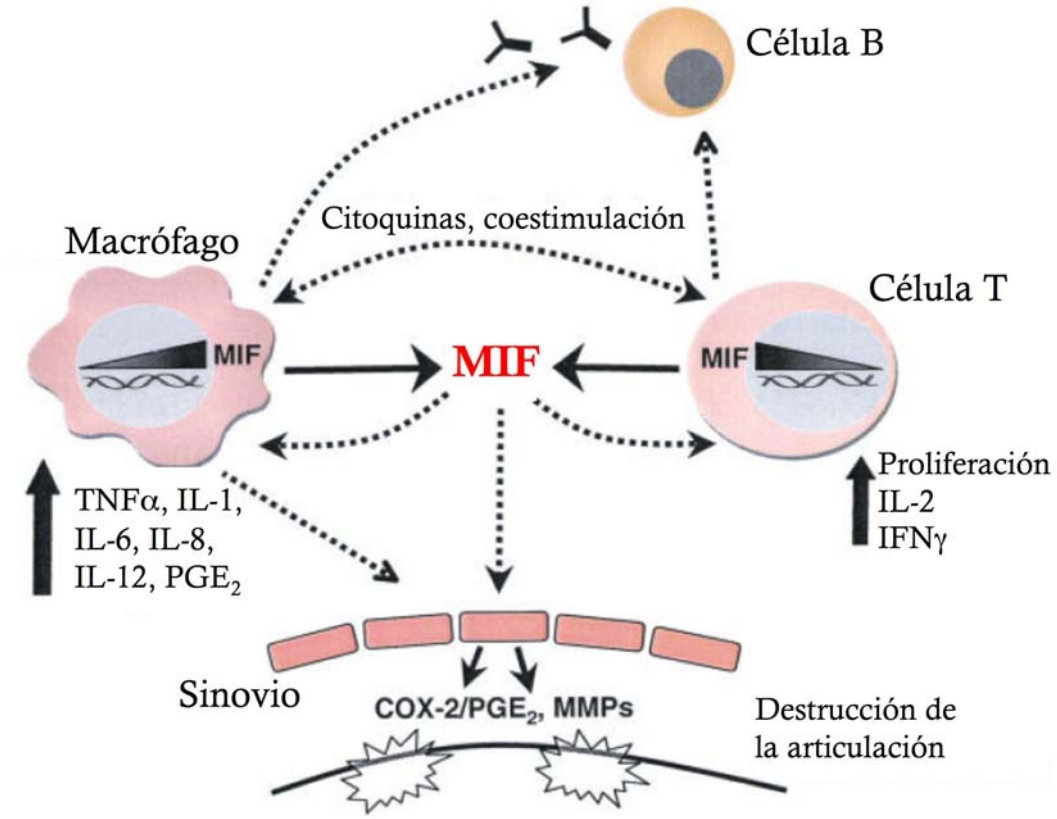

Figura 5.15. Esquema del papel de MIF en AR.

Dada la importancia de MIF en enfermedades autoinmunes humanas y otras condiciones patológicas, el gen ha sido estudiado ampliamente, lo que ha originado relevantes datos. Dos polimorfismos en el promotor de *MIF* destacan por su relevancia funcional: un microsatélite $(CATT)_{5\text{-}8}$ y un SNP -173G→C, cuyos alelos $(CATT)_7$ y -173C se encuentran en desequilibrio de ligamiento [243] (Figura 5.16). La presencia del alelo C del SNP provoca una mayor producción de MIF, debido a que crea un sitio de unión para el factor de transcripción AP4. Además, en el primer estudio de asociación realizado para *MIF*, se vio que este alelo predispone a AIJ [243].

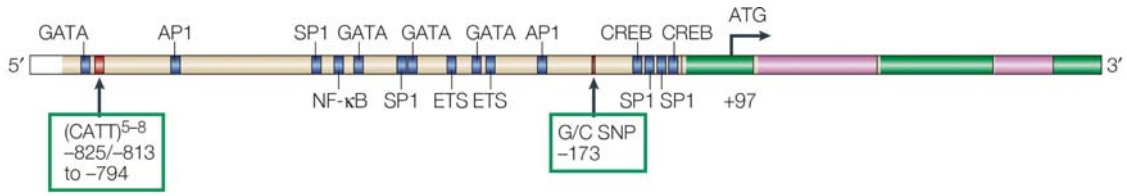

Figura 5.16. Estructura del gen *MIF* y localización de los polimorfismos $(CATT)_{5-8}$ y -173G→C.

Decidimos, en colaboración con el de la Dra. Urcelay, estudiar estos dos polimorfismos con relación a la susceptibilidad a AR, dado el importante papel de *MIF* en la patogénesis de la AR, y su localización en una región de ligamiento (22q11) [244]. Encontramos que en nuestra población, el alelo -173C está significativamente aumentado en pacientes de AR con respecto a controles sanos (*P*= 0.01, OR= 1.3; 95% CI 1.06-1.62). Sin embargo, no observamos diferencias en la distribución de los distintos alelos del microsatélite $(CATT)_n$. Aunque vimos que, como en otras poblaciones, los alelos -173C y $(CATT)_7$ están en alto LD (D´= 1), el coeficiente r^2 es bajo (0.6), por lo que se explica que no encontráramos asociación con el microsatélite.

Como *MIF* se había asociado previamente a AIJ, quisimos ver si el SNP tenía relación con la edad de comienzo de la AR. Pudimos comprobar que el alelo -173C se encuentra más fuertemente asociado a AR en el grupo de pacientes con menor edad de comienzo, mientras que en los pacientes con aparición tardía de la enfermedad no existe asociación.

Diversos estudios habían investigado ambas variantes de *MIF*, -173G→C y $(CATT)_{5-8}$, con relación a predisposición a AR previamente, pero los resultados obtenidos habían sido aparentemente contradictorios. Mientras que dos estudios encontraron asociación con la susceptibilidad a AR [245, 246], otros dos encontraron que *MIF* estaba asociado sólo a la severidad de la enfermedad [247, 248], e incluso otros reportaron que *MIF* no está asociado a AR [249, 250]. Estos resultados discordantes podrían ser debidos a la heterogeneidad genética que existe entre poblaciones, incluso entre Europeos [251]. De hecho, las frecuencias de los polimorfismos de *MIF* que encontramos en nuestra población eran bastante distintas a las encontradas por ejemplo en alemanes [246]. Sin embargo, a la vista de nuestros resultados, podríamos sugerir otra explicación, dado el impacto que la edad parece tener en la susceptibilidad dada por -173G→C a la enfermedad. La heterogeneidad en la distribución por edades entre cohortes podría explicar las diferencias observadas en los estudios previos, de tal manera que, si la cohorte está compuesta principalmente por individuos que desarrollaron la enfermedad más tardíamente, el efecto de susceptibilidad de *MIF* podría pasar desapercibido.

Los polimorfismos de *MIF* se han relacionado con susceptibilidad a otras enfermedades autoinmunes, aparte de AIJ y AR, como LES [252], enfermedad celiaca [253] y EII [254].

El relevante papel que MIF parece jugar en AR ha llevado a proponer que antagonistas de MIF podrían tener aplicación terapéutica, ya que, como se comentó anteriormente, la administración de anticuerpos anti-MIF inhibe el desarrollo y la severidad de modelos animales de AR [239, 255]. En éste sentido, recientemente se han obtenido resultados esperanzadores con una vacuna de DNA dirigida hacia MIF, al menos en modelos murinos de AR [256]. Curiosamente, los glucocorticoides, que son utilizados en el tratamiento de la AR, son capaces de inducir la expresión de MIF, y éste es capaz de inhibir los efectos de estas drogas mediante un mecanismo que aún no ha sido dilucidado por completo [257]. Por lo tanto, es probable que una terapia anti-MIF sea efectiva en aquellos pacientes que se vuelvan resistentes al tratamiento con glucocorticoides. Sin embargo, los polimorfismos de *MIF* -173G→C y $(CATT)_{5\text{-}8}$ no están asociados con la respuesta a glucocorticoides ni anti-TNFα en pacientes con AR [258].

En conclusión, nuestros datos sugieren que el alelo *MIF* -173C está asociado con la predisposición a padecer AR, probablemente a través de la elevada producción de MIF que provoca este alelo, y sobre todo en pacientes con edad de comienzo de la enfermedad más temprana. Sin embargo, un mayor número de estudios replicativos serían útiles para poder establecer firmemente cómo las variantes genéticas de *MIF* influyen en la AR en distintas poblaciones.

5.2. Genes implicados en la respuesta de las células T.

El desarrollo y diferenciación de las células T, así como sus funciones efectoras, están finamente controlados por la activación de varias rutas de señalización intracelular en respuesta a la activación del receptor de la célula T (TCR) y de receptores de moléculas co-estimuladoras [259, 260]. Anormalidades en las rutas de señalización del TCR pueden resultar en autoinmunidad, a través de efectos en la proliferación de las células T, apoptosis, cambios en el citoesqueleto, producción de citoquinas, diferenciación o anergia, por mencionar sólo unos pocos de los procesos regulados por el TCR en células T tímicas y periféricas [261]. Por tanto, la señalización mediante TCR ha sido objeto de estudio en AR [16] y en muchas otras enfermedades autoimunes. Por ejemplo, se ha observado que una mutación en el gen que codifica para ZAP-70, una importante molécula en la transducción de señales en células T, resulta en la aparición espontánea de artritis autoinmune en

ratones [262]. Por lo tanto, genes relacionados con la activación y diferenciación de las células T son candidatos obvios para la búsqueda de marcadores de susceptibilidad a AR.

5.2.1. *PTPN22*

A pesar de que un gran número de evidencias apoyan la implicación de múltiples factores genéticos en AR, desde la identificación de la relación entre la enfermedad y los genes HLA de clase II hace 30 años, ningún gen no-HLA convincente había surgido como marcador de susceptibilidad a AR de manera inequívoca. Sin embargo, hace ahora tres años, el panorama de la genética de la AR experimentó un giro gracias a la identificación de *PTPN22* como gen de susceptibilidad a AR.

En un estudio pionero que adoptaba una estrategia de genes candidatos, Bottini y col propusieron a las proteín tirosín fosfatasas (PTPs) como potenciales genes de suscepibilidad a diabetes tipo 1. Encontraron un polimorfismo funcional (1857C→T, R620W, rs2476601) en el gen proteín tirosín fosfatasa no receptor 22 (*PTPN22*) fuertemente asociado a diabetes tipo 1 [263]. Las PTPs son reguladores críticos de la transducción de señales en las células T [264]. Junto a las proteín tirosín quinasas (PTKs), las PTPs regulan la fosforilación reversible de proteínas en residuos de tirosina. Este es un mecanismos fundamental en la transducción de señales y la regulación de un amplio grupo de procesos fisiológicos, incluyendo la regulación de la respuesta inmune[265]. Recientes estudios han puesto de manifiesto que incluso pequeñas alteraciones en las PTPs pueden provocar una disfunción inmunológica y enfermedades en humanos [265]. En concreto, *PTPN22* codifica para la fosfatasa específica de linfocitos LYP, que inhibe la señalización del TCR junto a la kinasa intracelular CSK (Figura 5.17). Esta actividad combinada inhibe la activación de LCK, una PTK que está involucrada en los eventos tempranos de la señalización del TCR. Cuando el TCR reconoce el antígeno y se activa, CD45 desfosforila a LCK en su tirosina Y505, y ésta se autofosforila en la posición Y394. Esto inicia la cascada de transducción de señales que resultará en la activación de la célula T. Sin embargo, LYP (o PTPN22) es capaz de regular negativamente a LCK, eliminando el fosfato activador en Y394, junto a CSK, que vuelve a fosforilar a LCK en Y505. De ésta manera, LYP inhibe la activación de la célula T [15]. El polimorfismo R620W destruye el sitio de unión de LYP a CSK, que es fundamental para su función [263].

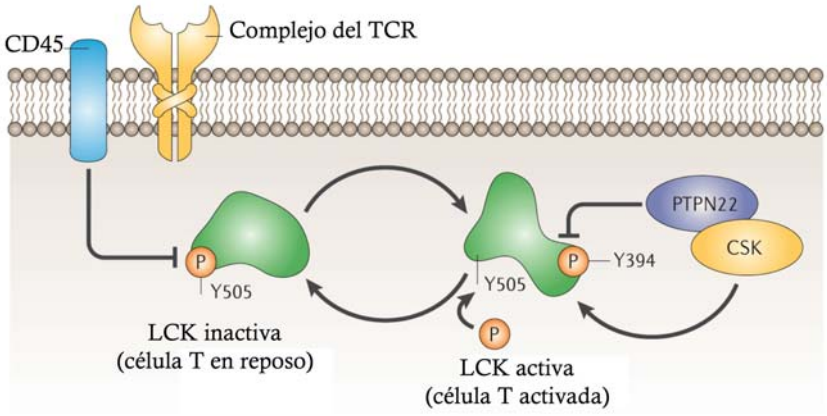

Figura 5.17. Función de LYP en la transducción de señales del TCR.

Como comentamos anteriormente, la regulación de la respuesta T es fundamental en la patogénesis de la AR, así que pensamos que *PTPN22* 1857C→T podría ser un marcador de susceptibilidad común a AR y diabetes. Además, este gen se localiza en la región de ligamiento a AR 1p13 [61], y ratones knockout para Ptpn22 tienen alteraciones en la señalización por TCR y un aumento en la producción de auto-anticuerpos [266]. Por lo tanto, decidimos genotipar esta variante en nuestra cohorte, encontrando que el alelo 1858T estaba fuertemente asociado a AR en nuestra población española [*P*= 0.001, OR (95% CI) 1.45 (1.15-1.83)] [72]. Cuando estábamos preparando estos resultados para su publicación, un grupo norteamericano reportó la asociación de este SNP con AR, lo que estaba de acuerdo con nuestros resultados [71]. Lo interesante de éste estudio es que, mediante un estudio a gran escala examinando multitud de SNPs potencialmente funcionales, Begovich y col encontraron de manera independiente el mismo polimorfismo que el previamente había sido descrito para diabetes tipo 1 en un estudio de genes candidatos.

Después de estos estudios iniciales, numerosas replicaciones de la asociación del polimorfismo 1857C→T del gen *PTPN22* con la AR han sido publicadas [267-273]. En todos los estudios la dirección de la asociación fue la misma: existe un aumento en la frecuencia del alelo T en pacientes de AR en comparación con la población sana, variando la OR para la AR conferida por este alelo T entre 1.38 a 2.04 [274]. Un reciente meta-análisis incluyendo gran parte de los estudios realizados en caucásicos ha revelado una fuerte asociación global [$P<10^{-5}$, OR (95% CI) 1.68 (1.53-1.84)] [275]. Juntos, todos estos

datos han consolidado la asociación del polimorfismo 1857C→T del gen *PTPN22* con la AR como la más robusta y reproducible fuera de la región HLA hasta la fecha. Finalmente, la relación de *PTPN22* con la AR también se ha demostrado en un estudio de ligamiento en familias [276]. Es más, en recientes GWAS se ha confirmado la asociación de *PTPN22* con AR [76, 77].

Además del importante papel que el polimorfismo de *PTPN22* tiene en la susceptibilidad a AR y la diabetes tipo 1, este SNP se ha visto asociado a muchas otras enfermedades autoinmunes, como LES [72], AIJ [267], enfermedad de Graves [277], enfermedad autoinmune tiroidea [278] y miastenia gravis [279], lo que ha llevado a proponer este polimorfismo como marcador general para muchas enfermedades autoinmunes. Sin embargo, otros estudios no han detectado asociación de este marcador con EII [280], enfermedad celiaca [281], esclerosis múltiple [282] y espondilitis anquilosante [283]. A la vista de estas discordancias, se ha sugerido que *PTPN22* 1857C→T predispone a enfermedades autoinmunes donde existe un fuerte componente humoral, es decir, donde los anticuerpos juegan un papel fundamental en la patogénesis, y por el contrario, no sería importante en enfermedades seronegativas. Con respecto a esto, se ha intentado investigar la relación entre el SNP y la presencia en suero del FR en pacientes de AR, generando resultados contradictorios. Nosotros no encontramos ninguna relación entre la presencia del factor reumatoide y el polimorfismo de *PTPN22* en nuestra población [72], lo que ha sido corroborado por otros estudios [267, 268, 270]. Sin embargo, otros grupos encontraron que *PTPN22* sólo se asocia a AR en pacientes RF+ [71, 284]. Estas discrepancias podrían simplemente reflejar la heterogeneidad clínica en los pacientes de AR entre poblaciones, pero también podrían ser debidas a los diferentes métodos de detección del FR, o a los diferentes estadios de la enfermedad en los que el FR fue medido.

A pesar de que los datos funcionales apoyan que *PTPN22* 1857C→T es el SNP causante de la enfermedad, la asociación podría deberse al LD con variantes adicionales del gen. Por eso Carlton y col realizaron un estudio de mapeo fino del gen, encontrando dos polimorfismos asociados con AR de manera independiente a 1857C→T [285]. Sin embargo, varios estudios posteriores han podido comprobar que 1857C→T es el único polimorfismo que puede explicar la asociación con AR dentro del gen *PTPN22* [168, 286, 287].

El alelo de riesgo de *PTPN22* produce una sustitución de un triptófano por una arginina en el aminoácido 620 (R620W) en el dominio de unión rico en prolina SH3. Este polimorfismo tiene dos consecuencias funcionales: la unión entre LYP y CSK se interrumpe [71, 263] y la actividad enzimática de LYP aumenta [288, 289]. El efecto de estos cambios bioquímicos parece ser un aumento en el umbral de estimulación que se

requiere para la señalización del TCR (Figura 5.18). En el timo, esto podría provocar la selección positiva de timocitos que en situación normal serían eliminados, apareciendo en la periferia células T potencialmente autorreactivas. Un segundo mecanismo podría suponer una menor activación de las células T reguladoras, lo que provocaría una deficiente regulación de células T autorreactivas. Estos mecanismos podrían llevar a un estado de susceptibilidad a autoinmunidad [290]. Un reciente estudio con linfocitos de pacientes ha demostrado que el alelo 1858T origina un aumento de células T de memoria CD4+, y además, una producción de citoquinas alterada [289]. De manera interesante, también han observado que no sólo las células T se encuentran alteradas por la presencia de la mutación, sino que también las células B responden menos a la estimulación vía BCR. Los autores sugieren que esto podría resultar finalmente en la producción aumentada de autoanticuerpos, lo que apoya que *PTPN22* 1857C→T se asocie con enfermedades típicamente seropositivas, como la AR. Sin embargo, son necesarios más estudios funcionales para poder llegar a comprender el complejo efecto que esta variante genética puede producir en el sistema immune.

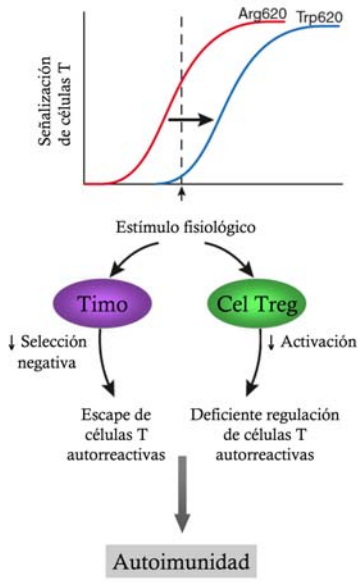

Figura 5.18. Posible papel funcional del polimorfismo Arg620Trp de LYP.

Una característica importante del polimorfismo *PTPN22* 1857C→T es la heterogeneidad en su distribución entre distintas poblaciones. El alelo 1858T se encuentra con una frecuencia casi nula en poblaciones asiáticas [291], y por otro lado, en poblaciones europeas existe un gradiente sur-norte en el aumento de la frecuencia de este alelo [292].

Estas diferencias no quieren decir que *PTPN22* no esté involucrado en la patogénesis de la AR en asiáticos. Valga como ejemplo el hecho de que la mayoría de los pacientes afroamericanos de AR no son portadores de la secuencia del EC en el gen HLA-DRB1 [293], sin que esto ponga en duda la importancia de estos alelos en la AR. La heterogeneidad genética supone que menos del 60% de los SNPs comunes evaluados por la fase I del proyecto HapMap fueron polimórficos en las tres poblaciones representadas en la figura 5.19: japonesas, africanas y blancas [294].

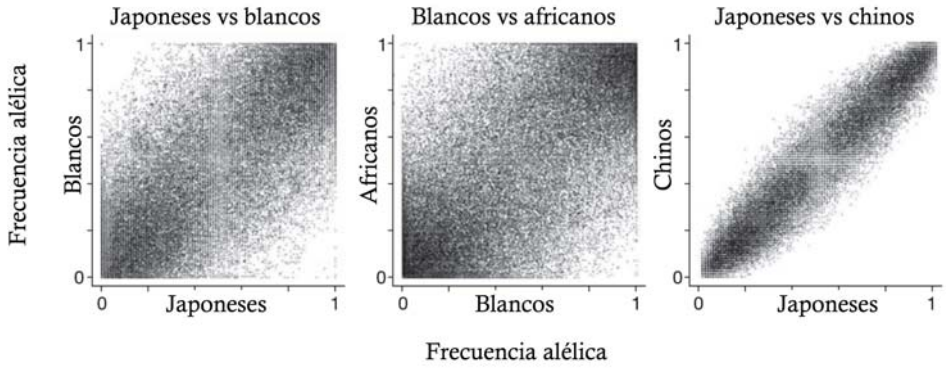

Figura 5.19. Gráficos representando la frecuencia alélica de los SNPs comunes del proyecto HapMap que muestra la heterogeneidad entre distintos grupos.

Como hemos comentado previamente, los marcadores más fidedignos de predisposición y severidad para AR hasta la fecha son los genes *HLA-DRB1* y *PTPN22*, junto con la presencia de los autoanticuerpos FR y anti-CCP. Recientemente, se ha propuesto que los alelos del EC *HLA-DRB1* se asocian exclusivamente con el subgrupo de pacientes de AR que son seropositivos para anti-CCP [52]. Además, un estudio ha observado que la combinación de la variante *PTPN22* 1858T y la presencia de anti-CCP es 100% específica para la AR y es capaz de predecir la aparición de la enfermedad [295]. A la vista de estos interesantes datos, decidimos analizar el valor de la combinación del polimorfismo *PTPN22* 1857C→T, los alelos *HLA-DRB1* del EC y la presencia de autoanticuerpos como marcadores de susceptibilidad para el desarrollo de AR. Para ello utilizamos una cohorte de artritis inflamatoria, de la que, tras seguimiento, se comprobó que 191 pacientes sufrían de AR y los restantes 184 individuos padecían otras artropatías [296]. De acuerdo con estudios publicados previamente [297, 298], vimos que los alelos del EC se asocia fuertemente a la presencia en suero de anticuerpos anti-CCP y que estos alelos no se asocian con la AR como tales, sino más bien con fenotipo anti-CCP definido.

También vimos que la combinación del alelo 1858T del gen *PTPN22* y la presencia de anti-CCP estaba fuertemente asociada a AR y es altamente específica para la enfermedad.

Teniendo en cuenta datos anteriores, Van Gaalen y col formularon un modelo para la patogénesis de la AR en dos pasos, que incorpora una nueva hipótesis del EC "citrulinado" [53]. En éste modelo, el primer paso sería la inducción de los anti-CCP. La citrulinación de proteínas es un proceso fisiológico que ocurre durante la apoptosis, el cual facilita el acceso a las proteasas. Éste proceso probablemente no lleva a una respuesta inmune contra antígenos citrulinados. Sin embargo, la citrulinación puede ocurrir durante la inflamación, o por influencia de un factor ambiental, lo que lleva a la inducción de una respuesta de células T sólo en un individuo EC positivo. Ésta respuesta T alterada también podría deberse al alelo *PTPN22* 1858T. Las células T activadas podría inducir una respuesta de células B patogénica específica para antígenos citrulinados, una vez que los antígenos citrulinados son generados en la articulación y los anti-CCP son capaces de entrar en ella. El segundo paso del modelo es la expresión de antígenos citrulinados en la articulación inflamada. El reclutamiento de granulocitos y monocitos en la articulación, seguido de su muerte, probablemente resulta en la activación de la enzimas PADI4 y PADI2, permitiendo la citrulinación de proteínas. Su reconocimiento por los anti-CCP llevaría a la formación de inmunocomplejos, perpetuando la respuesta inmune y la inflamación.

Como conclusión, podemos decir que la combinación del alelo *PTPN22* 1858T junto a la presencia de anticuerpos anti-CCP, preferentemente en un individuo EC positivo, está asociada al desarrollo de AR. En el futuro, esta combinación junto a otros factores, podría ser utilizada como marcador de pronóstico en AR.

5.2.2. *CTLA4*

La activación de la célula T se rige por un mecanismo que incluye dos señales. La primera señal la proporciona la interacción del complejo péptido-MHC con el TCR. Una alteración en la ruta de transducción de señales que se activa mediante esta primera señal puede conducir a situación patológica, como hemos visto anteriormente con el gen *PTPN22*. La segunda señal es proporcionada a la célula T mediante la ruta de coestimulación B7-1/B7-2—CD28/CTLA-4 (Figura 5.20) [299].Cuando CD28, que se expresa constitutivamente en las células T, se une a B7-1 o B7-2 se produce una activación del linfocito T. Sin embargo, CTLA-4 puede competir con CD28 por la unión de B7-1 o B7-2, inhibiendo la activación de la célula T. En consecuencia, una alteración en la función de CTLA-4 podría generar una mayor estimulación de las células T , fenómeno que está

presente en la autoinmunidad. Una particularidad de la función de CTLA-4 es que en células T_{reg}, donde se expresa constitutivamente, es activadora. Por tanto, una alteración de CTLA-4 podría suponer una disminución en la actividad de las células T_{reg}, propiciando así mismo la autoimunidad. Varios estudios apuntan a que esta ruta de coestimulación parece ser de vital importancia en el desarrollo de enfermedades autoinmunes [300]. De hecho, ratones deficientes en B7-1/B7-2 presentan autoinmunidad exacerbada [301-303].

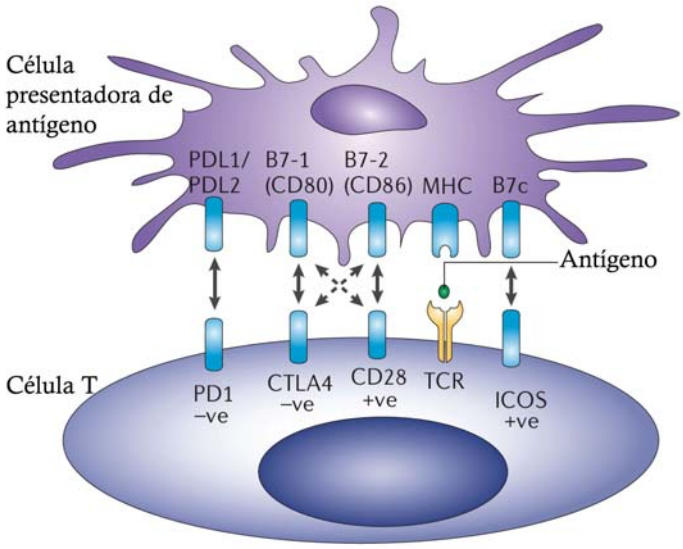

Figura 5.20. Moléculas coestimuladoras y coinhibidoras que intervienen en la activación de la célula T.

En éste sentido, varios polimorfismos de *CTLA4* se han visto asociados con diversas enfermedades autoinmunes [304]. Esta asociación fue descrita inicialmente para la enfermedad de Graves [305], y más tarde diabetes tipo 1 [306], centrándose la mayoría de los estudios en el polimorfismo +49A/G. *CTLA4* se localiza en una región del cromosoma 2q que también codifica para otras dos moléculas coestimuladoras, CD28 e ICOS, por lo que fue sugerido inicialmente que la asociación con *CTLA4* podría reflejar desequilibrio de ligamiento con otros genes candidatos en la región [304] (Figura 5.21, a). Sin embargo, un análisis de SNPs extensivo de esta zona redujo la región de susceptibilidad a un segmento de 6.1 kb en la región 3´UTR de *CTLA4* [307]. Se observó que un haplotipo formado por cuatro SNPs estaba asociado a diabetes tipo 1, enfermedad de Graves e hipotiroidismo autoinmune, de entre los que el llamado CT60 mostraba una asociación más fuerte. De manera interesante, el haplotipo de riesgo estaba asociado con una menor producción de la forma soluble secretada de CTLA-4 (sCTLA-4) con respecto a la forma completa (flCTLA-4) (Figura 5.21, b). Ésta alteración podría llevar a una menor ocupación de las moléculas

CD80 y CD86 por sCTLA-4, lo que permitiría una mayor activación de la célula T a través de CD28.

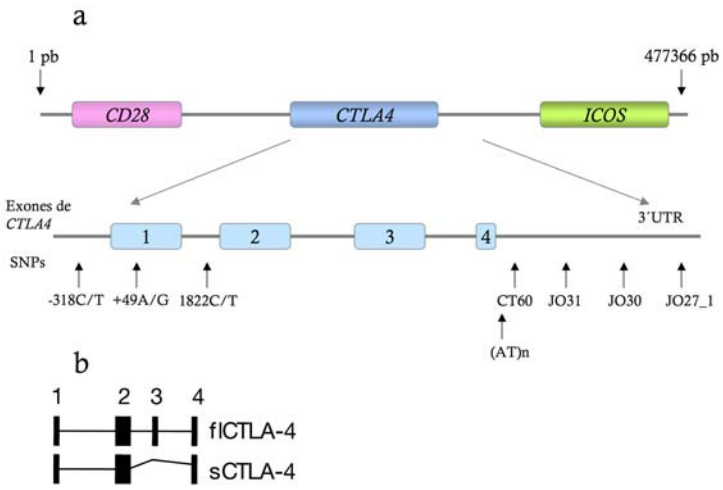

Figura 5.21. a. Estructura de la región 2q33 y localización de los SNPs de *CTLA4* más estudiados en autoimunidad. b. Distintas isoformas de la molécula CTLA-4.

A la vista de la importancia que la activación de las células T tiene en AR, y el papel de CTLA-4 como molécula inhibidora de este proceso, decidimos estudiar el polimorfismo CT60 en AR, por su potencial papel funcional y por estar fuertemente asociado a otras enfermedades autoinmunes. Además tuvimos en cuenta que el gen *CTLA4* mapea en una región de ligamiento a AR, 2q33 [57]. De manera interesante, uno de los fármacos usados extensamente en AR, abatacept, consiste en la administración de CTLA4-Ig, lo que pone de manifiesto la relevancia de este gen en la enfermedad [308]. A pesar de lo prometedor del marcador, encontramos que *CTLA4* CT60 no estaba asociado a AR en nuestra población [309]. Aunque varios estudios en poblaciones europeas han encontrado al igual que el nuestro que este SNP no está asociado a AR [310-312], dos estudios han encontrado asociación en chinos y norteamericanos [250, 313]. Se ha llevado a cabo un meta-análisis de la asociación de CT60 con AR incluyendo nuestros datos y los obtenidos en poblaciones británica, sueca y norteamericana, encontrando un modesto efecto de este polimorfismo (P= 0.01) [250]. Sin embargo, si se elimina del estudio la población nortemericana, se pierde la asociación. Otros polimorfismos de *CTLA4* han sido estudiados en AR, con resultados igual de controvertidos [314]. En el estudio más extenso realizado para el gen *CTLA4* en AR, no se encontraron evidencias de que ningún SNP ni haplotipo confiriera susceptibilidad a AR [310], lo que fue corroborado más tarde por Zhernakova y

col [311]. A la vista de estos resultados podemos concluir que los polimorfismos de *CTLA4* estudiados hasta la fecha no parece contribuir a la susceptibilidad genética a AR, y en el caso de que tuviera algún efecto, éste sería pequeño.

Aunque *CTLA4* se ha propuesto como un gen común de susceptibilidad a autoinmunidad, numerosos estudios muestran una gran inconsistencia en los resultados obtenidos, en enfermedades como LES, enfermedad de Graves, enfermedad celiaca y otras, incluyendo a la AR como ya hemos comentado. En un extenso estudio abarcando el locus *CD28-CTLA4-ICOS* con 80 SNPs, se ha visto que esta región posee un complicado patrón de bloques de desequilibrio de ligamiento y que los SNPs más asociados a LES se encuentran más alejados de la región 3´UTR de *CTLA4* que la previamente reportada por Ueda y col [307, 315]. Un reciente estudio ha corroborado el largo alcance de los haplotipos extendidos en esta región resaltando además las grandes variaciones en la distribución de haplotipos entre distintas poblaciones [316]. Estos datos sugieren que sería necesaria una re-evaluación de este locus en relación a la susceptibilidad a autoinmunidad con análisis de haplotipos más fidedignos. Quizá los estudios que ofrecen resultados controvertidos están detectando una señal producida por el LD con el marcador etiológico de susceptibilidad, y como se ha visto, este patrón de LD puede variar de una población a otra.

5.2.3. *BTNL2*

Con respecto a la ruta de coestimulación de las células T, otros genes han sido estudiados en AR, sin encontrar evidencias de asociación. Por ejemplo, no se ha encontrado asociación con los polimorfismos de los genes *CD80* y *CD86* (B7-1 y B7-2) y *CD28* [317], y el estudio de la posible asociación de polimorfismos del gen *PD1* con AR ha generado resultados conflictivos [15].

En un estudio de mapeo fino de la región 6p se encontró una fuerte asociación de un polimorfismo del gen *BTNL2* (molécula similar a butirofilina 2) con sarcoidosis [318]. Este SNP parecía tener relevancia funcional, ya que origina un codón de parada prematuro que impide la localización en la membrana de la proteína. En el momento de publicación de este artículo se desconocía la función de BTNL2, pero su alta homología con B7-1 hacía pensar que estaría involucrada en la regulación de la activación de la célula T. El gen *BTNL2* se localiza en la región HLA de clase II, muy cercano al gen *HLA-DRB1* (Figura 2.11), donde el alto grado de LD hace difíciles los estudios genéticos. Sin embargo, Valentonyte y col encontraron que la asociación de *BTNL2* con sarcoidosis era independiente del LD con *HLA-DRB1*, es más, era incluso más fuerte [318]. Éste hecho,

junto al posible papel de *BTNL2* en la ruta coestimuladora de las células T, nos llevó a estudiar el SNP en nuestra población de AR [319]. Encontramos una fuerte asociación del SNP de *BTNL2* con AR, pero llegamos a la conclusión que ésta no era independiente de HLA, sino que era debida al fuerte LD con los haplotipos DR-DQ previamente descritos como de susceptibilidad a AR. Además, en el mismo estudio, encontramos idénticos resultados para LES y diabetes tipo 1. De acuerdo con nuestros datos, no se ha podido encontrar asociación de *BTNL2* de manera independiente a HLA en varias enfermedades, como esclerosis múltiple [320], enfermedad de Graves [321], y colitis ulcerosa [322]. Por lo tanto, parece ser que el polimorfismo de *BTNL2* no es relevante en autoinmunidad, sino que se encuentra señalando el efecto de los conocidos alelos HLA asociados clásicamente a estas enfermedades.

Sin embargo, recientemente se ha confirmado el papel de BTNL2 en la fisiología de la célula T. Dos estudios ponen de manifiesto que BTNL2 es capaz de inhibir la activación del TCR y la proliferación de las células T, a pesar de que no se une a ningún receptor de B7 conocido, como CD28, CTLA-4, PD-1 o ICOS [323, 324]. Sorprendentemente, BTNL2 se expresa abundantemente en el tracto digestivo, además de en tejidos linfoides. Por lo tanto, serían necesarios más estudios para determinar cuál es el receptor de BTNL2 y qué papel juega en la inmunidad intestinal.

5.2.4. *STAT4*

En el más reciente estudio de WGS para AR, se identificó el loci 2q como región putativa de ligamiento [65], precisamente donde mapea el conflictivo locus *CD28-CTLA4-ICOS*. A continuación, se realizó un estudio de mapeo fino de la región utilizando una selección de genes candidatos, para identificar los marcadores etiológicos [325]. La figura 5.22 muestra los resultados obtenidos en la primera exploración de éste trabajo. Como se puede observar, la asociación encontrada para *CTLA4* fue bastante modesta en comparación con la encontrada para el gen *STAT4* (signal transducer and activator of transcription 4). Por lo tanto, podría ser que la señal que se había detectado en este locus anteriormente fuera debida en mayor medida a *STAT4*, y que *CTLA4* tuviera una contribución menor.

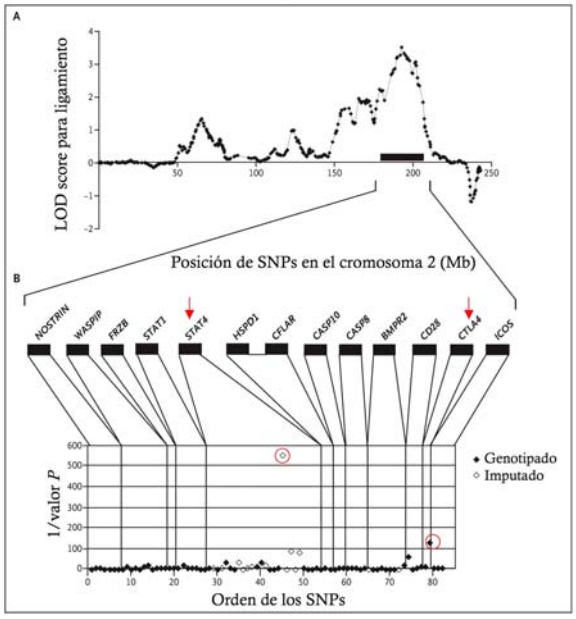

Figura 5.22. Pico de ligamiento en el cromosoma 2q y posición de los SNPs del mapeo fino realizado por Remmers y col [235].

Al realizar un estudio detallado de la región *STAT1-STAT4* en poblaciones norteamericana y sueca, Remmers y col identificaron un haplotipo en el gen *STAT4* formado por 4 SNPs fuertemente asociado a AR, que eran los responsables de la señal en 2q. Éstos marcadores están en alto LD, siendo el SNP rs7574865 el que define el haplotipo y el que mostraba una asociación más fuerte con AR [325]. Además, en el mismo trabajo, observaron que éste SNP también está asociado a LES.

De manera interesante, *STAT4*, identificado como gen de susceptibilidad a AR mediante una aproximación libre de hipótesis, tiene una importante función en la regulación del sistema inmune, y más concretamente, en la proliferación de las células T. La ruta de las kinasas Janus-STAT (Jak-STAT) es la diana de señalización de multitud de citoquinas, incluyendo IFNγ, IL-2, IL-4, IL-6, IL-7, IL-10, IL-12 e IL-15, todas ellas con funciones biológicas importantes en la inflamación sinovial en AR [326]. Tras la unión de la citoquina a su receptor, las kinasas Jak se activan. Entonces, fosforilan los receptores de citoquinas en residuos de tirosina, lo que permite el reclutamiento de STATs. Éstos son a su vez fosforilados por las Jaks, dimerizan y translocan al núcleo, donde modulan la expresión de genes diana [327] (Figura 5.23). En concreto, STAT4 transmite las señales inducidas por IL-12, IL-23 e interferones tipo I [328]. Como ya se ha comentado anteriormente en esta tesis, una de las principales funciones de IL-12 e IL-23 es promover la diferenciación y proliferación de las células T_H1 y T_H17, respectivamente. Por tanto, *STAT4* participa en la regulación de la respuesta T_H1 y T_H17 y, ya que estos dos tipos

celulares tienen un importante papel en la etiopatología de la AR, este gen podría ejercer su influencia en la AR a través de una señalización alterada en esta ruta. Además, la expresión de *STAT4* se encuentra incrementada en el sinovio de pacientes de AR [329-331], y varios estudios con modelos murinos de AR han puesto de manifiesto que la presencia de *STAT4* es necesaria para el desarrollo de la enfermedad [332, 333].

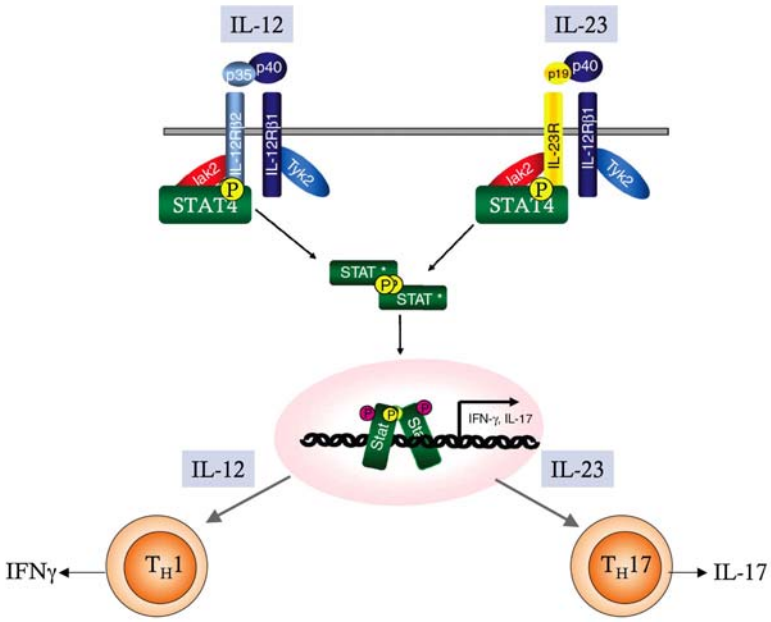

Figura 5.23. Señalización de IL-12 e IL-23 a través de STAT4.

Dada la potencial relevancia que *STAT4* puede tener en la patogénesis de la AR, se hacían imprescindibles estudios replicativos de la asociación reportada por Remmers y col [325]. Cuando comparamos la frecuencia del SNP rs7574865 entre pacientes de AR y controles de nuestra población española, observamos que el alelo T estaba fuertemente asociado a AR [P= 0.001, OR (95% CI) 1.26 (1.09-1.45)], corroborando los resultados previamente descritos [334]. Es más, pudimos replicar la asociación en dos cohortes más procedentes de Suecia y Holanda, siendo la asociación global para las tres poblaciones altamente significativa (P= $9.8 \cdot 10^{-6}$). Realizamos un meta-análisis incluyendo los datos referentes a todas las poblaciones caucásicas estudiadas hasta la fecha para el polimorfismo de *STAT4* (norteamericanos, españoles, holandeses y dos cohortes distintas de Suecia), encontrando una fuerte asociación global [P= 10^{-5}, OR (95% CI) 1.25 (1.18-1.33)]. De manera muy interesante, la asociación de *STAT4* con AR también ha sido replicada en coreanos [335]. Así que incluimos este estudio en el meta-análisis, y la asociación se mantuvo altamente significativa y muy similar a la encontrada para caucásicos solamente [P= 10^{-5}, OR (95% CI) 1.25 (1.19-1.33)]. Aún serían necesarios más estudios replicativos en

distintas poblaciones asiáticas, pero el hecho de que *STAT4* se asocie con AR tanto en caucásicos como en asiáticos con un riesgo similar es de gran relevancia, dada la heterogeneidad genética encontrada en anteriores estudios de asociación entre estos grupos étnicos [294]. Este tema ya se ha comentado con anterioridad en esta tesis, como en el caso de los genes *PTPN22* o *FCRL3*.

Recientemente se ha identificado que en pacientes de AR seropositivos para el FR, existe un tipo específico de células dendríticas que expresan STAT4 en abundancia [331, 332]. Por tanto, quisimos ver si la asociación que habíamos encontrado con el polimorfismo de *STAT4* se restringía al grupo de pacientes con FR positivo. También testamos si existía relación con los otros autoanticuerpos relevantes en AR, los anti-CCP. Sin embargo, vimos que la asociación se produce de manera independiente al estatus de autoanticuerpos [334]. En pacientes coreanos se observó el mismo efecto [335].

Finalmente, los polimorfismos de *STAT4* también se han encontrado asociados a una enfermedad autoimune relacionada, el LES, en individuos norteamericanos [325]. En nuestro laboratorio hemos replicado recientemente esta asociación con LES en población española (Sánchez y col, 2007, datos no publicados). Sería muy interesante estudiar este gen en relación a otras enfermedades, por su posible papel como marcador general en autoinmunidad, ya que su función como regulador de la respuesta T le hace ser un potencial candidato.

5.2.5. *MHC2TA*

Las moléculas MHC de clase II tienen un papel fundamental en el mantenimiento de la tolerancia y en la inducción y regulación de la respuesta inmune adaptativa. Como ya se ha comentado, estas moléculas tienen un importante papel en la patogénesis de la AR, y la asociación genética más fuerte con la enfermedad la proporcionan alelos del gen *HLA-DRB1*. Además, se ha observado que la expresión diferencial de los genes HLA de clase II se asocia con la susceptibilidad y la progresión de la AR [336]. Por tanto, la regulación de la expresión de éstas moléculas podría ser un mecanismo relevante en el desencadenamiento de la AR.

La producción de las moléculas MHC de clase II se regula fundamentalmente a nivel transcripcional. Todos los promotores *MHC II* contienen cuatro elementos llamados cajas W (o S), X, X2 e Y (Figura 5.24). La unión del grupo de proteínas llamado enhanceosoma a sus respectivas cajas está finamente regulado por el transactivador de clase II (CIITA), codificado por el gen *MHC2TA* (major histocompatibility complex class II transactivator), que se une sobre el enhanceosoma pero no se une directamente a los

elementos del promotor del gen. CIITA se expresa en distintos tipos celulares, en respuesta a citoquinas o durante distintos estadios de diferenciación [337, 338]. Por tanto, *MHC2TA* es un buen gen candidato de susceptibilidad a AR, por su función, y porque mapea en una región de ligamiento, el loci 16p13 [57, 59, 62, 64].

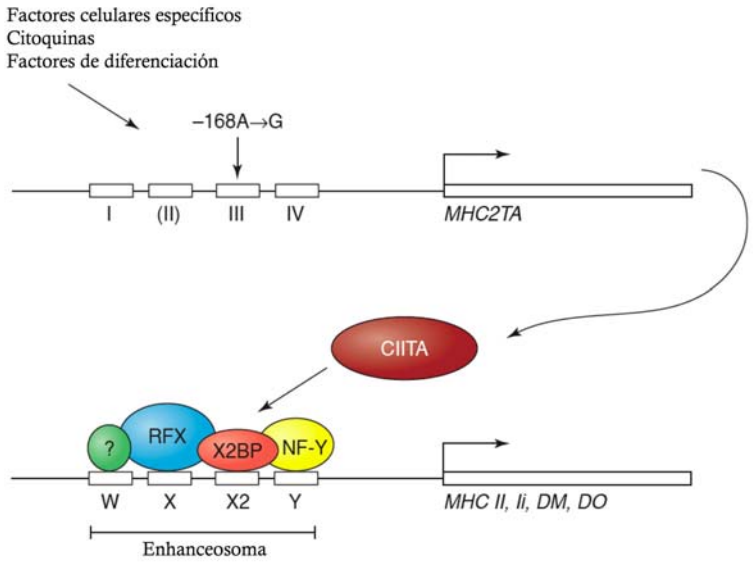

Figura 5.24. Regulación de la expresión de las moléculas MHC de clase II por CIITA, y posición del polimorfismo -168A→G en el gen *MHC2TA*.

Swanberg y col encontraron que un polimorfismo en el promotor del gen *MHC2TA* (-168A→G) estaba asociado a AR, esclerosis múltiple e infarto de miocardio en población nórdica [339]. Vieron además, que éste SNP producía una menor expresión de *MHC2TA*, sugiriendo que esto podría resultar en una inducción menos eficiente de células Treg. Por tanto, intentamos replicar esta asociación en nuestra cohorte española de AR. Encontramos que la variante genética estaba asociada con AR, pero el alelo de susceptibilidad que encontramos era el contrario al reportado anteriormente en población nórdica [340]. Así que para profundizar más en el estudio de este SNP utilizamos dos cohortes adicionales distintas procedentes de Argentina y Suecia, pero no encontramos asociación entre el SNP y la AR. Un meta-análisis conjunto de las tres cohortes que estudiamos mostró que *MHC2TA* -168A→G no está asociado a AR. Otros trabajos estudiado este SNP en AR han sido publicados. Sólo un estudio ha encontrado asociación de éste SNP con AR en población japonesa [341], mientras que el resto no ha replicado la asociación [342-345]. Vimos que existía heterogeneidad en la distribución de esta variante entre poblaciones, lo que quizás pudiera explicar esta falta de replicación del resultado original. Pero un reciente meta-análisis incluyendo los diez estudios realizados para este

SNP en distintas cohortes de AR, ha mostrado que no existe asociación de *MHC2TA* -168A→G con AR, por lo que, probablemente, la primera asociación reportada en nórdicos fuese una sobreestimación de un mínimo efecto. Estos resultados no excluyen la posibilidad de que otros polimorfismos aun no estudiados de *MHC2TA* estén asociados a AR, pero sí indican que -168A→G no está implicado en el desarrollo de la enfermedad.

Con respecto a otras enfermedades, no se ha podido replicar la asociación con esclerosis múltiple que se reportó inicialmente [342, 346], y además *MHC2TA* -168A→G no ha mostrado asociación con LES [347, 348], AIJ [346], narcolepsia y granulomatosis de Wegener [342].

5.2.6. *FOXP3*

El mantenimiento de la tolerancia inmune en la periferia consiste en un balance entre linfocitos autorreactivos y mecanismos reguladores que los contrarrestan. Las células T reguladoras (T_{REG}) $CD4^+CD25^+$ tienen un importante papel modulando la actividad de células autorreactivas influenciando la migración de efectores a órganos diana o nódulos linfáticos, mediante la prevención de la activación de la respuesta inmune actuando en las células presentadoras de antígeno, la inducción de anergia en potenciales efectores y la prevención de la adquisición de funciones efectoras en células T, NK o presentadoras de antígeno [349]. Diversos estudios muestran que un defecto en la actividad reguladora de las células T_{REG} puede provocar autoinmunidad [350, 351].

Se han observado alteraciones en las células T_{REG} en pacientes de AR [352]. De manera interesante, se ha visto que en estos pacientes la frecuencia de células T_{REG} es mayor en el tejido sinovial que en sangre periférica, y que el pronóstico es mejor en los pacientes con mayor cantidad de T_{REG} en la periferia. Notablemente, estas células reguladoras aisladas de la articulación inflamada, poseen actividad supresora *in vitro*, lo que aparentemente resulta contradictorio. Lo que se piensa que ocurre es que estas células T_{REG} migran a la articulación inflamada y allí son inactivadas por los mediadores inflamatorios (Figura 5.25). El sinovio reumatoide está enriquecido en mediadores inflamatorios como TNF, IL-6 e IL-17, los cuales son capaces de inhibir la función supresora de T_{REG}. Esto se confirma con la reversión del defecto supresor con el tratamiento anti-TNF en pacientes de AR. Además, parece ser que el balance T_{REG}/T_H17 en el lugar de la inflamación es crucial en el resultado de la respuesta inmune. En la AR éste balance se desequilibra debido al aumento de la secreción de TGF-β e IL-6, que favorece la diferenciación de las células T_H17 patogénicas [349].

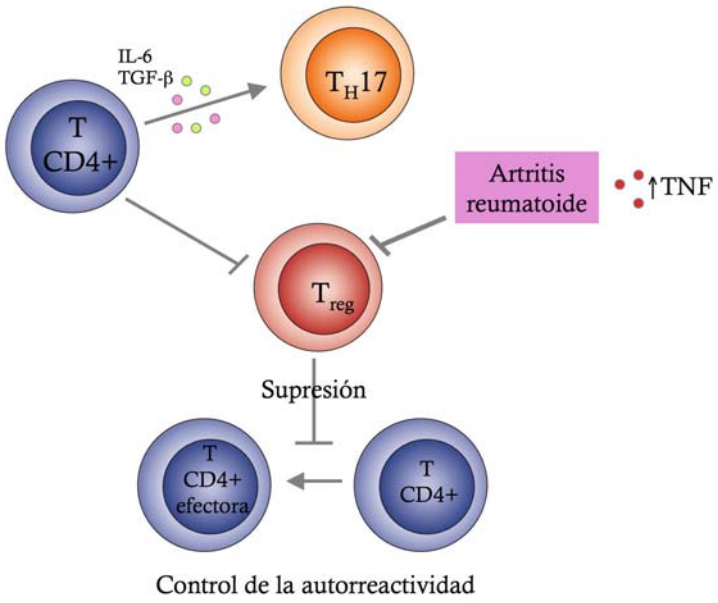

Figura 5.25. Disfunción de las células T_{REG} en la AR.

FOXP3 (forkhead box P3) es un factor de transcripción expresado únicamente por células T_{REG} que es crítico en el desarrollo de éstas. Mutaciones en el gen *FOXP3*, localizado en el cromosoma Xp11.23, pueden provocar graves enfermedades, como el síndrome IPEX (desregulación inmune, poliendocrinopatía, incluyendo diabetes tipo 1, enteropatía y síndrome ligado al cromosoma X) [353]. Por tanto, parece que *FOXP3* es un interesante candidato como gen de susceptibilidad a autoinmunidad. En este sentido, Bassuny y col observaron que un microsatélite $(GT)_n$ funcional estaba asociado a diabetes tipo 1 en japoneses [354]. Por tanto, decidimos estudiar este polimorfismo, pero encontramos que el microsatélite no está asociado a AR [355]. En el mismo estudio pudimos comprobar que esta variante genética tampoco está asociada a LES, EII y enfermedad celiaca. Encontramos que las frecuencias del microsatélite eran bastante distintas a las reportadas en población japonesa, por lo que podríamos pensar que esta falta de asociación sea debida a la heterogeneidad genética. Sin embargo, estudios posteriores no han podido replicar la asociación inicial del microsatélite de *FOXP3* con diabetes tipo 1 [356, 357], incluso en población japonesa [358]. Además, se ha estudiado el gen con mayor profundidad utilizando varios SNPs y no se han encontrado evidencias de asociación con diabetes tipo 1 [356]. Éstos datos, y el hecho de que no se haya encontrado asociación entre *FOXP3* y otras enfermedades como las comentadas LES, EII y enfermedad celiaca, AIJ [359] o enfermedad de Crohn [360] sugieren que los polimorfismos del gen *FOXP3* no juegan un papel relevante en autoinmunidad.

6. CONCLUSIONES

1. Los SNPs Arg677Trp y Arg753Gln de *TLR2* no son polimórficos en la población española. Además, los SNPs Asp299Gly y Thr399Ile de *TLR4*, que se encuentran en alto desequilibrio de ligamiento, no están asociados a AR en nuestra población.

2. Las variantes genéticas -94ins/delATTG y $(CA)_n$ del gen *NFKB1* y M55V de su regulador *SUMO4* no parecen estar implicadas en la susceptibilidad a AR.

3. No hemos replicado la asociación de los polimorfismos de *FCRL3* con AR previamente descrita en población japonesa. Esta falta de replicación se debe a la heterogeneidad genética, ya que el gen parece ser importante en la susceptibilidad a AR en asiáticos, pero no en caucásicos, como se ha demostrado recientemente en dos meta-análisis.

4. Tampoco hemos podido replicar la asociación de los genes *SLC22A4* y *RUNX1* con AR que Tokuhiro y col habían encontrado en población japonesa. En éste caso, la heterogeneidad genética no parece ser la causa de la falta de replicación, ya que no se ha encontrado asociación en una población japonesa similar a la que se había incluido en el estudio de Tokuhiro y col. Estos datos sugieren que la asociación descrita entre *SLC22A4* y *RUNX1* con AR podría ser un falso positivo.

5. Los polimorfismos más estudiados de los genes de la familia de la IL-12 *IL12B*, *IL12RB1* e *IL23R* no parecen jugar un papel relevante en la genética de la AR en nuestra población.

6. El alelo funcional *MIF*-173C productor de mayores cantidades de la citoquina proinflamatoria MIF está asociado a AR en población española. Además, parece ser que esta asociación se produce principalmente en pacientes con menor edad de comienzo, lo que podría ayudar a entender la controversia en torno a la asociación de este SNP con AR.

7. El SNP 1858C→T del gen *PTPN22* es un potente marcador de susceptibilidad a AR. Ésta es la asociación con AR más robusta y reproducible fuera de la región *HLA* hasta la fecha para poblaciones caucásicas. Además, la combinación del alelo 1858T junto a la presencia de los alelos del EC y anticuerpos anti-CCP está fuertemente asociada a AR y resulta altamente específica para la enfermedad, por lo que podría ser de utilidad como marcador de pronóstico.

8. Los polimorfismos CT60 y rs2076530 de los genes implicados en las rutas de coestimulación de la células T *CTLA4* y *BTNL2*, respectivamente, no parecen estar asociados a AR en nuestra población.

9. Hemos replicado la asociación del SNP rs7574865 del gen *STAT4* con AR. Esta asociación es de especial interés, ya que parece que la variante de *STAT4* es un marcador de susceptibilidad a AR común para poblaciones caucásicas y asiáticas.

10. La asociación encontrada en población nórdica del SNP -168A→G de *MHC2TA* no ha sido replicada en población española. Otros estudios replicativos y un meta-análisis apoyan nuestros resultados, por lo que podemos descartar este polimorfismo como marcador de susceptibilidad a AR.

11. El marcador (GT)n de *FOXP3* no parece influir la susceptibilidad a autoinmunidad en nuestra población.

7. PERSPECTIVAS

En los últimos años ha sido posible un gran avance en el conocimiento de las bases genéticas que predisponen a la AR. Sin embargo, buena parte de los genes que influyen en la susceptibilidad a la enfermedad permanecen aun por descubrir. Con este trabajo hemos pretendido contribuir a la identificación de nuevos marcadores de susceptibilidad a AR y, aun siendo nuestra aportación modesta, hemos podido ayudar en el establecimiento de marcadores de susceptibilidad a AR tan importantes como *PTPN22*, *STAT4* y *MIF*. Además, también hemos podido contribuir en un mejor conocimiento de las bases genéticas de la AR descartando polimorfismos como marcadores de la enfermedad. En éste sentido pudimos descartar tanto variantes que fueron estudiadas por primera vez en nuestro laboratorio, como polimorfismos que se habían visto asociados a AR y que, gracias a estudios replicativos como los nuestros, finalmente se ha podido concluir que no influyen en la susceptibilidad a AR.

Cada vez parece estar más claro que múltiples genes, que intervienen en un amplio rango de rutas, están implicados en la predisposición a AR, cada uno de ellos contribuyendo modestamente. Además, aún no está clara la extensión de la variabilidad en alelos y loci entre distintas poblaciones y grupos étnicos con respecto a la susceptibilidad a AR, que podría explicar las diferencias en las manifestaciones clínicas y la severidad entre distintas cohortes. Por tanto, es necesario continuar con la búsqueda de marcadores de AR y el análisis de éstos en distintas poblaciones.

Hasta ahora, la mayoría de los estudios encaminados a la identificación de las bases genéticas de la AR, y las enfermedades autoinmunes en general, han estado basados en estudios de asociación de genes candidatos. Como hemos podido comprobar en el presente trabajo, problemas derivados de falta de poder estadístico, falta de replicación, falsos positivos y heterogeneidad genética y clínica, deben ser sorteados para poder alcanzar un conocimiento más amplio y fidedigno de la susceptibilidad a la AR. Por tanto, se hace imprescindible incluir en estos estudios cohortes cada vez más amplias que incluyan un alto número de pacientes y controles con objeto de alcanzar el mayor poder estadístico posible para detectar riesgos modestos, así como poblaciones distintas para poder llevar a cabo estudios replicativos que confirmen los resultados. Además, la correcta caracterización de cada cohorte y la minimización de heterogeneidad dentro de ella son imprescindibles. Para ello es necesario que distintos grupos de investigación colaboren y aúnen sus fuerzas en la búsqueda de éstos marcadores, como ya se está demostrando, por ejemplo en nuestro estudio del SNP de *STAT4*, donde la colaboración entre grupos de distintos países ha resultado en una replicación robusta. El establecimiento de consorcios para el estudio de la AR y otras enfermedades autoinmunes, como el norteamericano NARAC, el sueco EIRA o el británico WTCCC han permitido un gran avance en el conocimiento de la genética de ésta enfermedad.

Actualmente nos encontramos al comienzo de una prometedora nueva etapa en la búsqueda de marcadores genéticos de susceptibilidad a AR, donde es posible abordar el estudio desde un punto de vista tan amplio como el análisis del genoma completo. Los estudios de asociación de genoma completo están siendo ampliamente aplicados al problema de las enfermedades complejas como la AR y ya están dando sus frutos, gracias al Proyecto Internacional HapMap y las técnicas de genotipado a gran escala, que están evolucionando rápidamente. El siguiente objetivo tras la identificación de regiones de susceptibilidad será la identificación de los marcadores etiológicos en cada región, la replicación en distintas poblaciones y la realización de estudios funcionales.

Uno de los siguientes retos a los que nos enfrentaremos será el estudio de variantes poco frecuentes o raras. Hasta ahora parece ser que la mayoría de los alelos de riesgo son comunes en la población, pero podría ser que un gran número de alelos raros estuvieran contribuyendo juntos al riesgo de la enfermedad. Otro reto para futuros estudios será el estudio de interacciones gen-gen y gen-ambiente, que son necesarias para entender al completo el riesgo genético.

Por tanto, un largo camino queda aún por recorrer en la identificación de las bases genéticas de la AR. Pero afortunadamente, el comienzo de ese camino está ya abierto.

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